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CD47 does not mediate amyloid-β(1–42) protofibril-stimulated microglial cytokine release

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ABSTRACT

Neuroinflammation triggered by accumulation of amyloid-β protein (Aβ) is a significant component of the Alzheimer's disease (AD) brain. Senile plaques composed of Aβ attract and activate microglia cells resulting in cytokine secretion and a proinflammatory environment. The mechanism by which Aβ activates microglia is complex and involves numerous cellular components. One receptor potentially involved in Aβ recognition and the ensuing microglia proinflammatory response is CD47. Since there is significant interest in soluble aggregated Aβ species, we sought to determine if CD47 plays a key role in microglia cytokine release stimulated by soluble Aβ(1–42) protofibrils. Pretreatment of primary murine microglia with the CD47 antagonist peptide 4N1K significantly and potently inhibited both tumor necrosis factor-α (TNFα) and interleukin-1β (IL-1β) secretion stimulated by Aβ(1–42) protofibrils. 4N1K displayed toxicity to the microglia but only at concentrations much higher than the observed inhibition. Surprisingly, 4N1K also potently inhibited TNFα secretion triggered by lipopolysaccharide which is not known to signal through CD47. Treatment of the microglia with a neutralizing anti-CD47 antibody failed to block the Aβ protofibril response even though comparable samples were completely inhibited by 4N1K. Finally, Aβ(1–42) protofibrils stimulated similar levels of secreted TNFα production in both wild-type and $CD47^{-/-}$ microglia and 4N1K still potently inhibited the Aβ protofibril response even in the $CD47^{-/-}$ microglia. The overall findings demonstrated that the microglial proinflammatory response to Aβ(1–42) protofibril is not dependent on CD47 and that 4N1K exhibits CD47-independent inhibitory activity.

1. Introduction

Alzheimer's disease (AD) is a neurodegenerative disease triggered and compounded by aberrant protein aggregation. The clinical manifestations at the later stages include severe impairment of memory, language and higher cognitive function [1]. The histopathological signature of AD includes extraneuronal senile plaques composed primarily of fibrillar amyloid-β protein (Aβ) and intraneuronal neurofibrillary tangles (NFTs) formed by the protein tau [2]. Several studies indicate that Aβ aggregation and accumulation occurs prior to, and has a bearing on, NFT formation [3–5]. Much of the evidence suggests that Aβ aggregation is the initiator of AD while tau aggregation propels the clinical symptoms prevailing in the later stages of AD [6]. While the senile plaques are a prominent pathological feature, the recent research emphasis has shifted towards soluble oligomeric forms of Aβ as a result of their increased neurotoxicity compared to insoluble fibrillar Aβ and their effects on synaptic function [7,8].

Aβ is derived from proteolytic cleavage of the amyloid precursor protein (APP) [9]. The C-terminal cleavage site is variable resulting in the production of peptide fragments of differing length. The two most commonly-studied fragments are Aβ(1–40) and Aβ(1–42) (reviewed in [10,11]). In vitro studies show that Aβ can undergo nucleation-dependent aggregation, whereupon unstructured monomers self-assemble non-covalently into a variety of β-sheet-rich soluble oligomeric species [12–16]. Many of these soluble species ultimately progress to insoluble fibrils [17,18]. Protofibrils are a well-characterized member of this growing group of soluble Aβ aggregates and may be an important AD therapeutic target [19]. Aβ(1–42) is much more prone to aggregation [12]

Abbreviations: AD, Alzheimer's disease; Aβ, amyloid-β protein; aCSF, artificial cerebrospinal fluid; HFIP, hexafluoroisopropanol; IL-1β, interleukin-1β; SEC, size exclusion chromatography; ThT, thioflavin T; TNFα, tumor necrosis factor α.

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and has the ability to form a more morphologically diverse range of aggregated species than Aβ(1–40) [15].

