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Research paper

Minocycline attenuates Aβ oligomers-induced pro-inflammatory phenotype in primary microglia while enhancing Aβ fibrils phagocytosis

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HIGHLIGHTS

- oAβ activates murine microglia towards a proinflammatory M1 phenotype.
- oAβ or LPS-induced microglial inflammatory response results in enhanced fAβ uptake.
- Minocycline attenuates oAβ-induced M1 polarization of murine microglia.
- Minocycline enhances microglial phagocytosis of fAβ.

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ABSTRACT

Microglia, the brain innate immune cells, are activated in response to amyloid beta (Aβ) resulting in neuroinflammation in AD brains. Recently, two phenotypes have been described for microglia: the pro-inflammatory classical and the anti-inflammatory alternative. Changes in microglia phenotype that control their phagocytic function are yet to be determined. The highly neurotoxic Aβ oligomers (oAβ) formed at an early disease stage induce pro-inflammatory microglia activation releasing neurotoxic mediators and contributing to neurodegeneration. A novel strategy for AD treatment is to attenuate microglia-induced inflammation while maintaining efficient Aβ clearance. Minocycline effectively crosses the blood-brain barrier and has widely reported neuroprotective effects. Yet, its exact mechanism of neuroprotection and its effects on microglia are still unknown. The aim of this study is to investigate the effect of minocycline on the phagocytic uptake of fAβ by primary microglia in relation to their activation state in an inflammatory milieu generated by oAβ or LPS. The study shows that minocycline is able to attenuate oAβ-induced neuroinflammatory response of microglia by inhibiting their pro-inflammatory phenotype activation. In addition, a significant enhancement of fAβ phagocytosis by minocycline- treated microglia is reported for the first time, providing novel insight into its neuroprotective role in AD.

1. Introduction

Currently available therapies are ineffective to halt the neurodegenerative process in AD urging the need for novel therapeutic strategies. Two aggregation forms of Aβ are observed in AD: the insoluble fibrils that constitute senile plaques and the more toxic soluble oligomers that are formed at an early disease stage. oAβ levels were found to be a more accurate indicator of the severity of cognitive impairment in transgenic mouse models [1,2]. Both oligomers and fibrils are potent activators of the brain's innate immune cells known as microglia [3]. They cluster in their activated state around amyloid plaques in postmortem AD brains and in transgenic mouse models [4]. Being the brain resident macrophages, microglia are able to phagocytize and degrade Aβ aggregates and represent an essential clearance mechanism of Aβ from the brain. It was found that the phagocytic activity of microglia is impaired in AD causing Aβ deposition [4]. Microglia exhibit great phenotypic and functional heterogeneity that underlie their multifaceted role in AD [5]. Recently, two distinct activation states of microglia have been defined: classical and alternative activation. Since microglia are derived from the same lineage as macrophages, the same terminology used in

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phenotyping macrophages will be used in the present study to describe the activation states of microglia; M1 being the pro-inflammatory and M2 the anti-inflammatory phenotype. Classically-activated M1 microglia release pro-inflammatory and neurotoxic molecules that contribute to neuronal loss. Alternatively-activated M2 microglia release anti-inflammatory cytokines and neurotrophic factors, that aid in central repair and regenerative processes. A novel therapeutic strategy would be to modulate microglial activity to promote their neuroprotective effects and attenuate their neurotoxic inflammatory responses. A promising candidate is the neuroprotective antibiotic minocycline, which was found to possess neuroprotective properties in various animal models of neuropathological and neurodegenerative conditions including AD [6]. Different mechanisms of neuroprotection by minocycline have been reported in CNS disorders including inhibition of poly (ADP-ribose) polymerase-1, down regulation of protein kinases, free radical scavenging and protection against oxidative stress in addition to inhibition of apoptosis, suppression of neuroinflammation and modulation of protein aggregation [7]. Although AD is characterized by an inflammatory reaction at sites of Aβ aggregation, the phagocytic clearance of these aggregates is insufficient. Therefore, it would be beneficial to find a treatment that attenuates microglial inflammatory response, while maintaining an adequate phagocytic activity. It was our interest in the current study to explore the modulatory effect of minocycline on microglia. Specifically, this study aimed at investigating minocycline's impact on microglial phagocytic response towards fAβ under *in vitro* inflammatory conditions triggered by lipopolysaccharide (LPS) or oAβ. Moreover, minocycline's effect on oAβ-mediated M1 microglial inflammatory response was investigated.

