Full Length Article

The NLRP3 inflammasome is involved in the neuroprotective mechanism of neural stem cells against microglia-mediated toxicity in SH-SY5Y cells via the attenuation of tau hyperphosphorylation and amyloidogenesis

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ABSTRACT

The cognitive impairment caused by Alzheimer's disease (AD) is associated with beta-amyloid (Aβ) and tau proteins, and is accompanied by inflammation. Recently, a novel inflammasome signaling pathway has been uncovered. Inflammasomes are implicated in the execution of inflammatory responses and pyroptotic death leading to neurodegeneration. Thus, the inflammasome signaling pathway could be a potential therapeutic target for AD. Neural stem cells (NSCs) are multipotent cells that can self-renew and differentiate into distinct neural cells. NSC therapy has been considered to be a promising therapeutic approach in protecting the central nervous system and restoring it following damage. However, the mechanisms involved remain unclear. The aims of this study were to investigate the protective effects of NE4C neural stem cells against microglia-mediated neurotoxicity and to explore molecular mechanisms mediating their actions. NE4C decreased the levels of caspase-1 and IL-1β, and attenuated the level of the NLRP3 inflammasome and its associated protein adapter, apoptosis-associated speck-like protein containing a C-terminal caspase recruitment domain (ASC) in LPS-stimulated BV2 microglial cells, possibly by regulating the phosphorylation of p38α MAPK. The conditioned media obtained from co-culture of LPS-stimulated BV2 and NE4C cells exhibited protective effects on SH-SY5Y cells against microglia-mediated neurotoxicity; this was associated with an attenuation of tau phosphorylation and amyloidogenesis and accompanied by down-regulation of GSK-3β and p38α MAPK signalling pathways. In conclusion, the present study suggested that NSC therapy could be a potential strategy against microglia-mediated neurotoxicity. NSCs regulate NLRP3 activation and IL-1β secretion, which are critical in the initiation of the inflammatory responses, hence preventing the release of neurotoxic pro-inflammatory factors by microglia. This eventually reduces tau hyperphosphylation and amyloidogenesis, possibly through the regulation of GSK-3β and p38α MAPK signalling pathways, and thus protects SH-SY5Y cells against microglia-mediated neurotoxicity.

1. Introduction

Alzheimer's disease (AD) is a devastating neurodegenerative disease characterised by widespread neuronal cell death and progressive dementia. Genetic and molecular studies have confirmed the central role of amyloid-β production in the pathogenesis of AD (Halle et al., 2008; Salminen et al., 2008; Tan et al., 2014). However, therapies at eliminating Aβ from AD have failed to halt disease progression (Castello et al., 2014). Recently, the association of several immune responsive genes with increased AD risks has suggested that inflammatory

mechanisms are also powerful pathogenic forces in the process of neurodegeneration (Sastre et al., 2011; Meraz-Rios et al., 2013).

In the central nervous system (CNS), microglia are known as the primary mediators of the innate immune response. Chronically-activated microglia stimulate deleterious effects through the release of pro-inflammatory cytokines and reactive oxygen species (Lampron et al., 2013). Increased interleukin (IL)-1β, a member of the IL-1 cytokine family, has been implicated in the response to Aβ deposition (Parajuli et al., 2013). IL-1β was shown to be up-regulated not only in specimens from patients with AD, but also in Aβ-treated neurons and transgenic AD mice (Salminen et al., 2008; Tan et al., 2014; Parajuli et al., 2013). In order to exert its functions, pro-IL-1β must be processed into its mature active form by the protease caspase-1, which itself is activated by cytosolic multiprotein complexes called inflammasomes. Inflammasomes contain i) NOD-like receptors (NLRs), ii) adaptor protein recruiting the effector proteins to the complex and iii) inflammatory caspases as effectors (Stutz et al., 2009; Tschopp and Schroder, 2010). According to their NLRs, inflammasomes can be divided into several types, including NLRP1, NLRP2 and NLRP3 which have been identified in the CNS in neurons, astrocytes and microglia respectively (Vaccari et al., 2014). Once activated, inflammasomes induce an inflammatory cell death mode termed as pyroptosis (Xie and Zhao, 2014). The most intensively studied is the NLRP3 inflammasome; this is formed when, upon activation, NLRP3 associates with procaspase-1 and the adapter apoptosis-associated speck-like protein containing a C-terminal caspase recruitment domain (ASC). The NLRP3 complex is suggested to play a role in AD, as its activation by Aβ in the microglia triggers neuroinflammation (Masters and O'Neil, 2011; Saresella et al., 2016). The NLRP3 inflammasome was shown to be activated in monocytes of AD patients (Saresella et al., 2016); moreover, deficiency of the NLRP3 inflammasome in the APP/PSI model of AD favoured the differentiation of microglia to an M2 (anti-inflammatory) phenotype (Heneka et al., 2013). Thus, these findings support the suggestion that NLRP3 is involved in the pathogenesis of AD, and that modulation of the NLRP3 inflammasome in microglia cells could be a promising therapeutic approach for AD.

Neural stem cells (NSCs) can give rise to rapidly dividing neural progenitor cells (NPCs) which produce neurons, astrocytes and oligodendrocytes, and functionally contribute to cognition and repair processes after injury (Liu et al., 2013; Lee et al., 2015). Recently, it was shown that human NSCs transplanted into an AD mouse model improved spatial memory, decreased tau phosphorylation and amyloid-β42 levels, and also reduced microgliosis and astrogliosis (Lee et al., 2015). However, the mechanisms underlying these effects of NSC therapy remain unclear. Other work showed that a reduced proportion of M1 macrophages in mice transplanted with NSCs led to reprogramming of the local inflammatory cell microenvironment, and thus promoted healing of injured spinal cord (Cusimano et al., 2012). Therefore, considering that inflammasomes mediate an innate immune response and that NSCs may protect against this, NSCs might produce their beneficial effects through regulation of inflammasomes in microglia cells. A better understanding of the potential interaction between NSCs and inflammasomes will provide new understanding on the crosstalk between neurons and microglia, and facilitate the development of new therapeutic modalities. In this study, we investigated the effects of the conditioned media obtained from a co-culture of LPS-stimulated BV2 microglial and NE4C neural stem cells on the viability of SH-SY5Y neuroblastoma cells, as well as on tau hyperphosphorylation and amyloidogenesis in this cell line.

