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Curcuminoids enhance amyloid-β uptake by macrophages of Alzheimer's disease patients

Laura Zhang[a], Milan Fiala[a,*], John Cashman[b], James Sayre[c], Araceli Espinosa[d], Michelle Mahanian[a], Justin Zaghi[a], Vladimir Badmaev[e], Michael C. Graves[f], George Bernard[d,g] and Mark Rosenthal[a]
[a]*Department of Medicine, Greater LA VA Medical Center and UCLA School of Medicine, Los Angeles, CA 90095, USA*
[b]*Human BioMolecular Institute, San Diego, CA 92121, USA*
[c]*Department of Biostatistics, UCLA School of Public Health, Los Angeles, CA 90095, USA*
[d]*Department of Neurobiology, UCLA School of Medicine, Los Angeles, CA 90095, USA*
[e]*Applied Pharmacology, Sabinsa Corporation, Piscataway, NJ 08854, USA*
[f]*Department of Neurology, UCLA School of Medicine, Los Angeles, CA 90095, USA*
[g]*Division of Oral Biology and Medicine, UCLA School of Dentistry, Los Angeles, CA 90095, USA*

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Abstract. Treatment of Alzheimer's disease (AD) is difficult due to ignorance of its pathogenesis. AD patients have defects in phagocytosis of amyloid-β (1-42) (Aβ) *in vitro* by the innate immune cells, monocyte/macrophages and in clearance of Aβ plaques [5]. The natural product curcuminoids enhanced brain clearance of Aβ in animal models. We, therefore, treated macrophages of six AD patients and 3 controls by curcuminoids *in vitro* and measured Aβ uptake using fluorescence and confocal microscopy. At baseline, the intensity of Aβ uptake by AD macrophages was significantly lower in comparison to control macrophages and involved surface binding but no intracellular uptake. After treatment of macrophages with curcuminoids, Aβ uptake by macrophages of three of the six AD patients was significantly ($P < 0.001$ to 0.081) increased. Confocal microscopy of AD macrophages responsive to curcuminoids showed surface binding in untreated macrophages but co-localization with phalloidin in an intracellular compartment after treatment. Immunomodulation of the innate immune system by curcuminoids might be a safe approach to immune clearance of amyloidosis in AD brain.

Keywords: Alzheimer's disease, amyloid-β, phagocytosis, curcuminoids, immunomodulation

1. Introduction

In AD brain macrophages and microglia are inefficient in clearing amyloid-β (Aβ) plaques [6]. Despite mechanistic advances of the Aβ hypothesis [20], progress in AD therapy has been difficult, at least in part due to poor understanding of the mechanisms regulating Aβ processing and brain clearance [8]. Aβ accumulation in AD brain has been speculated to be related to abnormal cross-talk between Aβ – reactive T cells and microglia leading to differentiation of microglia into either phagocytes or antigen presenting cells with unclear neuroprotective role [15], ligation of CD40 on microglia by CD40L [21], and inhibition of complement activation [24]. Recently we have shown that macrophages and microglia of middle-aged and older normal subjects physiologically perform Aβ clearance but this function is defective in AD patients [4]. Monocyte migration across blood-brain barrier, macrophage differentiation, survival, and chemokine secretion [7] might also be abnormal in AD patients.

To promote Aβ clearance, a vaccine against Aβ was developed in transgenic animals overexpressing mu-

*Corresponding author: Milan Fiala, M.D., UCLA, CHS 73-084, 10833 Le Conte, Los Angeles, CA 90095-1668, USA. Tel.: +1 310 206 6392; Fax: +1 310 825 2042; E-mail: fiala@ucla.edu.

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tant form of Aβ-protein precursor [9]. The vaccine stimulates the adaptive immune system with induction of Aβ antibodies, which induce phagocytosis through the microglial Fc receptor [2]. The vaccine was tested in AD patients but its trial was discontinued due to meningoencephalitis in some patients [23]. The innate immune responses in vaccinated patients were not specifically tested, although brain regions devoid of plaques were found to be infiltrated by microglia [17] and macrophages [13]. It has been speculated that CNS inflammation in vaccinated individuals could be related to Aβ-reactive T cells, which are detectable in older humans and patients with AD [16], although the clinical data did not favor this hypothesis [15].

