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RESEARCH ARTICLE

Astrocytic Expression of the Immunoreceptor CD300f Protects Hippocampal Neurons from Amyloid-β Oligomer Toxicity *In Vitro*

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Abstract: ***Background:*** Astrocytes contribute to neuroinflammation that accompanies neurodegenerative disorders such as Alzheimer's disease (AD). In this sense, the toxicity of these diseases might be attenuated through the modulation of astrocytic inflammatory responses. Recently, the CD300f immunoreceptor was described as a new member of the CD300 immunoreceptor family, showing promising modulatory properties.

Objective: Here, we investigated whether overexpression of hCD300f (the human isoform of CD300f) in astrocytes protects hippocampal neurons against the degeneration induced by amyloid-beta (Aβ) oligomer.

Method: Astrocyte monolayers were transfected with hCD300f before seeding the hippocampal neurons, and then the co-culture was exposed to $A\beta_{1-42}$ oligomers (5 μM, 48h).

Results: hCD300f expression significantly abrogated the neuronal loss elicited by Aβ. This effect was dependent on neuron-astrocyte cell-cell interactions since no protection was observed using conditioned media from transfected astrocytes. Astrocyte modulation was dependent on the cytoplasmic signaling tail of hCD300f. Furthermore hCD300f expression did not affect the ability of astrocytes to uptake $A\beta_{1-42}$ oligomers by endocytosis, which discards the possibility that increased $A\beta_{1-42}$ clearance could mediate neuroprotection by hCD300f.

Conclusion: These results suggest that the astrocyte-directed expression of the hCD300f immune receptor can be a neuroprotective strategy in AD disease.

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1. INTRODUCTION

Alzheimer's disease (AD) is the most prevalent type of dementia worldwide, whose prevalence is expected to double every 20 years, resulting in 80 million subjects affected by 2040 [1]. AD is confirmed histologically by the presence of senile plaques, whose major protein content are amyloid-β peptides (Aβ) [2], which have been considered the key element that induces further pathological changes [3, 4]. In addition to its direct toxic effects, Aβ can also induce an inflammatory response, which in turn is believed to increase neurodegeneration in AD affected brains [5, 6]. The toxicity induced by inflammation depends on several cell types including astrocytes [7, 8], and specific targeting of the inflammatory phenotype of this cell type ameliorates neurological changes in AD models [9]. Recently, mutations of immunoreceptor TREM2 have been associated to increased susceptibility to AD and other neuropathologies [10]. CD300f (IREM-1, IgSF13, and CMRF-35A5 in humans and CLM-1, LMIR3, MAIR-V, and CD300LF in mice) is a member of the CD300 family of immunoreceptors. It displays two cytoplasmic tyrosine-based inhibitory motif (ITIM) [11] and also a Phosphatidylinositide 3-kinases (PI3K) activating motif [12], and presents a FcεRγ-mediated activating potential [13]. Upon activation, the CD300f ITIM-motifs become phosphorylated in their tyrosine residues, and the Src2 homology domain tyrosine phosphatase 1 (SHP1) is then recruited and propagates an anti-inflammatory signal over the cell [11]. Interestingly, astrocytes and pyramidal neurons are the cells that express the highest levels of SHP1

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in the brain, and the reduction of its activity induces basal astrocyte reactivity [14].

The role of CD300f in neuron-glia interactions is largely unknown. A putative CD300f ligand has been described in the normal brain and its expression is enhanced after an acute excitotoxic brain injury [15]. Moreover, the overexpression of CD300f in this excitotoxic model was neuroprotective. CD300f knockout mice show increased inflammation and a worsened outcome in experimental autoimmune encephalomyelitis [16], several models of allergy [17] and in a model of Systemic Lupus Erythematosus [18]. The mechanisms of neuroprotection by CD300f involves the modulation of inflammation, as the upregulation of nitric oxide and Interleukin-1 beta (IL1β) correlates with the increased severity of experimental autoimmune encephalomyelitis in CD300f knockout animals [16]. Moreover, through the ITIM motifs, CD300f inhibits Toll receptor signaling [19], which is neurotoxic after ischemia [20]. Taken together, these data suggest that the modulation of inflammatory reactions by CD300f in the central nervous system (CNS) may be an important component of both acute and chronic neurodegenerative conditions. In this context, the present work aimed to explore the potential of the expression of CD300f in astrocytes as a neuroprotective strategy against Aβ toxicity *in-vitro*.