2. Materials and methods

2.1. Cell culture

The BV2 immortalised murine microglia cells ICLC ATL03001 (Interlab Cell Line Collection, Banca Bilogica e Cell Factory, Italy) and SH-SY5Y neuroblastoma cells (ATCC® CRL-2266™) were cultured in Dulbecco's Modification of Eagle's Medium (DMEM; Gibco, USA) supplemented with 10% FBS and 1% penicillin/streptomycin (P/S). The murine NE4C neural stem cells (ATCC® CRL-2925™) were cultured in Minimum Essential Media (MEM; Gibco, USA) with 10% fetal bovine serum (FBS) and additional 2 mM L-glutamine (Gibco, USA). All cells were cultured at 37 °C in a humidified cell incubator under 95%/5% (v/v) mixture of air and carbon dioxide.

2.2. Transwell Co-culture of NE4C and BV2 cells

NE4C cells (5×10^5 cells/mL) were seeded onto poly-lysine coated 0.4 μm porous insert (upper chamber) of transwell permeable supports (Falcon, USA) and BV2 cells (5×10^5 cells/mL) were seeded onto six-well plate (lower chamber) in DMEM. Seeded cells were incubated separately overnight at 37 °C in a 5% CO_2 incubator to allow cells to adhere. Then, NE4C and BV2 cells were co-cultured in serum-free medium and simultaneously stimulated with 1 μg/mL lipopolysaccharide (LPS; Escherichia coli serotype 0111:B4; Sigma, USA) for 24 h. LPS pre-incubation was required to stimulate the intracellular accumulation of NLRP3 and pro-IL1β, thus allowing the assembly of fully functional complexes (Saresella et al., 2016). Mono-cultures of NE4C cells and BV2 cells were processed as controls. All cells were incubated at 37°C in a humidified 5% CO_2 atmosphere.

2.3. Neuroprotective assay

SH-SY5Y cells were seeded (2.5×10^4 cells/mL) in DMEM and incubated overnight at 37 °C in a CO_2 incubator. Then, the basal media in SH-SY5Y were replaced with conditioned media (CM) derived from supernatants of BV2 cells with and without co-culture with NE4C cells. Eight control/treatment groups were set up as listed in Table 1.

2.4. Real time quantitative PCR

Total RNA was isolated from BV2 cells using RNeasy plus mini kit (Qiagen, Germany) and subsequently reverse transcribed into cDNA using QuantiNova reverse transcription kit (Qiagen, Germany). The ratio of absorbance at 260 nm and 280 nm was used to assess the quality of RNA (a ratio of ~2.0 was accepted as pure RNA). Real time polymerase chain reaction (RT-qPCR) was performed in the Bio-Rad IQ5 real-time PCR system using QuantiNova SYBR green PCR master mix kit (Qiagen, Germany) and QuantiTect primer assays (Qiagen, Germany), namely IL-1β (QT01048355), NLRP3 (QT00122458) and GAPDH (QT01658692). The commonly used reference gene, GAPDH was selected as the endogenous control for BV2 cells (Das et al., 2015). Data were analysed using the comparative threshold (Ct) method and presented as ratios between the target gene and the endogenous control.

Table 1
List of control/treatment groups used in this study on SH-SY5Y cells.

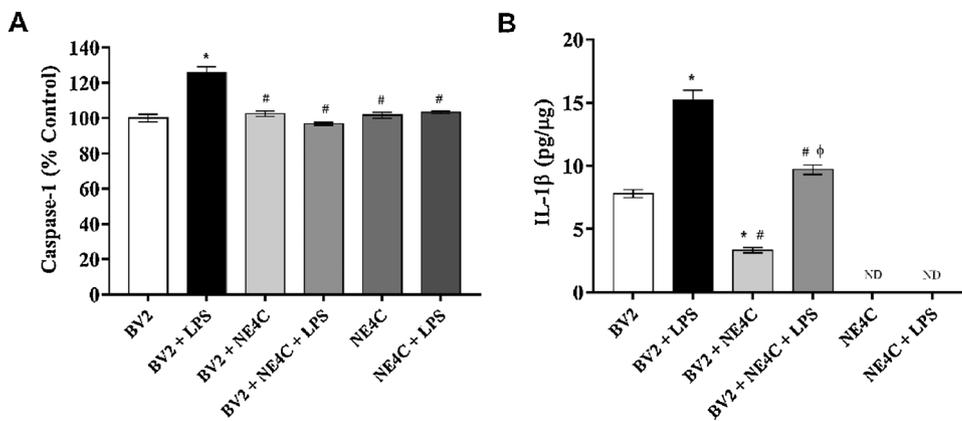| Group	Treatment	Description
1	CM(BV2)	CM from control BV2 cells
2	CM(BV2 + LPS)	CM from LPS-stimulated BV2 cells
3	CM(NE4C)	CM from NE4C cells
4	CM(NE4C + LPS)	CM from LPS-stimulated NE4C cells
5	CM(BV2 + NE4C)	CM from BV2 cells co-cultured with NE4C cells
6	CM(BV2 + NE4C + LPS)	CM from LPS-stimulated BV2 cells co-cultured with NE4C cells
7	SFM	Serum free media only (untreated control)
8	SFM + LPS	Serum free media with LPS (LPS-stimulated SH-SY5Y cells)

A

B

2.5. Caspase-1 colorimetric assay

The activity of caspase-1 was assayed with Caspase-1 colorimetric assay kit (Calbiochem; Merck Milipore, Germany) according to manufacturer's instruction. In brief, 50 μl of BV2 cell lysate (200 μg protein) were incubated for 2 h at 37 °C with 50 μl of reaction buffer containing 10 mM dithiothreitol (DTT) and 5 μl of 200 μM YVAD- p-nitroanilide (pNA) substrate. The absorbance was determined at 400 nm using a microplate reader (Tecan, Switzerland).

