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Basic research

# The synergic action of amyloid-β peptide and LPS in amyloid plaque formation

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## Abstract

**Introduction:** The current model which assume amyloid-β (Aβ) deposition to be an accidental process resulting from the abnormal behavior of an incidental product of catabolism, is now in contrast with the fact that Aβ, a key player in AD, may have a normal function because belongs to the group of AMPs also called host defense peptide. AMPs such as Aβ, are small molecule peptides which are not only intended to kill pathogens through their antimicrobial activity but they also have a high affinity for bacterial lipopolysaccharide (LPS) or membrane receptors. Once that Aβ is deposited can interact with the LPS which acts as a fibrillogenesis promoter; the positive charge of antibacterial peptides can increase the ability of binding to LPS. The interaction between LPS and Aβ occurs at molecular level, on the basis of their common surfactant properties and considering that detergents and fatty acids are able to form micelles.
**Material and methods:** SH-SY5Y (neuroblastoma cells) were treated with Aβ1–42 in the form of monomers at the starting concentration of 10 µg/ml, with LPS dissolved in order to obtain a starting concentration of1 µg/ml and their combination. Each experiment was performed for 24, 48, and 72 h at 37°C. Western blot analysis for Aβ1-42 monomers, Beclin-1 and Lamp-1; Cytokines (IL-1β) quantification with Elisa; Transmission electron microscopy (TEM) and Statistical analysis were performed.
**Results:** LPS leads to increased production and impaired degradation of Aβ mediated by upregulation of proinflammatory cytokines and inhibition of Aβ degrading enzyme, respectively intracellular accumulation at the endoplasmic reticulum and in lysosomes; the overexpression of IL-1 occurs at the beginning of the inflammatory process. Over-production of Aβ peptides directed against pathogenic neuroinvasion can cause accumulation of Aβ in plaques; Aβ deposits seem to trigger autophagy.
**Conclusions:** In this study authors observed the binding of the Aβ spherical oligomers to LPS micellar particles inside neuroblastoma cells, the incorporation of LPS to micellar particles occurs at an early stage of Aβ aggregation acting as a nucleation factor, these results are in agreement with the possibility that microorganisms could play a role in the formation of senile plaques in AD.

**Key words:** assumes amyloid-β, lipopolysaccharide, SH-SY5Y, Alzheimer's disease, antimicrobial peptides, TEM.

## Introduction

Literature data have indicated that the pathogenesis of inflammatory diseases is closely related to the activation of innate immune mechanisms [1, 2]. Within this context, Alzheimer's disease (AD) patients show pro-inflammatory profiles with enhanced tumor necrosis factor α (TNF-α) levels and a reduced immunosuppressive response indicating a lack of ability to control inflammatory responses which may contribute to neurodegeneration and AD pathology [3, 4].

In these last years, the important role of innate immunity in amyloid-β (Aβ) deposits' clearance has been demonstrated; a continuous aggregation of Aβ does not allow the resolution of inflammation, but increases the chronic reaction of the innate immune system [5]. This is in contrast with the immune response to microorganisms that ends once the pathogen has been removed [6]. The lack of efficiency in microglial Aβ degradation was later attributed to the low hydrolytic activity of endosomal and lysosomal enzymes in AD patients [6, 7].

Among the several factors that may trigger the cascade of events leading to chronic inflammation [8] and amyloid deposition in AD [9, 10] there are bacteria and bacterial debris [11, 12].

The hypothesis that Aβ is the result of abnormal behavior of an incidental product of catabolism [13] is now in contrast with the fact that amyloid may have a physiologic function as an antimicrobial peptide (AMP) [14]. Various models are proposed to explain the microbicidal activities of AMPs also called host defense peptides [14, 15] including the interaction with the negatively charged membrane composition of microbes resulting in pore formation. These biologically active molecules, potent broad spectrum bactericides [16] and well conserved across evolution [16], are found within the granules of neutrophils, epithelial layers and body fluids [17]. AMPs including LL-37 (human cathelicidin antimicrobial protein, hCAP18) activate chemokine release [2] to regulate inflammation and protect the organism against a wide range of infections [18]. Aβ has a high affinity for the lipopolysaccharide (LPS) present in the outer membrane of Gram negative bacteria; the positive charge of antibacterial peptides can increase the ability of binding to LPS which acts as a fibrillogenesis promoter [12].

A persistent or acute infection caused by pathogens in the central nervous system (CNS) may cause chronic impairment of clearance of Aβ [19], which accumulates with time, especially if the infection is followed by a period of latency and subsequent reactivations [9, 20, 21]. There are two main routes by which misfolded or toxic proteins may be cleared: the ubiquitin-proteasome system (UPS) localized in the cytoplasm, nucleus, subcellular regions [22] and autophagy-lysosomal pathways [23]; notably cellular aging is associated with a reduced autophagy process [24].

Intracellular accumulation of Aβ, neurofibrillary tangles and extracellular amyloid plaques are the characteristics of AD [25]. The extracellular aggregates consist of insoluble deposits of Aβ, a protein fragment generated from a larger protein called amyloid-β protein precursor (AβPP) following the cleavage operated by secretases; AβPP, β and γ-secretase are the three major players in the pathology of AD [25, 26]. The favorable conditions for Aβ accumulation in AD [23] are firstly the combination of increased autophagy induction and secondly the defective clearance of Aβ generating autophagy vacuoles (AVs). In contrast, in the healthy brain, efficient clearance of AVs and lysosomal degradation occurs, thus preventing accumulation or increase of Aβ. Aβ protein is able to associate with the lipid bilayers of the bacterial cell membranes [12] and to exert antimicrobial activity [14] by membrane permeabilization and by the alteration of calcium homeostasis [27]. Like Aβ, bacterial LPS too displays surfactant properties having characteristics such as a hydrophobic alkylic chain, and an anionic head group, which may aggregate into different physical structures such as micelles or bilayers [28]. Moreover, in combination with Aβ, LPS acts as a fibrillogenesis promoter in a time-dependent manner [12].

