






Soluble A β oligomers and protofibrils induce NLRP3 inflammasome activation in microglia

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Abstract

Alzheimer's disease (AD) is the most prevalent neurodegenerative disorder causing memory loss, language problems and behavioural disturbances. AD is associated with the accumulation of fibrillar amyloid- β (A β) and the formation of neurofibrillary tau tangles. Fibrillar A β itself represents a danger-associated molecular pattern, which is recognized by specific microglial receptors. One of the key players is formation of the NOD-, LRR- and pyrin domain-containing 3 (NLRP3) inflammasome, whose activation has been demonstrated in AD patient brains and transgenic animal models of AD. Here, we investigated whether A β oligomers or protofibrils that represent lower molecular aggregates prior to A β deposition are able to activate the NLRP3 inflammasome and subsequent interleukin-1 beta (IL-1 β) release by microglia. In our study, we used A β preparations of different sizes: small oligomers and protofibrils of which the structure was confirmed by atomic force microscopy. Primary microglial cells from C57BL/6 mice were treated with the respective A β preparations and NLRP3 inflammasome activation, represented by caspase-1 cleavage, IL-1 β production, and apoptosis-associated speck-like protein containing a CARD speck formation was analysed. Both protofibrils and low molecular weight A β aggregates induced a significant increase in IL-1 β release. Inflammasome activation was confirmed by apoptosis-associated speck-like protein containing a CARD speck formation and detection of active caspase-1. The NLRP3 inflammasome inhibitor MCC950 completely inhibited the A β -induced immune response. Our results show that the NLRP3 inflammasome is activated not only by fibrillar A β aggregates as reported before, but also by lower molecular weight A β oligomers and protofibrils, highlighting the possibility that microglial activation by these A β species may initiate innate immune responses in the central nervous system prior to the onset of A β deposition.

Abbreviations used: AD, Alzheimer's disease; AFM, atomic force microscope; ASC, apoptosis-associated speck-like protein containing a CARD; A β , amyloid beta; DAMP, damage-associated molecular pattern; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethylsulfoxide; EDTA, ethylenediaminetetraacetic acid; ELISA, enzyme-linked immunosorbent assay; FBS, fetal bovine serum; HFIP, hexafluoroisopropanol; IL-1 β , interleukin 1 beta; LDH, lactate dehydrogenase; LPS, lipopolysaccharide; NLRP3, NOD-, LRR- and pyrin domain-containing 3; PBS, Dulbecco's phosphate buffered saline; PFA, paraformaldehyde; PI, propidium iodide; PLL, poly-L-lysine; TNF- α , tumor necrosis factor alpha.

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KEYWORDS

Alzheimer, ASC speck, IL-1 β , microglia, NLRP3 inflammasome, s disease, soluble A β

1 | INTRODUCTION

Alzheimer's disease (AD) is the most prevalent neurodegenerative disorder of the central nervous system (dos Santos et al., 2018). AD is pathologically characterized by extracellular deposition of fibrillar amyloid- β (A β) and hyperphosphorylation of tau with subsequent intraneuronal formation of neurofibrillary tangles (Villemagne et al., 2013). Clinically, these changes are accompanied by progressive memory loss and cognitive decline (Shepherd et al., 2019). A β is generated by the sequential processing of the amyloid precursor protein by two aspartyl proteases: the β - and γ -secretases (Busciglio, Gabuzda, Matsudaira, & Yankner, 1993; Cai, Golde, & Younkin, 1993; Haass, Koo, Mellon, Hung, & Selkoe, 1992). Under normal conditions, A β peptides are removed from the brain through local degradation and by transfer into both the cerebrospinal fluid and blood vessels (Heneka, Golenbock, & Latz, 2015b). Previously it has been shown that fibrillar A β can activate the same immune receptors that respond to pathogen-associated molecular patterns including viral proteins or bacterial lipopolysaccharides (LPS) (Venegas and Heneka 2017), thereby contributing to the neuroinflammatory component of AD (Heneka, Carson, et al., 2015a). Less clear however, is whether other A β species such as protofibrils or oligomers are able to initiate a similar immune reaction and which inflammatory downstream signalling cascades are involved.

Fibrillar A β can initiate a neuroinflammatory immune response through activation of the NOD-like receptor (NLR) family, pyrin domain-containing 3 (NLRP3) inflammasome (Heneka et al., 2013). The NLRP3 inflammasome, in addition to the NLRP3 protein, also includes apoptosis-associated speck-like protein (ASC) and procaspase-1 (Ito et al., 2015; Shao, Xu, Han, Su, & Liu, 2015). Activation of the NLRP3 inflammasome leads to caspase-1 activity and consequently to the release of pro-inflammatory cytokines, such as interleukin-1 β (IL-1 β) and interleukin-18 (IL-18) (Shao et al., 2015).

Thus far, conflicting data exist regarding A β 's ability to induce a pro-inflammatory cytokine response (Maezawa, Zimin, Wulff, & Jin, 2011). One possible reason for this could be that the wide range of A β aggregates studied had different structures and origin, including those that were chemically synthesized or recombinant peptides. It has also been argued that some of the A β preparations used may have been contaminated with LPS, which could (at least partly) account for the observed inflammatory response. Importantly, the different physical and biochemical properties of A β may lead to diverse cellular reactions including differences in microglia activation (Ferrera, Mazzaro, Canale, & Gasparini, 2014; Lindberg, Selenica, Westlind-Danielsson, & Schultzberg, 2005).

Fibrillar A β induces NLRP3 inflammasome activation in microglia (Halle et al., 2008), but it remains unclear whether small A β oligomers and protofibrils, that seem to be more neurotoxic (Cizas et al., 2010; Umeda et al., 2011; Yang, Li, Xu, Walsh, & Selkoe, 2017), similarly

activate the NLRP3 inflammasome in microglia. Therefore, we selected soluble A β oligomers and protofibrils, with proven neurotoxicity (Deleglise et al., 2014; Fixman et al., 2017; Herzer, Meldner, Rehder, Gröne, & Nordström, 2016; Porter et al., 2016; Shin et al., 2014), to examine their ability to activate the microglial NLRP3 inflammasome by assessing caspase-1 activation, IL-1 β release and ASC speck formation.