2.6. Enzyme-linked immunosorbent assay (ELISA)

The levels of IL-1β in the supernatants of BV2 and Aβ$_{42}$ levels in the supernatants of SH-SY5Y cells were determined using IL-1β-ELISA kit (Cat. #336111; Qiagen, Germany) and human amyloid β$_{42}$ brain-ELISA kit (Cat. # EZHS42; Merck Millispore, Germany), respectively according to manufacturer's instructions. The data were normalised to total protein determined using the Bradford protein assay. The data are expressed as pg/μg and pg/mg for IL-1β and Aβ42 respectively.

2.7. Cell viability assay

After 24 h, treated SH-SY5Y cells were incubated with 20 μL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Calbiochem, USA) at 37 °C in a CO$_2$ incubator for 4 h. After incubation, MTT medium was aspirated from the wells and 100 μL of dimethyl sulfoxide (DMSO; Calbiochem, USA) were added to dilute the formed crystals. The absorbance was determined at 570 nm using a microplate reader (Tecan, Switzerland).

2.8. Western blot analysis

BV2 cells or SH-SY5Y cells were lysed using extraction buffer and protein concentration was determined using the Bradford assay. Samples with an equal amount of protein were separated on 10% sodium dodecyl sulfate polyacrylamide gels and transferred onto a polyvinylidene difluoride membrane. Transferred membranes were blocked with 5% bovine serum albumin for 1 h, incubated overnight at 4 °C with specific primary antibodies: rabbit monoclonal anti-NLRP3 (1:1000; Cat. # 15101S), mouse monoclonal anti-IL1β (1:1000; Cat. # 12242S), rabbit monoclonal anti-ASC (1:1000; Cat. # 67824S), rabbit monoclonal anti-p38 MAPK (1:1000; Cat. # 9212), rabbit monoclonal anti-Phospho-p38 MAPK (Thr180/Ytr182) (1:1000; Cat. # 9211), rabbit monoclonal anti-Phospho-GSK-3alpha/beta (Ser21/9) (1:1000; Cat. # 9331), rabbit monoclonal anti-GSK-3beta (1:1000; Cat. # 9315) and rabbit monoclonal anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (1:1000; Cat. # 5174 T) from Cell Signaling Technology as well as mouse monoclonal anti-Tau (Tau-5) (1:200; sc-58860) and

mouse monoclonal anti-p-Tau (PHF-6) (1:200; sc-32276) from Santa Cruz Biotechnology. Individual membranes were then incubated with a horse-radish peroxidase-conjugated secondary antibody anti-rabbit IgG (Cat. # 7074P2; Cell Signaling Technology) or anti-mouse IgG (Cat. # 7076P2; Cell Signaling Technology) for 1 h at room temperature. The blots were developed using enhanced chemiluminescence. Densitometry analysis of western blots was performed using Image Lab 4.0 software (Bio-Rad, USA). GAPDH was used as a loading control.

2.9. Statistical analysis

All statistical analyses were performed using the GraphPad prism software version 7. Data were analysed using one-way analysis of variance (ANOVA), followed by Tukey's post-hoc test. The differences were considered statistically significant when $p < 0.05$. Data are presented as mean ± standard error of mean (SEM).

3. Results

3.1. NE4C cells attenuated caspase-1 activation and IL-1β secretion in LPS-stimulated BV2 cells

LPS stimulated the activation of caspase 1 and IL-1β secretion ($p < 0.05$; Fig. 1) in BV2 cells. When NE4C cells were co-cultured with stimulated BV2 cells, the level of caspase-1 was close to that of control cultures (non-stimulated BV2 cells; Fig. 1A) and the secretion of IL-1β was markedly decreased ($p < 0.05$; Fig. 1B). However, IL-1β was undetectable either from LPS-stimulated or non-stimulated NE4C cells when cultured without BV2 cells (Fig. 1B).