2. Materials and methods

2.1. Materials

Lyophilized Aβ1-42 peptide was purchased from R&D systems (Wiesbaden, Nordenstadt, Germany) and lyophilized HilyteFluor™ 488-labeled Aβ1-42 peptide from Anaspec (Freemont, CA, USA). 111,333-hexafluoro-2-propanol (HFIP), Hanks' balanced salt solution 10× (HBSS), LPS 0127:B8, minocycline HCl, Percoll® and TRI Reagent® were all purchased from Sigma–Aldrich (Taufkirchen, Munich, Germany). CD11b microglia microbeads (human and mouse), Magnetic Separation columns, MiniMACS™ separator and MACS MultiStand were purchased from Miltenyi Biotec (Bergisch Gladbach, Germany). Chloroform, DMSO, ethanol, EDTA and isopropanol were purchased from Carl Roth (Karlsruhe, Germany) and Trypan blue from Serva (Heidelberg, Germany). DMEM/ Ham's F12mixture (1:1), phenol red free and Dulbecco's Phosphate Buffered Saline 10× (DPBS) without calcium or magnesium were supplied by Gibco (Grand Island, NY, USA). Fetal Bovine Serum (FBS), Penicillin/Streptomycin stock and Phosphate Buffered Saline 1× (PBS) without calcium or magnesium were purchased from Lonza (Basel, Switzerland).

2.2. Animals

Adult male Swiss albino mice, 8–12 weeks old weighing 20–30 g each, were used. Mice were purchased from the Holding Company for Biological Products and Vaccines Vacsera (Agouza, Giza, Egypt). Animals were housed at 20–25 °C with alternating 12 h dark/light cycles and were allowed free access to water and standard food. The study was approved by the Ethics Committee of the German University in Cairo and is in accordance with the recommendations of the Guide for Care and Use of Laboratory Animals, 8th edition (Washington (DC): National Academies Press (US); 2011).

2.3. Preparation of amyloid beta oligomers

Aβ1-42 peptide was used to prepare oAβ as previously described [8]. Briefly, the peptide was dissolved in cold HFIP at a concentration of 1 mM. After aliquoting and drying, resulting peptide films were stored at −20 °C. For oligomer preparation, dried peptide films were redissolved in DMSO at a concentration of 5 mM. The resulting solution was further diluted with DMEM/Ham's F12 nutrient mixture (1:1) to a final concentration of 100 μM and incubated for 24 h at 4 °C. Prepared oligomers were subsequently characterized through Western and dot blot analyses.

2.4. Preparation of fluorescent amyloid beta fibrils

Fluorescent fAβ were prepared from Hilyte 488-labeled Aβ1-42 peptide according to the manufacturer's instructions. The peptide was resuspended in 1% NH4OH to a final concentration of 2 mg/mL. The solution was diluted with PBS to a final concentration of 1.13 mg/mL, aliquoted and stored at −20 °C. For fibril formation, the peptide solution was stored at 37 °C for 1 week. fAβ were characterized through Western blotting.

2.5. Characterization of Aβ aggregation forms

2.5.1. Western blot analysis

1 μg of oligomeric or fibrillar preparation was resolved on 15% or 12% native polyacrylamide gel respectively and transferred to nitrocellulose membrane using the semi-dry transfer method. The membrane was blocked, incubated with primary mouse anti-Aβ 6E10 antibody, washed and then incubated with secondary goat anti-mouse HRP-conjugated IgG antibody. Bands were developed using the TMB membrane peroxidase substrate system.

2.5.2. Dot blot analysis

5 μg of the oligomers preparation were incubated with either anti-Aβ 6E10 antibody or anti-oligomer A11 antibody. The membrane was then washed and incubated with the secondary antibody either goat anti-mouse IgG antibody or goat anti-rabbit respectively. The membrane was washed and developed using the TMB membrane peroxidase substrate system.

