Short and long TNF-alpha exposure recapitulates canonical astrogliosis events in human-induced pluripotent stem cell-derived astrocytes

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Abstract
Astrogliosis comprises a variety of changes in astrocytes that occur in a context-specific manner, triggered by temporally diverse signaling events that vary with the nature and severity of brain insults. However, most mechanisms underlying astrogliosis were described using animals, which fail to reproduce some aspects of human astrogial signaling. Here, we report an in vitro model to study astrogliosis using human-induced pluripotent stem cells (iPSC)-derived astrocytes which replicate temporally intertwined aspects of reactive astrocytes in vivo. We analyzed the time course of astrogliosis by measuring nuclear translocation of NF-kB, production of cytokines, changes in morphology and function of iPSC-derived astrocytes exposed to TNF-α. We observed NF-kB p65 subunit nuclear translocation and increased gene expression of IL-1β, IL-6, and TNF-α in the first hours following TNF-α stimulation. After 24 hr, conditioned media from iPSC-derived astrocytes exposed to TNF-α exhibited increased secretion of inflammation-related cytokines. After 5 days, TNF-α-stimulated cells presented a typical phenotype of astrogliosis such as increased immunolabeling of Vimentin and GFAP and nuclei with elongated shape and shrinkage. Moreover, ~50% decrease in aspartate uptake was observed during the time course of astrogliosis with no evident cell damage, suggesting astroglial dysfunction. Together, our results indicate that human iPSC-derived astrocytes reproduce canonical events associated with astrogliosis in a time dependent fashion. The approach described here may contribute to a better understanding of mechanisms governing astrogliosis.
1 | INTRODUCTION

Astrocytes are the most abundant glial cells of the mammalian central nervous system (CNS). The astrocytic processes enwrap both presynaptic and postsynaptic elements and closely approach the synaptic cleft, thus modulating synaptic transmission (Perez-Alvarez, Navarrete, Coveo, Martin, & Araque, 2014; Ventura & Harris, 1999). Astrocytes are also known for their secretory potential, releasing neurotransmitters, ions, and other signaling molecules to the extracellular milieu in order to coordinate synaptic homeostasis (Benaroch, 2016; Verkhratsky, Matteoli, Parpura, Mothet, & Zorec, 2016).

As a cell population with heterogeneous morphology and functioning, astrocytes from rodents and humans show distinct features regarding morphology, gene expression, and functional competencies (Zhang et al., 2016). Roughly, the population of human cortical astrocytes is composed of larger and morphologically more complex cells when compared to cortical astroglia from rodents (Oberheim et al., 2009). Yet, multiple subclasses of astrocytes described in the human neocortex are not found in the same region of the murine brain (Oberheim et al., 2009).

Astrocytes are prompt to react after inflammatory insults, experiencing a continuum of temporally ordered physiological changes that culminates in a process called astrogliosis. Initially, mechanical or pathological injuries in the CNS trigger NF-κB signaling in astrocytes leading to an increased production of NF-κB-dependent cytokines, which potentially activate these cells (Lattke et al., 2017; Saggú et al., 2016). Once activated, astrocytes show progressive upregulation of intermediate filaments GFAP and Vimentin (Liu et al., 2014) and heterogeneous degrees of cell hypertrophy (Kang, Lee, Han, Choi, & Song, 2014). As long as the reactive phenotype is maintained, astrocytes may present impaired metabolic functions, such as disrupted recycling of neurotransmitters (Schreiner, Berlinger, Langer, Kafitz, & Rose, 2013) and energy metabolism (Gavillet, Allaman, & Magistretti, 2008). These biological events are intertwined and occur within a time course which determines the extension of the lesion (Burda & Sofroniew, 2014). However, it is unclear whether human astrocytes exhibit such response patterns since most of our knowledge about astrogliosis comes from murine systems. For instance, mouse and human astrocytes did not share the same responses to lipopolysaccharide and IL-1 (Tarassishin, Suh, & Lee, 2014). Besides interspecies differences regarding astrocytic responses, these cells can also exhibit different response patterns depending on the stimulus. It has been shown that mouse astrocytes activated by distinct stimuli exhibited different gene expression profiles (Hamby et al., 2012; Zamanian et al., 2012). Additionally, murine astrocytes exposed to distinct activation stimuli were classified as A1 and A2 according to their transcriptomic profile (Liddelow et al., 2017). Interestingly, together with other cytokines, TNF-α was able to induce both profiles. Indeed, TNF-α emerges as a key player on induction, maintenance, and profiling of astrogliosis.

