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Methods to monitor monocytes-mediated amyloid-beta uptake and phagocytosis in the context of adjuvanted immunotherapies

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ABSTRACT

Antibody-mediated capture of amyloid-beta (Aβ) in peripheral blood was identified as an attractive strategy to eliminate cerebral toxic amyloid in Alzheimer's disease (AD) patients and murine models. Alternatively, defective capacity of peripheral monocytes to engulf Aβ was reported in individuals with AD. In this report, we developed different approaches to investigate cellular uptake and phagocytosis of Aβ, and to examine how two immunological devices – an immunostimulatory Adjuvant System and different amyloid specific antibodies – may affect these biological events. Between one and thirteen months of age, APPswe X PS1.M146V (TASTPM) AD model mice had decreasing concentrations of Aβ in their plasma. In contrast, the proportion of blood monocytes containing Aβ tended to increase with age. Importantly, the TLR-agonist containing Adjuvant System $AS01_B$ primed monocytes to promote *de novo* Aβ uptake capacity, particularly in the presence of anti-Aβ antibodies. Biochemical experiments demonstrated that cells achieved Aβ uptake and internalization followed by Aβ degradation *via* mechanisms that required effective actin polymerization and proteolytic enzymes such as insulin-degrading enzyme. We further demonstrated that both Aβ-specific monoclonal antibodies and plasma from Aβ-immunized mice enhanced the phagocytosis of 1 μm Aβ-coated particles. Together, our data highlight a new biomarker testing to follow amyloid clearance within the blood and a mechanism of Aβ uptake by peripheral monocytes in the context of active or passive immunization, and emphasize on novel approaches to investigate this phenomenon.
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1. Introduction

Alzheimer's disease (AD) is a neurodegenerative disorder that manifests as memory deterioration and cognitive impairment. AD is the most common form of dementia worldwide, affecting over 37 million people (Rafii and Aisen, 2009), and is the sixth leading cause of death in the United States (Thies and Bleiler, 2013). Available treatments only temporarily alleviate the symptoms and have shown variable effectiveness (Thies and Bleiler, 2013). There is currently no remedy that stops or reverses disease progression (Thies and Bleiler, 2013).

Amyloid plaques and neurofibrillary tangles are histopathological hallmarks characterizing AD patients' brains (Small and Duff, 2008). The principal protein component of these amyloid plaques, the amyloid-beta (Aβ) peptide, is generated by the cleavage of the amyloid

Abbreviations: Aβ, amyloid-beta; AD, Alzheimer's disease; APP, amyloid precursor protein; $AS01_B$, adjuvant system 01_B; BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle medium; DPBS, Dulbecco's Phosphate Buffered Saline; EOAD, early-onset AD; Fig., figure; FMOs, fluorescence minus one; GMFI, geometric mean of fluorescence intensity; HRP, horseradish peroxidase; IDE, insulin-degrading enzyme; IM, intra-muscular; IV, intra-venous; LOAD, late-onset AD; LPS, lipopolysaccharide; MPL, monophosphoryl lipid A; PBS, phosphate-buffered saline; PiB, Pittsburgh compound B; PS-1, presenilin-1; PS-2, presenilin-2; ROS, reactive oxygen species; RPMI, Roswell Park Memorial Institute medium; TASTPM, T-alpha APPswe x PS1.M146V amyloid accumulating mouse model; TLR, Toll-like receptor.

