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Amyloid β plaque-associated proteins C1q and SAP enhance the $A\beta_{1–42}$ peptide-induced cytokine secretion by adult human microglia in vitro

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Abstract Pro-inflammatory cytokines released by activated microglia could be a driving force in Alzheimer's disease (AD) pathology. We evaluated whether the presence of complement factor C1q and serum amyloid P component (SAP) in Aβ deposits is related to microglial activation. Activated microglia accumulate in SAP- and C1q-immunoreactive fibrillar amyloid β (Aβ) plaques in AD temporal cortex. No clustered microglia are seen in SAP- and C1q-positive circumscript, non-fibrillar, tau-negative Aβ plaques in AD caudate nucleus and non-demented control temporal cortex. In addition, no clustered microglia were observed in C1q- and SAP-negative, irregular shaped, diffuse plaques in AD caudate nucleus and in non-demented control temporal cortex, which suggests that microglia are attracted and activated in Aβ deposits of certain fibrillarity that, in addition, have fixed SAP and C1q. Therefore, the effects of $A\beta_{1–42}$, SAP and C1q on cytokine secretion by human postmortem microglia in vitro were assessed. $A\beta_{1–42}$ alone had little to no effect. $A\beta_{1–42}$ peptides in combination with C1q or C1q and SAP increased microglial interleukin (IL)-6 secretion four- and eightfold, respectively. Tumor necrosis factor (TNF)-α, as well as intracellular IL-1α and IL-1β levels, also increased upon exposure of microglia to $A\beta_{1–42}$-SAP-C1q complexes. Combined with earlier findings, that amyloid and activated microglia accumulate at a relatively early stage of cognitive decline in AD patients, this suggests that clustering of activated, cytokine-secreting microglia in SAP- and C1q-containing Aβ deposits precedes neurodegenerative changes in AD, and thus may provide a "therapeutic window".

Keywords Alzheimer's disease · Amyloid-β · Microglia · C1q · Serum amyloid P

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Introduction

Activated microglia that express (pro)-inflammatory cytokines co-localize with amyloid β (Aβ) deposits in Alzheimer's disease (AD)-affected brain areas [4, 13, 16, 21]. Polymorphisms in interleukin (IL)-1α, IL-1β and IL-6 influence the risk for developing AD as well as the age of onset of AD pathology [34, 37]. Therefore, cytokines are considered an important driving force in the neuropathological cascade in AD (for reviews see [2, 22]). Not only the synthesis of amyloid precursor protein (APP) [20, 40] and its Aβ fragment [5], but also of the so-called amyloid-associated proteins, which may determine the rate of Aβ aggregation in the plaques [29], is regulated by cytokines (e.g., IL-1, IL-6). Differences in aggregation state of the Aβ deposits, the accumulation of Aβ-associated factors and the presence of activated glial cells probably all contribute to the formation of different cerebral Aβ plaque types. According to Aβ morphology, congophily and neuritic changes, two parenchymal Aβ plaque types can be classified: fibrillar and non-fibrillar Aβ plaques [11, 12, 36]. The fibrillar Aβ plaques, which are associated with neuritic and glial changes, comprise (1) the classic neuritic plaques with a dense Aβ amyloid core and are Congo red positive, and (2) the intermediate sized (20–60 μm), round-shaped, primitive plaques that lack a central core and show variable Congo red positivity. A third type of fibrillar plaque, is the rarely observed small (5–15 μm) compact (burnt-out) plaque that consist only of a Congo red-positive Aβ core and lacks neuritic changes.

The non-fibrillar plaque types, which all lack a central core, Congo red positivity and degenerative neurites, can be subdivided in three types: (1) large (10–200 μm) irregular-shaped diffuse plaques, (2) intermediate-sized (20–60 μm), circumscribed non-fibrillar plaques, and (3) small (2–20 μm), stellate plaques. The degree of aggregation or fibrillarity of Aβ within plaques seems to determine whether microglia will accumulate at sites of amyloid deposits [21, 41]. Increased numbers of clustered activated microglia in AD neocortex specimens correlate with Congo red positivity and with progression of dementia at early stages of AD, when tau pathology still is moderate [3].