Neuroinflammation is another feature of the AD brain and is propagated by glial cells. Microglia are commonly observed clustering around Aβ plaques and their activation in this environment causes the release of proinflammatory cytokines such as tumor necrosis factor-α (TNF-α) and interleukin (IL)-1β [20–22]. The ensuing inflammatory response to Aβ and the secreted cytokines likely contribute to neurodegeneration [23,24]. Microglial activation by Aβ has been recapitulated in cell culture many times, yet the mechanisms by which Aβ initially triggers a microglial inflammatory response are not completely understood. Multiple glial cell surface receptors and accessory proteins have been shown to mediate Aβ-induced proinflammatory events including Toll-like receptors (TLRs) 2, 4, and 6, CD14, CD36, CD47, and $\alpha_6\beta_1$ integrin (reviewed in [25]). Many of these studies have used TNFα and IL-1β secretion as a key measure of the proinflammatory response.

CD47 is an integrin-associated transmembrane protein expressed in a variety of cells types including those involved in innate immunity. CD47, along with ligands thrombospondin-1 (TSP-1) and SIRPα, is involved in numerous physiological and pathological processes including cell spreading, platelet activation, chemotaxis, adhesion, extracellular matrix organization, signaling, survival, proliferation, apoptosis, migration and ischemia–reperfusion injury (reviewed in [26–28]). A potential role for CD47 in AD has also been identified with studies reporting CD47 participation in the microglial proinflammatory response to, and uptake of, fibrillar Aβ [29–31]. Many of these studies have utilized the 4N1K peptide to presumably ligate CD47 and inhibit CD47-mediated processes, which include cytokine production in macrophages and dendritic cells (reviewed in [26]), and Aβ-microglia interactions [29–31]. 4N1K is derived from the carboxyl terminal domain of the natural CD47 ligand TSP-1, which acts as antagonist for CD47 receptor interaction with other ligands. Recently, our lab has demonstrated that Aβ(1–42) protofibrils, soluble precursors to fibrils, are robust stimulators of microglia [32]. In this study we investigated the possible role of CD47 receptor in Aβ(1–42) protofibril-induced cytokine production. Based on the findings from multiple techniques, we report that the microglial proinflammatory response to Aβ(1–42) protofibril is not dependent on CD47 and that caution should be used when interpreting inhibition results from the TSP-1-derived peptide, 4N1K.

2. Methods

2.1. Preparation of Aβ peptides

Aβ(1–42) was obtained from W.M. Keck Biotechnology Resource Laboratory (Yale School of Medicine, New Haven, CT) in lyophilized form and dissolved in 100% hexafluoroisopropanol (HFIP) at 1 mM. The solution was then divided into 1 mg aliquots in sterile microcentrifuge tubes and evaporated uncovered at room temperature overnight in a fume hood. The following day, aliquots were vacuum-centrifuged to remove any residual HFIP and stored in dessicant at −20 °C.

2.2. Size exclusion chromatography

Preparation and isolation of Aβ(1–42) protofibrils was done according to a previously published protocol [33]. Briefly, a stored aliquot of Aβ(1–42) was dissolved in 50 mM NaOH to yield 2.5 mM Aβ. The solution was further diluted to 250 μM Aβ in prefiltered artificial cerebrospinal fluid (aCSF, 15 mM $NaHCO_3$, 1 mM Na_2HPO_4, 130 mM NaCl, 3 mM KCl, pH 7.8). The solution was centrifuged at 18,000*g* for 10 min and the supernatant fractionated on a Superdex 75 10/300 GL column (GE Healthcare) using an AKTA FPLC system (GE Healthcare). The column was precoated with sterile bovine serum albumin to prevent any non-specific binding of Aβ to the column matrix. Aβ was eluted at 0.5 mL min^{-1} in aCSF and 0.5 mL fractions were collected and immediately placed on ice. Aβ(1–42) concentrations were determined in-line by UV absorbance using an extinction coefficient of 1450 cm^{-1} M^{-1} at 280 nm.

2.3. Primary microglia isolation

Primary murine microglia were obtained from wild-type (WT) C57BL/6 (Harlan Laboratories) or $CD47^{-/-}$ mice purchased from, and characterized by, Jackson Laboratories (C57BL/6 background strain). Microglia isolation was performed as previously described [32] from 3 to 4 day old mouse pups. Cells were cultured in complete DMEM at 37 °C in 5% CO_2 until confluent (1–2 weeks) and microglia were selectively harvested from the adherent astrocyte layer by overnight shaking of the flask at 37 °C in 5% CO_2 and collection of the medium. The flasks were replenished with fresh medium, and incubated further to obtain additional microglia.