2. MATERIALS AND METHODS

2.1. Animals

All primary cultures were made from Wistar rats, which were housed under standard laboratory conditions with light/dark cycle (12/12h), lights on at 7:00 AM, and food and water were provided *ad libitum*.

2.2. Cell Culture

Primary astrocytes cultures were prepared from 1-day-old rat pups according to the procedures of Saneto and De Vellis [21] with small modifications previously published [22]. The resulting astrocyte monolayers were 98% pure as determined by glial fibrillary acidic protein (GFAP) immunoreactivity.

In order to produce the co-cultures, primary hippocampal neurons were collected in embryonic day 18 and were prepared as adapted from Kaech and Banker [23]. Primary neurons were seeded over previously transfected (24 hours before) astrocytic monolayers and co-cultures were maintained in DMEM medium supplemented with 5% FBS and 5% horse serum. After 24 hours, co-cultures were exposed to $A\beta_{1-42}$.

2.3. Transfection

Primary astrocytes were transfected by lipofectamine according to manufacturer's protocol. The pcDNA3 plasmid was used empty in control conditions; or it encoded the full-length CD300f human isoform (hCD300f) complementary DNA (cDNA); or the hCD300f cDNA lacking the cytoplasmic domain (hCD300fΔcyt) [12]. The cytomegalovirus (CMV) promoter was used to control expression.

The expression of hCD300f was further confirmed using three different astrocytic cultures (N=3) and two different primary antibodies (UPD1 and UPD2 [11]. Transfection followed a similar procedure as described above, but, cells were suspended by trypsin incubation before 4% paraformaldehyde (PFA) fixation. Next cells were incubated with primary mouse anti-CD300f antibody (UPD1 or UPD2 1:30) followed by secondary anti-mouse antibody (1:200; Invitrogen, Cat. Nº A-11061). Then cells were analyzed by flow cytometry using a LSRFortessa (Beckton and Dickinson). Data were analyzed using the FCS Express Software (De Novo Software): The events were defined as the isolated population in the forward x side light scatter (FSC vs SSC) plot. The percentage of cells expressing CD300f was defined as the tail of fluorescence-intensity histogram from hCD300f-transfected cultures that exceeds the fluorescence-intensity histogram from control transfected cultures.

2.4. Amyloid-beta Peptide

Aβ oligomers were prepared as previously described [24].

These preparations were characterized by western-blot under low reducing conditions as previously published [25]. After electrophoreses proteins were transferred to a nitrocellulose membrane. The immunostaining of Aβ was carried out with primary monoclonal antibody (6E10, 1:1000, SIGNET) followed by a horseradish peroxidase-conjugated secondary antibody (1:20,000). Aβ bands were made visible by chemiluminescence ECL Kit.

2.5. Immunocytochemistry

Cells were fixed with PFA in 0.1 M PBS (pH 7.2) for 30 minutes and processed for immunocytochemistry as previously described [26]. The content of NeuN-immunostained cells defined the remaining neuronal population after Aβ exposure, hence reflecting the influence of previous transfection over neuronal degeneration. Neurons were counted as every NeuN-immunostained cell present in the central square covering 17.4% of the cultured well in 96 multiwell-plates. The cell counting was carried out by a blinded investigator for the treatments over digitalized images using the ImageJ software with the cell counter plug-in. To improve data homogeneity, neuronal survival after Aβ exposure was normalized by the neuronal content following correspondent transfection in the absence of Aβ. Data are presented as the average (±SEM) from 4 experiments (N=4) run in triplicate.

In order to detect the internalization of Aβ-coated fluorescent beads (Invitrogen, Cat. Nº F8781; wavelength: 365/415 nm), immunostaining with anti-Aβ was compared between 0.1% Triton-x100 permeabilized and non-permeabilized cultures. Briefly cell cultures were washed and incubated in blocking solution with primary antibody against Aβ (6E10, 1:1000), followed by fluorescent secondary antibody (1:1000; Invitrogen, Cat. Nº A-11061, wavelength: 578/603 nm), in the same conditions as described above. Cell culture images were taken by AxiocamMRm camera system (Carl Zeiss Inc., Thornwood, NY, USA) coupled to a fluorescent microscope (Axiovert 200M, Carl Zeiss Inc.). The location of Aβ was confirmed in images from 2 additional experiments (N=3).