2.6. Primary mouse microglia isolation and culture

2.6.1. Tissue preparation and mechanical dissociation

Microglia were isolated from mice brains as previously described [9] with some modifications. Briefly, mice were sacrificed by cervical dislocation and brains were obtained. The cerebellum was removed and cerebral cortices were mechanically homogenized in ice-cold 1× HBSS using Dounce homogenizer. The homogenate was triturated through glass Pasteur pipettes of decreasing diameters, and then filtered through 70 μm cell strainer to remove myelin and tissue debris. The resulting cell suspension was centrifuged at 1000 × *g* for 10 min at 4 °C and supernatant was discarded.

2.6.2. Density gradient separation

For microglia isolation, cell pellets were subjected to Percoll density gradient centrifugation. Stock isotonic Percoll (SIP) solution was prepared by mixing 9 parts of Percoll with 1 part of 10× DPBS. 25% and 75% Percoll solutions were prepared by diluting SIP solutions with 1× PBS. Cell pellets were resuspended in 75% Percoll, carefully overlayered with 25% Percoll followed by a layer of

1X PBS. After centrifugation at 800 × *g* for 25 min at 4 °C, the 25/75 interphase was carefully collected and diluted with ice-cold 1X PBS.

2.6.3. Immunomagnetic cell separation

Cell suspensions were pelleted at 1000 × *g* for 10 min at 4 °C and pellets were resuspended in 90 μL microbeads buffer (PBS containing 0.5% FBS and 2 mM EDTA, pH 7.2) and 10 μL CD11b microbeads were added. After incubation for 15–20 min at 4 °C, cells were washed with microbeads buffer and pelleted at 300 × *g* for 10 min at 4 °C. Pellets were resuspended in 500 μL microbeads buffer and applied onto magnetic columns. Effluents were discarded, columns were washed and then removed from the magnetic field and labeled microglia were eluted in 1 mL DMEM/F12 with 10% FBS and 1% PeniStrep. Viability of the isolated cells was checked and cells were seeded for further experiments.

2.7. Fluorescent Aβ fibrils phagocytosis assay

Fluorescent fAβ phagocytosis assay was adopted from the work of Pan et al. [10]. On day 1, microglia were isolated and seeded in DMEM/F12 with 10% FBS and 1% PeniStrep on glass cell culture inserts in 24-well plates at a density of 10^5 cells per well. On day 2, medium was changed to serum-free DMEM/F12 and cells were stimulated with either LPS (100 ng/mL) or oAβ (1 μM) in the presence or absence of minocycline (20 μM) for 24 h. On day 3, cells were treated with Hilyte 488 labeled fAβ (5 μM) for 30 min. After several washing steps with PBS, cells were fixed in 4% paraformaldehyde, glass inserts were mounted on microscopic slides and examined under AxioStar Plus fluorescence microscope (Zeiss, Germany). Images were taken using AxioCam ERc5s camera (Zeiss, Germany). 20–25 different fields were examined per sample. Up to 10 cells were randomly selected per field and the fluorescence intensity was individually measured for each cell. Fluorescence values were corrected by subtracting the background fluorescence. fAβ uptake was measured as the mean fluorescence intensity per cell for each sample. Image analysis and fluorescence measurements were performed using ZEN 2012 software package (Zeiss, Germany).

2.8. Quantification of CD86 gene expression

mRNA expression level of the pro-inflammatory marker CD86 was quantified using reverse transcription quantitative real-time PCR. On day 1, primary microglia were and seeded in a 24-well plate in DMEM/F12 with 10% FBS and 1% PeniStrep at a density of 3 million cells per well. On day 2, medium was changed to serum-free DMEM/F12 and cells were treated with oAβ (1 μM) in the presence or absence of minocycline (20 μM) for 24 h. On day 3, total RNA was extracted using TRI Reagent (Sigma–Aldrich, USA) and cDNA was synthesized using Maxima first strand cDNA synthesis kit for RT-qPCR (Thermo Scientific, USA) according to the manufacturer's instructions. RT-qPCR was performed using TaqMan® gene expression assays (Applied Biosystems, USA) for mouse CD86 as a marker gene for M1 activation and mouse β-actin as a reference gene. Template cDNA from each sample was amplified using Premix Ex Taq™ (Probe qPCR) kit (Takara, Japan). Thermocycling and fluorescence detection were performed using Mx3005P qPCR system (Agilent technologies, USA). Data acquisition and analysis was carried out using MxPro QPCR software (Agilent technologies, USA). The mean fold change in CD86 expression for each treatment group was expressed as $2^{-\Delta\Delta CT}$.