2.4. Cell stimulation assay

For cellular studies, WT and $CD47^{-/-}$ primary murine microglia were collected as described above and seeded in a sterile 96-well cell culture plate for 24 h at a density of 5×10^5 cells/mL in complete DMEM without granulocyte–macrophage colony-stimulating factor (GM-CSF). Prior to cell stimulation, the medium was replaced with complete DMEM without GM-CSF or serum. Cells were then treated with Aβ(1–42) protofibrils (15 μM) or lipopolysaccharide (LPS, 10 ng/mL, InvivoGen). The cells were incubated at 37 °C in 5% CO_2 for selected time periods. The conditioned medium after incubation was collected and stored at −20 °C for subsequent analysis by enzyme-linked immunosorbent assay (ELISA). For 4N1K inhibition studies, the 4N1K peptide (AnaSpec, Fremont, CA) was incubated with the microglia for 1 h prior to stimulation of the cells.

2.5. ELISA

Quantification of murine cytokines TNFα and IL-1β were determined by ELISA as previously described [32,34]. A standard curve of 15–16,000 pg/mL TNFα or IL-1β was constructed for each ELISA. When necessary, samples were diluted to fall within the standard curve.

2.6. Antibody neutralization assay

This procedure followed a previously described protocol [34] with little modification. Primary murine microglia were treated with either PBS, functional-grade neutralizing anti-mouse CD47 antibody (5 or 10 μg/mL), or functional-grade mouse IgG2a isotype control (eBioscience, San Diego, CA) for 1 h at 37 °C in 5% CO_2. After antibody pretreatment, the medium was replaced and SEC-purified Aβ(1–42) protofibrils were added to the wells and incubated for an additional 6 h. In some cases, the medium was not replaced prior to Aβ protofibril addition.

2.7. XTT cell viability assay

Mitochondria-mediated reduction of XTT [2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide] (Sigma Aldrich, St. Louis, MO) was used for determining viability of primary murine microglial cells as previously described [32].

2.8. Statistical analysis

Statistical analysis was performed for selected experiments to determine the confidence limit at which two measurements were statistically different. A one-tailed Student *t*-test was applied to each data set and *p*-values were obtained. Statistical differences with a *p*-value <0.05 were considered significant.

3. Results

3.1. 4N1K peptide inhibition of Aβ(1–42) protofibril-induced proinflammatory response

Aβ(1–42) protofibrils were prepared and isolated by SEC in a modified aCSF buffering system as previously described [33]. This protocol yields an enriched Aβ protofibril pool populated with curvilinear structures less than 100 nm in length, hydrodynamic radii (R_H) ranging from 10 to 40 nm, and significant thioflavin T fluorescence, albeit less than equimolar solutions of fibrils [33]. 4N1K was used in the current study to investigate the involvement of CD47 in Aβ(1–42) protofibril activation of primary murine microglia. Microglia were pre-treated with either 4N1K (100 μM) or buffer control for 1 h prior to treatment with Aβ(1–42) protofibrils (15 μM) for 6 h. Aβ protofibrils stimulated significant levels of secreted tumor necrosis factor α (TNFα) (Fig. 1A) and interleukin 1-β (IL-1β) (Fig. 1B) compared to buffer controls in the absence of 4N1K. However, the secreted TNFα and IL-1β response to Aβ(1–42) protofibrils was almost completely abolished in microglia pretreated with 4N1K. The inhibition by 4N1K suggested a role for CD47 in mediating Aβ protofibril-induced proinflammatory events.