The nature of the substance that triggers the plaque-associated microglia to become activated is still unknown. Stimulatory effects of Aβ, the major constituent of amyloid plaques in AD, have been extensively investigated in vitro with contradictory results. Most studies report no direct stimulatory effects of Aβ on microglial cytokine release in vitro [19], whereas in a recent study pre-aggregated $A\beta_{1–42}$ was shown to induce cytokine secretion by human microglia [28]. Mouse microglia secrete IL-1β, but not other pro-inflammatory cytokines, when exposed to $A\beta_{25–35}$ [33]. Human monocytes, on the other hand, secrete IL-1β only when exposed to Aβ in combination with the strong, pluripotent activator lipopolysaccharide (LPS) [27]. Likewise, the release of reactive oxygen species by microglia can be potentiated by Aβ, but is not induced by Aβ alone [32, 46]. Synergistic actions of, for instance, interferon-γ, phorbol esters or LPS and Aβ, combinations that probably are not physiological to AD brain, are needed.

More recently, a combination of factors more relevant to AD, the C5a activation product of complement together with fibrillar Aβ, was found to trigger the release of pro-inflammatory cytokines IL-6 and IL-1β by THP-1 cells as a surrogate for microglia [35].

Complement activation products co-localize with cerebral Aβ deposits [1, 2, 14, 15, 53]. These complement activation products may derive from direct activation of the complement system by Aβ, as was shown in vitro [7, 25, 39], and probably are involved in several key steps of amyloid plaque formation (e.g., Aβ aggregation, activation of microglia, Aβ phagocytosis) [7, 14, 49, 50]. Another factor that is present in virtually all kinds of amyloid deposits, including Aβ plaques in AD brain, is serum amyloid P component (SAP). SAP, which is locally produced in the brain and expression of which is up-regulated in AD-affected brain regions [31], is possibly essential in amyloid fibril formation and persistence [17]. In addition, similar to the direct activation of complement by Aβ peptides [25, 39], SAP can bind and activate complement C1 in vitro [8, 23, 52]. In a recent study [38], a palindromic compound that cross-links pairs of pentameric SAP in vitro was found to deplete SAP from the circulation in vivo. Moreover, this compound was found to markedly reduce the SAP content of major amyloid deposits in patients with systemic amyloidosis. This clinical trial has now been extended to see if long-term treatment of patients with systemic amyloidosis leads to destabilization and regression of amyloid deposits [38].

In the immunohistochemical part of the present study, the presence of activated microglia in AD cerebral amyloid deposits was found to be related to a certain degree of fibrillarity of the Aβ, as well as to the simultaneous presence of SAP and C1q. Therefore, the combination of $A\beta_{1–42}$, C1q and SAP was compared to $A\beta_{1–42}$ alone for its biological activity on microglia isolated from brain specimens from AD cases, and was found to have a significant stimulatory effect on (pro-inflammatory) cytokine secretion in vitro.

Materials and methods

Trypsin, gentamycin, streptomycin, penicillin, LPS (E55:B5), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), poly-L-lysine (PLL; Sigma, St. Louis, Mo.), bovine pancreatic DNase I (Boehringer Mannheim, Germany), Dulbecco's modified Eagle's medium (DMEM), nutrient mixture HAM F-10 (HAM-F10; GIBCO Life Technologies, Breda, The Netherlands) and fetal calf serum (FCS; ICN Biomedicals, Amsterdam, The Netherlands), were obtained as indicated. Microtiter plates (96 wells) for enzyme-linked immunosorbent assays (ELISA) were from Nunc (Roskilde, Denmark), 48-well plates and 80-cm^2 culture flasks for cell culturing from Costar (Corning, NY).

$A\beta_{1–42}$ (Bachem, Bubendorf, Switzerland) was dissolved (stock solution; 500 μM) in pyrogen-free water (Baxter B.V., Utrecht, The Netherlands), aliquoted and stored at –20°C until use. Serum amyloid P component was from Calbiochem (La Jolla, Calif.). Human C1q was either from Quidel (San Diego, Calif.), or isolated from the Cohn I fraction of pooled human plasma, followed by cation exchange chromatography (Bio-Rex 70; Bio-Rad) and gel-filtration (Bio-Gel A5; Bio-Rad) according to published protocols [45]. The C1q was homogeneous as judged by SDS-PAGE, and transferred from TRIS-HCl buffer to sterile PBS by passage over a PD10 column (Pharmacia Biotech). Glycerol (GIBCO BRL) was added to a final concentration of 40% (v/v) and the C1q preparation was subsequently passed over a 0.22-μm filter (Millipore, Bedford, Mass.), aliquoted and stored at –80°C.