Immunomodulatory therapies, such as those by curcumin complex [25] and insulin-like growth factor [3], enhance brain clearance of Aβ in animal models. Here we have tested immune modulation of the effector cells of the human innate immune system by curcuminoids. We have shown that a specific defect of Aβ phagocytosis by AD macrophages may be improved in approximately 50% of AD patients by curcuminoid treatment *in vitro*.

2. Methods

2.1. Reagents and antibodies

We purchased Aβ (1-42) and scrambled Aβ (1-42) (with the correct amino acids in random order) conjugated with fluorescein isothiocyanate (FITC) (AnaSpec, San Jose, CA); mouse anti-human CD68 (KP-1) (DAKO, Carpinteria, CA); anti-mouse and anti-rabbit IgG conjugated to Alexa 498 or Alexa 594, and fluorescein-labeled *E. coli* and Alexa Fluor 594-labeled *S. aureus* (Molecular Probes, Eugene, OR), and tetramethylrhodamine- phalloidin (Sigma, St. Louis, MO).

2.2. Patients and control subjects – Diagnostic Criteria, Blood Specimens, and Immune Studies

All subjects gave informed consent approved by the UCLA Institutional Review Board (IRB) for Human Studies. The diagnostic criteria for AD satisfied the National Institute of Neurological and Communicative Disorders and the Alzheimer's Disease and Related Disorders Association (NINCDS/ADRDA) criteria for probable Alzheimer's disease [14] as described [4]. Normal age-matched control subjects were recruited from UCLA Faculty and Alumni. Peripheral blood mononuclear cells (PBMC's) were separated from EDTA-anticoagulated blood by centrifugation on Ficoll-Hypaque gradient as described [4]. Eleven AD patients were chosen for testing but macrophage cultures could be established only from six.

2.3. Macrophage culture

100,000 PBMC's isolated by the Ficoll-Hypaque technique were cultured for 7–14 days at 37 °C in a 5% CO_2 humidified incubator in 0.5 ml RPMI medium with 10% autologous serum in the wells of a 8-chamber polystyrene vessel tissue culture treated glass slides (Becton Dickinson) sealed with parafilm with or without one change of medium. During the incubation, monocytes differentiated into adherent macrophages, whereas lymphocytes did not attach and were washed off. In comparison to control macrophages, AD macrophages appeared poorly differentiated and more loosely adherent.

2.4. Curcuminoid treatment

Differentiated macrophages were treated with curcuminoids (Curcumin 3 complex, lot C2118C, Sabinsa Corporation, Piscataway, NJ) (0.1 μM) in the medium overnight and were then exposed to FITC- Aβ (1-42), which was dissolved in DMSO and diluted in RPMI medium to 2.5 μg/ml, incubated for 24 or 48 h and examined by fluorescence or confocal microscopy. Fresh curcuminoid stock solution (100 mM) was prepared by dissolving 36.8 mg curcuminoids in 1 ml dimethylsulfoxide. Curcuminoids have intense orange color, but macrophages exposed to curcuminoids at the concentration used in the assay did not show any background fluorescence in the green or red emission spectrum.

2.5. Immunofluorescence microscopy and confocal microscopy

The cells were fixed in 4% paraformaldehyde and stored in 0.5% paraformaldehyde. They were washed, permeabilized with 0.1% Triton in PBS for 10 minutes, washed again with PBS, and blocked with 1% bovine serum albumin. Macrophages were visualized using anti-CD68 or fluorescent phalloidin. The preparations were examined using Olympus Bmax fluorescence microscope or using Zeiss 510 Meta confocal microscope.