2.6. Evaluation of Aβ Endocytosis

Primary astrocytes were cultivated in 6 multiwell plates. They were transfected to overexpress hCD300f or with the empty plasmid pcDNA3 (control). Forty-eight hours after transfection, cultures were incubated with Aβ-coated fluorescent beads for six hours and the intensity of beads' fluorescence in GFAP-positive cells was measured by flow cytometry as a result of Aβ endocytosis. Briefly, 0.02 µm FluoroSpheres were covered with Aβ according to manufacturer's protocol or they were simply stored in presence of bovine serum albumin (BSA). The coating occurred in a proportion of 7.6 molecules of $A\beta_{1\text{-}42}$ peptide per bead. FluoroSpheres mixture was concentrated by dialysis (Centricon) and it was finally sterilized by irradiation. At the end of exposure with fluorescent beads, cells were suspended and fixed with 4% paraformaldehyde and immunostained with anti-GFAP as described above. At last the cellular content of fluorescence was quantified in a FACS Calibur (BD). Data were computed as the sum of the fluorescence intensity from FluoroSpheres in all GFAP-positive cells. Analysis was carried out with FlowJo software and data are presented as the average from 3 experiments (N=3) run in uniplicate.

2.7. Statistical Analysis

Because data presented a normal distribution, they were analyzed by one way or factorial ANOVA followed by Duncan post-hoc test whenever appropriate. To all analysis, it was considered α=0.05 and only the analysis, whose statistical power were higher than 0.7, were considered relevant. Data are presented as mean±SEM (exceptions are indicated in the text).

All of the experimental protocols were approved by the Animal Care and Use Ethics Committee of UNIFESP and all procedures followed the eighth edition of the *Guide for the Care and Use of Laboratory Animals* (by The National Academy of Sciences)

3. RESULTS

3.1. hCD300f Protects Neurons from $A\beta_{1\text{-}42}$ Oligomer–induced Toxicity

Given the relevance of astrocytes to proinflammatory signaling and neuronal death induced by Aβ and their easiness to be transfected, they were elected as the cell model for a putative therapeutic overexpression of hCD300f. The transfection resulted in hCD300f expression in 22.25 % ± 4.45 (STD) of the astrocytes in a confluent culture (Fig. **1A**). Hippocampal neurons were seeded over hCD300f-overexpressing astrocytes 24 hours after transfection and, following additional 24 hours, the co-cultures were exposed to 5µM $A\beta_{1\text{-}42}$ oligomers for 48 hours. The oligomeric composition of $A\beta_{1\text{-}42}$ preparations was confirmed by western-blot (Fig. **1B**). The transfection of astrocytes by itself did not influence was not a statistically relevant factor influencing neuronal survival (data not shown , ANOVA, $F_{6/11}$=1.00, p=0.47). Notwithstanding, the content of transfection (*e.g.* the cDNA loaded in the pcDNA3 plasmid) determined the neurodegeneration induced by Aβ (N=4 in triplicate; ANOVA, $F_{5/10}$=3.49, p=0.04). As expected, the exposure of control-transfected co-cultures to 5µM of oligomeric $A\beta_{1\text{-}42}$ resulted in substantial neuronal toxicity, reducing neuronal survival to 63.88% ± 14.63% (Duncan post-hoc, p=0.008). Interestingly, this neurotoxicity was prevented in hCD300f-transfected co-cultures (as compared to control transfection; 96.01% ± 7.84% neuronal survival;Duncan post-hoc, p=0.04; Fig. **1C** and **D**). Nevertheless, the neuroprotective effect of hCD300f was not observed after the transfection with the mutant form of hCD300f lacking its cytoplasmic tail (hCD300fΔcyt, as compared to control transfection (81.13% ± 11.15% neuronal survival; Duncan post-hoc, p=0.62), and thus not capable of signaling through SHP1 or PI3K. Furthermore, to explore whether the protective activity of hCD300f against $A\beta_{1\text{-}42}$ was due to soluble factors released by astrocytes, conditioned medium from hCD300f transfected astrocytes was added to $A\beta_{1\text{-}42}$ treated co-cultures. This treatment also failed to significantly prevent neuronal death (as compared to control transfection; 77.89% ± 6.54% neuronal survival; Duncan post-hoc, p=0.36; Fig. **1D**).