Our previous data obtained in a previous cell-free study suggest that LPS may act through two possible mechanisms: 1) increasing the seeds necessary for the nucleation step and 2) stimulating fibril elongation without concomitant incorporation in growing filaments [12, 28]. The purpose of the present study was to verify whether the interaction between Aβ and LPS can also occur inside a neuronal cell. Indeed, the interaction between the Aβ fragment and the *Escherichia coli* endotoxin at different concentrations may influence Aβ fibrillization [12]. In this study we analyzed the effect of the co-treatment of $A\beta_{1-42}$ and LPS in human SH-SY5Y cells. We also examined beclin-1 since it is involved in the initial step of the autophagy process, taking part in double-membrane vesicle formation, and represents an early marker of its activation; in fact it is part of the PI3-kinase complex that is needed for autophagosome biogenesis [29]. Moreover, we analyzed Lamp-1 protein expression, which identifies mature lysosomal vesicles.

The effect of LPS, $A\beta_{1-42}$ and their combination on IL-1β induced release in human SH-SY5Y cells was also investigated, since inflammatory and autophagy processes interact within brain cells, as for example severe inflammation induced by IL-1β [24] activates autophagy in microglia [30].

## Material and methods

The LPS stock suspension from *E. coli* serotype 0128:BI2 (SIGMA-Aldrich Chemie, Germany), was dissolved in distilled water, in order to obtain a starting concentration 1 µg/ml for LPS.

The $A\beta_{1-42}$ fragment (SIGMA-Aldrich Chemie, Germany) was dissolved in distilled water (6 mg/ml) and then diluted in PBS, as suggested by different manufacturers, in order to obtain a starting concentration of 10 µg/ml.

### Cell culture

Human SH-SY5Y cells provided by the American Type Culture Collection (ATCC, Manassas, VA) were grown in Eagle's minimum essential medium, supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin, L-glutamine (2 mM), with the addition of non-essential amino acids (1 mM), and sodium pyruvate (1 mM) (all from Euroclone, Milan, Italy) at 37°C, in an atmosphere of 5% CO2 and 95% humidity.

SH-SY5Y cells were plated ($1.0 \times 10^6$) on 100 mm petri dishes; each experiment was performed for 24, 48, and 72 h at 37°C, 5% $CO_2$; $A\beta_{1-42}$ was in the form of monomers at the starting concentration of 10 µg/ml.

Cellular conditions: SH-SY5Y untreated (control), SH-SY5Y/ LPS (1 µg/ml), SH-SY5Y/Aβ1-42 (monomers) (10 µg/ml), SH-SY5Y/LPS (1 µg/ml)/ Aβ1-42 (monomers) (10 µg/ml).

After each treatment, cells were washed three times with PBS.

### Western blot analysis

Cells were homogenized with Cell Lysis Buffer 1X (Cell Signaling, Danvers, MA) to lyse the cells under non-denaturing conditions. Total protein content was measured via Bradford's method (Bradford, 1976). Proteins were separated by 12% SDS–polyacrylamide gel electrophoresis and processed as previously described [29]. Anti-BECN1 rabbit monoclonal antibody (dil. 1 : 1,000) and anti-Lamp-1 mouse monoclonal antibody (dil. 1 : 1,000) were purchased from Cell Signaling, and mouse monoclonal anti-α-tubulin (dil. 1 : 1,000) was purchased from Sigma-Aldrich (Milan, Italy). The native gel (Figure 1 A) was performed in the same conditions, but without SDS. $A\beta_{1-42}$ was separated by 12% native polyacrylamide gel electrophoresis and anti-$A\beta_{1-42}$ mouse antibody (dil. 1 : 500) was purchased from Sigma-Aldrich (Milan, Italy).

All antibodies were diluted in TBS buffer (10 mM Tris–HCl, 100 mM NaCl, 0.1% (v/v) Tween 20, pH 7.5), containing 6% (v/v) skimmed milk. Signals on nitrocellulose membranes were detected by chemiluminescence (ECL Advansta, Aurogene). Experiments were performed in duplicate for each different cell preparation, using α-tubulin as the loading control and for data normalization. Images were digitally acquired and processed using the Image J software.

### Cytokines (IL-1β)

IL-1β protein released in the medium from the different cell culture conditions was estimated by enzyme-linked immunosorbent assay (ELISA) using a commercially available kit (Thermo Fisher Scientific), following the manufacturer's instructions.

### Transmission electron microscopy (TEM)

TEM was performed by standard technique: samples were fixed in glutaraldehyde 2.5% and cacodylate sodium buffer pH 7.4 for 90′ at room temperature. Cells were then rinsed in cacodylate sodium buffer (pH 7.4) overnight and post-fixed in 1% aqueous osmium ($OsO_4$) for 90′ at room temperature. Dehydration was performed at increasing ethanol concentrations (50% to 100%) then samples were embedded in epoxy resin, Epon 812.

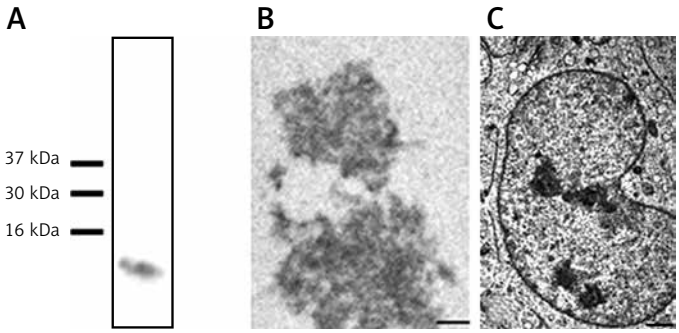


**Figure 1.** TEM. **A** – Representative native gel showing $A\beta_{1-42}$ in form of monomers; the anti-$A\beta_{1-42}$ antibody only detects a single band corresponding to $A\beta_{1-42}$ monomers. **B** – TEM of the aggregation state of the peptide after 48 h incubation, not yet in contact with human SH-SY5Y cells. **C** – human SH-SY5Y cells in control condition. Bar = 500 nm

Thin sections were counterstained; observations and micrographs were performed on a TECNAI G2 Spirit FEI TM operating at 80 kV.