LPS is a well-characterized proinflammatory molecule and its detection and signal transduction through the membrane is initially mediated by Toll-like receptor 4 (TLR4). The intracellular inflammatory pathway involves activation and translocation of NF-κB to the nucleus (reviewed in [35]). Despite the complexity of the pathway, there have been no reports of a role for CD47 in LPS-induced cytokine production. In the current study, the microglial LPS response was planned as a negative control for 4N1K. Surprisingly, 4N1K inhibited LPS-induced microglial TNFα secretion in a dose-dependent manner (Fig. 1C). Curve-fitting of the data produced an IC_{50} value of 0.52 μM. 4N1K also dose-dependently inhibited Aβ(1–42) protofibril-induced TNFα secretion (IC_{50} 2.5 μM). The data confirmed that 4N1K was an effective inhibitor of the Aβ protofibril-triggered proinflammatory response, but the LPS inhibition observation raised the possibility that 4N1K may exert its effects via other CD47-independent mechanisms. These findings lessened the certainty that CD47 was a mediator of Aβ protofibril-stimulated proinflammatory pathways. Based on a new report indicating that 4N1K may exert non-specific effects via IgG antibody binding [36], we tested our cytokine ELISAs in the absence and presence of 4N1K and found no change in the standard curves (data not shown).

In order to rule out the possibility that 4N1K inhibition was the result of toxicity to the microglia by the peptide alone or together with the protofibrils, an XTT cell viability assay was employed. In both the cases, no toxicity was observed by 4N1K up to 100 μM (Fig. 2). However, higher 4N1K concentrations significantly affected microglia viability. The cell viability results indicated that the 4N1K inhibition of Aβ protofibril-induced cytokine secretion was not due to toxicity to the microglia by 4N1K.

3.2. CD47 antibody neutralization studies

To further assess the role of CD47 in the Aβ(1–42) protofibril-mediated microglial TNFα response, we used an anti-mouse CD47 neutralizing antibody in an effort to disrupt a possible protofibril-CD47 receptor interaction. We have used this technique previously to determine the involvement of Toll-like receptors and CD14 in Aβ-stimulated monocyte activation [34]. Aβ(1–42) protofibrils stimulated significant secreted TNFα from primary microglia compared to aCSF controls (Fig. 3). Pretreatment of the microglia with 5 μg/mL anti-CD47 antibody, or the IgG2a negative control, had no effect on the protofibril response (Fig. 3A). An increase in the concentration of antibody used for pretreatment (10 μg/mL) did not alter the results (Fig. 3B). No difference in secreted TNFα was observed between protofibril-treated microglia and those pretreated with 10 μg/mL anti-CD47 antibody or IgG2a isotype control. In these experiments, the medium containing the antibody was replaced 1 h with fresh medium prior to the addition of Aβ protofibrils. Additional experiments leaving the medium (and antibody) in place did not change the results (data not shown).

Additional antibody neutralization experiments were carried out in parallel with 4N1K peptide treatments. Again, the anti-CD47 antibody (10 μg/mL) or IgG2a isotype control did not attenuate the Aβ(1–42) protofibril-induced TNFα (Fig. 3C) or IL-1β (Fig. 3D) secretion. However, pretreatment with 4N1K peptide (100 μM) significantly reduced the protofibril-stimulated TNFα and IL-1β response (Fig. 3C and D). These results supported the idea that microglia proinflammatory pathways triggered by Aβ protofibrils, and leading to TNFα and IL-1β secretion, may not be mediated by CD47. Furthermore, 4N1K again appeared to inhibit Aβ protofibril-induced cytokine production in a CD47-independent manner.

