Characterization of the cytokine secretion profile of highly purified, functional astrocytes

Hui Zhang, Sandy Reiß, Melanie Jungblut, and Andreas Bosio
Miltenyi Biotec GmbH, Bergisch Gladbach, Germany

This work is supported by NeuroKine – Marie Curie Initial Training Networks.

Introduction

The inflammatory response in the central nervous system (CNS) in multiple sclerosis (MS) is a dynamic multifactorial process that involves both the local resident immune cells, such as microglial cells and astrocytes, as well as infiltrating circulating immune cells such as macrophages. These immune cells communicate to each other by secreting different cytokines and chemokines, regulating and initiating their activities. Astrocytes, a population of CNS-resident cells, were found to play multiple roles in neuro-inflammation. However, there are no detailed and reproducible studies of cytokine and chemokine secretion, partly due to technical restrictions. Most astrocyte populations are not purified effectively and contain contaminating microglial cells, which can lead to erroneous interpretation of the results.

In the past we have set up an automated dissociation procedure for neonatal brain using the gentleMACS™ Octo Dissociator with an optimized enzymatic treatment. For isolation and culture of adult neural cells we have further improved the method and included a novel protocol for removal of debris and erythrocytes, yielding 2–4x10^6 living cells per mouse brain. After brain dissociation, astrocytes were isolated to a purity of >98.5% using MACS® MicroBeads coupled to antibodies against a specific pan astrocyte marker, ACSA-2 (astrocyte cell surface antigen-2).

We also characterized the cytokine secretion profiles of activated neonatal and adult astrocytes. Immunocytochemistry (ICC) analyses showed that neonatal and adult astrocytes expressed a broad range of cytokines and chemokines. When lipopolysaccharide (LPS) or conditioned media from LPS-stimulated microglia were added to neonatal astrocytes, an increased level of GM-CSF was detected with the MACSPlex Cytokine Kit (Miltenyi Biotec). In case of adult astrocytes distinct cytokine secretion profiles dominated either by GM-CSF or IL-23 were detected after activation with TNF-α/LPS or IFN-γ/LPS, respectively. This points to the presence of diverse astrocyte subtypes or to different cellular states, in which the cells are capable of reacting to different stimuli.

Results

1 Highly pure neonatal and adult astrocytes were obtained by MACS® Technology using Anti-ACSA-2 MicroBeads

Neonatal (P1–2) and adult (P9–12W) brains were dissected from CD1 or C57BL/6 mice, and dissociated with the Neural Tissue Dissociation Kit and Adult Brain Dissociation Kit, respectively. Astrocytes were isolated with Anti-ACSA-2 MicroBeads. Separated cells were stained with an Anti-ACSA-2-APC antibody for flow cytometry (MACSQuant® Analyzer) to determine astrocyte purity (fig. 1). High purities were achieved for both neonatal astrocytes (99.17%) and adult astrocytes (98.68%). Isolated astrocytes were cultivated for 5–14 days, then fixed and subjected to ICC using GLAST- (green) and GFAP (red)–specific antibodies (fig. 2; scale bar: 50 µm).

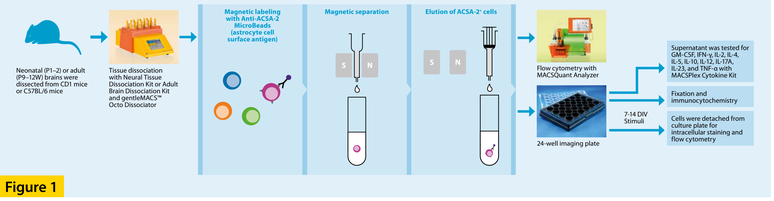


Figure 1

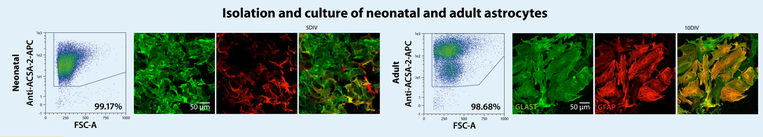


Figure 2

2 Neonatal and adult astrocytes express a broad range of cytokines and chemokines

Purified neonatal astrocytes were cultured for 5 days before fixation. Cells were stained intracellularly with antibodies against IL-17F, IL-12, IL-13, CCL-6, MIP-1α (CCL-3), IL-1β, TNF-α, G-CSF, IL-22, and IL-1α. Specific staining was observed for all the markers (fig. 3; scale bar: 50 µm), except IL-1α. Adult astrocytes were cultured for 10 days before fixation. Cells were stained intracellularly and specific staining was observed for the antibodies against TNF-α, CCL-3, IL-2, G-CSF, and IL-22 (fig. 4; scale bar: 50 µm). ELISA experiments also showed a wide range of cytokines expressed by activated adult astrocytes (data not shown). These results need to be confirmed by further experiments. Neonatal and adult astrocytes were cultured in MACS Neuro Medium supplemented with MACS NeuroBrew-21, L-glutamine, and pen/strep. Neonatal astrocytes were cultured on PLL-coated plates, while adult astrocytes were cultured on PLL/Laminin–coated plates.