### Statistical analysis

For statistical analysis, the GraphPad InStat statistical package (version 3.05 GraphPad software, San Diego, CA) was used. The data were analyzed by analysis of variance (ANOVA), followed, when significant, by an appropriate *post hoc* comparison test as indicated in the legends. Differences were considered statistically significant when *p*-values $\leq 0.05$.

## Results

### Transmission electron microscopy (TEM)

The aggregation state of the Aβ peptide, in control conditions, is shown in Fig. 1B; human SH-SY5Y without co-treatment in Figure 1 C.

$A\beta_{1\text{-}42}$ scattered short fibrils are formed, in contrast, after 48 h incubation with human SH-SY5Y; fibrils appear to be attached or near the neuro-

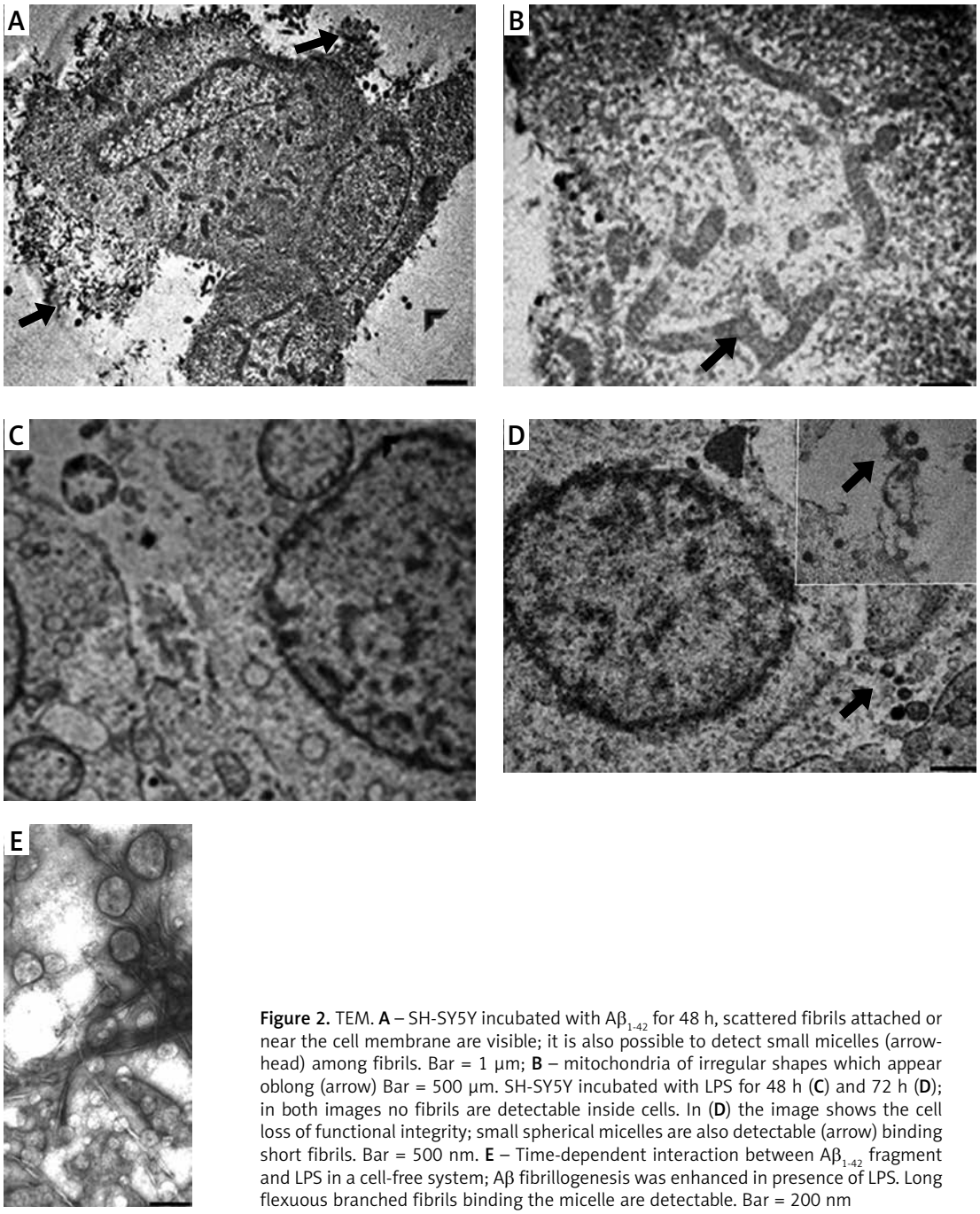


**Figure 2.** TEM. **A** – SH-SY5Y incubated with $A\beta_{1\text{-}42}$ for 48 h, scattered fibrils attached or near the cell membrane are visible; it is also possible to detect small micelles (arrowhead) among fibrils. Bar = 1 µm; **B** – mitochondria of irregular shapes which appear oblong (arrow) Bar = 500 µm. SH-SY5Y incubated with LPS for 48 h (**C**) and 72 h (**D**); in both images no fibrils are detectable inside cells. In (**D**) the image shows the cell loss of functional integrity; small spherical micelles are also detectable (arrow) binding short fibrils. Bar = 500 nm. **E** – Time-dependent interaction between $A\beta_{1\text{-}42}$ fragment and LPS in a cell-free system; Aβ fibrillogenesis was enhanced in presence of LPS. Long flexuous branched fibrils binding the micelle are detectable. Bar = 200 nm

blastoma cellular surface; little micelles are also detectable among fibrils (arrowhead). Spherical oligomers were present early during incubation, but as mature fibrils appeared spherical oligomers disappeared. At the time considered few fibrils were clearly detectable inside the cells (Figure 2 A).

