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Amyloid-β(1-42) protofibrils stimulate a quantum of secreted IL-1β despite significant intracellular IL-1β accumulation in microglia☆,☆☆

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ABSTRACT

Neuroinflammation is a characteristic feature of the Alzheimer's disease (AD) brain. Significant inflammatory markers such as activated microglia and cytokines can be found surrounding the extracellular senile plaques predominantly composed of amyloid-β protein (Aβ). Several innate immune pathways, including Toll-like receptors (TLRs) and the NLRP3 inflammasome, have been implicated in AD inflammation. Aβ plays a primary role in activating these pathways which likely contributes to the progressive neurodegeneration in AD. In order to better understand the complexities of this interaction we investigated the inflammatory response of primary microglia to Aβ(1-42) protofibrils. Aβ(1-42) protofibrils triggered a time- and MyD88-dependent process that produced tumor necrosis factor alpha (TNFα) and interleukin-1β (IL-1β) mRNA, and intracellular pro and mature forms of IL-1β protein. The accumulation of both IL-1β forms indicated that Aβ(1-42) protofibrils were able to prime and activate the NLRP3 inflammasome. Surprisingly, Aβ-induced accumulation of intracellular mature IL-1β did not translate into greater IL-1β secretion. Instead, we found that Aβ elicited a quantized burst of secreted IL-1β and this process occurred even prior to Aβ priming of the microglia suggesting a basal level of either pro or mature IL-1β in the cultured primary microglia. The IL-1β secretion burst was rapid but not sustained, yet could be re-evoked with additional Aβ stimulation. The findings from this study demonstrated multiple sites of IL-1β regulation by Aβ(1-42) protofibrils including TLR/MyD88-mediated priming, NLRP3 inflammasome activation, and modulation of the IL-1β secretory process. These results underscore the wide-ranging effects of Aβ on the innate immune response.

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1. Introduction

Alzheimer's disease (AD) is the most prevalent form of late-life dementia and has been defined by the presence of two pathological hallmarks, neurofibrillary tangles (NFTs) and senile (amyloid) plaques. The NFTs are intracellular lesions of insoluble, highly stable filamentous aggregates of the microtubule-associated protein, tau, whereas the senile plaques are extracellular lesions of insoluble, amyloid fibrils that are polymers of the amyloid-β protein (Aβ) [1,2]. However, Aβ is the protein that has been found to be most clearly associated to the cause of AD, while tau protein has shown to be most closely related to the clinical manifestations of AD [2]. The complexity of AD pathology reaches beyond NFTs and amyloid plaques. Recent findings have shown that the toxicity of Aβ may be due to soluble oligomeric intermediates found in the AD brain and cerebrospinal fluid rather than insoluble fibrils [3,4]. These soluble oligomeric Aβ forms appear to contribute to AD onset by causing neuronal and/or synaptic dysfunction [3,5].

There has been an array of different aggregate morphologies of Aβ found within the AD brain [6]. The core of the Aβ plaques is primarily composed of the 42-amino acid Aβ fragment, Aβ(1-42). However Aβ(1-40) has also been found colocalized with the Aβ(1-42) [7]. *In vitro*, Aβ monomer undergoes self-assembly by non-covalent interactions to form polydisperse mixtures of soluble oligomers [4] and protofibrils [8,9]. These soluble aggregates go through a conformational transition from predominately random coil to increasing amounts of β-sheet structure and ultimately produce insoluble fibrils [10,11]. As a precursor to mature fibrils, protofibrils have been fairly well characterized and have been shown to alter the normal physiology of cultured neurons [12], disrupt ion channels [13], block long-term potentiation (LTP), inhibit synapse remodeling and impair memory consolidation [14].

A prominent component of AD pathology is neuroinflammation found in the affected areas of the AD brain. Inflammatory markers, such as activated microglia and proinflammatory cytokines, are most

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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often observed surrounding the Aβ lesions within the AD brain [15]. Although the exact sequence of events leading to AD is still unclear, it is thought that Aβ accumulation initiates a pathological cascade of events leading to neuronal dysfunction and ultimately dementia. One part of this pathological cascade is an immune cell-mediated inflammatory response, involving proliferation and activation of microglia and astrocytes. The increase in inflammation-associated proteins and oxidative stress by-products is more than just a biomarker of AD [17]. Persistent inflammation can lead to a chronic inflammatory state, suggested to be an underlying mechanism of the progressive neurodegeneration in AD [16,17].

The innate immune system protects an organism by detection of invading pathogens via pattern recognition receptors (PRRs). Both microglia and astrocytes express Toll-like receptors (TLRs), which are a family of PRRs that recognize pathogen-associated molecular patterns (PAMPs) within specific molecules produced by bacteria, fungi, and viruses [18]. TLR engagement, with the exception of TLR3, leads to the recruitment of the adaptor protein myeloid differentiation protein 88 (MyD88), activation of the transcription factor nuclear factor-κB (NF-κB), and expression of a variety of genes involved in the immune response, such as tumor necrosis factor-α (TNFα) and interleukin-1β (IL-1β) [18]. Multiple TLRs and accessory proteins play a role in the pro-inflammatory response evoked by Aβ in monocyte/macrophages and microglia including CD14, TLR4, and TLR2 [19–23] and TLR4 and TLR6 in concert with CD36 [24]. In addition to TLRs, a multireceptor complex comprised of scavenger receptor class B (SR-B), CD36, $\alpha_6\beta_1$-integrin and the integrin-associated protein CD47 has also been shown to interact with Aβ and initiate a proinflammatory response [25].