2 | MATERIALS AND METHODS

2.1 | Materials

Dulbecco's modified Eagle's medium (DMEM; cat#61965059), fetal bovine serum (FBS; cat#26140079), penicillin/streptomycin (P/S; cat#15140122), Dulbecco's Phosphate Buffered Saline (PBS; cat#14040166) and 0.25% trypsin-EDTA solution (cat#25200056) were obtained from Gibco, Thermo Fischer Scientific. Cell culture plates: T75 culture flasks (cat#658170) were from Greiner Bio-One; IbiTreat μ -slides with 8-well (cat#80826) and 96-well μ -plates (cat#89626) were from Ibidi. LPS (cat#tlrl-eblps), nigericin (cat#tlr-nig) and MCC950 (cat#inh-mcc) were from InvivoGen. Materials for atomic force microscopy (AFM): TESP-V2 cantilevers were from Bruker; mica sheets were from SPI Supplies. LDH cytotoxicity detection kit (cat#11644793001) was from Roche Diagnostics and XTT Cell Viability Kit (cat#9095) from Cell Signaling Technology®. Propidium Iodide (PI; cat#638), Hoechst 33,342 (cat#639) and FAM-FLICA® Caspase-1 Assay Kit (containing FLICA reagent FAM-YVAD-FMK—caspase-1 inhibitor probe; cat#98) were obtained from ImmunoChemistry Technologies. Poly-L-lysine (PLL; cat#1524), paraformaldehyde (PFA; cat#158127), 1,1,1,3,3,3-Hexafluoro-2-propanol (HFIP; cat#105228) and Triton X-100 solution (cat#X100) were obtained from Sigma-Aldrich by Merck. Dimethylsulfoxide (DMSO; cat#A3672) was from PanReac AppliChem and the ITW Reagents. Membrane dye was from (cat#C10046, Invitrogen). Amyloid beta peptide (A β ; ID. No.:CM1804161) was from PSL Peptide Specialty Laboratories GmbH. IL-1 β /IL-1F2 DuoSet ELISA kit (cat#DY401) and TNF- α DuoSet ELISA kit (cat#DY410) were from R&D Systems. Normal goat serum (cat#S-1000) was from Vector Laboratories. The primary antibodies: rabbit anti-ASC (cat#AG-25B-0006; RRID: AB_2490440) from AdipoGen; rat anti-CD68 (cat#MCA1957; RRID:AB_322219) from Serotec by Bio-Rad; mouse anti-A β (clone 11E12) antibody was obtained from Dalgediene et al. (2013). Secondary antibodies: F(ab')₂-Goat anti-Mouse IgG (H + L) cross-adsorbed secondary antibody, Alexa Fluor 488 (cat#A11017; RRID:AB_143160), Goat anti-Rat IgG (H + L) cross-adsorbed secondary antibody, Alexa Fluor 594 (cat#A11007; RRID:AB_141374), F(ab')₂-Goat anti-Rabbit IgG (H + L) cross-adsorbed secondary antibody, Alexa Fluor 488 (cat#A11070; RRID:AB_142134) and Goat

anti-Mouse IgG (H + L) cross-adsorbed secondary antibody, Alexa Fluor 594 (cat#A11005; RRID:AB_141372) were obtained from Invitrogen, Thermo Fisher Scientific. Flow cytometry reagents: cell surface receptors FcγRIII (CD16) and FcγRII (CD32) blocking solution Purified Rat Anti-Mouse CD16/CD32 (Mouse BD Fc Block™) (cat#553141; RRID:AB_394656) was from (Becton, Dickinson and Company), rat APC anti-mouse/human CD11b Antibody (clone M1/70, cat#101212; RRID: AB_312795) was from BioLegend; F(ab')₂-Goat anti-Mouse IgG (H + L) cross-adsorbed secondary antibody, Alexa Fluor 488 (cat#A11017; RRID:AB_143160) was from Life Technologies, Thermo Fisher Scientific. Centrifugal filters with 3 kDa a cutoff—Amicon Ultra-0.5 Centrifugal Filter Unit (cat#UFC500396) was from Merck Millipore. Reagents for immunoblotting using Jess System (ProteinSimple; Bio-Techne): primary anti-caspase-1 antibody—anti-Caspase-1 (p20) (mouse), mAb (Casper-1) (cat#AG-20B-0042; RRID:AB_2490248) from AdipoGen; 12–230 kDa Jess/Wes Separation Module (cat#SM-W004) and secondary antibody—ready to use HRP-conjugated goat anti-mouse antibody (cat#042-205) were from ProteinSimple; Bio-Techne.

2.2 | Cell culture

All animals used for cell isolation were treated according to the legal and ethical requirements of the University of Bonn, Medical Center and the North Rhine-Westphalia (NRW) federal ministry for nature, environment and consumer protection (Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen, LANUV). Mouse breeding and husbandry were done according to the German animal welfare law.

For this experiment, mice were housed for primary microglia cell preparation only which did not require further approval. For microglia isolation, the brains of 221 0–3-day-old pups were used. The pups were killed by decapitation without anaesthesia. Primary microglia were isolated as described before with some adaptations (Giulian & Baker, 1986). Briefly, brains from neonatal (p0–p2, mixed gender cultures) C57BL/6 (WT) (purchased from Charles River Laboratories Inc., RRID: IMSR_JAX:000,664) mice were stripped of the meninges and dissociated using mechanical shearing and trypsin. Cells of two brains were plated on PLL-coated T75 culture flasks (Greiner Bio-One) and cultivated in DMEM supplemented with 10% heat-inactivated fetal bovine serum and 1% penicillin/streptomycin. On the next day, cells were washed three times with PBS to remove cellular debris and cultured with DMEM supplemented with 10% FBS, 1% P/S and 10% L929 conditioned medium as a source of growth factors. After approximately 8 days, loosely attached mature microglia were shaken off the astrocytic monolayer with a repetition of the harvesting procedure at every 2–3 days for up to three times. Cells were seeded at a density of 1 million/cm² in 1/2 old medium (condition medium from microglia shake) and 1/2 fresh DMEM supplemented with 10% FBS and 1% P/S and allowed to adhere overnight. On the next day, microglia were primed with 50 ng/ml LPS for 3 hr and treated with 2.5 μM Aβ aggregates for 6 hr in a serum-free DMEM containing 1% P/S. As a positive control, the inflammasome inducer nigericin was used at 10 μM concentration. MCC950,

which selectively inhibits the NLRP3 inflammasome, was used at 1 μM concentration. These in vitro protocols are used on a daily basis in the laboratory; therefore, we have not performed preliminary sample calculation. No blinding was applied in the cell culture treatment.

2.3 | Aβ preparation

Aβ oligomers and protofibrils were prepared according to the protocol of Stine (Stine, Dahlgren, Krafft, & LaDu, 2003). Briefly, Aβ was dissolved in ice cold HFIP to a final concentration of 221 μM. About 100 μl aliquots were incubated at 20°C for 1 hr and centrifuged in a speed vacuum centrifuge at 800 × g for 10 min until the HFIP had evaporated. The tubes with the peptide film were stored at –80°C. Aβ pre-treated with HFIP and was dissolved in DMSO to a final concentration of 2.5 mM and sonicated for 10 min. Aβ or the DMSO vehicle control was added to phenol red-free DMEM to a final concentration of 100 μM and incubated for 24 hr at 4 or 37°C. To confirm that the Aβ oligomers and protofibrils were intact during cell culture treatment further analysis was performed. Aβ preparations were diluted with DMEM from 10 to 2.5 μM and incubated for 6 hr at 37°C and 5% CO₂ to produce oligomers (*Protocol I*) and protofibrils (*Protocol II*), respectively. Aβ oligomers and protofibrils were then characterized by AFM. The buffers used for the preparation of Aβ served as controls for all cell culture experiments with Aβ oligomers or protofibrils. They are referred as oligomers control (Con. O) or protofibrils control (Con. P).

2.4 | Atomic force microscopy imaging

To assess the size and morphology of the Aβ oligomers and protofibrils, AFM measurements were performed using a Dimension Icon system (Bruker) in tapping mode in air. Imaging was performed at 20°C using TESP-V2 cantilevers with a nominal spring constant 40 N/m. Freshly cleaved mica sheets (grade 4, SPI Supplies) were modified with PLL for 10 min, and then rinsed with deionized water, and dried under a nitrogen stream. Before measurement 20 μL of a 2.5 μM Aβ solution was spotted on modified mica surface for 10 min, and then rinsed with deionized water, and dried under nitrogen stream. Images were acquired at scan rate 0.4 Hz in 512 × 512 pixel mode. Images were analysed by v4.0 Beta 9.1 WSxM (Horcas et al. 2007) and Nanoscope Analysis v1.40 (Bruker) software. The mean height of Aβ oligomers and protofibrils ("z-height") was estimated by determining step-height histograms.

2.5 | Cell viability/cytotoxicity assay

Cell viability/cytotoxicity was measured using three different assays: LDH release, XTT assay and PI/Hoechst nuclear staining.