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precursor protein (APP) (Small and Duff, 2008). Residues corresponding to the Aβ peptide within the native APP encompass part of the APP transmembrane domain (Haass and Selkoe, 2007). Therefore, APP proteolysis liberates the Aβ peptide and exposes hydrophobic amino acids and regions otherwise hidden in the intact protein (Sgarbossa, 2012). Self-association of free Aβ generates amorphous structures with granular morphology, oligomers, and fibrillar aggregates (Pryor et al., 2012; Sgarbossa, 2012). Interestingly, soluble Aβ intermediate assemblies, such as oligomers, were shown to interfere with long-term potentiation and cognitive performances in rat models (Haass and Selkoe, 2007; Selkoe, 2008). The accumulation of Aβ also facilitates the hyperphosphorylation and aggregation of the microtubule-associated protein tau, resulting in the formation of neurofibrillary tangles (Weiner and Frenkel, 2006; Holtzman, 2008; Small and Duff, 2008). Mutations in APP, as well as in presenilin-1 (PS-1) and -2 (PS-2), can trigger this sequence of biochemical events, which leads to early-onset AD (EOAD) (Small and Duff, 2008; Thies and Bleiler, 2013). Importantly, Jonsson et al. (2012) identified an amino acid substitution in APP that reduces APP processing and confers protection against AD (Jonsson et al., 2012), supporting a potential link between Aβ accumulation and AD. Even though the exact causes of late-onset AD (LOAD) have yet to be determined, Aβ is thought to play a critical role in the development of this disease.

The predominance of the amyloid hypothesis has stimulated the development of drugs preventing the proteolysis of APP, or the secretion or aggregation of Aβ (Rafii and Aisen, 2009). However, precluding Aβ production may not be the only strategy of treating AD. An effective alternative could be to stimulate the immune system in order to neutralize or eliminate a toxic overload of Aβ. Evidences supporting this possibility were reported by Mawuenyega et al. (2010), who demonstrated that LOAD patients have a general impairment in Aβ clearance rather than increased Aβ production (Mawuenyega et al., 2010). Several studies performed in murine AD models suggest that vaccination with Aβ antigens and injection of anti-Aβ antibodies could prevent the accumulation of Aβ molecules and the formation of plaques, and improve cognitive performance (Schenk et al., 1999; Games et al., 2000; Arendash et al., 2001; Bacskai et al., 2001; Zhang et al., 2003; Jensen et al., 2005; Maier et al., 2006; Spires-Jones et al., 2009). However, phase II clinical trials involving the vaccine AN1792 were curtailed because 6% of patients immunized with $A\beta_{42}$ presented symptoms of meningoencephalitis (Orgogozo et al., 2003). Immunogenic T-cell epitopes were identified in the central part of Aβ and were postulated as being responsible of these harmful effects (Monsonego et al., 2006; Toly-Ndour et al., 2011). Nevertheless, when these patients were followed up using the neuropsychological test battery, antibody responders were shown to have improved cognitive functions compared with placebo (Gilman et al., 2005; Vellas et al., 2009). In addition, *post-mortem* examination of patients treated with AN1792 showed reduced plaque formation and improved neurite morphology in the brain (Nicoll et al., 2003; Ferrer et al., 2004; Masliah et al., 2005; Holmes et al., 2008; Serrano-Pozo et al., 2010). These encouraging observations support further research into the design of an effective and safe immunotherapy for AD. CAD106, ACC-001 and Affitope AD02 are among the second-generation of Aβ-targeting active vaccines in humans, using short N-terminal amyloid antigens such as $A\beta_{1-6}$, and will, hopefully, minimize adverse side effects and improve disease prognosis (Panza et al., 2014; Arai et al., 2015).