Following incubation of SH-SY5Y with $A\beta_{1-42}$ for 72 h, the most evident cell ultrastructural alterations are mitochondria of irregular shapes that appear oblong with thin fragmented cristae (Figure 2 B). Bacterial LPS also displays surfactant properties and it may aggregate into different physical structures such as micelles or bilayers. Figure 2 C shows human SH-SY5Y incubated with LPS for 48 h. In Figure 2 D, after 72 h of incubation, the loss of functional integrity of cells is shown, little spherical micelles are detectable (arrow). In Fig-

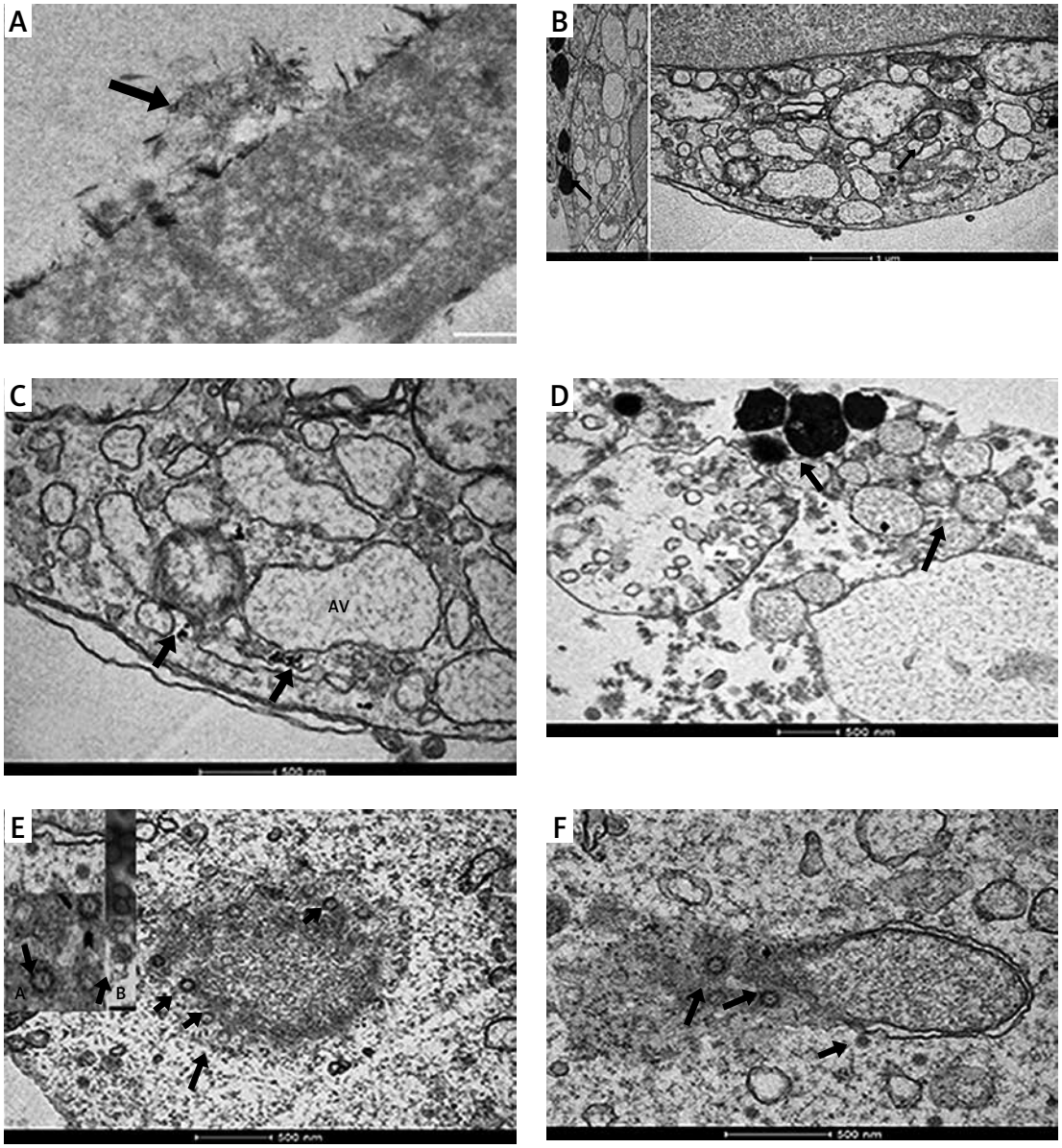


**Figure 3.** TEM. **A** – human SH-SY5Y incubated with $A\beta_{1-42}$ and LPS for 48 h at 37°C; short smooth twisted fibrils with small micelle outside cell membrane (arrow). **B**, **C** – show SH-SY5Y stimulated for 72 h with $A\beta_{1-42}$ and LPS. Osmiophilic lysosomal storage material known as lipofuscin (arrow). Some material may remain in the enlarged lysosome vacuole. Bar = 1 µm, (**C**) magnification of (**B**), Bar = 500 nm. **D** – depicting lipofuscin material in the cell cytoplasm. A cluster of defective lysosomes and autophagy vacuoles (AVs) (arrows) containing particles isolated from the cell's own cytoplasm. Bar = 500 nm. **E** – Aβ deposits appear as a network of randomly orientated fibrous material (arrows) localized in cell cytoplasm without limiting membrane. Micelles at different stages of formation are detectable (arrow). Bar = 500 nm. **F** – Fibrous material localized in an autolysosome; micelles at different stages are present. Bar = 500 nm. Inset E). Ultrastructure of micellar particles inside SH-SY5Y after 72 h co-treatment of $A\beta_{1-42}$ and LPS a) as a comparison, on the right, b) we report micelles formed following $A\beta_{1-42}$ and LPS co-treatment in the cell-free system (Asti et al., 2014). A) Bar = 200 nm

ure 2 E, micelles are formed in a cell-free system by an *in vitro* co-treatment with only $A\beta_{1-42}$ and LPS.

Indeed following incubation of SH-SY5Y with $A\beta_{1-42}$ and LPS for 48 h, short smooth twisted fibrils with little micelles are present outside cell membrane (Figure 3 A). Aβ and LPS also show affinity for lipid bilayers, and cell membrane stains positive for Aβ after Congo Red dye (not shown).

