Highly purified adult astrocytes display distinct stimulus-dependent activation and cytokine secretion patterns

Hui Zhang[1], Melanie Jungblut[1], Stefan Tomiuk[1], Silvia Rüberg[1], Sandy Reiss[1], Lisa Zatrieb[1], Lena Willnow[1], Stefan Wild[1], Richard Fekete[2], and Andreas Bosio[1]
[1]Miltenyi Biotec, Bergisch Gladbach, Germany | [2]Fluidigm Corp., South San Francisco, CA

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Introduction

Astrocytes are the most abundant cell type in the mammalian brain. However, little is known about their functions in inflammatory diseases such as multiple sclerosis. This is partly due to technical difficulties in isolating highly purified astrocytes from adult brains. In the past, we have set up an automated procedure for dissociation of 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 cell debris and erythrocytes, yielding 2–4×10⁶ living neural cells per adult mouse brain. To assess and compare neonatal and adult astrocyte diversity we isolated astrocytes using MACS® MicroBeads coupled to a specific pan-astrocyte marker, Anti-ACSA-2 (astrocyte cell surface antigen-2). We then analyzed their transcriptome by mRNA sequencing of single cells with the C1 Single-Cell Auto Prep System (Fluidigm®). The single-cell transcriptome analyses revealed particular molecular profiles for both neonatal and adult astrocytes. Next, we characterized the cytokine secretion profile of activated adult astrocytes. A distinct profile dominated either by GM-CSF or IL-23 was detected after activating adult astrocytes with TNF-α (or IL-1β) or IFN-γ, respectively. This points to diverse astrocyte subtypes capable of reacting to different stimuli.

Results

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

Neonatal (P0–2 days) or adult (P9–12 weeks) brains were dissected from CD1 mice or C57BL/6 mice, and dissociated with the Neural Tissue Dissociation Kit (P) and the Adult Brain Dissociation Kit (Miltenyi Biotec), respectively. Astrocytes were isolated with Anti-ACSA-2 MicroBeads (fig. 1). Separated cells were stained with Anti-ACSA-2 antibodies conjugated to APC for flow cytometry analysis (MACSQuant® Analyzer). High purities were achieved for both neonatal astrocytes (98.36%; SD = 0.4786; n = 5) and adult astrocytes (97.78%; SD = 0.8068; n = 7) (fig. 2). Cell viability was 87.67% for neonatal astrocytes (SD = 4.969; n =5), and 73.98% for adult astrocytes (SD = 8.798; n = 7). Isolated astrocytes were cultivated for 5–14 days, then fixed and subjected to ICC using GLAST- and GFAP-specific antibodies (fig. 2). The culture showed a dense layer of GLAST/GFAP-positive astrocytes.