Brain tissue specimens

Human brain specimens with a short postmortem delay were obtained at autopsy through the Netherlands Brain Bank (coordinator Dr. R. Ravid). The clinical diagnosis of AD was neuropathologically confirmed on formalin-fixed, paraffin-embedded tissue from different brain regions. Staging of AD neuropathology was evaluated according to the criteria of Braak and Braak [6]. Likewise, the brain specimens from non-demented control cases comprising the same age group but without any clinical history of dementia were neuropathologically examined. For immunohistochemistry, mid-temporal cortex specimens from 14 AD cases and 12 non-demented control cases were snap-frozen and stored in liquid nitrogen. Average age and postmortem delay of AD cases were 80.7 years (range 59– 90 years) and 4 h 55 min (range 1 h 30 min–6 h 40 min), respectively. Mean age and postmortem delay for non-demented controls were 71.8 years (range 41–98 years) and 8 h 28 min (range 4 h 45 min–16 h 5 min), respectively.

To get more insight into differences in C1q and SAP accumulation and microglial activation during AD progression, caudate nucleus specimens from 4 of the 14 AD cases were included in this study. The caudate nucleus shows no signs of neurodegeneration and is known [18, 36] to contain diffuse or low-fibrillar amyloid plaques only.

For cell culture purposes subcortical white matter and cortical gray matter cerebral cortex specimens from 10 AD cases with an average age of 84.9 years (range 61–95 years) and postmortem delay of 6 h (range 4 h 45 min–9 h 35 min) were collected in

DMEM/HAM-F10 (1:1) containing gentamycin (Gibco, Paisley, UK; 50 μg/ml).

Immunohistochemistry

Immunostaining for C1q, SAP and Aβ was performed on acetone-fixed cryostat sections (5 μm) of snap-frozen brain specimens from AD as well as non-demented control cases. Primary antibodies included rabbit polyclonal antibodies specific for $A\beta_{1-28}$ (gift Dr. W.E. van Nostrand, Stony Brook), human SAP (DAKO, Glostrup, Denmark), and human C1q (Zymed, San Francisco, Calif.), as well as mouse monoclonal antibodies specific for hyperphosphorylated tau (AT8; Innogenetics, Gent, Belgium), CD68 (KP1; DAKO) and the HLA-DP/DQ/DR β chain (CR3/43; DAKO) as microglial markers. Polyclonal antibodies were detected with biotinylated swine anti-rabbit IgG second antibody (DAKO), mouse monoclonal antibodies with biotinylated rabbit anti-mouse IgG (DAKO). Avidin-biotin-peroxidase complexes (ABC; Vector Laboratories, Burlingame, Calif.) and either the chromogen diaminobenzidine or 3-amino-9-ethylcarbazole (AEC) were used for visualization. For double stainings, acetone-fixed sections were incubated (4°C;18 h) with a cocktail of two primary antibodies. Bound (Aβ, SAP or C1q specific) rabbit antibodies were detected after biotinylated swine-anti-rabbit IgG, alkaline phosphatase-conjugated streptavidin and Fast Blue BB incubations. Residual biotin was then blocked with subsequent streptavidin and D-biotin incubation steps (10 min each), whereafter the monoclonal microglia-specific antibodies were detected with biotinylated rabbit anti-mouse IgG antibodies, followed by the ABC complex and AEC.

Cells

Microglial cells were isolated according to published protocols [10, 47]. In short, cell suspensions obtained after mincing and trypsin digestion (37°C; 20 min) of brain specimens, devoid of blood vessels and meninges, were passed through a nylon 130 μm mesh filter and spun on a Percoll gradient (1.03 g/ml) at 1,250 *g* to remove the myelin. After shock lysis of erythrocytes in NH_4Cl, the resulting cell suspension was plated in uncoated 80-cm^2 flasks and grown in DMEM/HAM-F10 (1:1) containing 10% (v/v) FCS, 2 mM L-glutamine, 100 IU/ml penicillin and 50 μg/ml streptomycin and, additionally, 25 μg/ml granulocyte-monocyte colony-stimulating factor (rHuGM-CSF; Leucomax, Sandoz, Uden, The Netherlands). Microglial cells had either a bipolar or an ameboid morphology and immunostained with monoclonal antibodies LeuM5 (CD11c) and LeuM3 (CD14) (Becton Dickinson, Erembodegem, Aalst, Belgium), whereas they were negative for the astrocyte marker glial fibrillary acidic protein (GFAP).