Neural cells derived from induced pluripotent stem cells (iPSC) have increasingly been used to model human diseases. In the last few years, efforts have been made to improve differentiation protocols in order to obtain and characterize human iPSC-derived astrocytes (Chandrasekaran, Avci, Leist, Kobolak, & Dinnyes, 2016; Emdad, D’Souza, Kothari, Qadeer, & Qadeer, 2012; Shaltouki, Peng, Liu, Rao, & Zeng, 2013; Tcw et al., 2017; Yan et al., 2013). Owing to the uniqueness of human astrocytes as well as their responses to insults and inflammation, we evaluated human iPSC-derived astrocytes regarding the canonical events associated with astrogliosis in response to short- and long-term TNF-α stimuli as a mimetic neuroinflammatory condition. In this study, human iPSC-derived astrocytes were obtained according to the protocol published by Yan et al. with modifications that allowed these cells to develop typical features of astrocytes (Yan et al., 2013). In order to provide not only a characterization of human astrocyte-secreted proteins following inflammatory stimulus, we also evaluated and characterized major events related to the time course of astrogliosis, such as: (a) TNF-α-mediated nuclear translocation of NF-kB p65 subunit, (b) increases in gene expression and secretion of inflammation-related cytokines, (c) alterations in cell morphology and shrinkage of astrocyte nuclei, and (d) dysfunctional aspartate/glutamate uptake. Although human iPSC-derived astrocytes have been shown to respond to inflammatory stimuli (Perriot et al., 2018; Santos et al., 2017), it was unclear whether human iPSC-derived astrocytes can reproduce in vitro the temporally ordered physiological changes typical of astrogliopathy.

2 | METHODS

2.1 | Generation of human iPSC-derived astrocytes

Human iPSC-derived astrocytes were differentiated from human iPSC-derived neural stem cells (NSC) obtained from iPSC of four healthy subjects. NSC were obtained according to a protocol described previously (Yan et al., 2013). These cell lines were used in other studies from our research group (Casas et al., 2018; Garcez et al., 2017; Ledur et al., 2020). One cell line was obtained from a female subject (Subject 1—available at Coriell Institute Biobank with the name GM23279A) and the other three from subjects whose cells...
were reprogrammed at the D’Or Institute for Research and Education (Subjects 2, 3, and 4). Reprogramming of human cells was approved by the ethics committee of Copa D’Or Hospital (CAAE number 60944916.5.0000.5249, approval number 1.791.182). Human cell experiments were performed according to Copa D’Or Hospital regulation. Table 1 exhibits summarized information regarding cell lines used in this study. NSCs were plated at density of 5 × 10^4 cells/cm² in 75 cm² culture flasks, precoated with Geltrex (A1413301—Thermo Fisher) in NSC expansion medium containing 50% Advanced DMEM/F12 (12634-010—Thermo Fisher Scientific, Waltham, MA), 50% fetal bovine serum (FBS) (21103-049—Thermo Fisher Scientific) and neural induction supplement (a16477-01—Thermo Fisher Scientific). On the following day, medium was replaced by astrocyte induction medium (AIM) composed of DMEM/F12 (11330-032—Thermo Fisher Scientific), N2 supplement (17502001—Thermo Fisher Scientific), and 1% fetal bovine serum (FBS) (12657029—Thermo Fisher Scientific). AIM was changed every other day for 21 days. During this period, when reaching confluence, cells were passed at a ratio of 1:4 using Accutase (A6964—Sigma Aldrich, St. Louis, MO) to new Geltrex-coated flasks. AIM was changed on the day after cell passaging. By the end of the 21 days of differentiation, cells were exposed to astrocyte medium containing 10% FBS in DMEM/F12. At this stage, cells were named human iPSC-derived radial glia-like cells due to their positive labeling for radial glia markers PAX6 and phosphorylated Vimentin, which decrease with time in culture (Garcez et al., 2017). From then on, after reaching confluence, cells were passed at a ratio of 1:2 in the absence of Geltrex. Medium was changed twice a week and on the following day after every cell passage. Human iPSC-derived radial glia-like cells were kept in culture for additional 4 weeks in order to expand and to enhance maturing of astroglial functions. All experiments described in this article were performed from Day 49 after the beginning of astrocyte differentiation (Figure 1a).

### 2.2 In vitro astrogliosis

Human iPSC-derived astrocytes were seeded in noncoated multiwell plates or 75 cm² flasks at densities varying from 2 × 10^4 cells/cm² to 6.25 × 10^3 cells/cm². Astrocyte medium was changed at Days 1 and 3 after each passage. At Day 4, astrocyte medium was replaced by serum deprived medium (DMEM/F12) for 24 hr and thereafter 1–50 ng/ml of human recombinant TNF-α (717904—BioLegend, San Diego, CA) or vehicle (DMEM/F12) were added directly to the cells. Cells were then cultured for an additional 1 hr, 24 hr, or 5 days and samples were collected/processed/analyzed according to the experiment (Figure 1b).