3.2. NE4C attenuation of NLRP3 inflammasome activation and pro-IL1β expression in LPS-stimulated BV2 cells

Stimulation with LPS produced a significant increase in the NLRP3 transcript ($p < 0.05$; Fig. 2A) by more than four-fold compared to non-stimulated BV2 cells. Protein levels of NLRP3 and ASC were consistently higher in LPS-stimulated BV2 cells. When LPS-stimulated BV2 cells were cultured with NE4C, the transcript ($p < 0.05$) and protein levels of NLRP3 were reduced. Furthermore, the level of ASC in stimulated BV2 cells when co-cultured with NE4C was close to that of control. LPS stimulated IL-1β mRNA expression in BV2 cells ($p < 0.05$) and increased production of pro-IL-1β (Fig. 2B). However, when stimulated BV2 cells were co-cultured with NE4C, the protein level of pro-IL1β was attenuated without inhibiting the level of the corresponding transcripts. NLRP3; IL-1β transcripts were undetectable when NE4C cells were cultured alone.
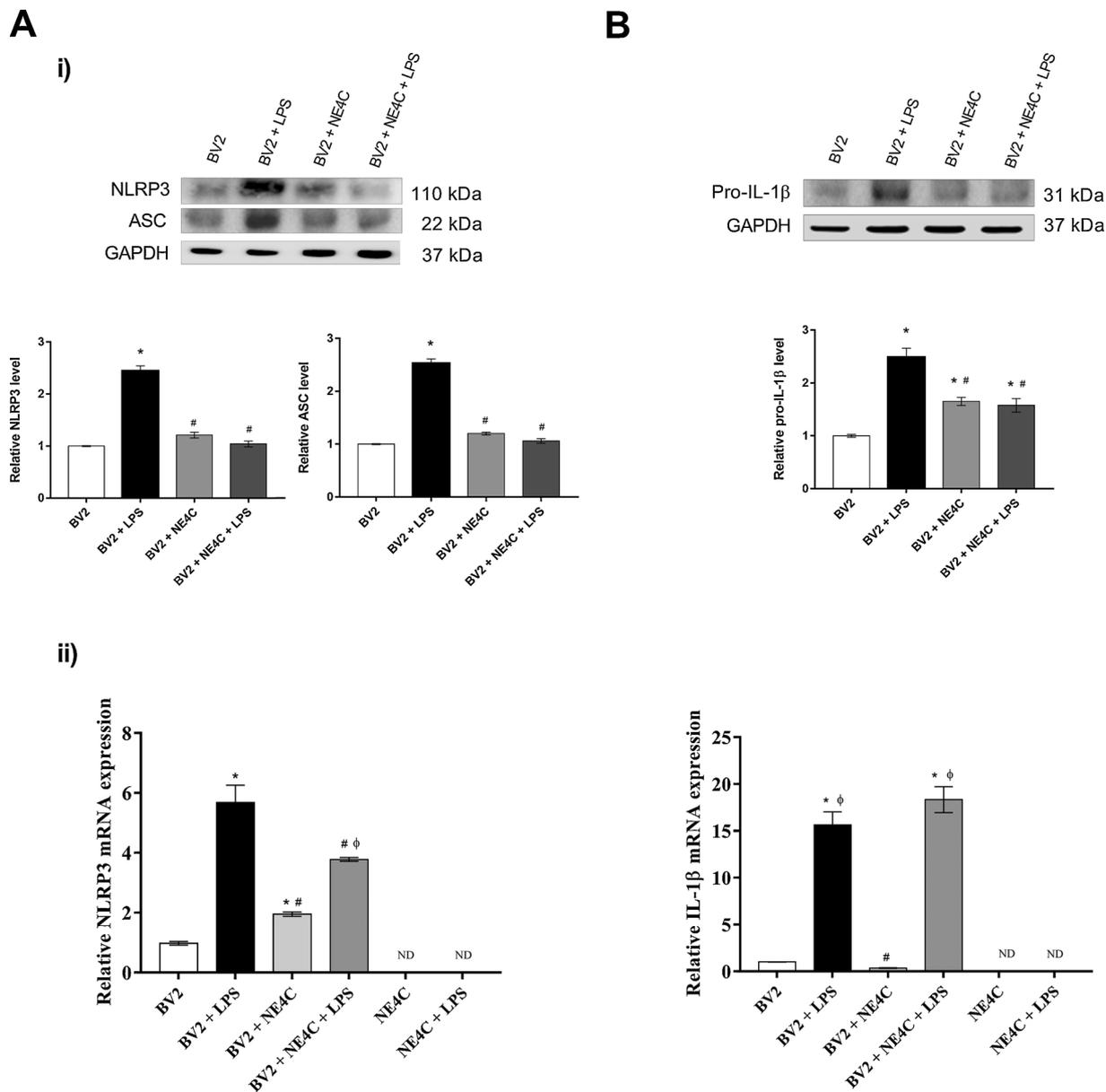
Fig. 2. NE4C attenuated (A) NLRP3 activation and (B) pro-IL1β expression in LPS-stimulated BV2 microglial cells. BV2 and NE4C cells were seeded at a 1:1 ratio. NE4C were co-cultured in serum free DMEM media with BV2 cells simultaneously with 1 μg/mL lipopolysaccharide (LPS) stimulation. At 24 h, i) expression of NLRP3, ASC and pro-IL-1β was determined using the Western blot analysis and ii) the mRNA expression of NLRP3 and IL-1β was determined using RT-PCR. Densitometry analysis of western blots was performed using Image Lab 4.0 software. The expression levels were normalised to GAPDH (endogenous control) levels. Data shown are representative of three independent experiments and presented as mean ± SEM. 'ND' denotes expression that was not detectable. Values of * $p < 0.05$ versus BV2; # $p < 0.05$ versus BV2 + LPS; and ɸ $p < 0.05$ versus BV2 + NE4C were considered as statistically significant.

3.3. p38α MAPK expression in LPS-stimulated BV2 cells co-cultured with NE4C

To further investigate the regulation of inflammasome-mediated neuroinflammation in microglial cells by NE4C cells, the activation of p38α MAPK was determined by detecting the presence of phosphorylated p38α MAPK (p-p38α MAPK) using Western blot analysis. Phosphorylated p38α MAPK was markedly increased in LPS-stimulated BV2 cells as compared with non-stimulated BV2 cells (Fig. 3). However, NE4C cells attenuated the activation of p38α MAPK when co-cultured with stimulated BV2 cells. Faint bands observed in co-culture of NE4C and BV2 cells, as well as in single cultures of either stimulated or non-stimulated NE4C cells indicated limited phosphorylation of p38α MAPK. In addition, there was no significant difference in the levels of p-p38α MAPK and total p38α MAPK between stimulated and non-

stimulated NE4C cells in single culture ($p > 0.05$).