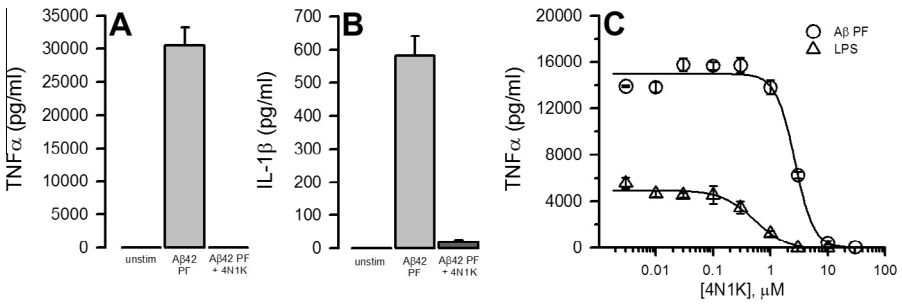

Fig. 1. Inhibition of Aβ(1–42) protofibril-induced TNFα and IL-1β secretion by 4N1K. Primary murine microglia were pretreated with 4N1K peptide (100 μM in Panels A and B; 0.01–100 μM in Panel C) or vehicle control in serum-free medium for 1 h followed by incubation with SEC-isolated Aβ(1–42) protofibrils (15 μM), LPS (10 ng/mL), or vehicle controls (unstimulated; aCSF or H_2O) for an additional 6 h as described in Section 2. The conditioned medium was removed and secreted TNFα (Panels A, C) and IL-1β (Panel B) protein levels were measured by ELISA. Data are expressed as the mean ± std error of triplicates for each concentration of 4N1K. Curves in Panel C were obtained by fitting the data to the following equation, $y = \min + [(\max - \min)/1 + (x/IC_{50})\ \text{slope}]$ using SigmaPlot 10.0.

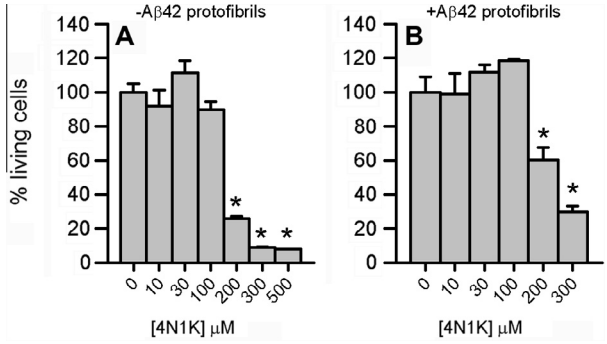

Fig. 2. Toxicity of 4N1K peptide to primary murine microglia. Primary microglia plated in a 96-well sterile plate were incubated with increasing concentrations of 4N1K for 1 h at 37 °C in 5% CO_2 followed by addition of aCSF (Panel A) or SEC-isolated Aβ(1–42) protofibrils (Panel B) for an additional 6 h. An XTT cell viability assay was then conducted on the microglia as described in Section 2. Microglia viability, as measured by reduced XTT absorbance, was compared between microglia exposed to 4N1K and/or Aβ(1–42) protofibrils and those with no exposure to either. Data is presented as the percentage of reduced XTT absorbance for treated cells compared to untreated cells (no 4N1K or protofibrils) and reported as % living cells. Data bars represent the mean ± std error of $n = 3$ replicates. Statistical differences ($p < 0.05$) in cell viability caused by 4N1K are denoted with an asterisk.

3.3. CD47 KO microglia studies

We used a more direct approach to investigate whether CD47 has a role in mediating Aβ(1–42) protofibrils-stimulated proinflammatory events. Primary microglia were isolated from both wild-type (WT) and $CD47^{-/-}$ mice and the cytokine response to Aβ(1–42) protofibrils was assessed. We have previously investigated the time course for protofibril-induced TNFα secretion in primary microglia and found that 6 h is an appropriate time frame for time-dependent studies [37]. The protofibril response in the current study was consistent with our previous work in WT microglia showing little TNFα secretion at 2 h but a significant increase after 6 h of microglia exposure to Aβ(1–42) protofibrils (Fig. 4A). Notably, the same time course and levels of TNFα secretion were observed in $CD47^{-/-}$ microglia upon Aβ protofibril stimulation. A subsequent experiment examined the effectiveness of 4N1K in $CD47^{-/-}$ microglia. Again, protofibrils stimulated significant TNFα in both WT and $CD47^{-/-}$ microglia (Fig. 4B). Pretreatment of either WT or $CD47^{-/-}$ microglia with 4N1K completely attenuated TNFα secretion elicited by Aβ(1–42) protofibrils (Fig. 4B). These results strengthen our conclusion that CD47 does not mediate Aβ(1–42) protofibril-stimulated microglia cytokine release and clearly show that 4N1K is inhibiting the process by a mechanism other than CD47 antagonism.