To assess the cytotoxicity of Aβ, an LDH cytotoxicity detection kit was used. A quantity of 50 μL of cell supernatants was used to



perform the cytotoxicity assay according to the manufacturer's protocol. Absorbance was measured at 450 nm using a microplate reader.

The metabolic activity of primary microglia was quantified using the XTT Cell Viability Kit. Primary microglia were seeded at 1 million cell/ml into 96-well plates and treated with 100 μ l medium per well. After treatment with the various stimuli, the XTT Reagent and Electron Coupling Solution were added directly to the cell culture medium according to the manufacturer's protocol and incubated for 4 hr. Absorbance was measured at 450 nm with a microplate reader.

To determine cell viability PI/Hoechst nuclear staining was used. Nuclei were stained with 1.25 μ g/ml PI and 1 μ g/ml Hoechst33342 in cell culture medium for 30 min. The cells were washed with PBS and fixed with 4% PFA. The fluorescent signal was determined using a fluorescence microscope. Images were taken using a 20 \times objective. Viability was quantified according to a ratio of PI (dead cells) and Hoechst (all cells) normalized to control cells.

2.6 | Measurement of IL-1 β and TNF- α release

NLRP3 inflammasome activation by A β was determined by measuring the IL-1 β secretion using the mouse IL-1 beta/IL-1F2 DuoSet ELISA kit. To assess the extent of the inflammatory response, tumor necrosis factor alpha (TNF- α) release was determined using the mouse TNF- α DuoSet ELISA kit. Primary microglia cells were treated with 1 ml medium per well in 6-well plates. After the LPS priming and A β treatment with relevant controls, the cell culture supernatants were harvested and frozen at -80°C . Supernatants were thawed once and assayed according to the manufacturer's protocol. The optical density was determined at 450 nm photometrically with a microplate reader (Infinite M200; Tecan). The concentration of cytokine released was quantified using the relevant standard curves.

2.7 | Immunocytochemistry

Cells were stained in IbiTreat 8-well μ -slides or 96-well μ -plates. After stimulation with the various NLRP3-inflammasome-inducing reagents and appropriate controls, cells were washed with PBS and fixed in 4% PFA dissolved in PBS for 15 min and permeabilized with 0.1% Triton X-100 prepared in PBS for 10 min. Blocking solution—PBS containing 5% normal goat serum (1:200) was applied for 30 min followed by two washing steps. The primary antibodies rabbit anti-ASC (1:200; clone AL177), rat anti-CD68 (1:1,000; Clone FA-11) and mouse anti-A β (3.3 μ g/ml; clone 11E12, Dalgediene et al. 2013) were added to the blocking solution and incubated overnight. The following secondary antibodies were used, respectively: goat anti-rabbit (1:600), goat anti-rat (1:600) and goat anti-mouse (1:600). The secondary antibodies were applied for 2 hr followed by two washing steps. Hoechst33342 was used for nuclear staining at 1 μ g/mL for 30 min in PBS. ASC speck images were taken using a 60 \times oil-objective. The uptake of A β oligomers and protofibrils was measured by imaging with 40 \times objective. CD68 was used as a microglia and lysosomal marker.

2.8 | FLICA assay (active caspase-1)

Active caspase-1 was detected using the FLICA assay according to manufacturer's protocol. Briefly, cells were stained in IbiTreat μ -slides with eight wells. FLICA reagent (FAM-YVAD-FMK—caspase-1 inhibitor probe) was added after the 6 hr treatment with A β and incubated for 1 hr. Cells were washed three times and stained with Hoechst 33,342 at 1 μ g/ml and membrane dye (1:1,000). After washing the cells were fixed with 4% PFA and washed again with PBS. Caspase-1 activation was analysed directly after staining. The signal was visualized by fluorescence microscopy using a 60 \times oil-objective. FLICA-caspase-1 intensity signal was measured and normalized to cell number. In every experiment, the calculated value of FLICA-caspase-1 intensity was normalized to the value of untreated cells (control).

2.9 | Microscopy

All experiments were examined using a Nikon Eclipse Ti fluorescence microscope (Nikon). Acquired images were processed using NIS-elements 4 (Nikon) and ImageJ (Wayne Rusband; National Institute of Health) by a person who was blind for the experimental conditions.

2.10 | Flow cytometry

To evaluate A β phagocytosis flow cytometric analysis was performed. Microglia that were incubated with various stimuli were collected by detaching with 0.25% trypsin-EDTA solution for 10 min and the trypsin was then inhibited with DMEM supplemented with 10% FBS and 1% P/S. After washing the cells were fixed with 4% PFA for 10 min and permeabilized with 0.03% Triton X-100 for 5 min. After washing the cell surface receptors, Fc γ RIII (CD16) and Fc γ RII (CD32) were blocked with Fc block (anti-mouse CD16/32) solution. The cells were incubated with primary antibodies against A β (clone 11E12) overnight at 4 $^{\circ}\text{C}$. After washing, the cells were stained with rat anti-mouse CD11b-APC (1:100; clone M1/70) together with the secondary antibodies goat anti-mouse (1:600) for 1 hr at 4 $^{\circ}\text{C}$. After washing, microglia were resuspended in staining solution (2% FBS in PBS). Flow cytometric data was acquired on a BD FACSCanto II flow cytometer (Becton, Dickinson and Company) and analysed using FlowJo software (FlowJo, LLC).

2.11 | Immunoblotting

The quantity of cleaved caspase-1 was determined by Western-blot using the Jess fully automated system (ProteinSimple; Bio-Techne) following the suggested protocol of the system. Primary microglia were cultured in 6-well plates and treated at a density of 2 million cells/ml per 9.5 cm 2 growth area. The protein content of the supernatants was concentrated 10 \times using centrifugal filters with

3 kDa cutoff. The chemiluminescence assay was used following ProteinSimple instructions. Samples were prepared as following: First, 5× master mix was prepared using reagents provided by Bio-Techne (EZ Standard Pack 1, cat. no.: S-ST01EZ-8). 1/2 10× Sample buffer was mixed with 1/2 400 mM DTT solution; cell culture supernatant was mixed 1–4 with 5× master mix, heated at 95°C for 5 min and stored on ice. Here 12–230 kDa Jess/Wes Separation Module was used and 3 µl of each sample was loaded for 9 s. The incubation time of the primary and the secondary antibodies was 30 min. As a primary antibody, anti-caspase-1 was used at a concentration of 20 µg/ml (1:50 dilution). For the secondary antibody, ready to use HRP-conjugated goat anti-mouse antibody was used.

2.12 | Statistical analysis

Data were evaluated using Graph Pad Prism software Version 6.0 and presented as box plots (showing minimum, first quartile, median, third quartile and maximum) of at least four independent

experiments. Independent experiments referred to as n means the number of independent cell culture preparations. Kolmogorov–Smirnov normality test was carried out to test if the values come from a Gaussian distribution. Statistical comparisons of vehicle controls versus treatment were performed with one-way ANOVA in conjunction with a Bonferroni's multiple comparison test. In other cases, a Kruskal–Wallis test with Dunn's post hoc test was used for non-parametric data. Levels of significance are indicated as * $p < .05$; ** $p < .01$; *** $p < .001$, **** $p < .0001$.