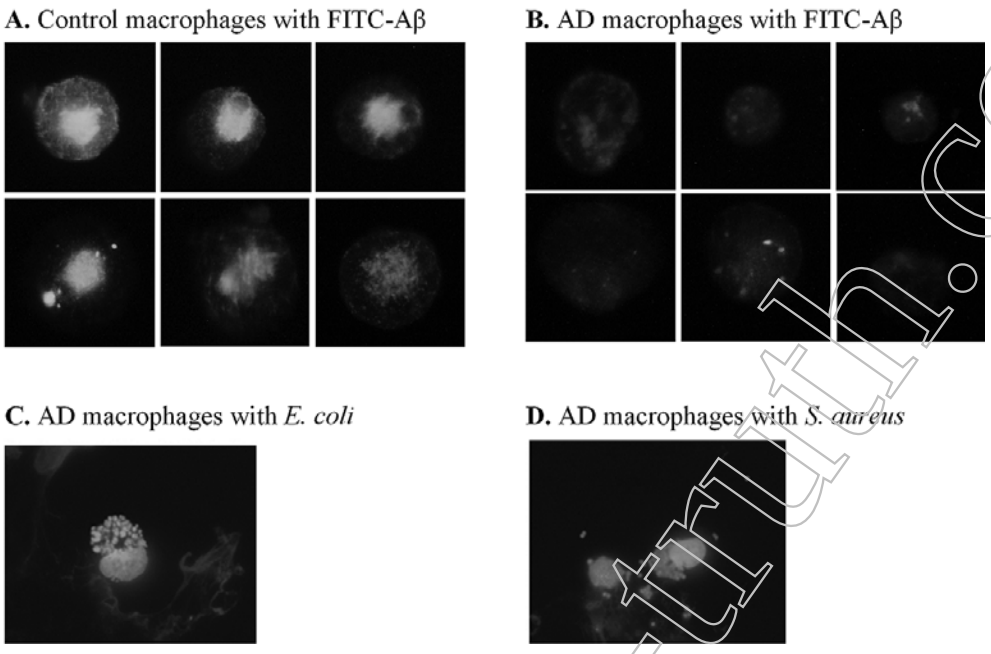


Fig. 1. FITC- amyloid-β uptake at baseline by control macrophages is much greater in comparison to AD macrophages (6 control macrophages (A); 6 AD macrophages (B)) (fluorescence microscopy, 100x). Uptake of fluorescein-labeled *E.coli* (C) and Alexa Fluor 594-labeled *S. aureus* (D) in AD macrophages was similar to control macrophages.

2.6. Data acquisition and statistical analysis

Six individual macrophages selected in a vertical strip in the middle of each chamber were photographed in a Bmax Olympus fluorescence microscope with 100x objective. The intensities of intracellular Aβ were obtained by digital scanning using the program Image-Pro (Media Cybernetics, Silver Spring, MD). The significance of data was determined by (a) t-test analysis for equality of means with equal variances not assumed when Levene's test for equality of variances was found significant, or with equal variances assumed when Levene's test was not significant. Statistical testing was performed with the statistical software SPSS, Version 10.0 (SPSS, Chicago).

3. Results

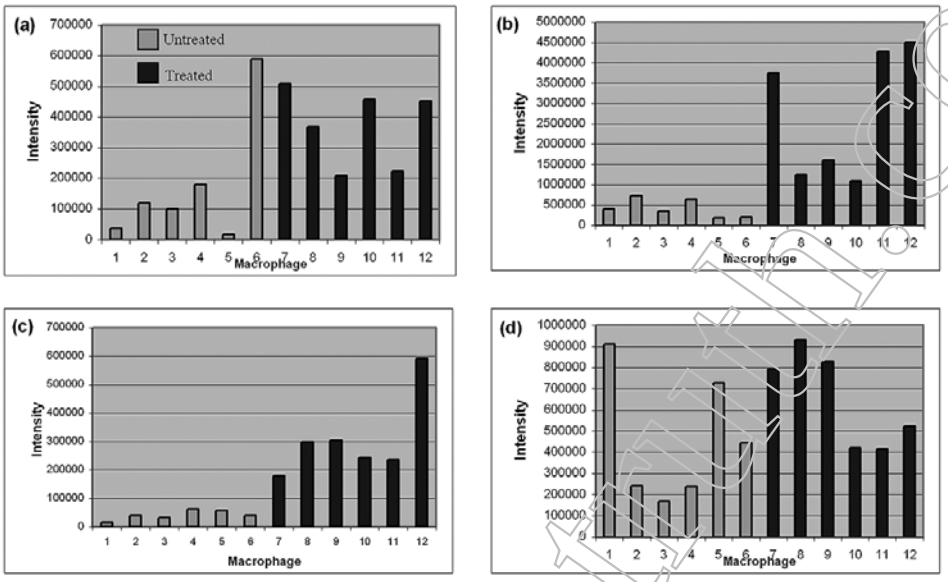
3.1. Amyloid-β phagocytosis by AD macrophages is defective