The ultrastructure of SH-SY5Y cells after co-treatment with LPS and $A\beta_{1-42}$ for 72 h reveals an insoluble brownish lysosomal stored material as curvilinear profiles, or granular osmiophilic scattered deposits in the cytoplasm (arrow) known as lipofuscin-like material (Figures 3 B–D). This material was not observed after incubation with LPS or with $A\beta_{1-42}$ after 72 h incubation. Notably, in Alzheimer's disease patients pathologic accumulation of lipofuscin-like material may often be found; the increase of lipofuscin is caused by a decline in intralysosomal degradation and age-related enhancement of autophagocytosis. A cluster of lysosomes and AVs containing particles isolated from the cell's own cytoplasm are also detectable. Figure 3 D depicts an additional image of SH-SY5Y after 72 h incubation with $A\beta_{1-42}$ and LPS showing a swollen cell abundantly filled with vacuolar structures, including lysosome-dense bodies which reflect upregulated synthesis of components of the lysosomal system; in autophagic vacuoles Aβ aggregates were sequestrated together with cellular organelles.

In Figures 3 B and C, at greater magnification, there is shown a phagophore formed by a nucleation site and the expansion of an isolation membrane, the edge fused to form a double-membraned vesicle, the autophagosome (arrow), that sequesters the cytoplasmic material and organelles. Defective mitochondria are also found (Figures 3 B, C); in this case the molecular damage is enhanced by the increased reactive oxygen species (ROS) production. Following 72 h co-treatment of human SH-SYH5 cells with $A\beta_{1-42}$ and LPS, Aβ deposits appear as a network of randomly orientated fibrous material localized in cell cytoplasm without limiting membrane (Figure 3 E); micelles (arrow) are observed in the cytoplasm. This is consistent with the reported Aβ and LPS common surfactant properties that are able to form micelles at active concentrations (Figures 3 E, F, 2 E). The incorporation of LPS occurs at an early stage of Aβ aggregation acting as a nucleation factor or seed (inset B Figure 3 E); LPS increases the seed necessary for the fibril elongation step (Figure 2 E).

In Figure 3 F fibrous material is localized in an autolysosome; micelles at different stages of formation are present near the membrane (arrow).

Inset A in Figure 3 E shows micelles formed by co-treatment with $A\beta_{1-42}$ and LPS after 72 h; in inset B micelles are formed in a cell-free system by *in vitro* co-treatment with only $A\beta_{1-42}$ and LPS [12]. LPS is able to accelerate the Aβ peptide assembly; Figure 2 E shows long smooth branched helical fibrils; considerable potentiation of Aβ fibrillogenesis has occurred; fibril binding to micellar particles is more marked.

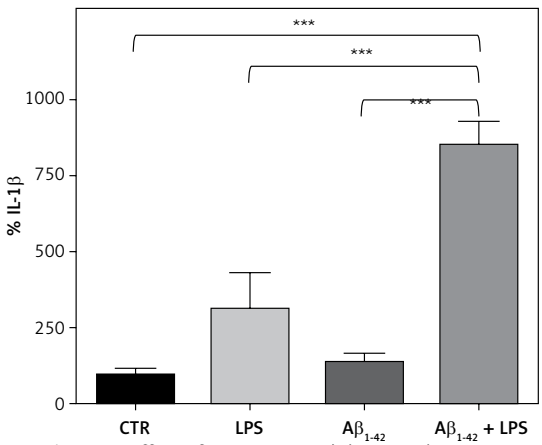


**Figure 4.** Effect of LPS, $A\beta_{1-42}$ and their combination on IL-1 β release in human SH-SY5Y cells. IL-1β was detected in the medium from human SH-SY5Y cells stimulated for 72 h with amyloid-$\beta_{1-42}$, LPS or the two combined stimuli. Data are expressed as mean percentages ± SEM with respect to control value (100%)

****p < 0.001 (Tukey's multiple comparisons test, n = 3–5).*

## Cytokine quantification

IL-1β cytokine quantification (Figure 4) shows a significant increase in the medium from human SH-SY5Y cells following $A\beta_{1-42}$ and LPS co-treatment for 72 h not only with respect to the control, but also in comparison with the two stimuli administered alone.

## Western blot analysis for $A\beta_{1-42}$ monomers, beclin-1 and Lamp-1

In control conditions, after 48 h incubation of a representative native gel, the anti-$A\beta_{1-42}$ antibody only detects a single band corresponding to $A\beta_{1-42}$ monomers (Figure 1 A).

LPS (1 µg/ml) alone does not affect beclin-1 protein levels, while $A\beta_{1-42}$ treatment (10 µg/ml) induces a significant increase in its expression (+107%). The addition of LPS to $A\beta_{1-42}$ does not further significantly increase beclin-1 protein expression, indicating that the addition of LPS does not induce further enhancement of autophagy activity (Figure 5).

Mature lysosomal vesicles are easily identified by staining for proteins such as lysosome-associated membrane protein 1 (Lamp-1). To this aim,

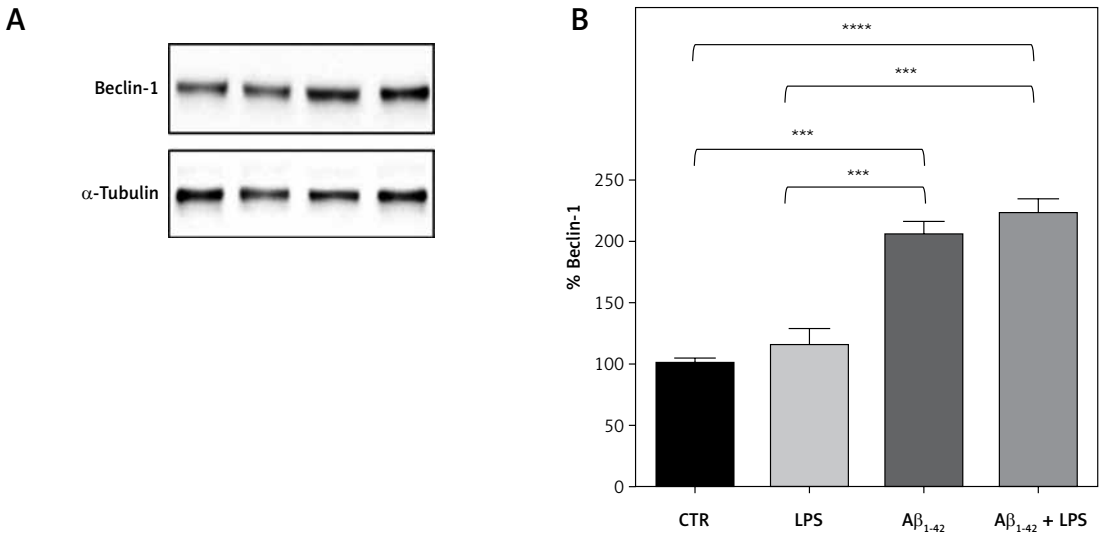


**Figure 5.** Effect of $A\beta_{1-42}$, LPS and their combination on beclin-1 protein levels. Representative western blots of beclin-1 and the respective α-tubulin (upper panel) in human SH-SY5Y cells exposed for 72 h to amyloid-$\beta_{1-42}$, LPS or the two combined stimuli. Densitometric analysis (lower panel), where α-tubulin was used to normalize the data. The values are expressed as mean percentages ± SEM with respect to control value (100%)