### 2.3 Immunocytochemistry and cell morphology analyses

Human iPSC-derived astrocytes were seeded at a density of 6.25 × 10^3 cells/cm² on 96-multiwell Cell-Carrier plates (PerkinElmer, São Paulo/Brasil). Four days after plating, cells were exposed to serum-deprived medium and in the following day, concentrations of TNF-α ranging from 1 to 50 ng/ml were added. For NF-kB translocation experiments, cells were fixed 1 hr after TNF-α exposure. For GFAP, Vimentin and morphology analyses, cells were fixed 5 days after TNF-α stimulus. Cells were fixed with 4% paraformaldehyde (Sigma Aldrich, São Paulo/Brasil) in phosphate-buffered saline for 20 min, permeabilized with 0.3% Triton X-100 (Sigma Aldrich, São Paulo/Brasil) and then exposed to blocking solution containing 2% bovine serum albumin (Sigma Aldrich, São Paulo/Brasil). Next, an overnight incubation at 4°C with primary antibodies was performed. We have used the following antibodies in this study: mouse anti-NF-kB p65 (1:100; sc-8008—Santa Cruz Biotechnology, Dallas, TX), rabbit anti-Vimentin (1:2,000; ab92547—Abcam, Cambridge, UK), mouse anti-GFAP (1:200; MO15052—Neuromics, Edina, MN). Subsequently, samples were incubated with the following secondary antibodies: goat anti-rabbit Alexa Fluor 594 IgG (1:400; A-11037), goat anti-mouse Alexa Fluor 488 IgG (1:400; A-11001), and goat anti-mouse Alexa Fluor 594 IgG (1:400; A-11032, all from Thermo Fisher Scientific, São Paulo/Brasil). Nuclei were stained with 0.5 μg/ml 4’-6-diamino-2-phenylindole (DAPI) for 5 min. Images were acquired with the Operetta high-content imaging system using 10x and 20x objectives in a nonconfocal mode (PerkinElmer, São Paulo/Brasil). Total number of

### Table 1: Cell lines used in the study

| Cell name | Cell line | Gender | Age of collection | Primary cells | Institution | Previous publications | Use in experiments |
|-----------|-----------|--------|-------------------|---------------|-------------|-----------------------|-------------------|
| GM23279A  | Subject 1 | Female | 20                | Dermal fibroblasts | Coriell Biobank | Casas et al. (2018) and Ledur et al. (2020) | Figures 2–6 and Supplementary Figure S1 |
| CF1       | Subject 2 | Male   | 37                | Dermal fibroblasts | D’Or Institute for Research and Education | Casas et al. (2018) and Ledur et al. (2020) | Figures 2–6 and Supplementary Figure S1 |
| CF2       | Subject 3 | Male   | 31                | Dermal fibroblasts | D’Or Institute for Research and Education | Casas et al. (2018), Garcez et al. (2017), and Ledur et al. (2020) | Figures 2–6 and Supplementary Figure S1 |
| C15       | Subject 4 | Female | 16                | Urine cells     | D’Or Institute for Research and Education | Ledur et al. (2020) | Figures 3–5 and Supplementary Figure S1 |
cells was calculated by DAPI stained nuclei counting. Densitometry and cell morphology analyses were performed using software Harmony 5.1 (PerkinElmer, São Paulo/Brasil). Eleven different fields from triplicate wells per experimental condition were used for quantification.

2.4 | Quantitative real-time PCR

Cultures of human iPSC-derived astrocytes were seeded at a density of 2 x 10^4 cells/cm² in T-75 culture flasks. Four days after plating, cells were exposed to serum-deprived medium. After 24 hr of serum deprivation, 10 ng/ml TNF-α was added for an additional period of 1.5, 3, 4.5, 6, and 24 hr. Cells were then detached with Accutase (Merck, Darmstadt, Germany), centrifuged at 300 g for 5 min after which the supernatant was discarded and the resulting pellet was submitted to RNA extraction. Total RNA was isolated using TRIzol reagent, according to the manufacturer’s instructions (Thermo Fisher Scientific, São Paulo/Brasil). The total RNA was resuspended in a final volume of 12 μl of nuclease-free water and quantified by absorbance at 260 nm in a spectrophotometer (NanoDrop 2000; Thermo Fisher Scientific). Then, 1 μg of total RNA was reverse transcribed in a 20 μl reaction volume using M-MLV (Thermo Fisher Scientific, São Paulo/Brasil), the generated cDNA was diluted 5× and a quantitative PCR was performed using Eva Green PCR Mix (Biotium, Fremont, CA) in the StepOne Plus Real-Time PCR Platform (Applied Biosystems, Foster City, CA). RT-qPCR was carried out to detect transcripts of the following genes: tumor necrosis factor α (TNF-α; forward: 5'-CTGCACTTTGGAGTGATCGG-3'; reverse: 5'-TGAGGGTTTGCTACAACATGGG-3'); interleukin 1 β (IL-1β; forward: 5'-CACGATGCACCTGTACGATCA-3'; reverse: 5'-GTTGCTCCATA TCCTGTCCTC-3'); interleukin 6 (IL-6; forward: 5'-TACCCCCAGGAG-AAGATTCC-3'; reverse: 5'-GCCCTCAACGACCACTTTG-3'); glyceraldehyde-3-phosphate dehydrogenase (GAPDH; forward: 5'-GGCCCTCAACGACCACTTTG-3'; reverse: 5'-CCACCACCTGTG GCTTAG-3'); hypoxanthine phosphoribosyltransferase 1 (HPRT-1; forward: 5'-CGTGGATATTGAGTGATGAAG-3'; reverse: 5'-AGAGGGCTACAATGTTGACG-3'); ribosomal protein lateral stalk subunit P0 (RPLP0; forward: 5'-TCCGAACCTTGGGGAATTT-3'; reverse: 5'-ATCTGCTTGGAGCCCACATT-3'); and importin-8 (IPO8; forward: 5'-TCCGAACTATTATCGACAGGACC-3'; reverse: 5'-GTTCAAAGAGCCGAGCTACAA-3'). All primers used in this study were validated by serial dilution to allow reaction efficiency calculation. Efficiencies from all tested primers ranged from 90 to 110%, and the maximum allowed difference in efficiencies for primers used to estimate fold change of each gene was 10% (data not shown). RT-
qPCR data were calculated by the 2^(-ΔΔCt) method using the geometric mean of RPLPO, GAPDH, and IPO8 (IL-1β) or GAPDH and HPRT1 (IL-6) to normalize results.