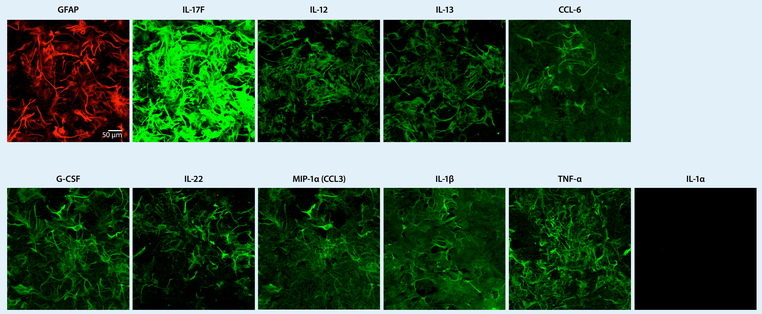


Figure 3

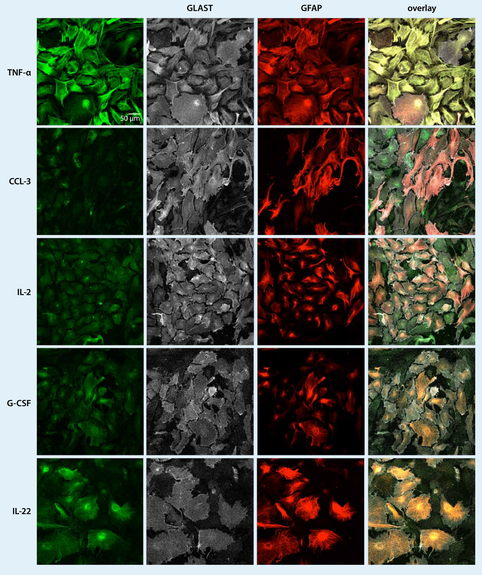


Figure 4

3 Activation of neonatal astrocytes

To validate the activation and detection methods for glial cells *in vitro*, stimulation of microglial cell cultures was first tested as a positive control. Neonatal microglial cells were first isolated with CD11b (Microglia) MicroBeads (Miltenyi Biotec) and cultured for 4 days. Subsequently, the cells were treated with different concentrations of LPS O127:B8 (LPS1) or LPS O111:B4 (LPS2), with or without addition of IFN-γ, for 24 hours. Supernatants were collected and analyzed with a MACSPlex Cytokine Kit (Miltenyi Biotec) to investigate cytokine secretion. Significantly increased TNF-α and IL-10 levels were detected in the supernatant of LPS-treated neonatal microglia cultures (fig. 5A; one-way ANOVA plus Dunnett's multiple comparisons test, SEM, **** p < 0.0001, *** p < 0.001, n = 3).

Neonatal astrocytes were treated with different concentrations of LPS O127:B8 (LPS1) or LPS O111:B4 (LPS2), with or without addition of IFN-γ, for 24 hours. An increased level of GM-CSF was detected when neonatal astrocytes were treated with very high concentrations of LPS (15 µg/mL) (fig. 5B; one-way ANOVA with Dunnett's multiple comparisons test, SEM, ****p < 0.0001, **p < 0.01, *p < 0.05, n = 3). In contrast, no difference in the TNF-α level was detected in the supernatant of LPS-treated neonatal astrocyte cultures.

Neonatal astrocytes were also treated with microglia-conditioned media (MCM), with or without additional LPS treatment, for 24 hours. Significantly increased GM-CSF levels were observed in the supernatants when astrocytes were treated with MCM (fig. 5C; one-way ANOVA with Dunnett's multiple comparisons test, SEM, ****p < 0.0001, ***p < 0.001, **p < 0.01, *p < 0.05, n = 3.). However, no significant changes in TNF-α levels were seen (fig. 5D; one-way ANOVA with Dunnett's multiple comparisons test, SEM, n = 3).