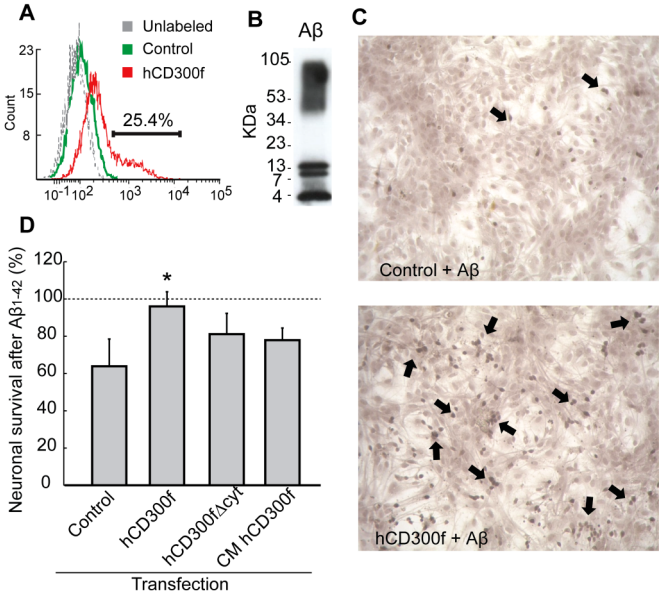

Fig. (1). The overexpression of hCD300f prevents $A\beta_{1\text{-}42}$ induced neurotoxicity. (A) A representative histogram of cell counting according to the fluorescence intensity from hCD300f immunolabeling as assessed by flow cytometry. The grey dashed line represents astrocytes which were not immunolabeled. The green and red lines represent immunolabeled astrocytes transfected with control plasmid and with hCD300f, respectively. (B) Oligomeric composition of $A\beta_{1\text{-}42}$ was checked by western blot before samples were added to co-cultures. (C) Representative images of NeuN-immunostaining from co-cultures whose astrocytes were previously transfected with pcDNA3 empty plasmid (control; upper panel) or hCD300f cDNA (lower panel). The neuronal survival after 5µM oligomeric $A\beta_{1\text{-}42}$ exposure is illustrated in D as group mean and SEM. The columns are labeled according to the cDNA used in transfection. The neuronal content that followed Aβ in each condition is indicated as a percentage of neuronal counting following the same transfection without Aβ. The horizontal dashed line represents a reference for the absence of neurodegeneration. hCD300fΔcyt represent hCD300f cDNA lacking the cytoplasmic domain. CM hCD300f column represents co-cultures whose astrocytes were not transfected, but were treated with conditioned medium from hCD300f-transfected astrocytes simultaneously with the $A\beta_{1\text{-}42}$ exposure. * indicates p<0.05 as compared to control transfection, according to Duncan *post-hoc* test.

3.2. hCD300f Maintains $A\beta_{1-42}$ Endocytosis

The toxicity of Aβ is utterly dependent of its concentration in brain parenchyma, which is determined by the balance between synthesis, cleavage and clearance of the peptide. Astrocytes, the most abundant cell population in human CNS, play an important role in endocytosis-mediated clearance of Aβ and ITIM-bearing immune receptors have been implicated in the modulation of phagocytosis [27-29]. Therefore, we explored the influence of hCD300f signaling over the phagocytosis of $A\beta_{1-42}$ oligomers by astrocytes wondering whether increased $A\beta_{1-42}$ internalization could account for the neuroprotective effect observed after hCD300f overexpression.

Astrocytes were incubated for six hours with $A\beta_{1-42}$-coated or BSA-coated fluorescent beads 48 hours after transfection with hCD300f or a control (empty pcDNA3) plasmid. Immunocytochemistry against Aβ confirmed that $A\beta_{1-42}$-coated fluorobeads were mostly internalized by astrocytes, as an intense signal was observed in the presence of the permeabilizing agent Triton X-100, whereas very faint staining was seen in its absence (Fig. **2A**). Moreover, both astrocyte cultures transfected with the control plasmid or with hCD300f internalized higher amounts of $A\beta_{1-42}$-coated beads than those incubated with BSA (ANOVA, $F_{1/14}$=8.40, p=0.01 Fig. **2B**). Notwithstanding, the internalization of $A\beta_{1-42}$ after the overexpression of hCD300f was similar that after control transfection (ANOVA, $F_{1/14}$=0.23, p=0.64).