3.4. Neuroprotective effects on SH-SY5Y cells against LPS-stimulated microglia-mediated neurotoxicity

When SH-SY5Y cells were treated with conditioned media (CM) from LPS-stimulated BV2 cells (Group 2), there was a significant decrease in cell viability (approximately 25%; $p < 0.05$; Fig. 4) when compared to the control (Group 1). However, when the SH-SY5Y cells were treated with CM from LPS-stimulated BV2 co-cultured with NE4C cells (Group 6), the cell viability recorded for SH-SY5Y cells was not significantly different from that of control (Group 1). CM obtained from single culture of LPS-stimulated (Group 4) or non-stimulated (Group 3) NE4C cells did not affect the viability of SH-SY5Y cells. LPS had no direct effect on the viability of SH-SY5Y cells (Group 8) as compared to
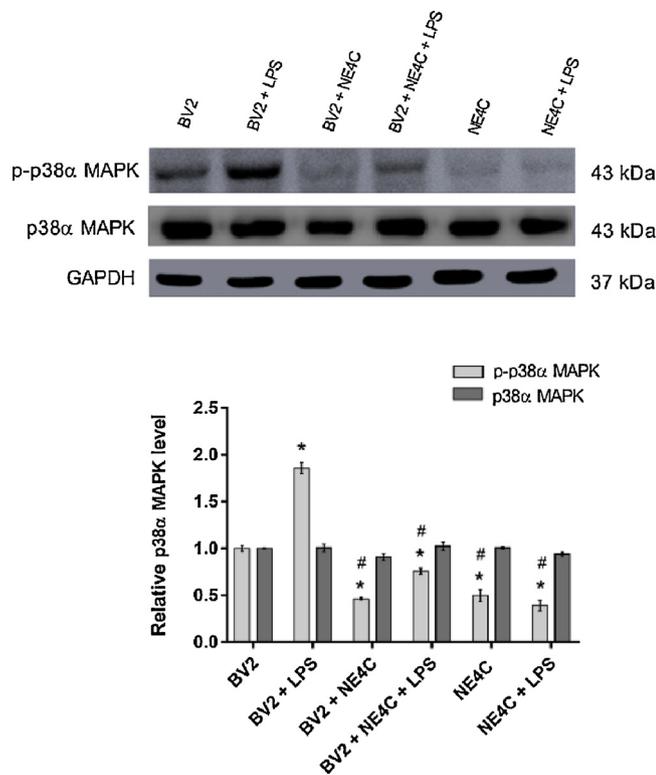
Fig. 3. NE4C reduced the level of LPS-stimulated phosphorylated-p38α MAPK in BV2 microglial cells. BV2 and NE4C cells were seeded at a 1:1 ratio. NE4C were co-cultured with BV2 simultaneously with 1 μg/mL lipopolysaccharide (LPS) stimulation. At 24 h, presence of total p38α MAPK and phosphorylated p38α MAPK (p-p38α MAPK) in BV2 cells or monoculture of NE4C cells was detected using the Western blot analysis. Densitometry analysis of western blots was performed using Image Lab 4.0 software. The expression levels were normalised to GAPDH levels (endogenous control). Data shown are representative of three independent experiments and presented as mean ± SEM. Values of * p < 0.05 versus BV2; and # p < 0.05 versus BV2 + LPS were considered as statistically significant.

untreated SH-SY5Y cells (Group 7).

3.5. Effects of conditioned media (CM) from Co-culture of NE4C and BV2 cells on the production and phosphorylation of tau proteins in SH-SY5Y cells

CM from LPS-stimulated BV2 cells (Group 2) increased the phosphorylation of tau proteins (pT231) and p38α-MAPK (as indicated by the level of p-p38α MAPK), without modifying the levels of p38α-MAPK proteins, or significantly increasing the level of total tau (p > 0.05) in SH-SY5Y cells when compared with SH-SY5Y cells treated with CM from BV2 cells alone (Group 1; Fig. 5). Although there was no significant difference in the levels of phosphorylated GSK-3β at serine 9 (S9) in SH-SY5Y exposed to CM from BV2 cells stimulated by LPS (BV2 + LPS) (Group 2), the expression of total GSK-3β protein was markedly upregulated (Fig. 5B). When SH-SY5Y cells were treated with CM from LPS-stimulated BV2 cells co-cultured with NE4C cells (Group 6), the pT231 levels were downregulated, although there were no significant changes in the levels of total tau when compared to Group 2. Furthermore, total GSK-3β as well as phosphorylated p38α-MAPK proteins were downregulated in Group 6 when compared with Group 2.

On the other hand, CM from a single culture of either stimulated (Group 4) or non-stimulated (Group 3) NE4C, as well as CM from co-culture of NE4C and BV2 (Group 5), increased the levels of pT231, although their total tau proteins were lower than (Group 5), or similar to (Group 3 and Group 4) those found in untreated SH-SY5Y cells (Group 7). LPS stimulation also increased the levels of pT231 in SH-
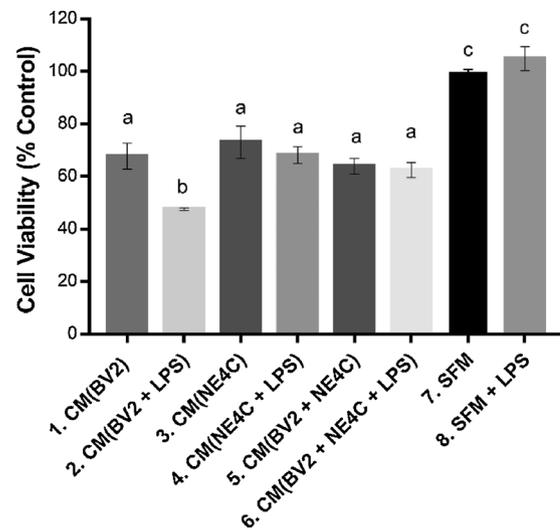

Fig. 4. Neuroprotective effects of NE4C cells against neurotoxicity of microglial-conditioned media. The viability of SH-SY5Y cells was analysed using MTT assay after 24 h of incubation with conditioned media (CM). Eight control/treatment groups were set up as follows: 1) CM(BV2): CM from control BV2 cells; 2) CM(BV2 + LPS): CM from LPS-stimulated BV2 cells; 3) CM(NE4C): CM from NE4C cells; 4) CM(NE4C + LPS): CM from LPS-stimulated NE4C cells; 5) CM(BV2 + NE4C): CM from BV2 co-cultured with NE4C; 6) CM (BV2 + NE4C + LPS): CM from LPS-stimulated BV2 cells co-cultured with NE4C cells; 7) SFM: SH-SY5Y cells in serum free media as untreated control; 8) SFM + LPS: LPS-stimulated SH-SY5Y cells. Data shown are representative of three independent experiments and presented as mean ± SEM. Values between each group were significantly different (p < 0.05) except between those marked with the same letter.