2.9. Statistical analysis of data

Data analysis was performed using GraphPad Prism 5 software (GraphPad Software Inc., CA, USA). In the phagocytosis assay, data were expressed as mean fluorescence intensity of internalized fAβ per cell ± standard error of mean (SEM) and analyzed using one-way ANOVA with Tukey's post-test multiple comparison. Values are representative of four independent experiments ($n = 4$). In gene expression experiments, data were expressed as the mean fold change in CD86 expression relative to β-actin expression as a reference gene and analyzed using Student's t-test. Data is presented as mean ± SEM for three independent experiments ($n = 3$).

3. Results

3.1. Characterization of Aβ oligomers and fibrils

A band of oligomers around 30 kDa and a smear of high molecular weight oligomers (>95 kDa) were detected. Oligomers were 6E10-positive and A11-negative, which shows their fibrillar nature. Fluorescent fAβ1-42 had a size above 315 kDa (data not shown).

3.2. Effect of Aβ oligomers, LPS and minocycline on microglial phagocytosis of Aβ fibrils

LPS treatment (17.79 ± 0.6013, $n = 4$) resulted in a highly significant increase of about 61% ($p < 0.001$) in microglial phagocytosis of fAβ relative to untreated controls (11.05 ± 0.2864, $n = 4$). Similarly, treatment with oAβ (13.58 ± 0.3128, $n = 4$) resulted in a significant increase of around 23% in fAβ uptake compared to control ($p < 0.05$). However, the fAβ uptake in the LPS group was significantly greater than oAβ group ($p < 0.001$). Minocycline treatment (16.25 ± 0.4714, $n = 4$) significantly increased fAβ uptake by 39% with respect to control ($p < 0.001$). Treatment with minocycline in combination with LPS (15.37 ± 0.3529, $n = 4$) attenuated LPS-induced increase in phagocytic activity by 14%, however, the phagocytic activity under this combination was still significantly higher than control. Combined treatment of minocycline with oAβ (17.18 ± 0.3835, $n = 4$) caused a 27% increase in fAβ phagocytosis relative to oAβ alone ($p < 0.001$) and a 55% increase relative to control ($p < 0.001$). Thus, fAβ uptake was significantly higher in the three different minocycline treatment groups: minocycline alone, minocycline/oAβ and minocycline/LPS compared to control ($p < 0.001$). Nonetheless, there was no statistical difference in fAβ uptake between the three groups (Fig. 1).

3.3. Effect of Aβ oligomers and minocycline on M1 microglial activation

The effect of oAβ on M1 microglial activation was assessed in the presence or absence of minocycline by measuring the gene expression level CD86. Results are represented as the mean fold change in CD86 expression ± SEM for three independent experiments ($n = 3$). Treatment with oAβ (2.338 ± 0.3345, $n = 3$) caused significant upregulation of CD86 mRNA expression ($p = 0.0497$) relative to control (1.094 ± 0.2965, $n = 3$). Co-treatment with oAβ and minocycline (1.220 ± 0.2607, $n = 3$) resulted in a remarkable decrease in CD86 expression relative to oAβ alone ($p = 0.05$) and showed no significant difference compared to control ($p = 0.7649$). Minocycline treatment alone (1.423 ± 0.3437, $n = 3$) had no effect compared to control ($p = 0.5090$) (Fig. 2).