4N1K dose-dependently inhibited TNFα release in response to both LPS and Aβ(1–42) protofibril in $CD47^{-/-}$ primary microglia (Fig. 4C). Estimated IC_{50} values for 4N1K inhibition of either LPS or Aβ were similar (≈3 μM) indicating perhaps a common non-CD47 target in the proinflammatory signaling pathways of the two molecules. LPS-induced secreted TNFα levels were similar in WT and $CD47^{-/-}$ microglia (data not shown). Conspicuously, the potency of 4N1K inhibition of Aβ protofibril-induced TNFα secretion in $CD47^{-/-}$ microglia (Fig. 4C) was nearly identical to that in WT microglia (Fig. 1C). In addition to TNFα, IL-1β secretion triggered by Aβ(1–42) protofibrils was also significantly attenuated by 4N1K in $CD47^{-/-}$ microglia (Fig. 4D). These results confirmed the earlier findings showing a CD47-independent inhibitory mechanism for 4N1K and argue against a major role for CD47 in soluble Aβ(1–42) protofibril proinflammatory signaling.

4. Discussion

A variety of receptors and receptor complexes have been shown to mediate Aβ-stimulated proinflammatory cytokine secretion in monocyte/macrophage and microglia cells. These include CD14, TLR4, and TLR2, a CD36/TLR4/TLR6 complex, and a CD36/$\alpha_6\beta_1$ integrin/CD47 complex (reviewed in [25]). In addition to Aβ proinflammatory signaling, CD47 plays a role in Aβ uptake by microglia [30,31]. While many of these studies have focused on fibrillar Aβ, a previous investigation from our laboratory found that isolated Aβ(1–42) fibrils were not effective stimulators of microglia [32]. Instead, Aβ(1–42) protofibrils were robust stimulators and we have recently demonstrated that protofibril-induced TNFα secretion occurs predominantly via the TLR/MyD88 innate immune pathway [37]. The objective of the current investigation described in this report was to determine if CD47 played a significant role in mediating the microglia proinflammatory response to protofibrils. Utilizing pharmacological inhibition by 4N1K, anti-CD47 antibody neutralization, and CD47 knockout microglia, we determined that CD47 does not mediate Aβ protofibril-induced cytokine secretion. We also found that 4N1K exerted its inhibitory action in a CD47-independent manner. The 4N1K peptide was a potent inhibitor of Aβ signaling even in $CD47^{-/-}$ microglia (Fig. 4) and almost completely blocked the microglia response to Aβ protofibrils when an anti-CD47 neutralizing antibody had no effect (Fig. 3). Since the previous studies exploring the microglia response to Aβ utilized 4N1K to establish a role for CD47 [29–31], caution is advised in the interpretation of those data. A newly-published report also raised questions regarding the specificity of 4N1K for CD47 supporting the cautionary recommendation [36]. In conclusion, while there are likely numerous cellular components involved in the microglial recognition of Aβ protofibrils and subsequent transduc-

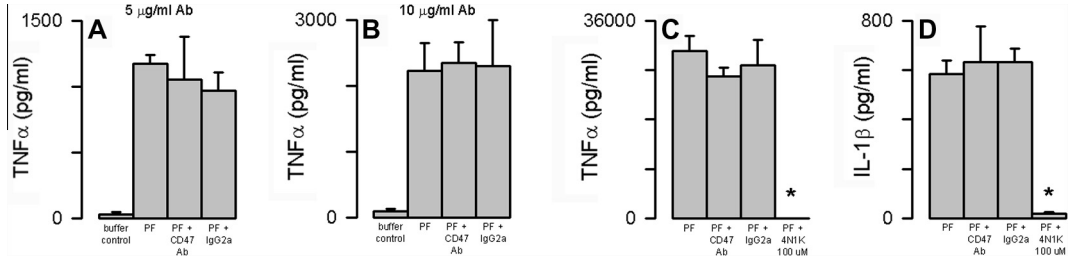

Fig. 3. Neutralizing CD47 antibodies do not inhibit the Aβ(1–42) protofibril induced proinflammatory response. Primary murine microglia were pretreated with 5 μg/mL (Panel A) or 10 μg/mL (Panel B) anti-mouse CD47 antibody or the corresponding isotype control (IgG2a) for 1 h followed by replacement of the medium and addition of SEC-isolated Aβ(1–42) protofibrils (15 μM) for 6 h. Secreted TNFα (Panels A–C) or IL-1β (Panel D) levels were determined by ELISA in the conditioned medium. Additional experiments in Panels C and D directly compared CD47 antibody neutralization and 4N1K inhibition of Aβ(1–42) protofibril-induced cytokine production. Data are expressed as the mean ± std error for $n = 3$ replicates for each treatment. Statistical differences ($p < 0.05$) in secreted TNFα or IL-1β elicited by Aβ protofibrils in the absence or presence of 4N1K are denoted with an asterisk. No statistical differences were found in the absence or presence of antibody or isotype control.