3 | RESULTS

A β aggregates of different size and structure were used to investigate NLRP3 inflammasome activation in primary murine microglia. Microglial cells were primed with LPS to induce translation of the NLRP3 inflammasome components and subsequently treated with various A β aggregates. NLRP3 inflammasome assembly and activation was analysed by detection of cleaved caspase-1, ASC speck

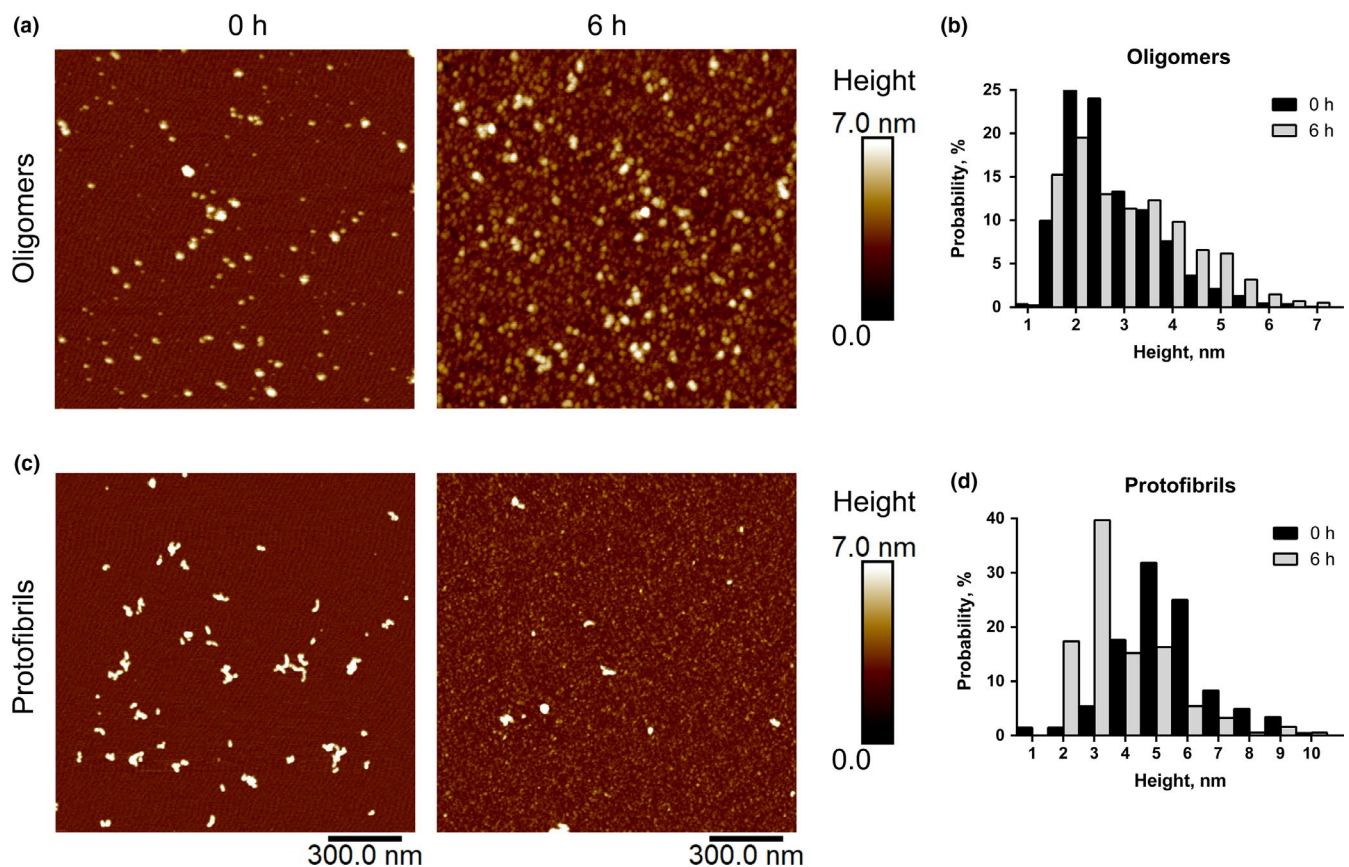


FIGURE 1 A β characterization by atomic force microscope and confirmation that A β oligomers do not aggregate to fibrils during the cell treatment period. Representative images of A β oligomers and protofibrils prepared according to different protocols: (a) Oligomers—prepared using *Protocol I*; mainly 1–5 nm in size. (c) Protofibrils—prepared using *Protocol II*. (b) and (d) Histograms of height distribution of A β particles, $n = 3$ independent A β preparations. After preparation of A β oligomers and protofibrils, they were diluted with cell culture medium to 2.5 µM and incubated for 6 hr at 37°C under cell-free conditions. A β oligomers and protofibrils dissociated into smaller particles. After additional incubation of protofibrils, a higher fraction of small oligomers, about 1–2 nm in size was detected. Oligomers dissociated into small particles as the scanned surface was fully covered with very small A β particles. The scanned size of the images is 1.5 x 1.5 µm. All images are represented in height scale from 0 to 7 nm