*****p < 0.0001; ***p < 0.001 (Tukey's multiple comparisons test, n = 4).*

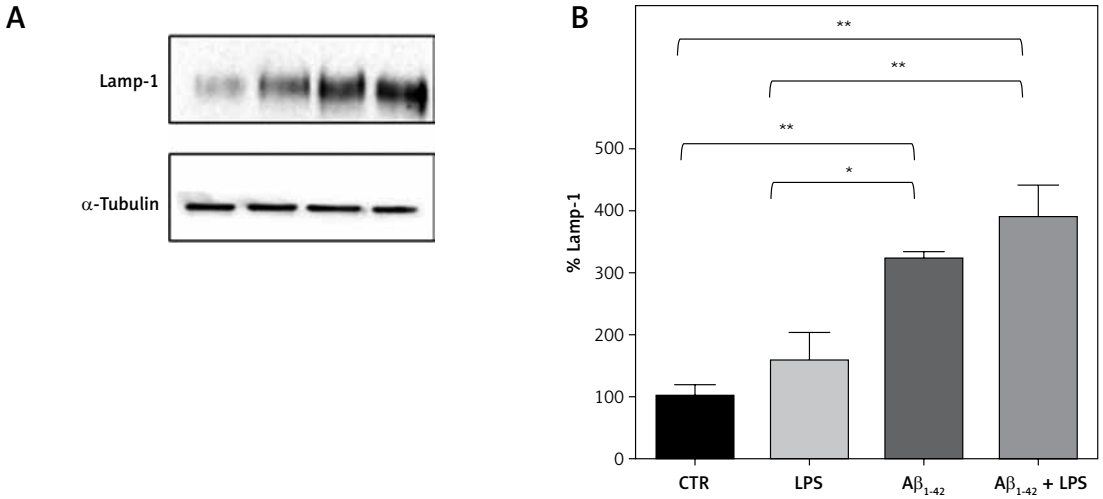


**Figure 6.** Effect of $A\beta_{1-42,}$ LPS and their combination on Lamp-1 protein levels. Representative western blots of Lamp-1 and the respective α-tubulin (upper panel) in human SH-SY5Y cells exposed for 72 h to amyloid-$\beta_{1-42}$, LPS or the two combined stimuli. Densitometric analysis (lower panel), where α-tubulin was used to normalize the data. The values are expressed as mean percentages ± SEM with respect to control value (100%)

****p < 0.001, **p < 0.01, *p < 0.05 (Tukey's multiple comparisons test, n = 3).*

we assessed the effect of $A\beta_{1-42}$ and LPS treatments on Lamp-1 protein expression. The results indicate that $A\beta_{1-42}$ significantly increases Lamp-1 content, but its levels are not further increased by LPS addition (Figure 6), thus suggesting an engulfment of lysosomal maturation.

## Discussion

Epidemiologic studies have shown an association between bacterial infections and AD. Indeed bacterial infections lead to increased production of Aβ, creating a persistent inflammatory state [27] that is proposed to be involved in the causal pathway of AD. In this process we observe inhibition of Aβ degrading enzyme [22, 31] and intracellular accumulation at the endoplasmic reticulum and in lysosomes (Figure 3 B). Over-production of Aβ peptides directed against pathogenic neuroinvasion [15, 32] can cause accumulation of Aβ in plaques; Aβ deposits seem to trigger autophagy. If infection is lifelong or repeated in different hosts, according to their general health, pharmacological treatments, genetic background ($APO_{E1}$) or concomitant disease, it enhances the expression of inflammatory mediators [9]; Toll-like receptors

recognize pathogen-associated microbial patterns (PAMPs). Exaggerated antigenic stimulation [13] may represent an important role in the development of amyloid fibrils [20] because it disturbs the immune system at the microcirculation level.

Since the fibrillogenesis process requires a nucleation step and micelles are regions of high peptide concentration, they can act as sites for the nucleation of Aβ fibrils that can successively grow by irreversible binding of Aβ monomers to fibril ends. The incorporation of LPS into Aβ micelles suppressed their ability to generate nuclei of fibrils to the point that heterogeneous nucleation dominated the nucleation process (Figure 2 E). This is the condition where the nucleation mainly occurs on non-Aβ seeds; the resulting fibrils are indistinguishable from those nucleated through micelles (Figure 2 E).

Possible mechanisms of action: cationic AMPs such as Aβ undergo strong electrostatic interactions with the negatively charged LPS, this enables them to get closer and neutralize the negative charge [33]; secondly, the hydrophobicity of Aβ makes it easy to embed LPS micelles, increasing the seed necessary for the fibril elongation step and stimulates fibril elongation without concomitant incorporation in the growing filament [12, 28]. AMPs are usually small molecule peptides, which are not only intended to kill pathogens through their antimicrobial activity but also have a high affinity for LPS or membrane receptors; the positive charge of antibacterial peptides can increase the ability of binding to LPS [33].

One of the first models proposed by Lomakin [34] for Aβ protein is a kinetic model where the aggregation of free monomers into stable ordered nuclei is a fast process which starts with the formation of micelles (Figures 2 A, E), consisting of oligomers in a disordered state [34] followed by rearrangement of the monomers inside micelles to form stable ordered nuclei. Spherical amyloid intermediates [35] (Figures 2 A, D, E; 3 E, F) have been identified in many other types of amyloids, suggesting that they represent a common state of assembly and aggregation for many different amyloids [11].