Stimulation experiments

Stimulation experiments were performed essentially as described [47]. Isolated microglial cells, after trypsinization and scraping with a cell scraper (Costar, Corning, NY), were transferred to 48-well plates at cell densities of 2×10^4 cell/well, and allowed to settle for 24 h. At the start of the experiment, medium was replaced by DMEM/HAM-F10 containing 0.1% FCS, with or without $A\beta_{1-42}$ peptides, C1q, SAP, or combinations of these. For this, aliquots of $A\beta_{1-42}$ (500 μM), SAP (4,38 μM) and C1q (2,7 μM) stock solutions were diluted in DMEM/HAM-F10 containing 0.1% FCS to final concentrations of 10 μM ($A\beta_{1-42}$), 85 nM (SAP) and 5 nM (C1q). As a positive control, LPS was added to the cells at a concentration of 1 μg/ml. No detrimental effects of peptide and C1q or SAP treatment on cell viability, as assessed in an MTT assay, were observed in control experiments.

Culture supernatants from microglia were collected 24 h after the start of the treatment, centrifuged to remove cells, aliquoted and stored at –20°C, until assayed for the presence of cytokines. To determine intracellular cytokine levels, cells remaining in the wells were washed in PBS and lysed in PBS containing 0.5% of the nonionic detergent Nonidet (NP)-40 (100 μl/well).

ELISA

Cytokine levels in cell culture supernatants were measured in commercial enzyme immunoassay (ELISA) kits according to the instructions of the manufacturer (CLB, Amsterdam, The Netherlands, for IL-6, IL-1β, TNF-α, and BioSource Europe SA, Nivelles, Belgium, for IL-1α, respectively). In these sandwich ELISAs, 96-well ELISA plates (PolySorp; Nunc, Roskilde, Denmark) were coated with IgG fractions of cytokine-specific monoclonal antibodies in 50 mM carbonate buffer pH 9.6 at room temperature for 18 h, and subsequently blocked by incubation with PBS containing 2% (v/v) milk for 45 min. Standards, human recombinant cytokines, and samples were diluted in PBS 2% (v/v) milk. Washing in between incubation steps was with PBS containing 0.05% Tween 20. Bound cytokines were detected after subsequent incubations with the respective biotinylated antibodies and with streptavidin polyhorseradish peroxidase (CLB; Amsterdam). 3,5,3',5'-Tetramethylbenzidine (TMB; Sigma) at a concentration of 100 μg/ml in 0.11 M sodium acetate pH 5.5 containing 0.003% (v/v) H_2O_2 (100 μl final volume) was used as a substrate. Color development was stopped by adding an equal volume of 2 M H_2SO_4, whereafter the absorption at 450 nm was determined in a microculture plate reader (Titertek Multiscan; Flow labs). Detection limits of the ELISAs were 3.8, 0.4, 0.2 and 1 pg/ml for IL-1α, IL-1β, IL-6 and TNF-α, respectively.

Electron microscopy

Ultrastructural investigation was carried out on $A\beta_{1-42}$ peptide, C1q and SAP alone and on a mixture of $A\beta_{1-42}$ and C1q and SAP at the same concentrations as in stimulation experiments (i.e., 10 μM, 5 nM and 85 nM, respectively). All samples were diluted in 50 mM TRIS-HCl pH 7.0, and incubated at 37°C for 1 and 24 h. At each time point 5 μl of suspension was applied to formvar carbon-coated nickel grids for 6 min. Grids were negatively stained with uranyl acetate for 5 min and examined with a Zeiss EM109 electron microscope.

Statistical methods

SPSS (release 9.0.1) for Windows was used to assess statistical significance of differences between levels of cytokines expressed by microglial cells in vitro upon treatment with Aβ peptides, SAP, C1q, combinations of these, LPS or medium alone. Analysis of variance (ANOVA) was used to analyze repeated measures data. *P* values <0.01 were considered significant.

Results

Aβ and tau immunostaining

Two types of fibrillar amyloid deposits, classic neuritic plaques with a dense congophilic core and primitive plaques consisting of compacted Aβ, both containing tau (AT8)-positive dystrophic neurites, were predominant in the temporal cortex of AD cases (not shown).