As previously published [4], control macrophages internalize Aβ into a perinuclear site, which is stained using Lysotracker (Molecular Probes, Eugene, OR) (unpublished data), whereas AD macrophages bind Aβ only on the surface and show defective phagocytosis. Accordingly, at baseline uptake of Aβ by control macrophages was shown to be significantly greater than that by AD macrophages (Fig. 1) and the uptake by AD macrophages appeared only as surface binding when examined by confocal microscopy (Figs 3(A) and 3(E)). The uptake was specific for correctly folded Aβ (1-42), since FITC- scrambled Aβ (1-42) (which comprises identical amino acids in a random order) was not phagocytized by either control or AD macrophages (data not shown). The phagocytic deficit was selective for Aβ; phagocytosis of *E. coli* and *S. aureus* by AD macrophages was normal (Fig. 1C and D).

3.2. Curcuminoids reverse defective phagocytosis of amyloid-β by macrophages of AD patients

To reverse the defect in phagocytosis, we treated macrophages with curcuminoids during overnight FITC-Aβ phagocytosis. Initially we tested a range (0.01 to 10 μM) of curcuminoid concentrations and determined that the optimal concentration enhancing phagocytosis was 0.1 μM. We then treated with 0.1 μM curcuminoids the macrophages of 6 AD patients and 3 controls. In each case, six macrophages selected in a pre-determined order were photographed and scanned. The analysis by t-test for equality of means of the intensities of Aβ uptake showed significant (P <0.001

A.



Subject**	Age	Sex	MMSE	p*
AD1	71	F	Not Known	0.081
AD2	80	F	20	N.S.
AD3	71	M	22	0.005
AD4	53	M	28	<0.001
AD5	71	M	21	N.S.
AD6	84	F	20	N.S.

*Significance of uptake of amyloid-β treated vs. untreated macrophages
**Uptake by macrophages of patients AD1 through 4 is shown in (a) through (d) respectively

B.

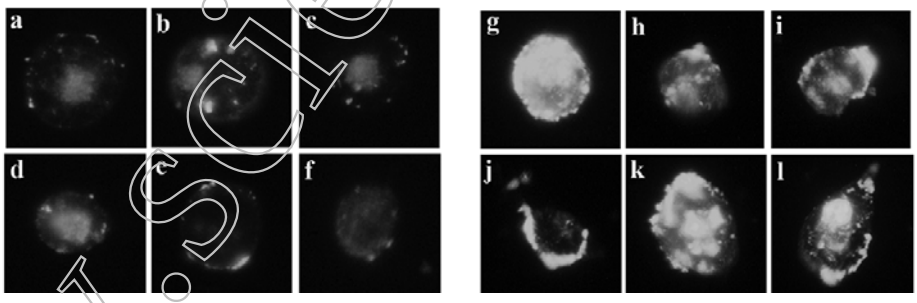


Fig. 2. Curcuminoid treatment increases uptake of amyloid-β by AD macrophages.
A. Analysis of amyloid-β uptake by macrophages of 6 patients. Replicate macrophage cultures in 8- chamber slides of 6 AD patients were exposed overnight to FITC- amyloid-β (2.5 microg/ml) and either no drug or curcumin complex (0.1 μM). In each subject, six macrophages in a vertical strip in the middle of each well were photographed at 100x magnification in a Bmax Olympus microscope. Image Pro scanning determined the intensity (density x area) of Aβ fluorescence. The panels (a) to (d) show the intensities of 4 patients' macrophages (intensities of treated macrophages in dark blue and those of untreated macrophages in light blue). The table shows the result of analysis by t-test of the intensities of macrophages of 6 patients. Significant differences in amyloid-β uptake between treated and untreated macrophages were shown in 3 patients (AD1, AD3, AD4), whereas the remaining patients did not show a response. Three control subjects' macrophages were treated with curcuminoids but the effects on FITC- amyloid-β uptake were not significant.
B. Fluorescence microscopic pictures of untreated and curcuminoid-treated AD macrophages of the patient AD3. The photographs (100x) show FITC-amyloid-β in macrophages, which were scanned in Fig. 2(Ac) and were either untreated (a to f) or treated with curcuminoids (g to l).