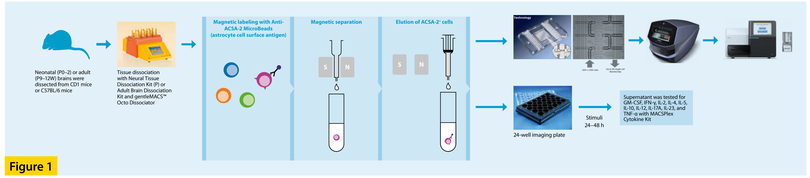


Figure 1

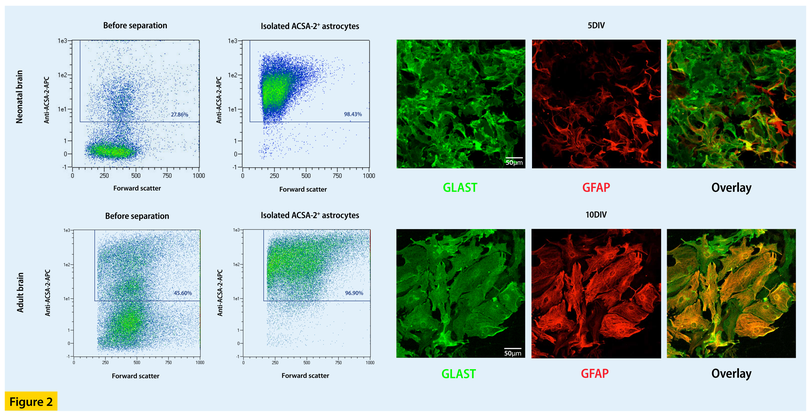


Figure 2

2 Single-cell RNA sequencing and data processing

A single-cell suspension (480,000 cells/mL) of ACSA-2⁺ astrocytes was loaded on a C1 Single-Cell Auto Prep IFC microfluidic chip designed for cells with sizes of 5–10 µm. The chip was then processed on a Fluidigm C1 instrument for single-cell capture, lysis, and cDNA amplification (fig. 1). After each capture experiment, the individual chamber in the integrated fluidics circuits (IFC) chip was examined under a light microscope. Only chambers with one single healthy-looking cell were further processed. In total we collected 435 cells (170 neonatal and 265 adult) for single-cell RNA sequencing (RNA-seq). Sequencing libraries were prepared from a fraction of collected cells using the Nextera® XT DNA Library Preparation Kit and Nextera XT Index Kit (Illumina®). For sequencing the MiSeq® Reagent Kit v3 (150 cycle, Illumina) was used to generate 75-bp paired-end reads (fig. 1). Data were processed to remove low-quality reads and samples (fig. 3A). On average, 2,647 genes were detected in adult astrocytes and 3,937 genes in neonatal astrocytes (tpm ≥ 1) (fig. 3B). An extrapolation of detectable genes in relation to an *in silico* increased number of read pairs illustrates different saturation plateaus depending on transcript abundance. For example, if only transcripts of high abundance (tpm ≥ 100) are of interest, a minimum number of 40–50,000 read pairs is required (fig. 3C).

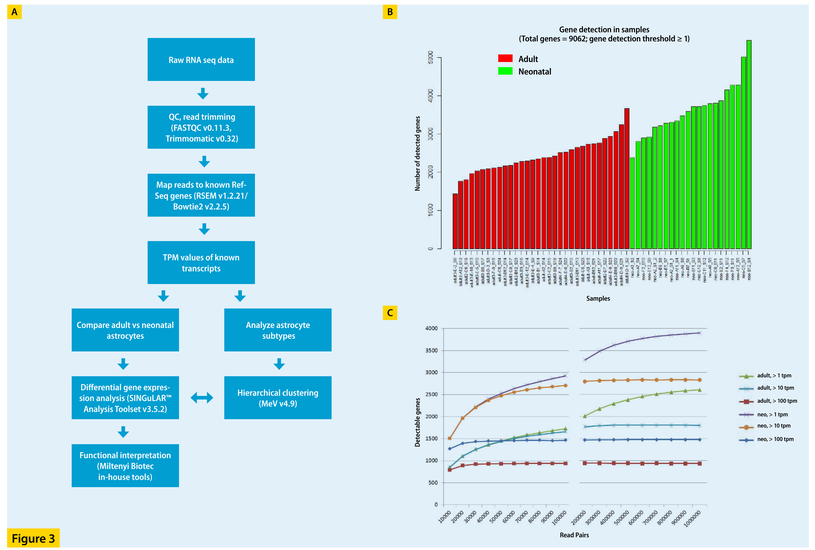


Figure 3

3 Neonatal and adult astrocyte heterogeneity quantified by single-cell RNA sequencing

Up to now, we have profiled 22 neonatal and 32 adult ACSA-2⁺ cells. We identified differentially expressed genes (Fluidigm SC toolset) and conducted annotation enrichment analyses (Miltenyi Biotec in-house tools) (fig. 3A). Based on a combination of selection criteria (ANOVA $p \leq 0.01$; average effect size ≥ 3; more than 50% of samples with tpm ≥ 10 in the group with higher expression) 821 genes were expressed at a significantly higher level in neonatal ACSA-2⁺ cells relative to adult cells. In contrast, 109 genes were expressed at a significantly higher level in adult ACSA-2⁺ cells relative to neonatal cells. Among the genes with higher expression levels in neonatal cells, the terms translation, nucleotide metabolism, intracellular import/export, and others were enriched with high significance (fig. 4A, B). Subgroups of neonatal and adult ACSA-2⁺ cells were identified by hierarchical clustering. Neonatal ACSA-2⁺ cells clustered into two groups. 244 genes were expressed at significantly higher levels in group 1, and 453 genes in group 2 (fig. 5A; ANOVA $p \leq 0.01$; average effect size ≥2; more than 50% of samples ≥10 tpm). Significantly differentially expressed genes mainly belong to the categories protein translation, precursor & energy metabolism, cellular import/export, and intracellular trafficking (fig. 5A).

Adult ACSA-2⁺ cells were also clustered into two groups. 92 genes were expressed at a significantly higher level in group 1, and 185 genes in group 2 (fig. 5B; ANOVA $p \leq 0.01$; average effect size ≥2; more than 50% of samples ≥10 tpm). Significantly differentially expressed genes mainly belong to the categories cellular import/export, precursor & energy metabolism, and intracellular trafficking (fig. 5B).