Next to the midtemporal cortex, also caudate nucleus sections from the same AD case were examined for differences in expression of Aβ, C1q, SAP and microglial activation markers (HLA class II and CD68). The striatum is known [18, 36] to predominantly contain diffuse Aβ plaques that lack fibrillar amyloid and degenerating

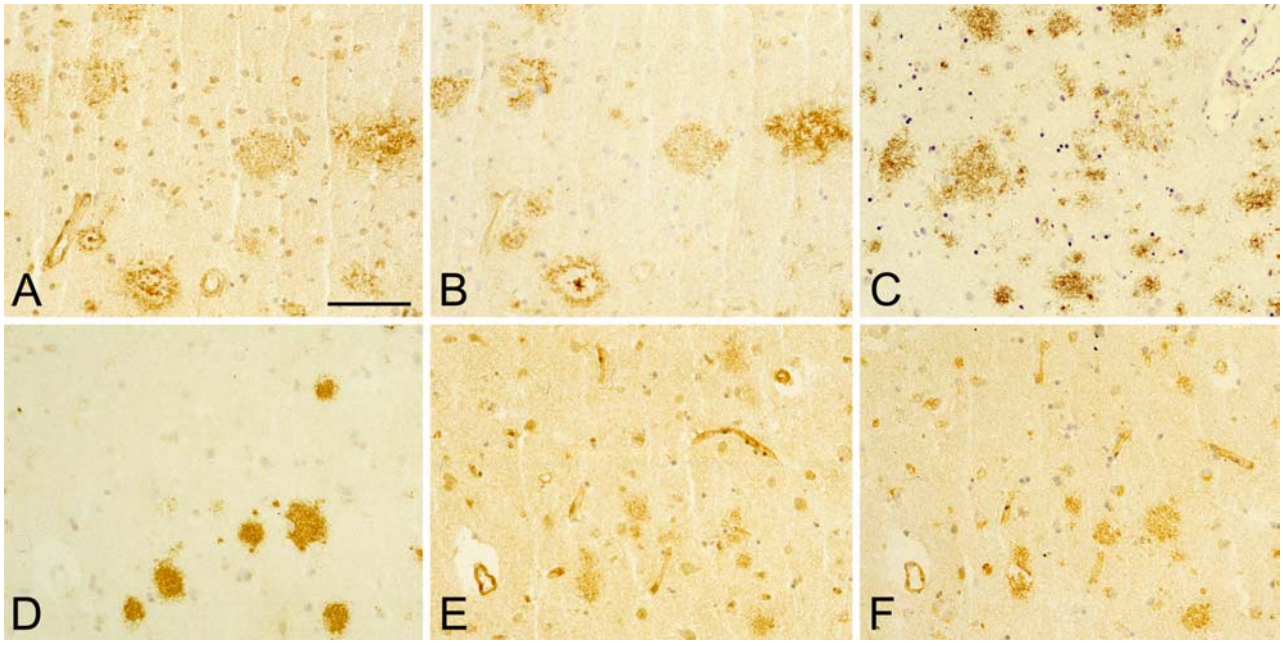

Fig. 1A–F Serial cryostat sections of AD midtemporal cortex and caudate nucleus were immunostained for tau (not shown), Aβ, SAP and C1q. Classic as well as primitive type Aβ plaques in the temporal cortex (AD case Braak stage 4/C) are immunoreactive for SAP (**A**) as well as C1q (**B**). The AD (Braak stage 6/C) caudate nucleus contains diffuse-type Aβ plaques (**C**) that are SAP and C1q negative, as judged from immunostainings on adjacent sections (not shown). In an equal number of AD cases also more compacted and circumscript, tau-negative (not shown) Aβ-plaques are observed in the caudate nucleus. These circumscript Aβ plaques (**D**) are immunoreactive for SAP (**E**) and C1q (**F**) (AD case Braak stage 5/C) (*AD* Alzheimer's disease, *SAP* serum amyloid P component). *Bar* 20 μm

neurites. The diffuse-type Aβ plaques in the caudate nucleus of the AD cases studied were not associated with tau pathology (not shown). In two of the four caudate specimens from AD cases, next to the diffuse Aβ plaques, more condensed, circumscribed Aβ plaques were also seen (Fig. 1D) that were tau-negative (not shown).

No Aβ plaques were observed in temporal cortex samples of 6 of 12 non-demented control cases. The other 6 had large numbers of, mainly diffuse-type, Aβ plaques. The diffuse-type plaques were Congo red negative and were devoid of neuritic changes, when immunostained with the tau-specific antibody AT8. Next to the relatively large, irregular-shaped diffuse Aβ plaques, smaller, circumscript Aβ plaques were also observed, which had a somewhat stronger immunostaining intensity for Aβ, and were Congo red and AT8 negative. Occasionally, dense-core (classic type-like) Aβ plaques were seen that were Congo red positive, but AT8 negative.