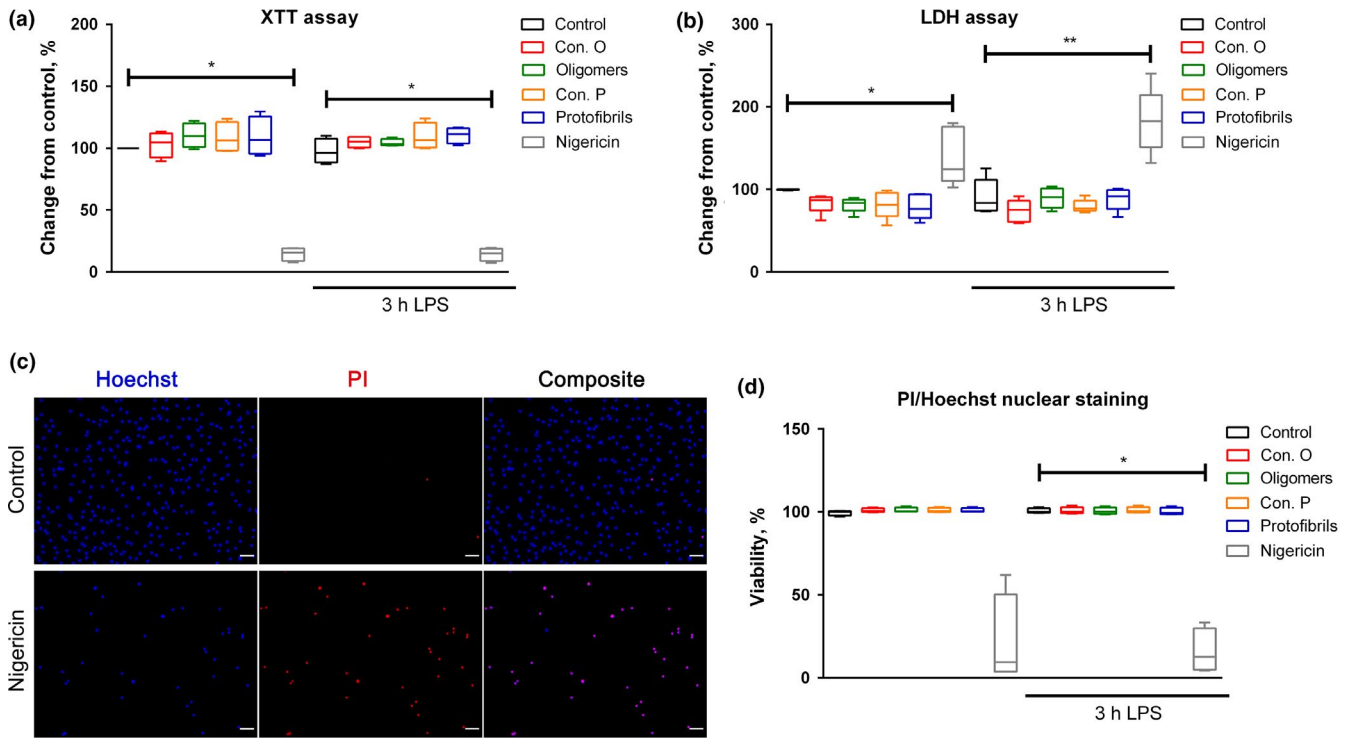


FIGURE 2 A β does not affect microglial viability. Microglia were primed with 50 ng/ml lipopolysaccharide (LPS) and treated with A β oligomers or protofibrils for 6 hr at a concentration of 2.5 μ M. Nigericin (10 μ M) was used as a positive control. To assess A β -induced cytotoxicity (a) metabolic activity—XTT assay ($n = 4$ independent cell culture preparations), (b) Lactate dehydrogenase assay ($n = 5$ independent cell culture preparations), and (c, d) PI and Hoechst nuclear staining ($n = 4$ independent cell culture preparations) were performed. (c) Representative images of PI and Hoechst nuclear staining; PI stains dead cells, Hoechst—all cells. The images were taken using 20x objective. The scale bars indicate 40 μ m. (d) Quantification of PI and Hoechst nuclear staining. Con O.—oligomer control, Con P.—protofibrils control. Data are represented using box plots, * $p < .05$, ** $p < .01$, Kruskal–Wallis test in conjugation with a Dunn's post hoc test

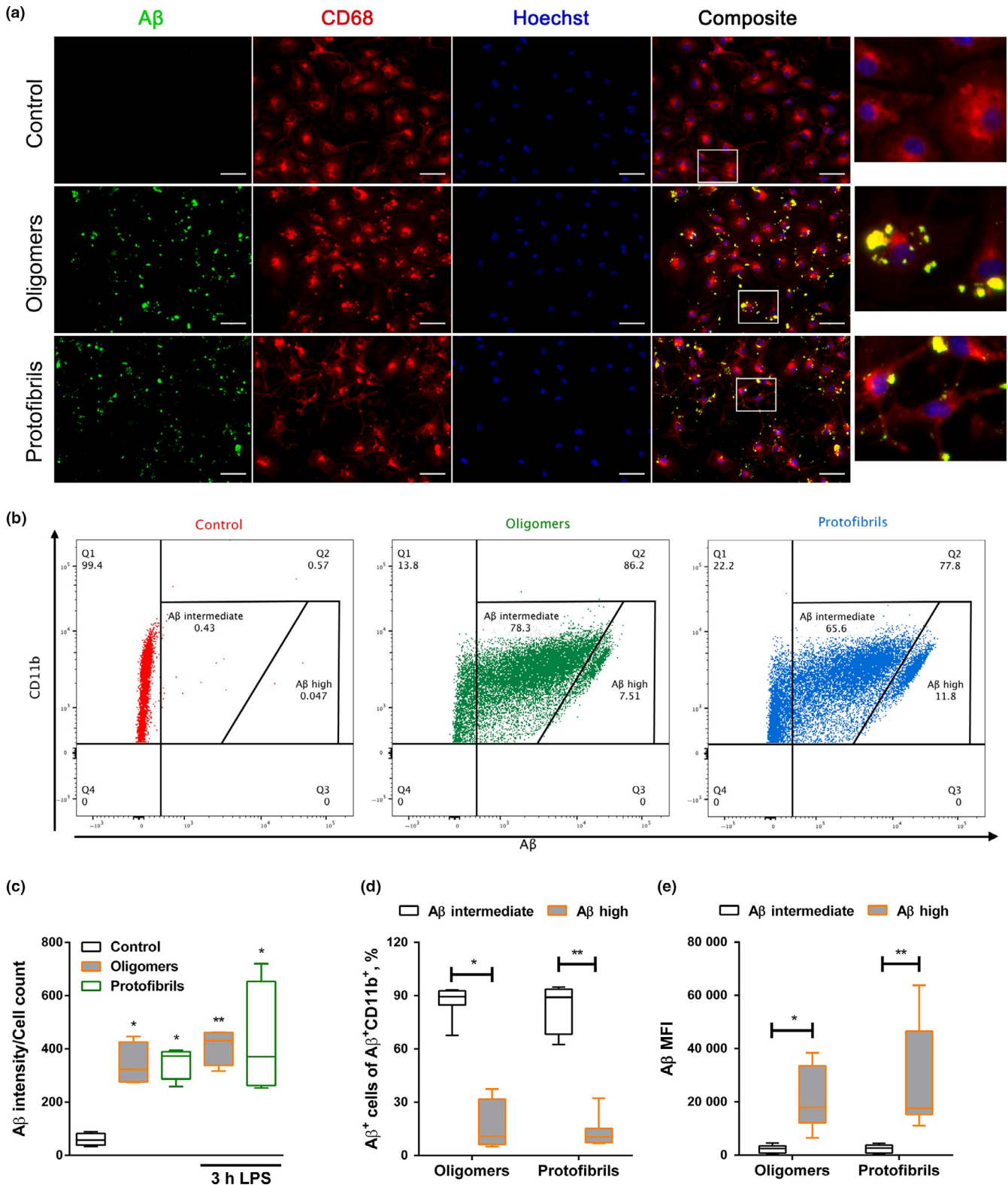
formation, and IL- β release. Additionally, the NLRP3 inflammasome inhibitor MCC950 was used and nigericin was included as a positive control.

3.1 | Characterization of A β structure

The morphology of the prepared A β structures was revealed by AFM. Oligomers of 1–5 nm in height were observed after preparation according to *Protocol I*, and in preparation according to *Protocol II* protofibrils ~5 nm in height were found. To confirm that the oligomers were intact and did not form fibrils during cell culture treatment at 37°C, further analysis was performed. The solution of 2.5 μ M A β

was incubated in cell culture medium for 6 hr at 37°C and 5% CO $_2$ atmosphere to mimic the culture conditions during microglia treatment. The morphology of the prepared A β species was analysed before and importantly, after an incubation period of 6 hr (Figure 1). A β aggregates dissociated after dilution from 100 to 2.5 μ M and 6 hr incubation at 37°C. It should be noted that A β monomers and dimers were observed by AFM in all A β preparations analysed. If A β aggregates are very large, they overshadow monomers and small oligomers. After the additional incubation step, the mica sheets were prepared in the same way as before, however, the results shown in Figure 1 revealed that the background was fully covered with very small A β particles, demonstrating that A β oligomers do not aggregate to fibrils during the cell treatment period chosen for the stimulation experiments.

FIGURE 3 Microglia uptake of A β oligomers and protofibrils. Microglia were primed with 50 ng/ml lipopolysaccharide (LPS) and treated with A β oligomers or protofibrils for 6 hr at 2.5 μ M concentration. Cells were immunostained with anti-A β (green), anti-CD68—microglia and lysosomal marker (red), nuclear stain Hoechst (blue) and analysed by fluorescent microscope (a, b). (a) Representative images of one experiment. The images were taking using 40x objective. The scale bars indicate 40 μ m. In the composite images rectangles show magnified parts. (b) Quantification of A β uptake—A β co-localization with CD68 was calculated, $n = 4$ independent cell culture preparations. A β uptake was also analysed by FACS (c, d and e). Cells were immunostained with anti-A β , and anti-CD11b was used to detect microglia. CD11b $^+$ A β $^+$ cells were analysed. (c) Representative dot plots of one experiment. (d) and (e) Quantification of A β uptake. CD11b $^+$ A β $^+$ population highly rich in A β was referred as A β high. Other A β positive cells were referred as A β intermediate. (d) Fraction of CD11b $^+$ A β $^+$ cells in A β intermediate and A β high populations, (e) MFI—median fluorescent intensity, $n = 7$ independent cell culture preparations. Data are represented using box plots, * $p < .05$, ** $p < .01$ and the Kruskal–Wallis test in conjugation with a Dunn's post hoc test for microscope analysis data, one-way ANOVA followed by Bonferroni's multiple comparison test for FACS analysis data



3.2 | A β had no influence on cell viability

Persistent NLRP3 inflammasome activation is often characterized by the induction of an inflammatory pyroptotic cell death. To test whether the A β oligomers or protofibrils affected microglial

viability over the treatment period of 6 hr, several cytotoxicity assays were performed. Nigericin was used as a positive control. After treatment with A β oligomers and protofibrils, no significant changes in metabolic activity (Figure 2a) or LDH release (Figure 2b) were found. To detect cell viability changes at an individual single cell level PI and Hoechst nuclear staining was used (Figure 2c and d).