4. DISCUSSION

The present work shows that the astrocytic overexpression of the hCD300f receptor can be a neuroprotective strategy against Aβ-induced toxicity *in vitro*. This neuroprotective effect was dependent on the cytoplasmic tail of hCD300f and on neuron-astrocyte cell-cell interactions. In addition, the overexpression of hCD300f did not affect endocytosis of $A\beta_{1-42}$ peptide by astrocytes, and thus an increased clearance of $A\beta_{1-42}$ was not the neuroprotective mechanism of hCD300f.

The present work is however limited by the absence of endogenous basal expression of CD300f by astrocytes *in vivo*. Its expression is known to take place mainly in the surface of the cells of myeloid lineages such as macrophages, neutrophils, dendritic cells, granulocytes and mast cells [30]. In addition, CD300f has been found to be expressed in cultivated CNS cells such as microglia, oligodendrocytes and neurons, but not in astrocytes under basal conditions [15]. Additional studies should evaluate the safety of astrocyte-targeted expression of CD300f as well as its neuroprotective potential *in vivo*. An interesting possibility in addition to the astrocytic expression of CD300f- could be the modulation of microglial/macrophage CD300f signaling for neuroprotection against Aβ.

The exposure of hippocampal astrocyte-neuron co-cultures to 5μM oligomeric $A\beta_{1-42}$ resulted in approximately 40% neurodegeneration. Despite their well-known neurotrophic support, astrocytes can become activated by diverse proinflammatory stimuli, including Aβ, leading to a phenotypic switch that is responsible for an active neurotoxicity [31, 32]. Accordingly, the modulation of this proinflammatory phenotype of astrocytes has been assumed to be able to decrease neuronal degeneration [7, 22]. In agreement with the beneficial modulation of the astrocytic phenotype, here the overexpression of hCD300f immunoreceptor in this cell type was able to abolish the $A\beta_{1-42}$-induced neurotoxicity.

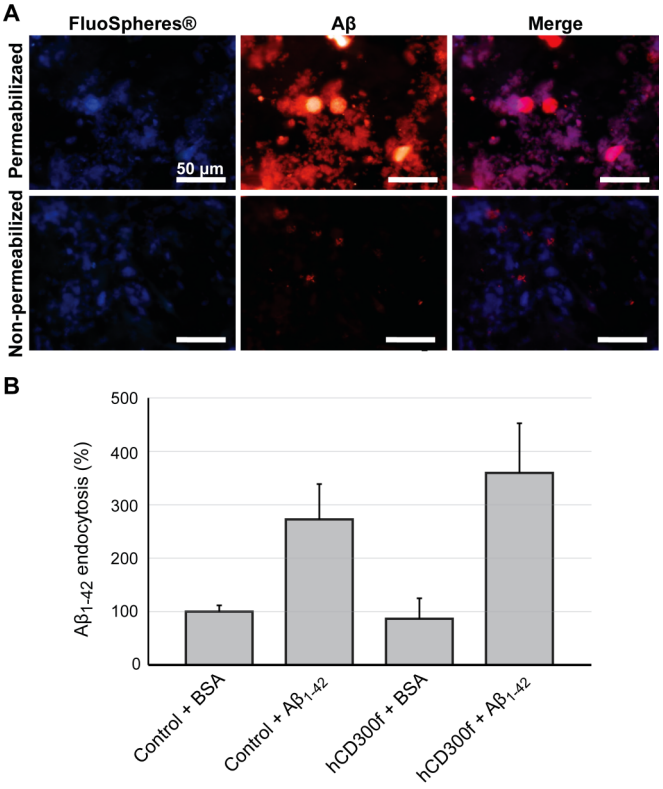

Fig. (2). Effect of hCD300f overexpression on the endocytosis of beads coated with $A\beta_{1-42}$ oligomer. Astrocyte monolayers were transfected with the empty plasmid pcDNA3 (control) or encoding hCD300f cDNA, and 48 hours after they were exposed to $A\beta_{1-42}$ or BSA- coated fluorescent beads for 6 hours. (A) To distinguish intra and extracellular location of $A\beta_{1-42}$-coated beads, the cultures were immunostained for Aβ in Triton X-100 permeabilized (higher panels) and non-permeabilized cultures (lower panels). Scale bar = 10μm. (B) FACS analysis represented as the sum of fluorescence intensity from all Aβ-coated beads internalized by GFAP-positive cells as a percentage of Control + BSA group. Vertical bars illustrate group means (±SEM) relative to Control+BSA.