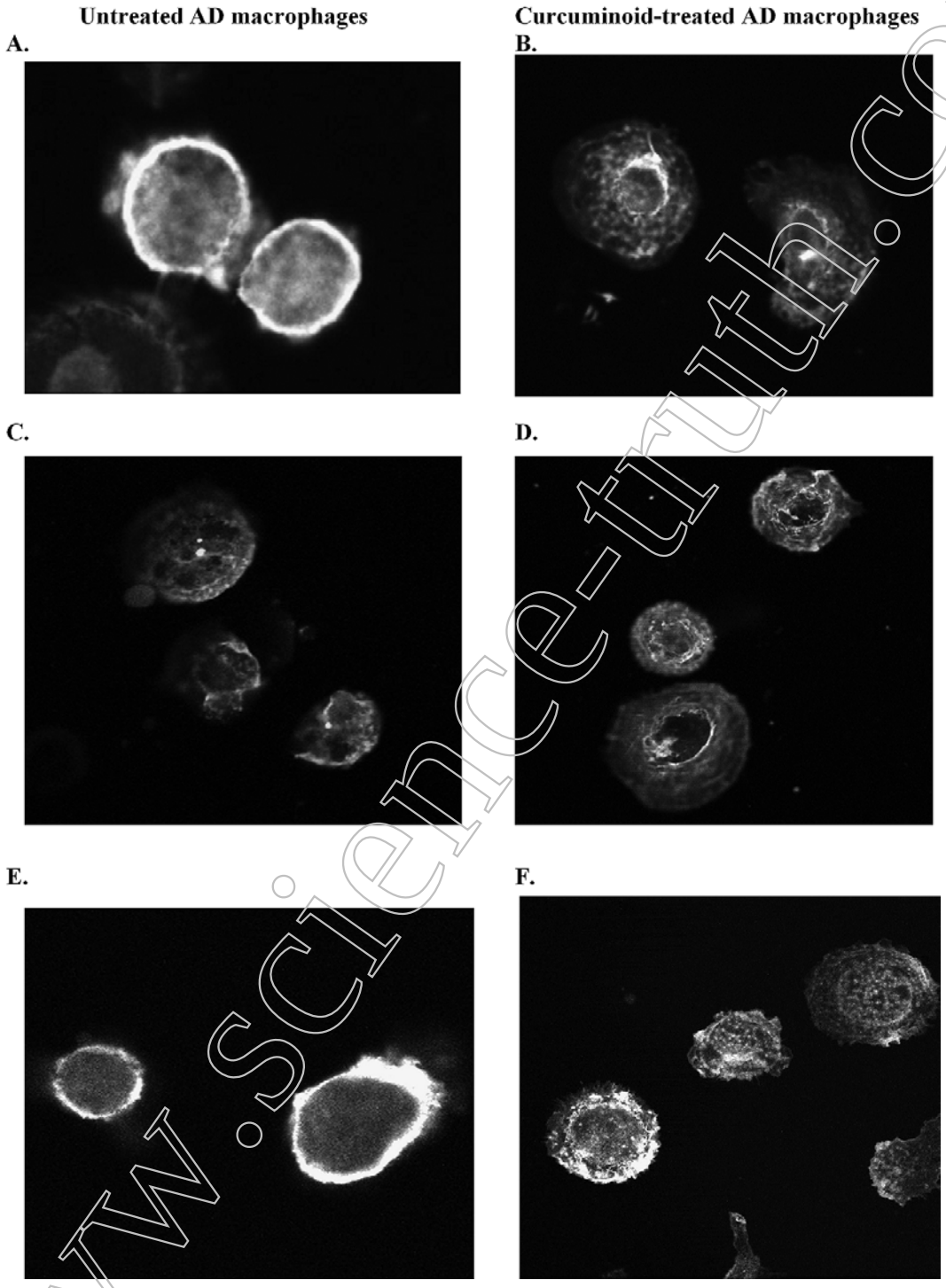


Fig. 3. Confocal microscopy of untreated and curcuminoid-treated macrophages.
Macrophages of patients AD1, AD3 and AD4, which were either untreated (A, C, E) or treated with curcuminoids (B, D, F), were examined by confocal microscopy. In untreated macrophages FITC-Aβ binds on the surface of phalloidin-Texas Red-stained untreated macrophages (the co-localization is shown in yellow) (A, E). In curcuminoid-treated macrophages, Aβ is intracellular in perinuclear location (B, F) (40x, confocal microscopic overlay of 20 sections).

to 0.081) increase in uptake in three patients (Fig. 2A (a) (b) (c)). Most importantly, the increase in uptake was through induction of intracellular phagocytosis by curcuminoids, as shown by confocal microscopy, as opposed to surface binding in untreated macrophages (cf. Fig. 3A to 3B and 3E to 3F). Control macrophages had a high uptake at baseline and were not improved by curcuminoids (data not shown).