4. Discussion

Minocycline has demonstrated neuroprotective effects in animal models of neurodegeneration owing to its antiapoptotic, anti-inflammatory and inhibitory action on protein aggregation [7]. The current study has explored the modulatory effect of minocycline on primary murine microglia by testing its effect on their

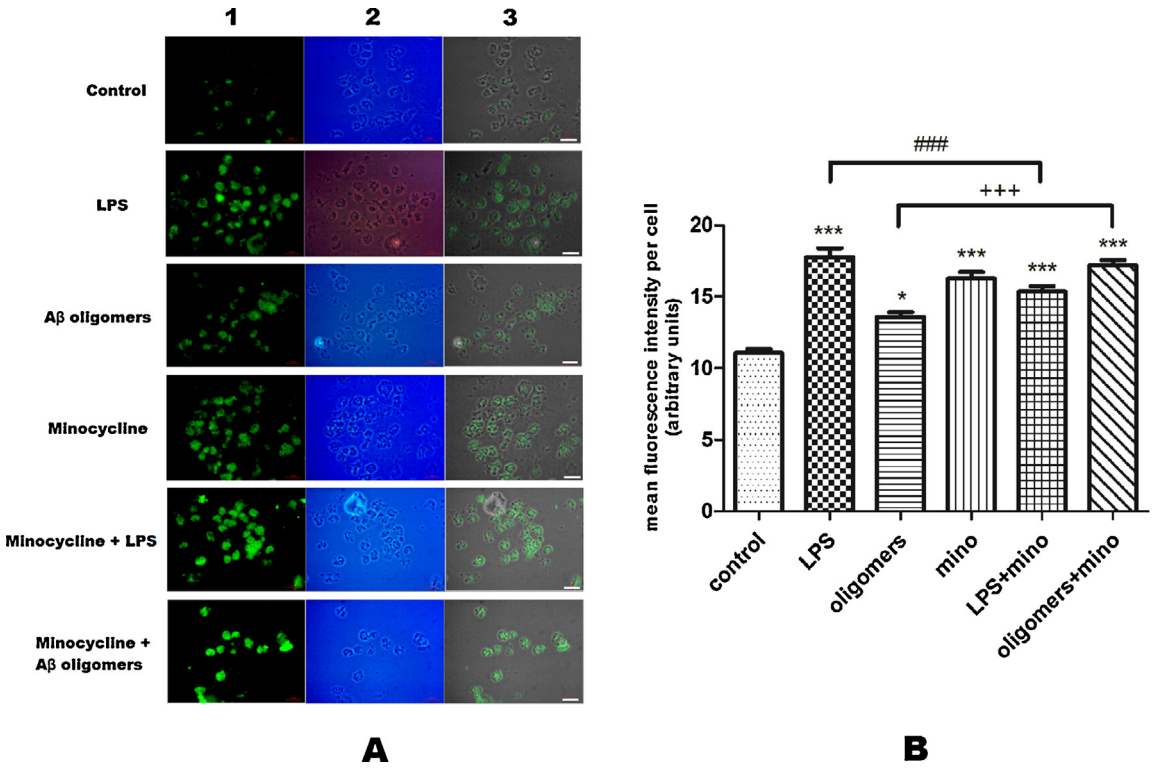

Fig. 1. Effect of LPS, oAβ and minocycline on microglial phagocytosis of fAβ. Microglia were pretreated with LPS (100 ng/mL), oAβ (1 μM), minocycline (20 μM), a combination minocycline/LPS or minocycline/oAβ for 24 *h* with one untreated control group. Cells were treated with fluorescent fAβ1-42 fibrils (5 μM) for 30 min and fixed with 4% formaldehyde. (A-1) Images of fluorescent fAβ aggregates emitting green fluorescence. (A-2) Phase contrast images (1000×, ph3). (A-3) Merged images show microglia with engulfed fluorescent fAβ. Bar, 10 μm. (B) fAβ uptake was quantified as mean fluorescence intensity per cell (arbitrary units). One-way ANOVA with Tukey's multiple comparison test was performed and data are expressed as mean ± SEM for four independent experiments ($n = 4$). $^{*}p < 0.05$ or $^{***}p < 0.001$ with respect to control group, $^{\#\#\#}p < 0.001$ with respect to LPS group and $^{+++}p < 0.001$ with respect to oAβ group.