The exposure of co-cultures to $A\beta_{1-42}$ in presence of conditioned medium from hCD300f-transfected astrocytes failed to prevent neuronal death. This results suggest that neuron-astrocyte cell-cell contacts were fundamental for the neuroprotection to occur and that this process was not mediated solely by trophic factors released by hCD300f-transfected astrocytes. This cannot however exclude the role of all soluble factors involved in the neuroprotection by hCD300f, but rather indicate that these factors, by their own, were not enough to sustain a statistically significant effect. Moreover, the neuroprotection induced by the transfection with the plasmid encoding the full-length form of the hCD300f gene did not persist in the absence of hCD300f's cytoplasmic tail (hCD300fΔcyt transfection). Thus, the mechanism of neuroprotection may involve at least one of its cytoplasmic recruiting motifs (*e.g.* SHP1, Growth factor receptor-bound

protein 2 - Gbr2, and/or PI3K), rather than only the activation of putative un-known ligand(s) in other cells by CD300f extracellular region. The recruitment of SHP1 seems a probable requirement for the observed neuroprotection, given its high expression in astrocytes [14] and its role in inhibitory signaling. However, it is not possible to discard that the PI3-Kinase and Grb2 domains may also participate in the neuroprotective signaling of astrocytes. This far, the described ligands for hCD300f are sphingomyelin and ceramide [33], but these ligands do not propagate signaling events once they interact with the extracellular domain of hCD300f. However, it is not possible to discard any influence from all various ligands based on present results, which is limited to indicate that neuroprotection requires hCD300f's cytoplasmatic domain. Altogether, these results indicate that the therapeutic strategies aimed to take benefit of CD300f neuroprotection should target the activation of this receptor rather than to modulate its ligand(s).

The increase of Aβ clearance from brain parenchyma rises as a promising strategy to reduce amyloidosis in CNS [34, 35]. An important route for Aβ elimination is mediated by the phagocytosis of the peptide, which is carried out by microglia [36] and markedly by astrocytes [37]. The relevance of astrocyte-mediated clearance becomes more important in light that astrocytes constitute the most frequent cell type in the human CNS [38]. Glial activation by pro-inflammatory reactions represents a physiological mechanism to enhance phagocytic capacity of the cell [35], as it is induced by Triggering receptor expressed on myeloid cells 2 (TREM2) [29]. However, the overexpression of hCD300f in astrocytes leads to a similar endocytosis of $A\beta_{1\text{-}42}$-coated beads as compared to cells transfected with control plasmid. This fact excludes the possibility that the neuroprotective effect of hCD300f would be rather a consequence of reduced Aβ content in culture medium promoted by increased phagocytosis. Nevertheless, the statement that CD300f does not affect overall Aβ clearance still needs further investigation that addresses other mechanisms, such as the secretion of Aβ proteases neprilysin and insulin degrading enzyme [39, 40].

The interaction between neurons and glia is a well-known phenomenon. For example, the immunoreceptor CD200R expressed on microglial cells has been shown to interact with its ligand CD200, expressed on neurons, prompting an anti-inflammatory signaling [41]. In addition, TREM2 was shown to interact with an unknown ligand on the surface of apoptotic neurons [42]. Another type of immunoreceptors, the Sialic acid-binding Ig superfamily lectin-11 (Siglec-11), which are expressed in microglial cells, binds to the polysialylated neuronal cell adhesion molecule (PSA-NCAM), originating a protective response to neurons in co-cultures [43]. These examples endorse the therapeutic modulation of the interaction between neuron and glial cells may be oriented towards recovering the homeostatic microenvironment in the brain.

CONCLUSION

The evidences presented here place CD300f as a piece of this newly unraveled immunoreceptor-ligand interaction network of neuron and glial cells, whose properties could be directed to favor the therapy of neurodegenerative diseases. In particular, the astrocyte-directed overexpression of hCD300f may constitute a gene therapy strategy for AD, thus demanding further *in vivo* studies to explore this interesting possibility.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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Declared none.

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