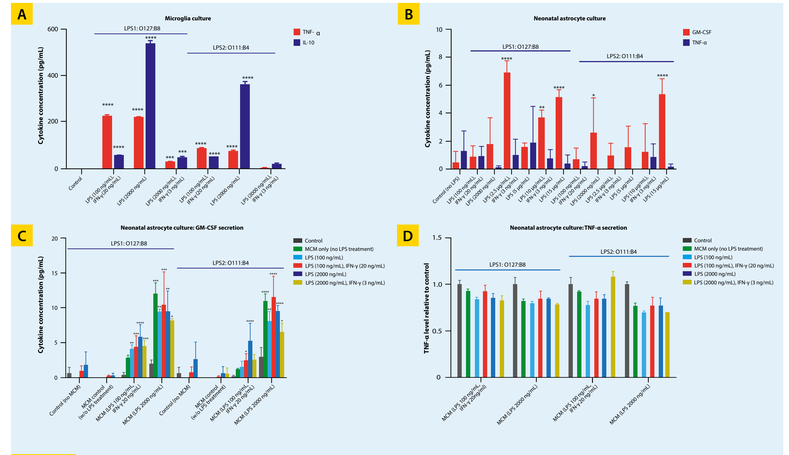
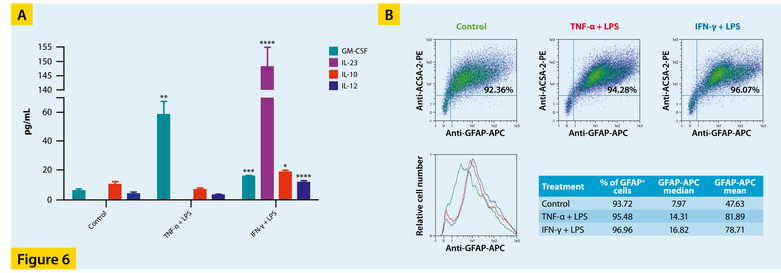


Figure 5

4 Activation of adult astrocytes

Adult astrocytes were cultured for 14 days and then treated with cytokines and LPS for 48 hours. Astrocytes showed a significantly increased GM-CSF level when treated with TNF-α/LPS, and a significantly increased IL-23 level when treated with IFN-γ/LPS. The latter was accompanied by slightly increased GM-CSF, IL-10 and IL-12 (fig. 6A; one-way ANOVA with Dunnett's multiple comparisons test, SEM, ****p < 0.0001, ***p < 0.001, **p < 0.01, *p < 0.05, n = 3). Similar to TNF-α, IL-1β treatment also led to a significantly increased level of GM-CSF, but not IL-23 (data not shown).

To investigate the status of astrocytes after culture and stimulation, activated astrocytes were detached from the culture plates, and stained with Anti-ACSA-2-PE and Anti-GFAP-APC antibodies for flow cytometry analysis (MACSQuant Analyzer). More than 92% of the cells were ACSA-2$^+$GFAP$^+$ (fig. 6B). Astrocytes treated with TNF-α/LPS or IFN-γ/LPS showed a higher GFAP fluorescence intensity (median, mean) compared to untreated astrocytes (fig. 6B), indicating that these astrocytes were in a more active state.



Treatment	% of GFAP$^+$ cells	GFAP-APC median	GFAP-APC mean
Control	93.72	7.97	47.63
TNF-α + LPS	95.48	14.31	81.89
IFN-γ + LPS	96.96	16.82	78.71

Figure 6

Summary and outlook

- We present a novel technology to purify functional astrocytes from neonatal and adult mouse brain.
- Neonatal and adult astrocytes express a broad range of cytokines and chemokines as shown by ICC.
- A significantly increased level of GM-CSF was detected in cultures of neonatal astrocytes when the cells were treated with microglia-conditioned media or with higher concentrations of LPS. In contrast, there was no significant change in the TNF-α level.
- Distinct cytokine secretion profiles dominated either by GM-CSF or IL-23 were detected following activation of adult astrocytes with TNF-α/LPS or IFN-γ/LPS, respectively. This suggests that diverse astrocyte subtypes are present or that the cells are in different states and therefore capable of reacting to different stimuli.
- Further experiments will focus on the isolation of astrocytes from EAE mouse brain to gain insight into the *in vivo* cytokine secretion profile of astrocytes in neuroinflammation.

Unless otherwise specifically indicated, Miltenyi Biotec products and services are for research use only and not for therapeutic or diagnostic use. gentleMACS, MACS, and MACSQuant are registered trademarks of Miltenyi Biotec GmbH. Copyright © 2015 Miltenyi Biotec GmbH. All rights reserved.