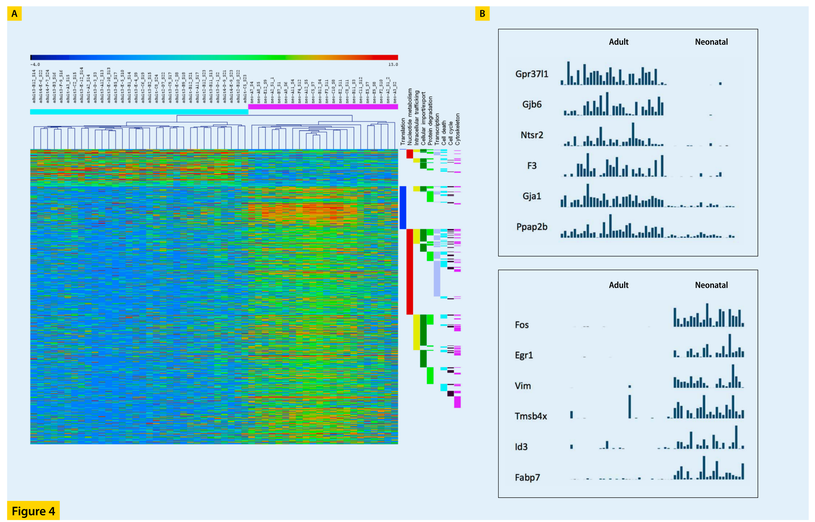


Figure 4

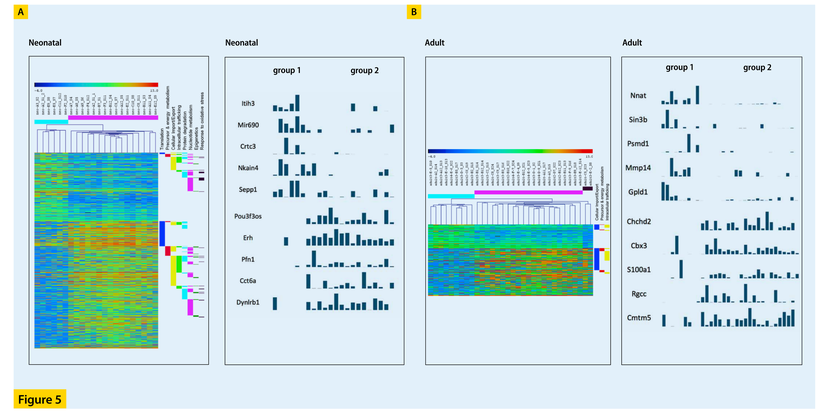
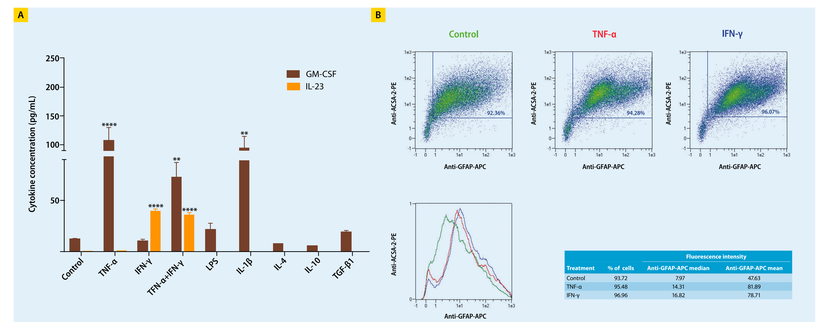


Figure 5

4 Heterogeneity of activated adult astrocytes

Adult astrocytes from C57BL/6 mice (P9–12W) were cultured for 10–14 days and then treated with different cytokines or LPS for 24–48 hours (fig. 1). To increase cytokine secretion from the activated astrocytes, PMA and ionomycin were added 16 hours before collecting the supernatant for analysis with the MACSPlex Cytokine Kit. Astrocytes showed a significantly increased GM-CSF level when treated with TNF-α or IL-1β, and a significantly increased IL-23 level when treated with IFN-γ (fig. 6A; one-way ANOVA with Dunnett's multiple comparisons test; SEM; ****$p < 0.0001$; **$p < 0.01$; *$p < 0.05$; n = 3). Treatment of astrocytes with LPS alone, IL-4, IL-10, or TGF-β1 showed no significant increase in cytokine secretion.

To investigate the status of adult astrocytes after culture and stimulation, activated astrocytes were detached from the culture dishes, 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 or IFN-γ 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 cells	Fluorescence intensity: Anti-GFAP-APC median	Anti-GFAP-APC mean
Control	93.72	7.97	47.63
TNF-α	95.48	14.31	81.89
IFN-γ	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 paving the way for detailed and comprehensive molecular and cellular analyses. Transcriptome sequencing of single cells demonstrated distinct expression profiles for neonatal and adult ACSA-2⁺ astrocytes. Preliminary data suggest at least two different subgroups within neonatal and adult ACSA-2⁺ astrocytes.
- Distinct cytokine secretion profiles dominated either by GM-CSF or IL-23 were detected following activation of adult astrocytes with TNF-α (or IL-1β) or IFN-γ, respectively. This points to diverse astrocyte subtypes capable of reacting to different stimuli.
- We will extend our single-cell sequencing data for a comprehensive classification of subgroups within adult and neonatal astrocytes. In addition, the characterization of astrocyte polarization will help to further understand and modulate the inflammatory response in the CNS in diseases such as multiple sclerosis.

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