2.5 | Multiplex analysis of cytokines and brain-derived neurotrophic factor

Cultures of human iPSC-derived astrocytes were seeded at a density of 2 × 10⁶ cells/cm² in 75 cm² culture flasks. Four days after plating, cells were exposed to 24 hr of serum-deprived medium. Then, 10 ng/ml TNF-α was added for an additional period of 24 hr. After this incubation time, conditioned medium was collected, aliquoted, and immediately frozen and stored at −80°C. ProcartaPlex bead-based multiplex immunoassays were performed for simultaneous detection and quantitation of multiple protein targets in cell culture supernatant. The platform MAGPIX was used for simultaneous detection of brain-derived neurotrophic factor (BDNF), INF-γ, IL-1β, IL-10, IL-13, IL-2, IL-4, IL-6, IL-8, and TNF-α in a single sample according to the manufacturer’s instructions. Briefly, 50 μl/well of coated magnetic beads solution (Luminex Corporation) were added to a 96-well plate. The plate was inserted onto a magnetic plate washer; beads were allowed to accumulate on the bottom of each well and were then washed. Samples or standards (50 μl) were added to the wells and then incubated at room temperature for 2 hr on a Capp 18-X multiwell plate shaking platform (500 rpm). The plate was washed again twice and the detection antibody mixture was added and incubated at room temperature for 30 min on a shaking platform (500 rpm). The streptavidin solution was added to each well and incubated at room temperature for 30 minutes (500 rpm). After washing, reading buffer was added into each well and incubated at room temperature for 5 min (500 rpm). Data were acquired on MAGPIX.

2.6 | d-Aspartate uptake

Cultures of human iPSC-derived astrocytes were seeded at a density of 2 × 10⁶ cells/cm² on 24-well plates. Four days after plating, cells were exposed to serum-deprived medium. After 24 hr of serum deprivation, 10 ng/ml TNF-α was added for an additional period of 24 hr or 5 days. Cultures were then incubated with 1 μCi/ml of 2,3-[3H]-d-aspartate (11.3 Ci/mmol) in Hanks saline solution (HBSS) for 15, 30, and 60 min. The competitive inhibitor of the excitatory amino acid transporters (EAATs) family, 100 μM DL-threo-benzoyloxyaspartic acid (DL-TBOA), was added 10 min prior to 2,3-[3H]-d-aspartate. After incubating periods, uptake was terminated by washing cells with HBSS in order to remove non-incorporated 2,3-[3H]-d-aspartate, followed by cell lysis. Aliquots were taken for intracellular radioactivity quantified by scintillation counting. Experiments were performed with two replicate per experimental group in all time points analyzed and the average value of both replicate was used for statistical analyses.

2.7 | Cell death assay

Cell death was assessed 5 days after exposing cells to 10 ng/ml TNF-α. Cells were loaded with the fluorescent dye ethidium homodimer (2 μM—LIVE/DEAD Cell Imaging Kit, Thermo-Fisher Scientific, São Paulo/Brasil) for 30 min. Live cell images were acquired with the Operetta high-content imaging system using a 20x objective (PerkinElmer, São Paulo/Brasil). Cell death was determined by the ratio of ethidium homodimer labeling per DAPI labeling. High-content image analysis Harmony 5.1 was the software used for data analysis (PerkinElmer, São Paulo/Brasil). Seven different fields from duplicate wells per experimental condition were used for quantification.

2.8 | Statistical analyses

Statistical comparisons between two experimental groups were performed by unpaired Student’s t test. NF-kB translocation and asparagine uptake experiments were analyzed by one-way analysis of variance (ANOVA) followed by Tukey’s post hoc test. Statistical significance was considered for p < .05. RT PCR experiments were analyzed by one-way ANOVA followed by Dunnett’s post hoc test. GraphPad Prism v8.02 (GraphPad Software, San Diego, CA) was used for data analyses and graphics.

3 | RESULTS

3.1 | TNF-α induces NF-kB p65 subunit nuclear translocation in iPSC-derived astrocytes