SY5Y cells (Group 8). The total quantities of p38α MAPK and p-GSK-3β in SH-SY5Y cells were not affected by LPS-stimulation, but p-p38α MAPK and total GSK-3β were reduced.

3.6. Effects of conditioned media (CM) from Co-culture of NE4C and BV2 cells on the production of aβ in SH-SY5Y cells

Compared with control (Group 1), the Aβ levels were statistically higher in SH-SY5Y cells treated with CM from LPS-stimulated BV2 cells (Group 2; p < 0.05; Fig. 6). However, the Aβ levels in SH-SY5Y cells decreased when treated with CM from LPS-stimulated BV2 cells co-cultured with NE4C cells (Group 6). Aβ levels observed in Group 1 were not statistically different from Group 7 (untreated SH-SY5Y). Although Aβ levels in Group 5 (CM(NE4C)) and Group 6 (CM(NE4C + LPS)) were significantly lower than Group 7 (p < 0.05), there was no significant different in the Aβ levels between the two groups (p > 0.05).

4. Discussion

The aberrant activation of the NLRP3 inflammasome signalling pathway is implicated in neuroinflammation (Guo et al., 2015; Song et al., 2017). Tight regulation of NLRP3 activation by NSCs may thus present a promising therapeutic target for the treatment of neuroinflammation-associated neurological diseases. The present study demonstrated that NE4C neural stem cells negatively regulated NLRP3 inflammasome activation in LPS-stimulated BV2 microglial cells, possibly mediated through p38α MAPK, and conferred neuroprotective effects against microglia-mediated neurotoxicity. Additionally, this study showed that the neuroprotective effects of NE4C against LPS-stimulated microglia-mediated neurotoxicity in SH-SY5Y cells, as well as the underlying molecular mechanism, were associated with a reduction in tau phosphorylation and in amyloidogenesis.

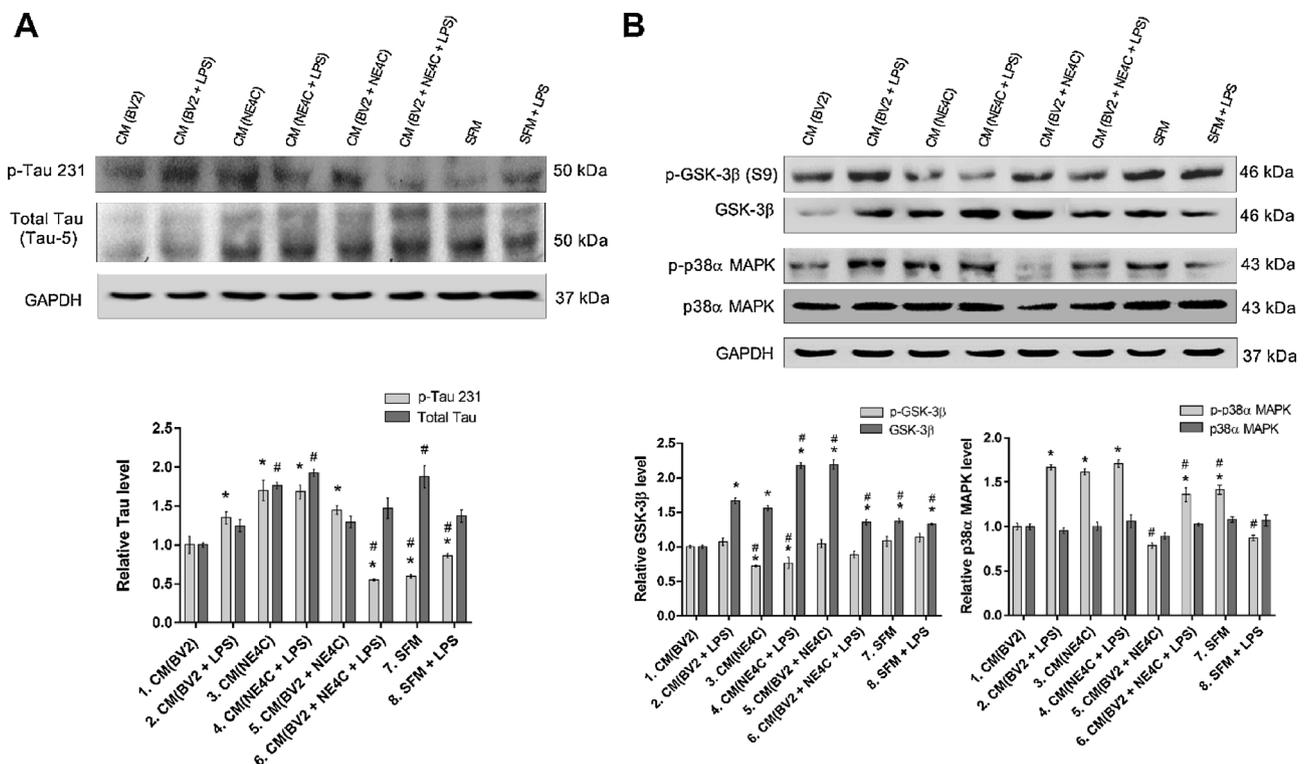The NLRP3 inflammasome is highly expressed in microglia and

Fig. 5. Inhibitory effects of conditioned media (CM) from co-culture of NE4C and BV2 cells on levels of (A) phosphorylated tau (pT231), total tau proteins and (B) the p38α MAPK/GSK3 signaling pathways in SH-SY5Y cells. The culture media from SH-SY5Y cells was replaced with CM from BV2 cells or monoculture of NE4C cells. Western blot analysis was performed using specific antibodies against phospho-tau (pT231), total tau (Tau-5), phospho-GSK-3β, GSK-3-β, phosphor-p38α MAPK and p38α MAPK. Densitometry analysis of western blots was performed using Image Lab 4.0 software. GAPDH was used as the loading control. Eight control/treatment groups were set up as follows: 1) CM(BV2): CM from control BV2 cells; 2) CM(BV2 + LPS): CM from LPS-stimulated BV2 cells; 3) CM(NE4C): CM from NE4C cells; 4) CM(NE4C + LPS): CM from LPS-stimulated NE4C cells; 5) CM(BV2 + NE4C): CM from BV2 co-cultured with NE4C; 6) CM(BV2 + NE4C + LPS): CM from LPS-stimulated BV2 cells co-cultured with NE4C cells; 7) SFM: SH-SY5Y cells in serum free media as untreated control; 8) SFM + LPS: LPS-stimulated SH-SY5Y cells. Results are representative of three independent experiments and presented as mean ± SEM. Values of * $p < 0.05$ versus CM(BV2) and # $p < 0.05$ versus CM (BV2 + LPS) were considered as statistically significant.