The Aβ peptide was detected in plasma samples from patients examined in several studies (Tamaoka et al., 1996; Mayeux et al., 1999, 2003; Assini et al., 2004; Schupf et al., 2008; Devanand et al., 2011). Furthermore, individuals with high baseline $A\beta_{42}$ levels were more likely to develop AD (Mayeux et al., 1999, 2003; Schupf et al., 2008). Among participants who developed AD, plasma levels of $A\beta_{42}$ diminished concomitantly with the onset of cognitive defects as well as the increased binding of Pittsburgh compound B (PiB) to the brain (Schupf et al., 2008; Devanand et al., 2011). The presence of Aβ in the plasma implies a peptide outflow from the brain, whereas blood was proposed to serve as a source of Aβ that would flow into the encephalon to elicit toxicity (Clifford et al., 2007). This possible dynamic between the blood-stream and the brain led to the peripheral sink hypothesis. Accordingly, peripheral administration of anti-Aβ antibodies to PDAPP (APP^{V717F}) transgenic mouse led to a rapid increase of Aβ in the plasma, which reflected levels of amyloid deposition in the brain (DeMattos et al., 2001, 2002). This potential dynamic between blood and brain Aβ levels may be critical for efficient active and passive immunization, as well as other therapies involving the capture of disseminating Aβ. Nonetheless, the molecular sequestration of Aβ may not be sufficient for its complete elimination or for amelioration of the disease prognosis. Interestingly, macrophages from AD patients have shown defective phagocytosis of Aβ (Fiala et al., 2005). Alternatively, monophosphoryl lipid A (MPL)-mediated Toll-like receptor 4 (TLR4) stimulation promoted the expansion of monocytes and their uptake of Aβ, which correlated with the reduction of cerebral Aβ load and improved cognitive performance in murine models (Michaud et al., 2013). Strategies aimed at capturing and stimulating cell-mediated elimination of Aβ may therefore also be important in generating an effective immunotherapy. Notably, assessing and following the cellular activities involved in Aβ clearance will benefit the development and characterization of such treatment.

Here, we propose novel procedures to explore cellular Aβ uptake mechanisms and phagocytosis of larger amyloid particles. Using *in vivo*, *ex vivo* and *in vitro* systems, we observed that amyloid specific antibodies promoted efficient uptake and phagocytosis of Aβ. In addition, we explored the effect of combining anti-Aβ antibodies with the MPL-containing Adjuvant System $AS01_B$. Importantly, $AS01_B$ treatment activated blood monocytes to multiply their Aβ uptake activity in presence of antibodies. Also, our data show that cells engulfing Aβ are likely capable of degrading this peptide. This phenomenon involved the protease insulin-degrading enzyme (IDE). We believe that our approach will benefit the development of future immunotherapies for AD and of blood biomarkers to monitor their efficiency.

2. Materials and methods

2.1. Reagents and cell culture

Chemicals were from Sigma-Aldrich, Fisher Scientific and Thermo Scientific; Aβ peptides from AnaSpec; and paraformaldehyde, from Electron Microscopy Sciences (EMS). To characterize cells by flow cytometry, we used the following antibodies from BD Pharmingen: Fc block (rat anti-mouse CD16/CD32, clone 2.4G2, cat # 553142), PE rat anti-mouse Ly-6G (clone 1A8, cat # 551461), PE mouse-anti mouse NK-1.1 (clone PK136, cat # 553165), PE rat anti-mouse CD45/B220 (clone RA3-6B2, cat # 553090), PE hamster anti-mouse CD3e (clones 145-2C11, cat # 553064), V450 rat anti-mouse Ly-6C (clones AL-21, cat # 560594), APC rat anti-mouse CD11b (clone M1/70, cat # 553312) and PerCP-Cy5.5 rat anti-mouse I-A/I-E (MHC class II, clone M5/114.15.2, cat # 562363). The 2E7 anti-Aβ antibody was generated by GSK Pharma. This antibody is an IgG2a that recognizes the first seven amino acids of Aβ.

THP-1 cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 2 mM L-glutamine, 0.05 mM 2-mercaptoethanol, 100 U/mL penicillin, 100 μg/mL streptomycin and 10% fetal bovine serum (FBS), referred to as complete RPMI. BV2 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 1× sodium pyruvate, 100 U/mL penicillin, 100 μg/mL streptomycin and 10% FBS. Media and additives used in cell culture were from Gibco. Both cell lines were incubated at 37 °C in a 5% CO_2 humid atmosphere.

2.2. Animals

Procedures involving animals were executed accordingly to the guidelines of the Canadian Council on Animal Care (CCAC) in science.

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