First, we aimed to determine whether human iPSC-derived astrocytes were responsive to TNF-α by analyzing nuclear translocation of the transcription regulator NF-kB p65 subunit, a mandatory step on NF-kB-mediated gene expression regulation and astrogliosis induction (Brambilla et al., 2005; Li, Sibon, & Dijkers, 2018; Saggu et al., 2016). It can be noted that cells incubated for 1 hr with TNF-α ranging from 1 to 50 ng/ml showed nuclear translocation of NF-kB in all tested conditions (Figure 2a). The increase of NF-kB in the nucleus was concentration dependent, with 1, 5, 10, 20, and 50 ng/ml TNF-α promoting increases of NF-kB immunoreactivity around 97, 138, 156, 186, and 184%, respectively (Figure 2b). When the whole cell area was analyzed, a trend toward increasing was observed for NF-kB in all concentrations of TNF-α tested, although it did not reach statistical significance (Figure 2c). The translocation index of NF-kB (calculated by immunoreactivity of nuclei/cell area ratio) revealed that TNF-α induced a significant increase in the NF-kB translocation index in all concentrations tested. Similar to nuclei NF-kB immunoreactivity, the increase was dose dependent, as 1, 5, 10, 20, and 50 ng/ml TNF-α promoted an increase of NF-kB nuclear translocation around 74, 110, 160, 130, and 138%, respectively (Figure 2d). Despite the fact that cells were responsive to 1 ng/ml TNF-α for NF-kB nuclei translocation, cells that received 10, 20, or 50 ng/ml TNF-α were even more effective in recruiting NF-kB to the nucleus. Based on these
findings, 10 ng/ml TNF-α was chosen for further experiments in order to promote astrocytes activation.

3.2 Increased expression of inflammation-related cytokines genes after stimulation with TNF-α

The upregulation of IL-1β, IL-6, and TNF-α gene expression is a hallmark of inflammation within the CNS (Albrecht et al., 2016; Raghavendra, Tanga, & DeLeo, 2004). Quantitative RT PCR (qPCR) was conducted to verify the expression of these key cytokines in a time window of 24 hr following stimulation with TNF-α (Figure 3). As expected, cytokines expression for these three targets were particularly increased as early as 1.5 hr following stimulation and kept high 24 hr after stimulation. Both IL-1β and IL-6 genes showed ~6.2- and ~40.2-fold increased expression 1.5 hr following stimulation and ~5.0- and ~19.9-fold increase, respectively, in the time point of 24 hr after TNF-α exposure (Figure 3a,b). In addition, TNF-α expression was detected only in stimulated cells in all time points tested, but not in any replicate within the control group (Figure 3c).
In order to show that control samples were viable in terms of gene expression, we also analyzed the expression levels of housekeeping genes GAPDH, IPO8 and RPLP0, from the same samples used in Figure 3b,c. No differences were found among experimental groups (Supplementary Figure S1).

### 3.3 | Inflammation-related cytokines are increased after TNF-α stimulation

The increase in gene expression of inflammation-related cytokines is often followed by an increase in secretion of several immunomodulators. Therefore, we followed the presence of a broader spectrum of cytokines in the conditioned media from human iPSC-derived astrocytes activated by TNF-α using a multiplex analysis of cytokines. Based on the literature, cytokines were classified into three major categories related to their primary biological roles in inflammation: pro-inflammatory, modulatory or anti-inflammatory, and also BDNF. The secretion of pro-inflammatory cytokines IL-1β, IL-8, and IFN-γ was significantly stimulated by TNF-α. While the secretion was increased by 425% for IL-1β (from 12.32 ± 1.11 to 64.72 ± 14.05 pg/ml) and 254% for IL-8 (from 1.09 ± 0.16 to 3.85 ± 0.39 ng/ml), IFN-γ was increased by 890% (from 47.9 ± 22.5 to 474.3 ± 147.8 pg/ml) when compared to conditioned media from vehicle-treated cells (Figure 4a).

As expected, IL-1β, IFN-γ, and TNF-α were barely detectable in conditioned media of nonstimulated human iPSC-derived astrocytes. TNF-α was highly detected in the conditioned media from stimulated cells, probably as a remaining from the activation stimulus.

Regarding modulatory cytokines, conditioned media obtained after TNF-α stimulus presented an increase of 352% for IL-2 (from 0.23 ± 0.05 to 1.05 ± 0.14 ng/ml), of 704% for IL-4 (from 0.24 ± 0.11 to 2.35 ± 0.39 ng/ml), and of 311% for IL-6 secretion (from 1.19 ± 0.16 to 3.85 ± 0.39 ng/ml) when compared to conditioned media from vehicle-treated cells (Figure 4b). Among anti-inflammatory cytokines, secretion of IL-13 increased 618% (from 11.3 ± 7.4 to 81.4 ± 21.5 pg/ml) and BDNF increased 106% in the conditioned medium after TNF-α exposure (from 255.5 ± 41.4 to 527.5 ± 40.6 pg/ml) (Figure 4c), while IL-10 levels were unchanged.

### 3.4 | TNF-α induced morphological alterations and upregulation of intermediate filaments Vimentin and GFAP

The incubation of astrocytes with 10 ng/ml TNF-α during five consecutive daily (in serum-free conditions) triggered a typical phenotype of astrogliosis, as revealed by increased cellular immunoreactivity for Vimentin and GFAP (Figure 5a–c) as well as shrunken nuclei (Figure 5d). The incubation with 10 ng/ml TNF-α did not change measurements of cell area (Figure 5e). However, some Vimentin-stained astrocytes clearly showed a thin, elongated and polarized phenotype after treatment with 10 ng/ml TNF-α. To identify these cells, we calculated the aspect ratio (length to width ratio) of Vimentin-positive cells. A greater length/width ratio would indicate an increase in the amount of cells with polarized thin shapes (Figure 5f). We confirmed that TNF-α promotes an increase in cell polarization (25%), which is typically found under inflammatory conditions (Jang et al., 2013).