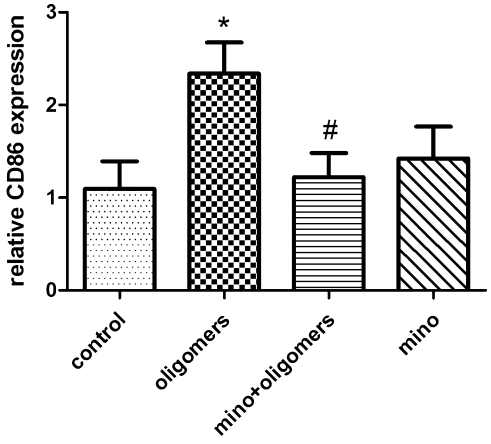

Fig. 2. Effect of oAβ and minocycline on M1 microglial activation. Microglia were treated with oAβ (1 μM), minocycline (20 μM) or a combination of both for 24 h with one untreated control group. mRNA expression of CD86 was analyzed by RT-qPCR. CD86 expression level was normalized to β-actin. Data are expressed as the mean fold change ± SEM for three independent experiments ($n = 3$). Data for each two treatment groups was analyzed by unpaired Student's t test. $^{*}p = 0.0496$ with respect to control and $^{\#}p = 0.05$ with respect to oAβ.

fAβ phagocytic capability and pro-inflammatory phenotype. In an attempt to simulate the *in vivo* inflammatory milieu present in early stages of AD, microglia freshly isolated from adult mice brains were stimulated with the highly neurotoxic oAβ and LPS and their ability to phagocytize fAβ was tested. Both treatments resulted in a significant increase in uptake of fluorescent fAβ. The significant increase in microglial phagocytosis induced by LPS can be related to the activation of TLR4-CD14 signaling, which is believed to play a key role in Aβ uptake by microglia. Activation of its downstream target p38 MAPK was shown to induce the expression of scavenger receptors and enhance phagocytosis of bacteria by macrophages [11]. Moreover, a direct molecular interaction between Aβ and CD14 was previously detected and CD14 deletion in microglia decreased Aβ uptake *in vitro* [12]. In agreement with the findings of the present study, Tahara et al. proved that activation of TLR2, 4 and 9 with receptor specific ligands resulted in significant increase in Aβ uptake as measured by Aβ1-42 specific sandwich ELISA [13]. In the same study, TLR4 mutation in transgenic AD mice increased both diffuse and fAβ deposits as well as levels of soluble and insoluble Aβ compared to TLR4 wild type mice. Similarly, a recent study showed that *in vitro* stimulation of BV2 cells with two TLR4 agonists namely LPS and monophosphoryl lipid A at a concentration of 1 μg/mL significantly increased the phagocytosis of fluorescent oAβ as detected by flow cytometry [14]. Moreover, intrahippocampal injection of LPS in APP/PS1 transgenic mice reduced Aβ load considerably as compared to saline injection [15]. In contrast to our study, Pan et al. [10] reported that treatment of with LPS (1 μg/mL) or oAβ (1 μM) for 12 h significantly suppressed phagocytosis of fluorescent fibrils. These opposing findings can be attributed to using different cell models. While Pan et al. used either immortalized murine BV2 cell line or primary microglia isolated from postnatal day 1C57BL/6 mice, primary microglia isolated from adult Swiss Albino mice were used in the present study. It has been shown that adult and postnatal microglia respond differently to fAβ where postnatal cells are more efficient at phagocytizing fibrils [16]. The study at hand is the first to explore the direct *in vitro* effect of minocycline on microglial uptake of fluorescent fibrils either alone or in the presence of LPS or oAβ. An important finding is that minocycline appears to directly enhance microglial phagocytosis