SAP, C1q and microglia marker immunostaining

AD temporal cortex neuritic plaques with amyloid cores immunostained for C1q and SAP. Staining intensity for SAP (Fig. 1A), and for C1q (Fig. 1B) was highest in the cores of the neuritic plaques. The majority of these plaques was found to be associated with clusters of activated [HLA-class II (CR3/43) or CD68 (KP1) positive] microglia. Similar results were seen with primitive Aβ plaques in AD cases; however, a lower percentage (approximately 75%) of these plaques immunostained for C1q and SAP. Clusters of activated microglia were seen in a minority of the primitive plaques, and on average consisted of lower numbers of microglia than the clusters seen in dense-cored amyloid plaques of AD cases (Fig. 2). Clustered, activated microglia were only seen in SAP- and C1q-positive primitive and neuritic plaques (Table 1).

The diffuse-type Aβ plaques in the caudate nucleus (Fig. 1C) did not immunostain for C1q and SAP, and were not associated with activated microglia (not shown), comparable to the diffuse-type plaques in the temporal cortex of non-demented controls. The more condense, circumscript Aβ plaques that were seen in two of the four caudate specimens from AD cases (Fig. 1D) exhibited a relatively intense SAP (Fig. 1E) and C1q (Fig. 1F) immunostaining. A few of these were decorated with low numbers, but not clusters of microglia (not shown).

At maximum 50% of the circumscribed Aβ plaques in temporal cortex specimens of non-demented control cases showed moderate immunostaining intensity for SAP and C1q. SAP and C1q were always found to co-localize, as judged from immunostainings on adjacent serial sections. Neither the SAP- and C1q-positive circumscript Aβ plaques, nor the SAP- and C1q-negative circumscript and irregular-shaped diffuse Aβ plaques in non-demented controls were found to be associated with clusters of activated microglia. Occasionally, more compacted or even classic-type amyloid plaques with a dense core were observed. These were tau negative, C1q and SAP positive and sometimes contained clusters of activated microglia (Table 1).