### 3.5 | Glutamate uptake is impaired in human iPSC-derived astrocytes exposed to TNF-α

Although conflicting results have been reported regarding gene expression and function of glutamate/aspartate transporters in different pathological conditions (Armbruster, Hampton, Yang, & Dulla, 2014; Pregnolato, Chakkarapani, Isles, & Luyt, 2019; van Landeghem, Weiss, Oehmichen, & von Deimling, 2006), the dysfunction of...
glutamate/aspartate transporters has been shown in situations where reactive astrocytes are present (Maragakis & Rothstein, 2004). In order to accurately measure uptake activity of these transporters in human iPSC-derived astrocytes, the nonmetabolized analog D-[3H]aspartate was used. Aspartate uptake was carried out 1 and 5 days after TNF-α incubation. At both time points, TNF-α was able to inhibit D-[3H]aspartate uptake by human iPSC-derived astrocytes (Figure 6a, b). At 60 min of aspartate uptake, a reduction of ~47% was found in cells exposed to TNF-α for 1 day (Figure 6a). When cells were exposed to TNF-α for 5 days, a reduction in aspartate uptake was observed at 5, 15, 30, and 60 min time points (61.5, 72.4, 73.3, and 51.5%, respectively) when compared to control cells (Figure 6b). Also, aspartate uptake activity by human iPSC-derived astrocytes was highly specific, since full inhibition with the competitive inhibitor of EAATs DL-TBOA was observed (Figure 6a, b). In the presence of DL-TBOA, no effect of TNF-α on transporters activity was observed (Figure 6a, b). Since the astrogliosis induction was carried out in the absence of serum, we assessed astrocyte viability after five consecutive days of TNF-α exposure. The removal of FBS from culture medium induced a small increment in cell death: 5.1% of cells incorporated ethidium bromide while only 0.7% of the cells were ethidium positive in serum-supplemented cultures (Figure 6c). Also, a small increment in cell viability loss (7.4%) was detected in the cultures treated with TNF-α. Although statistically significant as compared to vehicle-treated cultures, this increment in cell death seems too small to contribute to the TNF-α-induced effects described in the previous sections.

4 | DISCUSSION

In this study, human iPSC-derived astrocytes were characterized with regard to their responsiveness to short and long-term inflammatory conditions. Thus, human iPSC-derived astrocytes from healthy subjects were challenged with the pro-inflammatory cytokine TNF-α, which is able to initiate and sustain long-term features of astrogliosis (Cui, Huang, Tian, Zhao, & Zheng, 2011).

In both neural cells and immune-competent cells, the NF-kB p50/p65 heterodimer is usually in an inactive complex bound to the IkB family members located in the cytoplasm. Upon inflammation, dissociation and degradation of IkB proteins may lead to the nuclear translocation of the NF-kB complex, where gene upregulation of several cytokines and growth factors may occur (Kaltschmidt & Kaltschmidt, 2009; Liu, Zhang, Joo, & Sun, 2017; Qiu et al., 2004; Zaheer, Yorek, & Lim, 2001). We noticed that human iPSC-derived astrocytes were capable of responding to short term TNF-α exposure through the translocation of NF-kB to the nucleus, a predicted event that occurs within an hour after inflammatory stimuli (Nelson et al., 2004; Romano, Freundenthal, Merlo, & Routtenberg, 2006).

Secretoome analysis revealed that high levels of TNF-α were detected in the conditioned media 24 hr after exposing cells to this cytokine, but it was difficult to distinguish between residual levels arising from the stimulus or secretion. However, TNF-α was almost undetectable in the conditioned medium and cell extracts from non-stimulated astrocytes, confirming that TNF-α is essentially produced by stimulated astrocytes. Since TNF-α release via NF-kB activation triggers inflammatory events such as cytokines production (Hayden & Ghosh, 2014), we also found that upon short-term TNF-α stimulation (24 hr), either modulatory cytokines IL-2, IL-4, and IL-6 or the chemo-tactic and inflammatory cytokine IL-8 had their levels increased from pg/ml to ng/ml range, which is typical for inflammatory responses or pathological conditions (Stenken & Poschenrieder, 2015). However, IFN-γ and BDNF were also increased in the conditioned medium from astrocytes exposed to TNF-α, but to a lesser extent. It is important to take into account that over the course of acute inflammatory stimuli many factors and cytokines are expressed in a coordinated manner. Since IFN-γ is a pro-inflammatory factor, increases in its secretion may be important to induce and sustain astrogliosis (Yong et al., 2001). In a previous report, cultured rat astrocytes were exposed to different inflammatory stimuli, but only TNF-α was able to increase expression and secretion of BDNF via NF-kB activation (Saha, Liu, & Pahan, 2006). Even though the activity-dependent BDNF secretion is well documented in neuronal cells, the secretory nature of astrocytes suggests that increased BDNF secretion by TNF-α might be an attempt of stimulated astrocytes to provide a neurotrophic factor that would rescue viability following acute inflammatory stimuli.