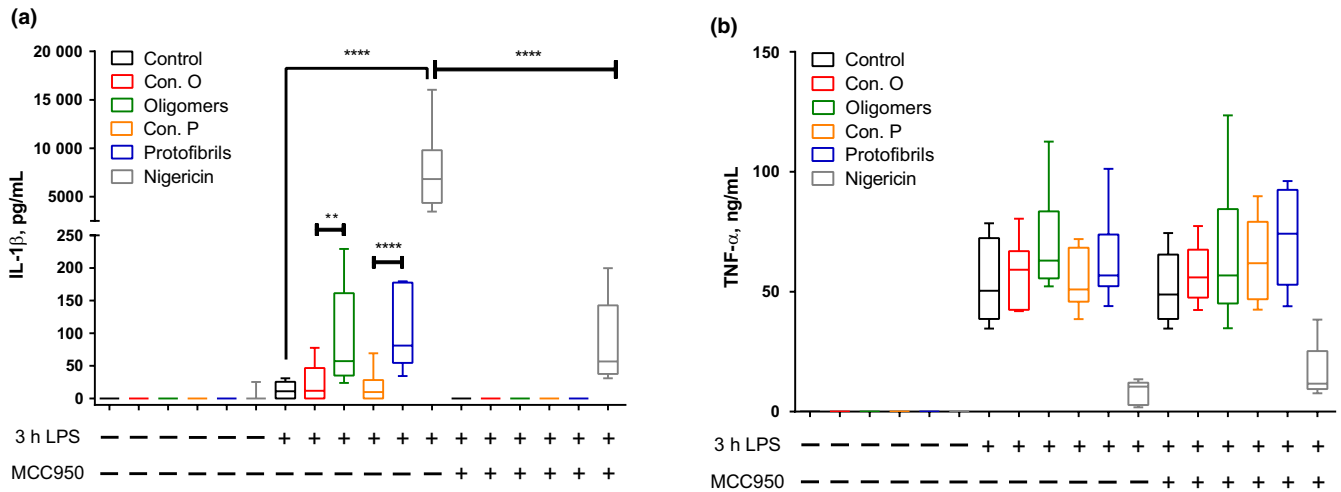


FIGURE 4 Aβ oligomers and protofibrils induce interleukin-1β (IL-1β) release in murine microglia. Microglia were primed with 50 ng/ml lipopolysaccharide (LPS) and treated with Aβ oligomers or protofibrils for 6 hr at 2.5 μM. MCC950 (1 μM) was added 20 min before Aβ treatment. Nigericin (10 μM) was used as a positive control. Supernatants were collected and analysed by ELISA, for (a) IL-1β release, *n* = 10 independent cell culture preparations and (b) Tumor necrosis factor alpha release, *n* = 6 independent cell culture preparations. Con O.—oligomer control, Con P.—protofibrils control. Data are represented using box plots, ***p* < .01, *****p* < .0001, one-way ANOVA followed by Bonferroni's multiple comparison test

While occasionally single dead microglia were detectable, no significant effect on cell viability was found in treatment groups when compared with controls. These data indicate the the respective Aβ preparations did not affect microglial viability (pyroptosis) under these experimental conditions.

3.3 | Aβ oligomers and protofibrils were phagocytosed at a similar extent by microglia

The uptake of Aβ by microglia was investigated by immunocytochemistry. To detect phagocytosis, it was investigated whether

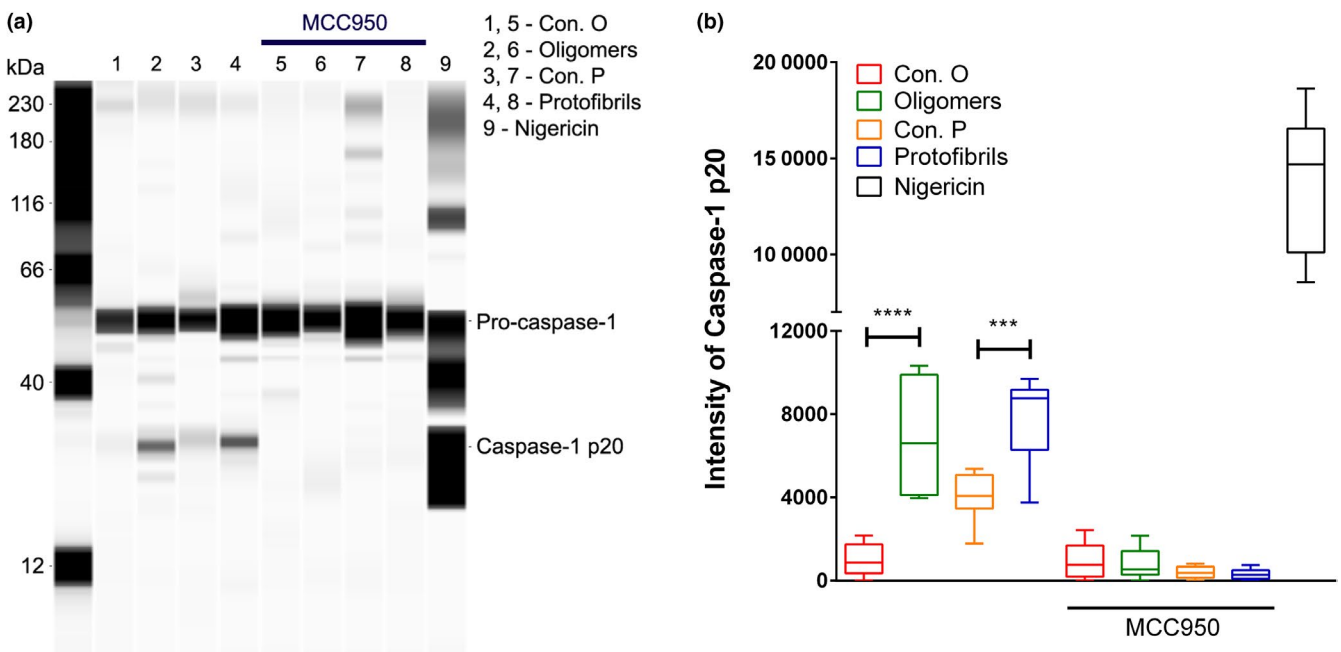


FIGURE 5 Aβ oligomers and protofibrils induced a significant increase in cleaved caspase-1 in microglial cells. Microglia were primed with 50 ng/ml lipopolysaccharide and treated with Aβ oligomers or protofibrils for 6 hr at 2.5 μM concentration. Inflammasome inhibitor MCC950 (1 μM) was added 20 min before Aβ treatment. Nigericin (10 μM) was used as a positive control. Cleaved caspase-1 was determined in cell supernatants using western blot performed with automated Jess system (ProteinSimple). (a) Representative image of western blot analysis. Cleaved Caspase-1 was significantly elevated after Aβ oligomers and protofibrils treatment. Con O.—oligomers control, Con P.—protofibrils control. (b) Quantification of Caspase-1 p20 determined by western blot. Caspase-1:50 kDa—pro-caspase-1; 20 kDa—cleaved caspase-1 (p20), *n* = 6 independent cell culture preparations. Data are represented using box-plots, ****p* < .001, *****p* < .0001, one-way ANOVA followed by Bonferroni's multiple comparison test