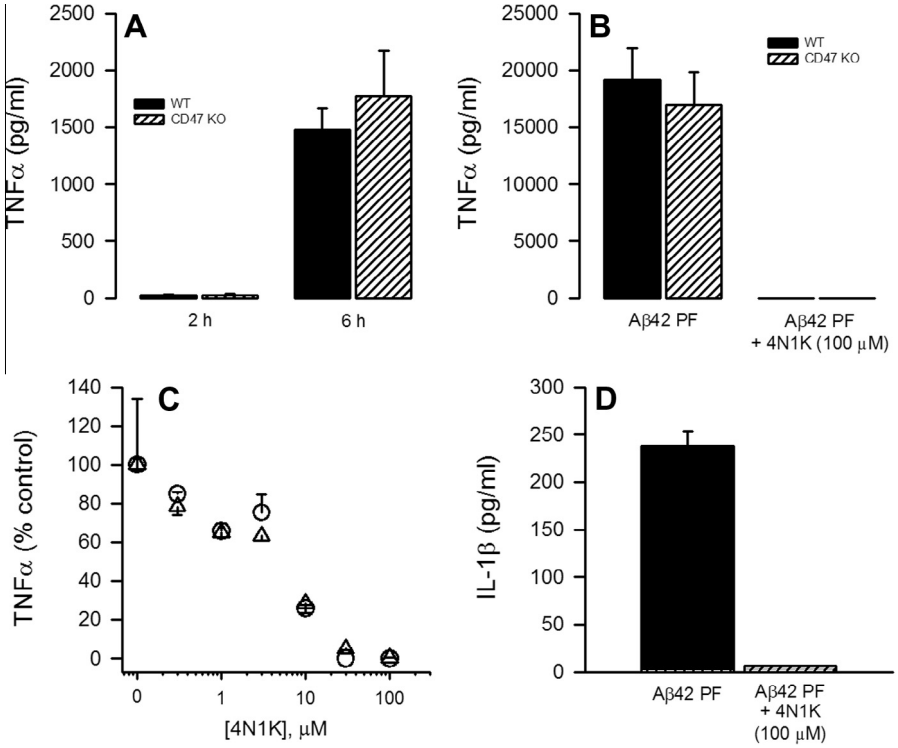

Fig. 4. Aβ(1–42) protofibrils stimulate similar levels of TNFα in both WT and CD47$^{-/-}$ microglia and 4N1K remains an effective inhibitor in CD47$^{-/-}$ microglia. WT and CD47$^{-/-}$ microglia were incubated with SEC-isolated Aβ(1–42) protofibrils (15 μM) for 2 h and 6 h in serum-free medium (Panel A) or pre-incubated with or without 4N1K (100 μM) for 1 h prior to protofibril addition for 6 h (Panel B). Secreted TNFα or IL-1β levels were determined in the conditioned medium in all figure panels by ELISA. (Panel C) Microglia isolated from CD47$^{-/-}$ mice were treated with either Aβ(1–42) protofibrils (15 μM) or LPS (10 ng/mL) for 6 h following a 1 h pretreatment with increasing concentrations of 4N1K (0.3–100 μM). Secreted TNFα levels are presented as % control (microglia treated with Aβ or LPS without 4N1K). (Panel D) CD47$^{-/-}$ microglia were treated with Aβ(1–42) protofibrils (15 μM) with or without 1 h 4N1K (100 μM) pretreatment. Data bars in all figure panels represent the mean ± std error of $n = 3$ replicates. Secreted TNFα elicited by Aβ protofibrils at 6 h in WT or CD47$^{-/-}$ microglia was not significantly different ($p > 0.05$) in Panels A and B.

tion of proinflammatory signaling, CD47 does not appear to be one of them.

Conflict of interest

The authors declare that they have no conflict of interest.

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