Interestingly, relatively high levels for IL-6 and IL-8 were detected in the conditioned medium from nonstimulated astrocytes (1.6 and 1.0 ng/ml, respectively). Of note, astrocytes are a major source of IL-6, which is important for synapse formation (Wei et al., 2011), maturation of dendritic spines (Wei et al., 2012), and sprouting of glutamatergic connections (Menezes et al., 2016), while IL-8 regulates angiogenesis by enhancing survival and proliferation of endothelial cells (Li, Dubey, Varney, Dave, & Singh, 2003). IL-6, IL-2, and IL-4 were described in this article as modulatory cytokines because they may induce and terminate inflammatory responses depending on the context of inflammation (Hoyer, Dooms, Barron, & Abbas, 2008; Luzina et al., 2012; Rothaug, Becker-Pauly, & Rose-John, 2016). These three cytokines are able to modulate microglial function (Akhmetzyanova, Kletenko, Mukhamedshina, & Rizvanov, 2019; Recasens, Shivastava, Almolda, Gonzalez, & Castellano, 2019; Rossi et al., 2018) as well as astrocyte activation (Alves et al., 2017; Brodie, Goldreich, Haiman, & Kazimirsky, 1998; Klein et al., 1997). Diapedesis and activation of other immunocompetent cells like neutrophils and lymphocytes within the brain can also be regulated by these modulatory cytokines (Ertu, Quintana, & Hidalgo, 1998; Gadani, Cronk, Norris, & Kipnis, 2012; Gao et al., 2017). These cytokines are potential targets for modulation of neuroinflammation. Further investigations are required to understand how different stimuli of stress affect the balance of cytokines and the dynamics of multiple cell types following brain inflammation.

As expected for mature astrocytes, almost all human iPSC-derived astrocytes were immunoreactive for GFAP and Vimentin, though not all astrocytes necessarily express GFAP in the human brain tissue (Kettenmann & Verkhratsky, 2011; Kimelberg, 2004). Both GFAP and Vimentin are considered markers of astrocytes and their overexpression characterizes primary features of reactive
astrogliosis, since their deletion reduces reactive gliosis and promotes synaptic regeneration (Wilhelmsson et al., 2004). TNF-α is fairly known to induce overexpression of GFAP and Vimentin (Perriot et al., 2018; Zhang et al., 2016) and our human iPSC-derived astrocytes were responsive to long-term TNF-α exposure by increasing GFAP and Vimentin. The long-term exposure to TNF-α was also able to alter astrocyte morphology in vitro, since they displayed an elongated shape, namely polarized astrocytes, resembling those in response to a wound (Peng & Carbonetto, 2012) or recruited to mold the glial scar around injured sites (Adams & Gallo, 2018).

The crucial role of astrocytes is well documented in maintaining glutamate homeostasis by continuous removal of extracellular glutamate from the synaptic cleft by glutamate transporters, a mechanism that avoids glutamate excitotoxicity (Rothstein et al., 1996). Recent evidences have shown that extracellular glutamate stimulates glutamate release from astrocytes in order to coordinate neuronal activity, pointing to an emerging role for astrocytes in modulating both glutamate uptake and release and extending their therapeutic potential (Mahmoud, Gharagozloo, Simard, & Gris, 2019). Recent advances in the characterization of A1/A2 astrocytes have contributed to understand the dual effects of reactive astrocytes (Liddelow et al., 2017), even though the reproducibility of these phenotypes has been challenged in vitro (Hyvarinen et al., 2019; Zhou et al., 2019). However, TNF-alpha is one of main factors involved in the signaling of both phenotypes. Once released by
microglia, this cytokine participates in the induction of neurotoxic dysfunctional phenotype A1 (Liddelow et al., 2017). According to these recent reports, long-term exposure to TNF-α impaired glutamate uptake by iPSC-derived astrocytes (Hyvarinen et al., 2019; Zhou et al., 2019). Here, we show that this impairment can occur just after short-term exposure to TNF-α as well. Of note, as astrogliosis progresses due to long-term TNF-α exposure, impairment of aspartate uptake confirmed their dysfunctionality indeed. Considering that reactive astrocytes lose their normal functions and gain others potentially toxic (Liddelow et al., 2017), the impairment of glutamate uptake by TNF-α intertwines both events in the context of astrogliosis, which is considered a complex response varying from hypertrophy, atrophy, alteration in astrocytes biomarkers and dysfunction (Pekny & Pekna, 2016). Our findings show that human iPSC-derived astrocytes can be functionally activated, mimicking previously reported physiological aspects of astrogliosis described in animals, human primary cultures, and postmortem brains. Astrogliopathology comprises both reactive astrogliosis and astrocytopathy that may co-exist after an insult by a series of changes that appear to be context- and disease-driven (Kim, Healey,
These changes vary over time with a continuum of progressive alterations that can result in either beneficial or detrimental effects on the surrounding tissue (Burda & Sofroniew, 2014). The inflammatory processes share similarities and differences between animal and human models, causing intense debate over the theme (Cauwels, Vandendriessche, & Brouckaert, 2013; Warren et al., 2015). For example, genomic responses to inflammatory challenges are divergent between mice and humans, although the same dataset has been subject of contradictory interpretations in two different studies (Kodamullil et al., 2017; Mestas & Hughes, 2004; Seok et al., 2013; Takaó & Miyakawa, 2015). Besides, although signaling pathways of neuroinflammation share similarities between both species, substantial differences were found for molecular and cellular interactions (Kodamullil et al., 2017). Astrogliosis has been systematically related to several CNS pathologies and the implementation of functional in vitro models of human neural cells has been subject of research in the last years. Therefore, a complete screening of inflammatory responses to different stressing stimuli by human astrocytes may shed light in the pathophysiology of neurodegenerative and psychiatric diseases. For instance, human iPSC-based model of astrogliosis may improve the understanding of neuroinflammation-related diseases and promote the development of new therapeutic strategies.