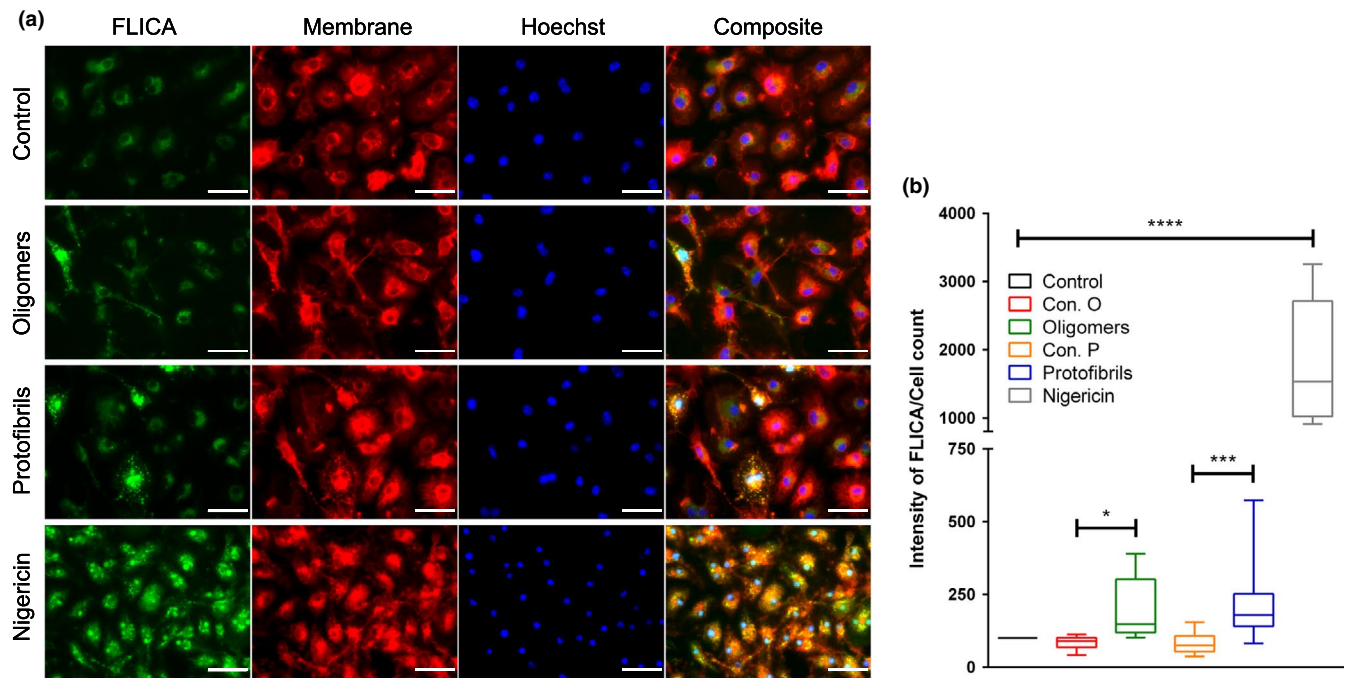


FIGURE 6 A β -induced caspase-1 activation. Microglia were primed with 50 ng/ml lipopolysaccharide and treated with A β oligomers or protofibrils for 6 hr at 2.5 μ M concentration. Inflammasome inhibitor MCC950 (1 μ M) was added 20 min before A β treatment. Nigericin (10 μ M) was used as a positive control. Cells were stained for activated caspase-1 using fluorescently labelled inhibitor of caspase-1 (FLICA, green), membrane stain (red) and nuclear stain Hoechst (blue). a) Representative images of the staining. b) Quantification of fluorescent intensity signal of caspase-1, $n = 12$ independent cell culture preparations. Con O.—oligomer control, Con P.—protofibrils control. Data are represented using box-plots, * $p < .05$, *** $p < .001$, **** $p < .0001$, Kruskal–Wallis test in conjunction with a Dunn's post hoc test. The images were taken using x60-oil objective. The scale bars indicate 40 μ m

the A β oligomers or protofibrils were co-localized with the lysosomal marker CD68. After 6 hr of incubation, the cells had phagocytosed a significant amount of the respective A β preparations (Figure 3a and b). However, there was no difference in the uptake of A β oligomers compared to protofibrils. Pre-stimulation with LPS did not significantly affect A β oligomer or protofibril phagocytosis.

Additionally, A β oligomer or protofibril uptake was analysed by FACS (Figure 3c and d). Again, both A β oligomers and protofibrils were phagocytosed to a similar extent by microglia. However, a small population of cells were highly immunopositive for A β (A β high) after treatment with both oligomers and protofibrils (Figure 3c), suggesting a heterogeneous phagocytic capacity across the microglial cell population.

3.4 | Both A β oligomers and protofibrils induced IL-1 β secretion

Next, the induction of pro-inflammatory cytokines in microglia was investigated. Both A β oligomers and protofibrils induced a significant increase in IL-1 β release (Figure 4a). This IL-1 β release was completely inhibited by co-incubation with the specific NLRP3 inflammasome inhibitor MCC950. The secretion of another pro-inflammatory cytokine, TNF- α , was also measured (Figure 4b), however, no significant changes in TNF- α release were detected after A β treatment in comparison to control cells. These results show that A β oligomers and

protofibrils activate the microglial NLRP3 inflammasome, resulting in IL-1 β production.

3.5 | A β -Induced activation of caspase-1

To investigate whether the A β -induced IL-1 β release occurred through activation of caspase-1, cleavage of caspase-1 to its p20 kDa subunit was analysed. A significant increase in cleaved caspase-1 secretion was detected after co-culture with A β oligomers or protofibrils (Figure 5). These results were confirmed using a fluorescent cell-permeable probe (FLICA) that covalently binds only to activated caspase-1 (Figure 6). A β oligomers or protofibrils significantly induced FLICA⁺ caspase-1 activation in microglia cells. These data demonstrate that the IL-1 β release induced by A β oligomers and protofibrils was mediated by the activation and cleavage of caspase-1.