The average Mini-Mental State Exam (MMSE) score in patients not responding to curcuminoid treatment was 20 and in those responding was 25. The average age of non-responders was higher than that of responders (78.3 vs. 62 years). There were no major differences in the use of prescription and non-prescription drugs; both responders and two of three non-responders used Aricept^{R}. These patients were enrolled in a double-blind study of oral Curcumin complex or placebo administration. The two responders were tested at visits 3 and 5, respectively; the three non-responders were tested at visits 1, 2 and 2. This study is on going, the drug or placebo assignment for each patient is not known.

We also treated with curcuminoids the macrophages of control patients, which had a high Abeta uptake at baseline, but their uptake was not further enhanced by curcuminoids.

4. Discussion

In agreement with previous findings, we are showing that macrophages of AD patients in different stages of the disease bind Aβ on the surface but do not appear to internalize Aβ, and usually have a low total uptake. Control macrophages usually have a high total uptake of Aβ, including surface binding and intracellular phagocytosis, although they also degrade intracellular Aβ, which decreases their intracellular Aβ content after 24–72 h. The defect in Aβ phagocytosis by AD macrophages is selective for Aβ; bacterial phagocytosis is adequate. We previously showed that AD macrophages are more susceptible to apoptosis on exposure to Aβ [4].

The salient result of the current study is that macrophages of 3 patients, 50% of those tested, showed significant increase in total Aβ uptake after curcuminoid treatment *in vitro*. The responding patients were younger and had higher MMSE score, suggesting that patients in less advanced stage of AD may respond better. However, the responders had been a longer time in a double-blind study of curcumin and thus may have been sensitized by *in vivo* curcuminoid administration (the code has not yet been broken). Further studies are needed to resolve the factors determining good response to curcuminoids. *In vitro* testing of curcuminoids in macrophage cultures may be useful in individualizing the treatment of AD patients. Unfortunately, macrophages of 5 AD patients were unsuitable for testing due to poor differentiation. Recently, we have increased successful macrophage cultivation by substituting human serum from young donors for the autologous AD serum.

The results of our study are in partial agreement with a recent study describing a general decline of immune responsiveness in AD, which involves defects in adaptive immune responses [18]. Previous studies have emphasized enhancement of phagocytosis by serum factors, complement and antibodies, but only few studies have been done with immune cells from AD patients and these studies have not analyzed the differences between controls and patients. The uptake of Aβ by microglia is enhanced through the Fc region of the anti-Aβ antibody and C1q. C1q is colocalized with Aβ plaques and its receptor, C1qR$_p$, exists on microglia [22]. Human postmortem microglia show enhanced reactivity with specific antibody-opsonized Aβ deposits [12]. However, in our studies the presence of autologous serum or fetal calf serum in RPMI medium during testing of AD macrophages did not correct defective phagocytosis.

Curcuminoid treatment enhanced not only the intensity of Aβ uptake but, crucially, induced intracellular phagocytosis, which leads to Aβ degradation. These favorable results *in vitro* extend the evidence of therapeutical efficacy of curcuminoids with respect to immunomodulatory activity in animals, including phagocytosis [1], and reduction of oxidative damage, interleukin-1β reactivity and microgliosis in APPsw transgenic mouse model [11]. Curcuminoids have anti-inflammatory properties [19] and anti- and pro-apoptotic properties [10], which may modulate excessive inflammatory responses by macrophages. Other beneficial properties of curcuminoids, such as inhibition of Aβ aggregation [25], could be also relevant to AD patients..

The enhancement of the innate immune function, phagocytosis of Aβ, by curcuminoids *in vitro* suggests that they could be used for immune modulation of the innate immune system. Immune modulation of the innate immunity may avoid stimulation of the adaptive immune system and inflammatory responses. Testing Aβ phagocytosis in AD macrophages might be helpful for assessing the ability of patients to respond to immunomodulatory therapy with curcuminoids.

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