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CONFLICT OF INTEREST
The authors declare no potential conflict of interest.

AUTHOR CONTRIBUTIONS
P.T. improved the protocol for obtaining and culturing of human iPSC-derived astrocytes. Aspartate uptake was carried out (a) 1 day or (b) 5 days after exposing cells to vehicle or 10 ng/ml TNF-α. The competitive inhibitor of glutamate transporters DL-threo-β-Benzoyloxyaspartic acid (DL-TBOA) was added 10 min prior to aspartate. Data are presented as means ± SEM of the percentage of counts per minute (cpm). (c) Cell viability was evaluated by ethidium incorporation. As a positive control for cell death, cells were lysed with Triton 2%. As a positive control for cell viability, cells grown in DMEM/F12 with 10% FBS were also evaluated. Data are presented as means ± SEM of the percentage of ethidium incorporation (arbitrary units of fluorescence). Data from three cell lines and experiments were performed in duplicates (a), (b) and quadruplicates (c). *p < .05; **p < .01; ***p < .001; one-way analysis of variance (ANOVA) followed by Tukey post hoc test. ns—nonsignificant

FIGURE 6 Impairment of [3H] D-aspartate uptake by TNF-α in human-induced pluripotent stem cells (iPSC)-derived astrocytes. Aspartate uptake was carried out (a) 1 day or (b) 5 days after exposing cells to vehicle or 10 ng/ml TNF-α. The competitive inhibitor of glutamate transporters DL-threo-β-Benzoyloxyaspartic acid (DL-TBOA) was added 10 min prior to aspartate. Data are presented as means ± SEM of the percentage of counts per minute (cpm). (c) Cell viability was evaluated by ethidium incorporation. As a positive control for cell death, cells were lysed with Triton 2%. As a positive control for cell viability, cells grown in DMEM/F12 with 10% FBS were also evaluated. Data are presented as means ± SEM of the percentage of ethidium incorporation (arbitrary units of fluorescence). Data from three cell lines and experiments were performed in duplicates (a), (b) and quadruplicates (c). *p < .05; **p < .01; ***p < .001; one-way analysis of variance (ANOVA) followed by Tukey post hoc test. ns—nonsignificant

Sepulveda-Orengo, & Reissner, 2018; Pekny & Pekna, 2016). These changes vary over time with a continuum of progressive alterations that can result in either beneficial or detrimental effects on the surrounding tissue (Burda & Sofroniew, 2014).

The inflammatory processes share similarities and differences between animal and human models, causing intense debate over the theme (Cauwels, Vandendriessche, & Brouckaert, 2013; Warren et al., 2015). For example, genomic responses to inflammatory challenges are divergent between mice and humans, although the same dataset has been subject of contradictory interpretations in two different studies (Kodamullil et al., 2017; Mestas & Hughes, 2004; Seok et al., 2013; Takaó & Miyakawa, 2015). Besides, although signaling pathways of neuroinflammation share similarities between both species, substantial differences were found for molecular and cellular interactions (Kodamullil et al., 2017). Astrogliosis has been systematically related to several CNS pathologies and the implementation of functional in vitro models of human neural cells has been subject of research in the last years. Therefore, a complete screening of inflammatory responses to different stressing stimuli by human astrocytes may shed light in the pathophysiology of neurodegenerative and psychiatric diseases. For instance, human iPSC-based model of astrogliosis may improve the understanding of neuroinflammation-related diseases and promote the development of new therapeutic strategies.

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AUTHOR CONTRIBUTIONS
P.T. improved the protocol for obtaining and culturing of human iPSC-derived astrocytes, performed cell morphology analyses and experiments for astrogliosis characterization. P.T. and E.C.L. cultured and adjusted human iPSC-derived astrocytes for all performed experiments. P.T., J.A.S., and I.M.O. designed and analyzed NF-kB experiments. J.G., C.T.R., J.C.F.M., and D.P.G. performed and analyzed secretome data. P.L.C., S.D., and F.M.R. performed and analyzed quantitative real-time PCR experiments. P.T., E.C.L., and A.L.M.V. conducted D-aspartate uptake assay and analysis. P.F.L. and P.L.C. designed schematic figures. P.T. and S.K.R. designed the research. P.T., P.F.L., J.A.S., L.O.P., and S.K.R. analyzed the data, discussed the results, and wrote the paper. All authors discussed results and validation steps.

DATA AVAILABILITY STATEMENT
The data analyses that support the findings of this study are openly available in figshare at http://doi.org/10.6084/m9.figshare.9249140.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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