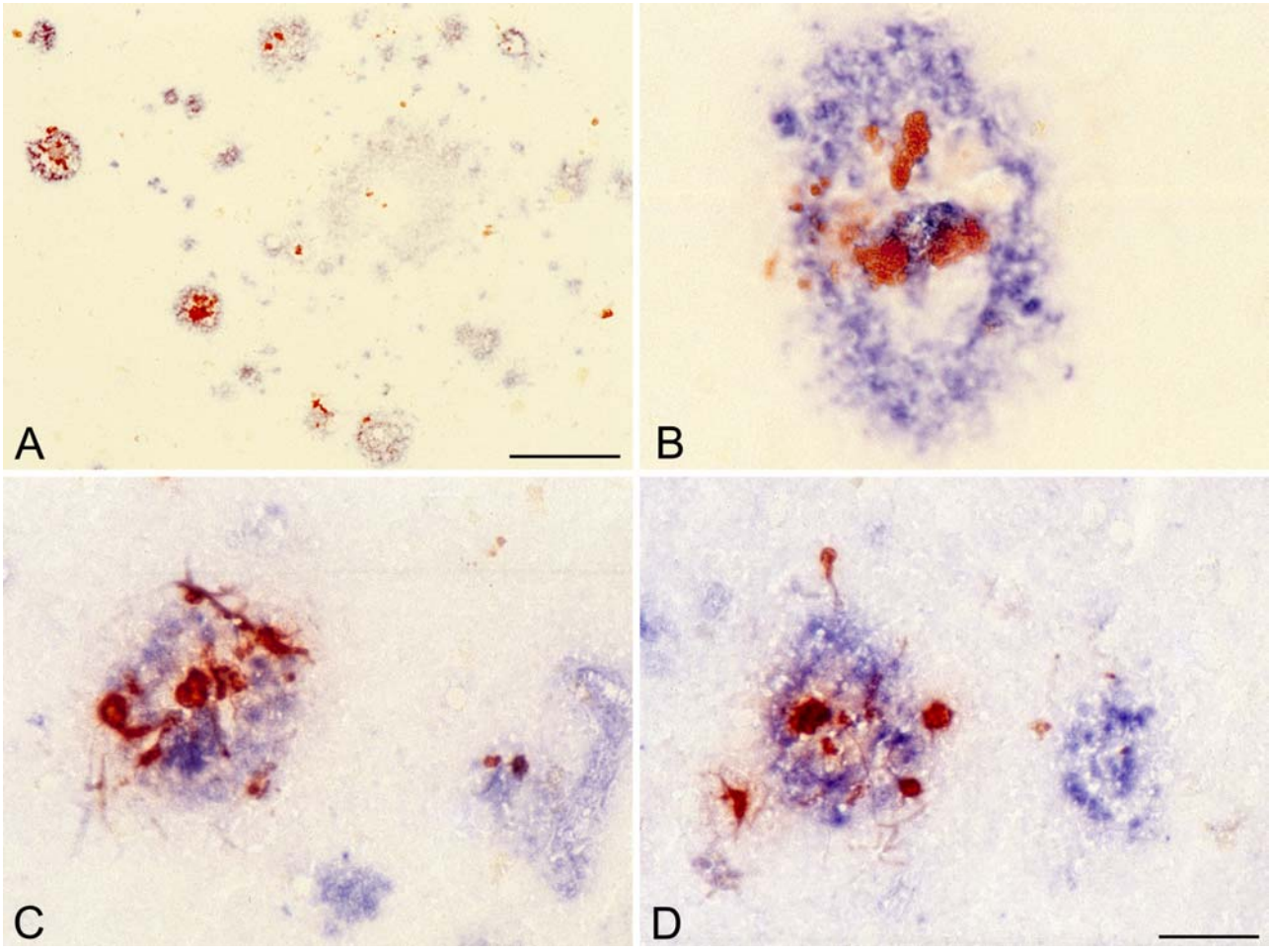

Fig. 2 Double staining for a microglial marker KP1 (CD68; *red*) and Aβ (*blue*) clearly shows the clustering of microglia in neuritic plaques with a dense core (**A**, **B**), not in more diffuse, large-sized Aβ deposits (**A**). Double labeling for either C1q (**C**) or SAP (**D**) and KP-1 on adjacent sections of the same AD case indicates that C1q and SAP positivity coincide and that all classic and part of the primitive (diffuse neuritic) C1q and SAP positive plaques are accompanied by clusters of microglia. **A** AD case Braak stage 4/B; **B**–**D** AD case Braak stage 4/C. *Bar* **A** 100 μm; **D** 35 μm (also for **C**), 20 μm for **B**

Table 1 Immunohistochemical distribution of SAP, C1q and activated microglia in morphologically distinguished cerebral Aβ plaque types; – none, ± maximally 50% of total, + >75% of total, ++ all plaques (*SAP* serum amyloid P component)

Immuno-staining	Aβ plaque type				
	Non-fibrillar		Fibrillar (neuritic)		
Aβ	Irregular shaped, diffuse	Circum-script (well demarcated)	Classic with dense core		Primitive neuritic plaques
			Core	Corona	
SAP	–	±	++	+	+
C1q	–	±	++	+	+
Tau (AT8)	–	–	+[a]		+
Clustered microglia[b]	–	–	+		±

[a]Classic Aβ plaques in non-demented controls lack tau immunoreactivity (AT8)
[b]Microglia markers KP-1 (CD68) and CR3/43 (HLA DP/DQ/DR)

Constitutive and LPS-induced cytokine release by microglia in vitro

To see, whether the simultaneous presence of Aβ, SAP and C1q was responsible for the presence of activated microglia, in vitro studies were performed in which a mixture of Aβ, SAP and C1q was added to the adult human microglia in culture. Human microglia isolated from post-mortem brain specimens used in this study displayed a bipolar to ameboid morphology. For stimulation experiments, microglia were transferred from culture flasks to 48-well plates (2×10^4 cells/well) and allowed to adhere and settle for 24 h. Microglia in culture secreted low to moderate levels of IL-6 and TNF-α (15.9±5.4 and 3.5± 0.6 pg/ml, respectively; mean ± SEM), as determined by ELISA in cell culture supernatant collected after 24 h (Table 2). Levels of secreted IL-1α and IL-1β were around or below detection limits of the ELISAs (3.8 and 0.4 pg/ml for IL-1α and IL1β, respectively).