plays a crucial role in the initiation of inflammation. A previous study showed that the NLRP3 inflammasome was activated by LPS, as evidenced by the upregulation of caspase-1 and IL-1β (Kauppinen et al., 2013). This observation was consistent with the results of the present study, where we found LPS to increase the activation of caspase in BV2 microglia and the secretion of IL-1β in these cells. However, activation of caspase and the increased secretion of IL-1β were attenuated when BV2 microglial cells were co-cultured with NE4C neural stem cells; co-culture with NE4C cells also reduced the secretion of IL-1β by BV2 cells even in the absence of LPS stimulation. These in vitro anti-inflammatory effects supported previous observations showing that NSCs decreased brain levels of pro-inflammatory mediators including IL-1β in vivo (Neri et al., 2011; Lee et al., 2015; Pang et al., 2017), as well as increasing the expression of anti-inflammatory factors, such as transforming growth factor B1 (TGFB1), interleukin-4 (IL-4) and interleukin-13 (IL-13) (Lee et al., 2015). Interestingly, the protein and mRNA expressions of NLRP3 were downregulated when LPS-stimulated BV2 cells were co-cultured with NE4C cells. This supported the inference of the current findings that NE4C cells can suppress IL-1β production by inhibiting the NLRP3 inflammasome. In the absence of LPS, the expression of NLRP3 was upregulated in the BV2 + NE4C co-culture compared to that seen in BV2 cells cultured alone, although the increase was much less than that seen in BV2 cells stimulated with LPS; this indicates that secretory factors from NE4C cells regulated NLRP3 expression in BV2 cells depending on the cell microenvironment. Appropriate activation of microglia can be beneficial for the reconstruction of the microenvironment, whereas excessive microglial activation is neurotoxic (Bussi et al., 2017).

During inflammasome formation, activation of the pattern

recognition receptor NLRP3 leads to recruitment of the adapter apoptosis-associated speck-like protein containing a C-terminal caspase recruitment domain (ASC). The NLRP3 inflammasome requires ASC for the conversion of pro-caspase-1 into its active form (Freeman et al., 2017). Our observation that co-culturing NE4C cells with LPS-stimulated BV2 cells (BV2 + NE4C + LPS) led to the downregulation of ASC protein levels, may indicate that the inhibitory effect of NE4C on caspase-1 and IL-1β production could be mediated via regulation of inflammasome complex assembly and activation. As well as regulating the NLRP3 inflammasome, NE4C cells attenuated the production of pro-IL-1β when co-cultured with LPS-stimulated BV2 cells, this being consistent with the demonstrated reduction in IL-1β secretion. However, the upregulation of pro-IL-1β mRNA transcripts was not modified, suggesting that inhibition of pro-IL-1β production by NE4C cells might be exerted at the translational level. Taken together, the current findings suggested that NE4C cells suppressed the production of the NLRP3 inflammasome and the pro-forms of IL-1β in LPS-stimulated BV2 cells, resulting in the attenuation of the release of mature IL-1β.

The activation of mitogen-activated protein kinase (MAPK) pathways is a characteristic of activated microglia. In addition to mediating the production of the central pro-inflammatory cytokines, such as interleukin 6 (IL-6) and tumour necrosis factor-α (TNF-α), activation of MAPK signalling cascades can prime NLRP3 inflammasomes and mediate the production of the pro-forms of IL-1β (Kauppinen et al., 2013). The p38 MAPKs, which are members of the MAPK superfamily, were reported to be activated by LPS in BV2 microglial cells (Bachstetter et al., 2011). Typically, MAPKs become activated through a phosphorylation cascade. p38 MAPK signalling regulates LPS-induced inflammasome activity and the production of both caspase-1 and IL-1β in
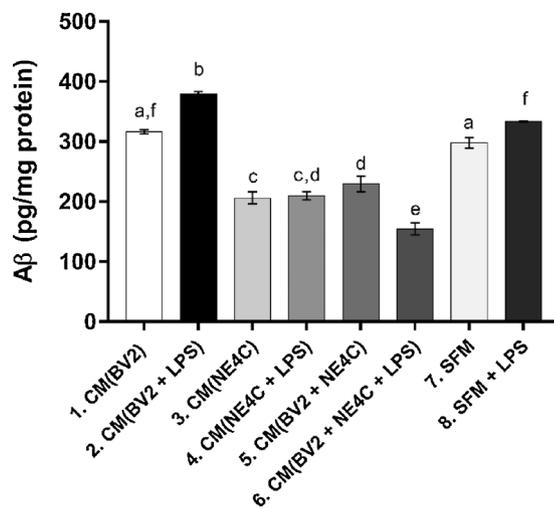
Fig. 6. Inhibitory effects of conditioned media (CM) from co-culture of NE4C and BV2 cells on levels of Aβ in SH-SY5Y cells. The culture media from SH-SY5Y cells were replaced with the CM from BV2 cells or monoculture of NE4C cells and the levels of Aβ were determined using ELISA Kit after 24 h. Eight control/treatment groups were set up as follows: 1) CM(BV2): CM from control BV2 cells; 2) CM(BV2 + LPS): CM from LPS-stimulated BV2 cells; 3) CM(NE4C): CM from NE4C cells; 4) CM(NE4C + LPS): CM from LPS-stimulated NE4C cells; 5) CM(BV2 + NE4C): CM from BV2 co-cultured with NE4C; 6) CM (BV2 + NE4C + LPS): CM from LPS-stimulated BV2 cells co-cultured with NE4C cells; 7) SFM: SH-SY5Y cells in serum free media as untreated control; 8) SFM + LPS: LPS-stimulated SH-SY5Y cells. Data shown are representative of three independent experiments and presented as mean ± SEM. Values between each group were significantly different (p < 0.05) except between those marked with the same letter.