The results we obtained in a previous study [12] suggest that LPS constitutes an important cofactor in Aβ fibrillogenesis and that the incorporation of LPS occurs at an early stage of Aβ aggregation, acting at the beginning as a nucleation factor or seed, and then in the elongation of the amyloid fibrils. The interaction between LPS and Aβ (Figures 3 E, F, 2 E) acts at the molecular level, on the basis of their common surfactant properties and considering that detergents and fatty acids are able to form micelles at active concentrations.

The intracellular pool of Aβ increases in endosomal-lysosomal systems; when released extracellularly, Aβ in soluble or aggregate form may interact with surface receptors, affect the membrane lipid bilayer or, after internalization, operate in endosomal-lysosomal compartments; there is a quantitative relationship between Aβ and autophagic vacuoles [36].

Ultrastructural features of autophagy (Figures 3 B–D) have often been found in human diseases, including infections, neurodegenerative diseases such as AD [7], transmissible spongiform encephalopathies, Parkinson's disease, and Huntington disease. As a consequence the hypothesis would be that autophagy is involved in the pathogenesis of these disorders [37].

The co-treatment with Aβ and LPS is also able to modulate the expression of beclin-1 [29] (Figure 5), a fundamental protein involved in the early stages of autophagy [29]. The addition of LPS and $A\beta_{1\text{-}42}$ to SH-SYH5 does not further significantly increase beclin-1 protein expression, indicating that the addition of LPS does not induce further enhancement of autophagy activity (Figure 5); hippocampal beclin 1 expression decreases after chronic LPS-induced inflammation.

Moreover, a reduced content of beclin-1 protein has been documented in the postmortem mid-frontal cortex and isolated microglia of AD patients [23].

Another neuropathological feature of AD is the dysfunction of lysosomal pathways [38] Accordingly, lysosomal vesicles accumulating around amyloid plaques have been documented in mouse AD models as well as in AD patients [39]. In agreement with this concept, in SH-SY5Y human neuronal cells, we detected an increase in Lamp-1 protein expression following $A\beta_{1\text{-}42}$ challenge (Figure 6). However, as observed for beclin-1, the addition of LPS to $A\beta_{1\text{-}42}$ does not further significantly increase Lamp-1 protein expression, possibly as an indicator of a defective lysosomal maturation, thus likely contributing to Aβ accumulation.

During aging and in a disease state in general, if proteins are partially degraded together with lipid they may accumulate to form lipofuscin [40] (Figures 3 B–D); this is due to the defect of the autophagy system. Lipofuscin is characterized by natural brown-yellow color, high electron density (Figure 3 C), and wide-spectrum autofluorescence [40].

Aβ and LPS are also implicated in the activation of cytokine release (Figure 4) [9]. After Aβ deposition, proinflammatory stimuli have been hypothesized to create a self-reinforcing positive feedback loop that increases amyloidogenic processing of AβPP, promoting further Aβ accumulation and neuroinflammation in AD [4]. The pattern of cytokine production in AD subjects is not simply an enhancement of the situation observed in elderly people.

The overexpression of interleukin (IL-1) occurs at the beginning of the inflammatory process (Figure 4) IL-1β is one of the earliest cytokines released after LPS exposure and persists, causing neuronal cell death [4]; IL-1β primarily appear in inactive forms that need to be activated via cleavage through caspase-1 or -8, ultimately contributing to AD pathological mechanisms in activated forms [5]; the caspase-1 activation complex [23, 30] and the activation of NLRP3 inflammasome are both inhibited by autophagy processes.

The role of IL-1β itself in AD is unclear; elevated IL-1β values were also found after brain injury in the CF and brain parenchyma [5].

Pharmacological treatment that can improve Aβ clearance, inhibit Aβ aggregate formation, reverse age-dependent decline of AD patients and the lysosomal clearance of autophagosome, would be of interest for the treatment of neurodegenerative diseases [26] and the prevention of aging [27]. It has also been shown that it is possible to increase the hydrolytic activity of endosomes and lysosomes by delivering additional enzymes through the mannose-6-phosphate receptor's enzyme uptake pathway [7]; but despite the accumulated knowledge, AD still remains incurable [24].

In this experiment, we confirm that LPS constitutes an important cofactor in Aβ fibrillogenesis; the incorporation of LPS occurs at an early stage of Aβ aggregation, acting as a nucleation factor; the interaction of Aβ with LPS to form heterogeneous micellar particles (Figures 2 E; 3 E and inset, F) indicates an affinity at the molecular level. Hence, these results are in agreement with the possibility that microorganisms could play a role in the formation of senile plaques in AD.

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## Conflict of iterest

The authors declare no conflict of interest.