3.6 | Both A β oligomers and protofibrils induced ASC speck formation

Oligomerization of the inflammasome adaptor protein ASC into a large 'ASC speck' complex is an essential inflammasome activation step, which is a platform for pro-caspase-1 recruitment and activation. To test whether A β oligomers or protofibrils induced ASC

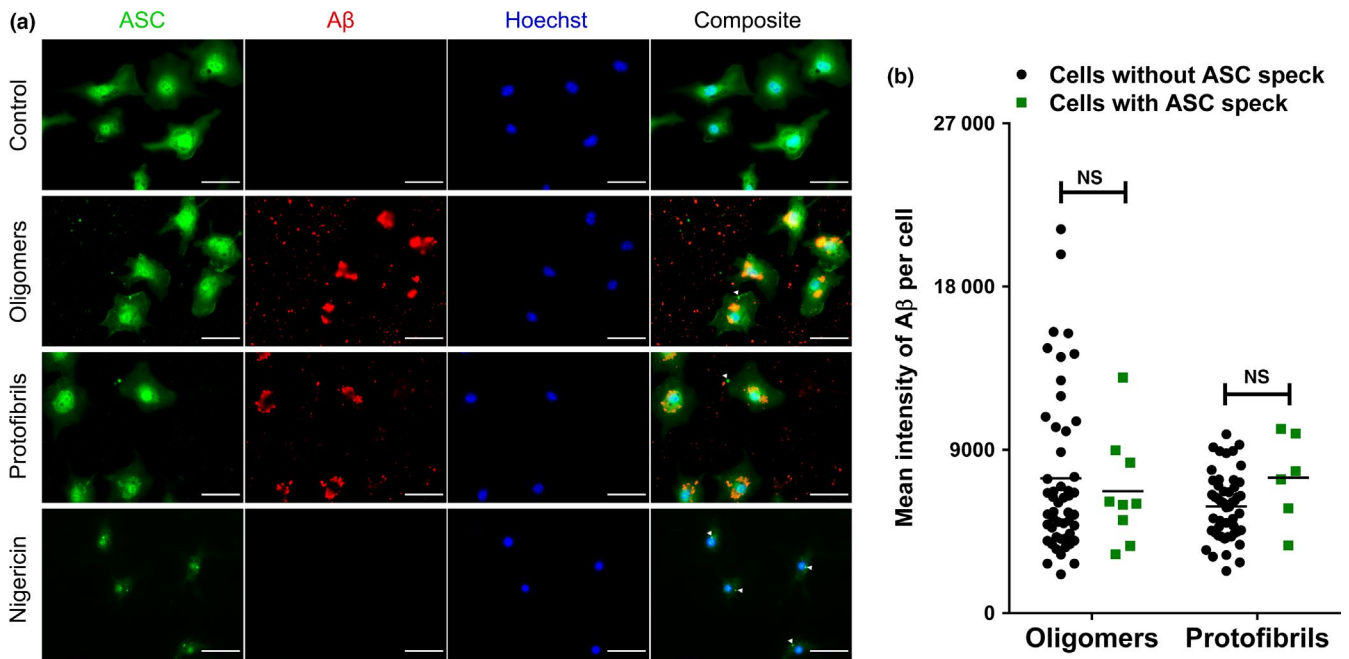


FIGURE 7 A β induced apoptosis-associated speck-like protein containing a CARD (ASC) speck formation. Microglia were primed with 50 ng/ml lipopolysaccharide and treated with A β oligomers or protofibrils for 6 hr at 2.5 μ M concentration. Nigericin (10 μ M) was used as a positive control. Cells were immunostained with anti-A β (red), anti-ASC (green) and nuclear stain Hoechst (blue). (a) Representative images of one experiment. White arrows show ASC specks. The images were taken using 60x-oil objective. The scale bars indicate 40 μ m. (b) Quantification of A β mean intensity per cell. Cells with and without ASC specks were quantified. In oligomers treatment, 59 cells were counted and 9 of these had incorporated ASC specks (15.2%); in protofibrils –6 cells out of the 57 counted had ASC specks (10.5%). There was no significant difference in A β uptake comparing ASC

speck formation in microglia, cells were immunostained with anti-ASC antibodies. Both A β oligomers and protofibrils induced ASC speck formation in 15.2% and 10.5% of cells, respectively, after 6h treatment. ASC specks were found inside the cells (Figure 7). This suggests that these A β preparations can act as potent inducers of ASC speck formation.

4 | DISCUSSION

Here we demonstrate that soluble, low molecular weight A β oligomers and protofibrils can activate the NLRP3 inflammasome in microglia without inducing a pyroptotic inflammatory cell death during the observation period. Importantly, the morphology of the oligomer and protofibril preparations used for cell stimulation were not altered during the incubation period as evidenced by AFM, therefore the NLRP3 inflammasome was indeed activated by small, soluble A β oligomers with a maximum size of 5 nm.

There are several studies that have shown both natural and synthetic A β oligomers exert neurotoxic effects in vitro (Yang et al., 2017; Yasumoto et al., 2019). However, some controversy exists on the particular morphology and length that accounts for these observations. Some studies have shown that A β 42 forms oligomers faster in comparison with other A β species and the subsequently formed A β oligomers had significantly higher cytotoxicity for neurons (Ferrera et al., 2014; Li et al., 2018). In addition, primary microglia cells internalized A β 42 protofibrils with a higher preference than A β 42 monomers (Gouwens,

Makoni, Rogers, & Nichols, 2016). In this study, we showed that small A β oligomers and protofibrils were able to activate murine microglia cells without affecting their viability. Together this suggests that the respective soluble A β species are able to induce an innate immune response prior to the deposition of A β in the so-called neuritic plaques.

We found that microglia phagocytosed both the A β oligomers and protofibrils to the same extent. Using FACS analysis, we could identify cells with a significantly higher A β content suggesting that some microglia have a higher phagocytotic activity than others. The reason for this difference in A β uptake is not yet known. It is important to note that even this phagocytic effect was independent of the A β size. In turn, an early compromise in the microglial phagocytic capacity may lead to an unfavourable increase of these A β species in the CNS, which could then aggregate further into dense A β -containing plaques over time.

Next, we investigated whether these soluble A β species were able to activate the NLRP3 inflammasome, a key element of innate immune activation in myeloid cells. One of the hallmarks of the NLRP3 inflammasome activation is the caspase-1 activity, which cleaves pro-IL-1 β to the mature and active IL-1 β . It is well known that fibrillary A β can act as a damage-associated molecular pattern and activate the NLRP3 inflammasome (Halle et al., 2008; Sheedy et al., 2013), however, whether soluble A β oligomers and protofibrils share this immunoreactive property has not yet been demonstrated. Here, we found that small A β oligomers and protofibrils induce caspase-1 activation and subsequent IL-1 β release, which was blocked by MCC950, a known NLRP3 inflammasome inhibitor (Coll et al., 2015).

Formation of the NLRP3 inflammasome and a subsequent caspase-1 activation also leads to nitric oxide formation and TNF- α release, which occurs downstream of IL-1 β release (Halle et al., 2008; Heneka, 2017). We could not detect any change in TNF- α release induced by A β oligomers or protofibrils. This is likely due to a difference in the timing of the experiments, as we assessed cytokine release after 6 hr, whereas other studies found that TNF- α was present 24 hr after A β treatment in primed cells. In addition, LPS priming already induced TNF- α release, which may have led to a ceiling effect with maximal TNF- α that could not be further augmented by subsequent exposure to A β oligomers or protofibrils within our 6 hr assay. However, recent studies revealed that A β protofibrils but not monomers are robust stimulators of TNF α release (Gouwens et al., 2016; Paranjape, Gouwens, Osborn, & Nichols, 2012).

As mentioned above, we found that A β oligomers and protofibrils induced ASC speck formation, which is an important step in NLRP3 inflammasome activation as the ASC complex is a platform for pro-caspase-1 recruitment and activation. ASC speck formation plays an important role in seeding for A β pathology where microglial-released ASC specks can bind to A β thereby increasing the peptides propensity to aggregate. ASC also had a key role in mediating APP/PS1 brain lysate-induced A β seeding, together suggesting that microglial release of ASC specks is one of the initiating mechanisms for A β deposition (Venegas et al., 2017).

This study shows that soluble A β oligomers and protofibrils are phagocytosed by microglia and can activate all important components of the NLRP3 inflammasome, including ASC speck formation and IL-1 β release. This early neuroinflammatory event can trigger further neuropathological changes and enhance progression or acceleration to AD-associated dementia.

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All experiments were conducted in compliance with the ARRIVE guidelines.

CONFLICT OF INTEREST

The other authors declare no conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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