microglial cells, as evidenced by the attenuation of their LPS-induced production in culture and in the mouse brain by pharmacological inhibition of p38 MAPK (He et al., 2017). This is consistent with our findings that NE4C cells inhibited the LPS-stimulated phosphorylation of p38α MAPK in BV2 cells, suggesting that the inhibition of the phosphorylation of p38α MAPK may attenuate the production of the inflammasome receptor NLRP3 as well as the production of pro-IL-1β. However, alternatively, activation of p38 MAPK could be induced by secreted proinflammatory cytokines including IL-1β. Further studies are required to show the temporal relationships between inhibition of LPS-induced p38 MAPK phosphorylation by NE4C cells and IL-1β transcription and secretion; the role of p3MAPK also needs exploring using pharmacological inhibition.

Elevated production of IL-1β by activated microglia could exacerbate neuronal cell death (Lopez-Castejon and Brough, 2011; Edwan et al., 2015). We found that when co-cultured with LPS-stimulated BV2 cells, NE4C cells attenuated the production of mature IL-1β. Moreover, conditioned media from NE4C cells co-cultured with LPS-stimulated BV2 cells prevented the neurotoxicity caused by LPS-stimulated BV2 cells in SH-SY5Y cells; this suggests a neuroprotective effect of NE4C cells via regulation of microglial neuroinflammation. Collectively, these findings suggested that NE4C cells co-cultured with LPS-stimulated BV2 cells could inhibit the NLRP3 inflammasome assembly and the production of pro-IL-1β, possibly through the p38α MAPK pathway, thereby attenuating the overproduction of caspase-1 and mature 1L-1β. Thus, the subsequent protection of SH-SY5Y neuronal cells from the indirect toxicity mediated by LPS-stimulated BV2 microglial cells may have been due to the inhibitory effect of NE4C cells on the overproduction of IL-1β.

There is accumulating evidence to suggest that microglial neuroinflammation promotes tau hyperphosphorylation (Metcalfe and Figueiredo-Pereira, 2014; Wang et al., 2015a,b). Transgenic AD mice overexpressing IL-1β showed an increased expression of intraneuronal

hyperphosphorylation of tau proteins (Ghosh et al., 2013). Furthermore, increased levels of tau phosphorylation at threonine 231 (Thr 231) were correlated with cognitive decline and AD progression in individuals with mild cognitive impairment (Buerger et al., 2002). Therefore, inhibiting tau hyperphosphorylation might be a common pathway to exert neuroprotective effects. Our observation that conditioned medium from LPS-stimulated BV2 cells co-cultured with NE4C cells attenuated tau phosphorylation may indicate that the neuroprotective effects of NE4C against microglia-mediated toxicity were related to the alteration in the levels of phosphorylated tau.

Phosphorylation of tau might be attributable to the activation of both glycogen synthase kinase 3 beta (GSK-3β) and p38α MAPK (Cherry et al., 2016). GSK-3β is deactivated through phosphorylation at S9 (Lee et al., 2015), while phosphorylation activates p38α MAPK (Maphis et al., 2016). Co-transfection of tau with GSK-3β in a cell culture model resulted in increased cell death (Gendron and Petrucelli, 2009). Moreover, the elevation of tau phosphorylation in transgenic mice was accompanied by the activation of MAPK, while selective suppression of p38α MAPK rescued late-stage tau pathology (Maphis et al., 2016). Thus, GSK-3β and p38α MAPK signalling pathways might be involved in the neuroprotective mechanisms of NE4C against microglia-mediated toxicity. Accordingly, we showed that conditioned medium from NE4C cells co-cultured with LPS-stimulated BV2 cells attenuated the expression of GSK-3β (active form; Fig. 5B) in SH-SY5Y cells; this suggested that regulation of NE4C on BV2 cells mitigated aberrant tau phosphorylation in SH-SY5Y cells via inhibiting GSK-3β production. Additionally, there was a decrease in phosphorylation of p38α MAPK. Previous work showed that deletion of p38α MAPK expression attenuated Aβ generation in the brain of AD mice and in SH-SY5Y cells (Schnoder et al., 2016). Interestingly, we found that conditioned medium from NE4C cells co-cultured with LPS-stimulated BV2 cells inhibited the production of Aβ. Taken together, these results suggest the involvement of GSK-3β and p38α MAPK in the mechanism by which conditioned medium from NE4C cells co-cultured with LPS-stimulated BV2 cells conferred neuroprotective effects in SH-SY5Y cells. However, more detailed molecular studies are required to fully clarify the mechanisms whereby neuronal stem cells modulate microglia-mediated tau phosphorylation and amyloidogenesis.

5. Conclusion

The present study has provided evidence that NSCs regulated the NLRP3 inflammasome, and inhibited the production of IL-1β and caspase-1 in activated microglia, as well as subsequently attenuating neurotoxicity caused by microglial neuroinflammation. The attenuation of NLRP3 assembly and production of pro-IL-1β in BV2 cells by NE4C cells could be mediated through the p38α MAPK pathway. Moreover, downregulation of phosphorylated tau, Aβ, GSK-3β and phosphorylated p38α MAPK in SH-SY5Y cells might be associated with the neuroprotective mechanisms of NE4C against microglia-mediated toxicity. Finally, these results suggested the role of NSCs as regulators of NLRP3 inflammasomes in microglia, which could confer neuroprotection by preventing tau phosphorylation and amyloidogenesis, adding to the inherent benefits of NSCs in AD treatment.

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