## References

1. McGeer PL, McGeer EG. The inflammatory response system of brain: implication for therapy of Alzheimer and other neurodegenerative diseases. Brain Res Brain Res Rev 1995; 21: 195-218.
2. Zaiou M. Multifunctional antimicrobial peptides: therapeutic targets in several human diseases. J Mol Med 2007; 85: 317-29.
3. Wang WY, Tan MS, Yu JT, Tan L. Role of pro-inflammatory cytokines released from microglia in Alzheimer's disease. Ann Transl Med 2015; 3: 136.
4. Rubio-Perez JM, Morillas-Ruiz JM. A review: inflammatory process in Alzheimer's disease, role of cytokines. Scientific World J 2012; 2012; 756357.
5. Heneka MT, Golenbock DT, Latz E. Innate immunity in Alzheimer's disease. Nature Immunol 2015; 16: 229-36.
6. Wisniewski HM, Wegiel J, Kotula L. Some neuropathological aspects of Alzheimer's disease and its relevance to other disciplines. Neuropathol Appl Neurobiol 1996; 22: 3-11.
7. Majumdar A, Chung H, Dolios G, et al. Degradation of fibrillar forms of Alzheimer's amyloid beta-peptide by macrophages. Neurobiol Aging 2008; 29: 707-15.
8. Ling YS, Mao HP, Zhong AC, Guo YC. The effects of Escherichia coli and its endotoxin on amyloidogenesis in ducks. Vet Pathol 1991; 28: 519-23.
9. Miklossy J, Martins RN. Chronic inflammation and amyloidogenesis in Alzheimer's disease: the emerging role of infection. J Alzheimers Dis 2008; 13: 357.
10. Mawanda F, Wallace R. Can infections cause Alzheimer's disease? Epidemiol Rev 2013; 35: 161-80.
11. Dobson CM. The structural basis of protein folding and its links with human disease. Philos Trans R Soc Lond B Biol Sci 2001; 356: 133-45.
12. Asti A, Gioglio L. Can a bacterial endotoxin be a key factor I the kinetic of amyloid fibril formation? J Alzheimers Dis 2014; 39: 169-79.
13. Ling D, Song OJ, Garza D, Neufeld TP, Salvaterra PM. Abeta42-induced neurodegeneration via an age-dependent autophagic-lysosomal injury in Drosophila. PLos One 2009; 4: e4201.
14. Soscia SJ, Kirby JE, Washicosky KJ, et al. The Alzheimer's disease-associated amyloid beta-protein is an antimicrobial peptide. PLos One 2010; 5: e9505.
15. Gosztyla ML, Brothers HM, Robinson SR. Alzheimer's amyloid-beta is an antimicrobial peptide: a review of the evidence. J Alzheimers Dis 2018; 62: 1495-506.
16. Dorin JR, Davidson DJ. Mammalian antimicrobial peptides: defensins and cathelicidins. Molecular Medical Microbiology Elsevier 2014; 539-65.
17. Boman HG. Peptide antibiotics and their role in innate immunity. Annu Rev Immunol 1995; 13: 61-92.
18. Durr HUS, Sudheendra US, Ramamoorthy ALL-37 the only human member of the cathelicidin family of antimicrobial peptide. Biochim Biophys Acta 2006; 1758: 1408-25.
19. Balin BJ, Gerard HC, Arking EJ, et al. Identification and localization of Chlamydia pneumoniae in the Alzheimer's brain. Med Microbiol Immunol 1998; 187: 23-42.
20. De Chiara G, Marcocci ME, Sgarbanti R, et al. Infectious agents and neurodegeneration. Mol Neurobiol 2012; 46: 614-38.
21. Asti A. Bacterial lipopolysaccharide (LPS) and Alzheimer's disease. In: Infection and Alzheimer's Disease. Miklossy J (ed.) IOS Press 2017.
22. Goldberg AL. Protein degradation and protection against misfolded or damaged proteins. Nature 2003; 426: 895-9.
23. Uddin MS, Stachowiak A, Al Mamun A, et al. Autophagy and Alzheimer's disease: from molecular mechanisms to therapeutic implications. Front Aging Neurosci 2018; 10: 04.
24. Rubinsztein DC, Marino G, Kroemer G. Authophagy and aging. Cell 2011; 146: 682-95.
25. Wang M, Jing T, Wang X, Yao D. Beta-secretase/BACE1 promotes APP endocytosis and processing in the endosomes and on cell membrane. Neurosci Lett 2018; 685: 63-7.

26. Nixon RA. Autophagy, amyloidogenesis and Alzheimer disease. J Cell Sci 2007; 120: 4081-91.
27. Bolintineanu D, Hazrati E, Kaznessis YN. Antimicrobial mechanism of pore- forming protegrin peptides: 100 pores to kill E. coli. Peptides 2009; 31: 1-8.
28. Chirita CN, Necula M, Kuret J. Anionic micelles and vesicles induce tau fibrillization in vitro. J Biol Chem 2003; 278: 25644-50.
29. Marchesi N, Osera C, Fassina L, et al. Autophagy is modulated in human neuroblastoma cells through direct exposition to low frequency electromagnetic fields. J Cell Physiol 2014; 229: 1776-86.
30. Francois A, Terro F, Janet T, Rioux Bilan A, Paccalin M, Page G. Involvement of interleukin-1beta in the autophagic process of microglia: relevance to Alzheimer's disease. J Neuroinflammation 2013; 10: 151.
31. Blasko I, Veerhuis R, Stampfer-Kountchev M, Saurwein-Teissl M, Eikelenboom P, Grubeck-Loebenstein B. Costimulatory effects of interferon-gamma and interleukin-1beta or tumor necrosis factor alpha on the synthesis of Abeta1-40 and Abeta1-42 by human astrocytes. Neurobiol Dis 2000; 7: 682-9.
32. Miklossy J. Emerging role of pathogens in Alzheimer's disease. Expert Rev Mol Med 2001; 13: e30.
33. Sun Y, Shan D. Inhibitory effects of antimicrobial peptides on lipopolysaccharide-induced inflammation. Mediators Inflamm 2015; 2015: 167572.
34. Lomakin A, Chung DS, Benedek GB, Kirschner DA, Teplow DB. On the nucleation and growth of amyloid beta-protein fibrils: detection of nuclei and quantitation of rate constant. Proc Natl Acad Sci USA 1996; 93: 1125-9.
35. Soreghan B, Kosmoski J, Glabe C. Surfactant properties of Alzheimer's A beta peptides and the mechanism of amyloid aggregation. J Biol Chem 1994; 269: 28551-4.
36. Pajak B, Songin M, Strosznajder JB, Orzechowski A, Gajkowska B. Ultrastructural evidence of amyloid beta-induced autophagy in PC12 cell. Folia Neuropathol 2009; 47: 252-8.
37. Levine B, Kroemer G. Autophagy in the pathogenesis of disease. Cell 2008; 132: 27-42.
38. Whyte LS, Lau AA, Hemsley KM, Hopwood JJ, Sargeant TJ. Endo-lysosomal and autophagic dysfunction: a driving factor in Alzheimer's disease? J Neurochem 2017; 140: 703-17.
39. Hassiotis S, Manavis J, Blumbergs PC, et al. Lysosomal LAMP1 immunoreactivity exists in both diffuse and neuritic amyloid plaques in the human hippocampus. Eur J Neurosci 2018; 47: 1043-53.
40. Terman A, Brunk UT. Lipofuscin. Int J Biochem Cell Biol 2004; 36: 1400-4.