of fibrils. The increase in fAβ uptake was consistently significant in all minocycline treatment groups when compared to control group. Interestingly, co-treatment with minocycline and oAβ further enhanced the phagocytic response compared to oligomers alone. These findings suggest that minocycline treatment at early disease stages, characterized by the presence of oligomers, can promote fAβ clearance potentially preventing plaque development. This hypothesis is further supported by the recent work of Ferretti and colleagues [17] who reported a beneficial role of minocycline in preventing and delaying AD neuropathology at early stages in transgenic mice. Contrary to LPS and oligomeric Aβ, such increase in phagocytic activity achieved by minocycline is not accompanied by unwanted inflammation owing to its wide spectrum of anti-inflammatory actions. A recent study showed that aged human brains develop robust pro-inflammatory microglial activation compared to relatively modest further changes in AD patients. They highlighted the critical early involvement of inflammation even in the preclinical stages of AD. These data suggest that an important strategy to maintain cognitive health involves reducing chronic innate immune activation that should be initiated early in the aging process [18]. For this purpose, relative young adults rather than aged mice were used in the current study, especially that microglia from aged mice were shown to lose their ability to phagocytize fAβ when compared to younger mice [19]. Although LPS-induced phagocytic response was significantly weaker in the presence of minocycline than in its absence, it was consistently higher than the control group. This implies that although minocycline inhibits LPS-induced microglial activation [20], [21], the phagocytic function of the cells was not compromised. This observation is supported by Malm et al. [22] who reported that minocycline does not inhibit LPS-induced clearance of hippocampal Aβ deposits following intrahippocampal LPS injection in a transgenic mouse model of AD. Unlike the potent anti-inflammatory steroid dexamethasone, minocycline does not inhibit clearance of amyloid plaques following intracranial injection of anti-Aβ antibodies in AD transgenic mice [23]. Moreover, minocycline was found to downregulate the production of pro-inflammatory cytokines by human monocytes and microglia without affecting their beneficial phagocytosis of fAβ as measured by flow cytometry [24]. Minocycline can significantly inhibit inflammatory reactions of microglial cells both *in vitro* and *in vivo* [6]. In their remarkable study, Ferretti et al. [17] showed that 1-month administration of minocycline to young APP transgenic mice prior to plaque formation downregulated the expression of several inflammatory markers that accompany M1 activation such as the inducible nitric oxide synthase (iNOS), COX2 and IL-1β. In other studies done on transgenic mouse models, the inhibition of neuroinflammatory reactions in mice brains by minocycline's administration was accompanied by marked improvement in their cognitive functions [25–27]. Interestingly, the anti-inflammatory effect of minocycline was specific to the hippocampus and the cortex, regions with high burden of intracellular oAβ [17]. This indicates that early signs of M1 microglial activation detected in the hippocampus and cortex are attributed to the formation of oAβ in these two areas at early stages of the disease. In another study, Kobayashi et al. [20] minocycline treatment significantly attenuated LPS induction of M1 markers in primary cultured mouse microglia. As previously depicted, the present study assessed M1 phenotype activation of microglia by measuring the expression level of the pro-inflammatory marker CD86. It was shown that CD86 upregulation in microglia treated with oAβ was significantly attenuated upon co-treatment with minocycline (Fig. 2). This confirms minocycline's inhibitory effect on oAβ-induced M1 polarization of primary murine microglia *in vitro* which agrees well with the aforementioned studies. Accordingly, it is suggested that early minocycline administration can potentially protect against pre-plaque neuroinflammatory changes and slow down disease progression most likely by inhibiting oAβ-induced M1 microglia activation. Currently, clinical trials are being conducted to determine the efficacy of minocycline in patients with very early stages of AD. Since the current study was conducted on mouse microglia in vitro, the effect of minocycline on microglial phagocytic activity needs further investigation in human brain. Moreover, further investigation is warranted to understand the mechanisms by which minocycline promotes the phagocytic activity of microglia. In summary, our study reveals a dual role of minocycline in AD where it suppresses microglia-mediated neuroinflammation and promotes their phagocytic clearance of Aβ. This provides novel insights into the neuroprotective mechanism of minocycline in neurodegenerative and neuroinflammatory conditions.

5. Conclusion

Recently, minocycline has received great attention because of its widely reported neuroprotective effects. The current study has addressed novel aspects of minocycline's neuroprotection by investigating its role as a modulator of microglial phagocytosis and classical microglial activation. Minocycline was shown to inhibit the pro-inflammatory phenotype of microglia challenged with oAβ. Moreover, minocycline was found for the first time to enhance phagocytosis of fAβ by untreated microglia as well as microglia treated with LPS or oAβ. Taken together, these results indicate that minocycline is an effective neuroprotective agent by inhibiting the pro-inflammatory phenotype of microglia, while enhancing their ability to clear fAβ deposits.

Competing interests

The authors declare that they have no conflict of interest.

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