Extracellular recognition of PAMPs by TLRs and downstream signaling events can trigger oligomerization of cytoplasmic Nod-like receptors (NLRs) to form a multisubunit inflammasome complex [26]. Different NLR family members vary in their N-terminal protein–protein interaction region. For example, NLRP3 contains a pyrin domain which is responsible for complexation with ASC, the adaptor molecule termed apoptosis-associated speck-like protein containing a caspase-recruitment domain (CARD) [27]. An important aspect of TLR signaling is production of IL-1β mRNA and the pro-form of IL-1β protein (pro-IL-1β). This event is considered "priming" of the inflammasome and represents Signal 1 of a 2-stage process [28]. Many molecules, including the classical TLR4 agonist lipopolysaccharide (LPS), stimulate priming of the inflammasome (Signal 1) but not activation. Inflammasome assembly via a CARD-CARD interaction brings caspase-1 to the complex and activation of the complex triggers caspase-1-catalyzed proteolytic cleavage of pro-IL-1β to mature IL-1β [29]. A second signal is required for the inflammasome activation step and subsequent production of mature IL-1β. Signal 2 may be provided by a growing number of endogenous human molecules, referred to as danger-associated molecular patterns (DAMPs). These molecules include certain pore-forming toxins, ATP, K^+ efflux, and crystalline particles such as silica and uric acid crystals [28].

Increasing data have demonstrated a significant role for the NLRP3 inflammasome in AD (reviewed in [30]. Double transgenic mice that overexpress human APP with familial AD mutations and have a deficiency in the NLRP3 inflammasome showed a reduction in AD pathology and were protected from the ensuing cognitive defects [31]. Earlier *in vitro* studies demonstrated that fibrillar Aβ stimulated NLRP3 inflammasome activation and IL-1β production in microglia and this process was shown to involve phagocytosis, lysosomal damage, and release of cathepsin B [32]. Another report confirmed fibrillar Aβ-stimulated inflammasome activation but demonstrated that pre-priming of the inflammasome was required [33]. However, it was recently shown that Aβ may provide both priming and activation signals [34].

We have recently demonstrated that Aβ(1-42) protofibrils, but not Aβ(1-42) fibrils, are robust activators of microglia [35] and that these soluble fibrillar precursors can trigger proinflammatory events via Toll-like receptors (TLRs) [20]. In the current study we investigated the role of MyD88 in both Aβ(1-42) protofibril-induced cytokine production and NLRP3 inflammasome activation. During this investigation, we identified multiple points of regulation by Aβ protofibrils in both pathways including new insights into the IL-1β secretion process.

2. Experimental procedures

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 stored at −20 °C. Aβ(1-42) peptides were dissolved in 100% hexafluoroisopropanol (HFIP) (Sigma-Aldrich, St. Louis) at 1 mM, separated into aliquots in sterile microcentrifuge tubes, and evaporated uncovered at room temperature overnight in a fume hood. The following day the aliquots were vacuum-centrifuged to remove any residual HFIP and stored in dessicant at −20 °C.

2.2. Size exclusion chromatography

Aβ protofibrils were isolated as previously described [35]. Briefly, yophilized Aβ was dissolved in 50 mM NaOH to yield a 2.5 mM Aβ solution followed by dilution 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 then centrifuged at 18,000 ×*g* for 10 min and the supernatant was fractionated on a Tricorn Superdex 75 10/300 GL column (GE Healthcare) using an AKTA FPLC system (GE Healthcare). Prior to injection of Aβ, the Superdex 75 column was coated with sterile bovine serum albumin (Sigma) to prevent any non-specific binding of Aβ to the column matrix. Following a 1 mL loading of the sample, Aβ was eluted at 0.5 mL min^{-1} in aCSF and 0.5 mL fractions were collected and immediately placed on ice. Aβ 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 MyD88$^{-/-}$ mice (gift from Dr. Tammy Kielian, University of Nebraska Medical Center). Microglia were isolated as previously described [35,36] from 3–4 day old mouse pups. Briefly, brains were isolated under sterile conditions, minced, and trypsinized. The brain tissue was then resuspended in complete DMEM containing 10% fetal bovine serum (FBS), 4 mM L-glutamine, 100 U/ml penicillin, 0.1 mg/ml streptomycin and 0.25 μg/mL amphotericin-B, OPI medium supplement (oxalocetate, pyruvate, insulin), and 0.5 ng/ml recombinant mouse granulocyte-macrophage colony-stimulating factor (GM-CSF). The cell suspension was filtered, centrifuged, resuspended in complete medium and seeded into 150 cm^2 flasks. Cells were cultured 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. Typically, this procedure was repeated 3–4 times for one flask without removal of the astrocyte layer.

2.4. Cell stimulation assay

For cellular studies, WT and MyD88$^{-/-}$ primary murine microglia were collected as described above by overnight shaking, collection of the cells, and seeding in a sterile 96-well cell culture plate for 24 h at a density of 5×10^5 cells/ml in growth medium with serum and GM-CSF (0.1 mL per well). Prior to cell treatment, medium was replaced with 0.1 mL medium lacking FBS and GM-CSF. Cells were then treated with Aβ (15 μM) or TLR stimuli ultra-pure lipopolysaccharide (LPS, 10 ng/ml

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