LPS, the pluripotent activator that was used as a positive control for the induction of microglial cytokine secretion, clearly enhanced the secretion of IL-6 (586±97.1 pg/ml)

Table 2 Effects of different combinations of $A\beta_{1–42}$, SAP and C1q on cytokine expression by adult human microglia in vitro

	IL-6	TNF-α	IL-1β (sup)	IL-1α (sup)	IL-1β (NP-40)	IL-1α (NP-40)
Medium	15.9±5.4 (*n*=27)	3.5±0.6 (*n*=27)	1.5±0.4 (*n*=27)	3.3±0.5 (*n*=21)	6.0±1.0 (*n*=26)	14.8±5.6 (*n*=26)
Aβ	22.7±6.7 (*n*=24)	10.2±2.2* (*n*=24)	2.0±0.3 (*n*=24)	5.2±1.1 (*n*=18)	10.2±2.1 (*n*=24)	26.4±11.2 (*n*=24)
Aβ + C1q	93.6±22.9*# (*n*=12)	8.6±2.1 (*n*=12)	1.1±0.3 (*n*=12)	3.5±0.9 (*n*=12)	27.7±9.3# (*n*=12)	5.2±1.7 (*n*=12)
Aβ + SAP	8.2±1.8 (*n*=3)	1.0±0.4 (*n*=3)	3.4±0.5 * (*n*=3)	7.2±6.3 (*n*=3)	50.8±25.8 (*n*=6)	11.5±5.1 (*n*=6)
SAP + C1q	75.7±18.9* (*n*=9)	15.4±5.9 (*n*=9)	3.9±1.7 (*n*=9)	1.2±0.8 (*n*=9)	26.8±7.1*# (*n*=9)	23.5±7.8 (*n*=9)
Aβ + SAP + C1q	205.2±48.0*#§ (*n*=21)	83.6±26.3*#§ (*n*=21)	2.4±0.4§ (*n*=21)	3.6±0.9 (*n*=15)	73.3±13.5*#§ (*n*=21)	60.0±12.7*§ (*n*=21)

Human microglia (2×10^4 cell/well) were exposed to $A\beta_{1–42}$ (10 μM), to combinations of $A\beta_{1–42}$ (10 μM), human SAP (85 nM) and human C1q (5 nM) or to medium alone in three replicate wells for 24 h. Cytokine levels (pg/ml) in cell culture supernatants or in Nonidet-P40 cell lysates were determined by ELISA. Data are mean ± SEM of nine independent experiments, each performed in triplicate. Due to the limited yield of adult human microglia, each experiment was performed with microglia from different cases and not all combinations of Aβ, SAP and C1q could be tested in one and the same experiment. *Asterisks*, statistically different from corresponding medium control values (*P<0.01), # from Aβ values (#P<0.01), and § Aβ-SAP-C1q values from Aβ-C1q values (§P<0.01).

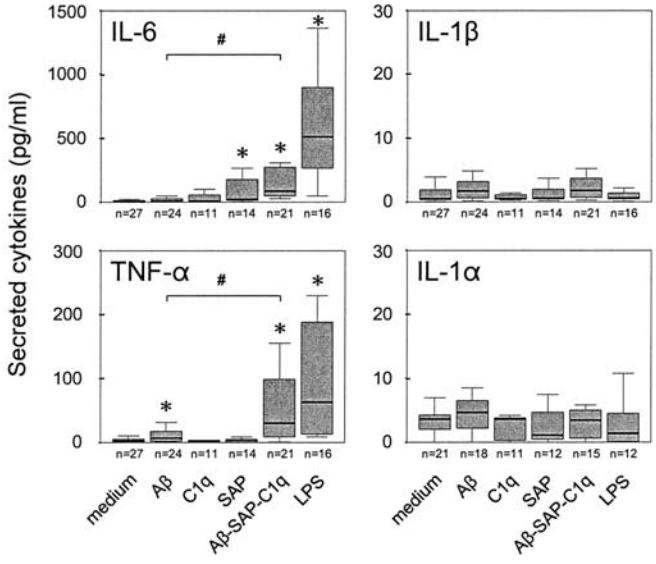

Fig. 3 Amyloid-β ($A\beta_{1–42}$) in combination with SAP and C1q induces the secretion of IL-6 and TNF-α, not of IL-1β and IL-1α, by adult human microglia. Human microglia (2×10^4 cell/well; 250 μl medium) were exposed to either medium alone, medium containing Aβ (10 μM), SAP (85 nM) or C1q (5 nM) or to Aβ in combination with SAP and C1q for 24 h. LPS (1 μg/ml) was added to the cells as a positive control. Results are from nine independent experiments performed in triplicate, each with microglia from different cases. Levels of secreted cytokines are depicted as *boxplots*. The *horizontal line* through the *box* represents the median, and the *top* and *bottom* of the *box* mark the upper and lower quartiles. *Tails* mark the actual range of all data in the group. Note the different scales on the *Y-axis* in the panels depicting IL-6, TNF-α, IL-1β and IL-1α data. ANOVA was performed to evaluate statistical significance (*P<0.01 versus medium; # P<0.01 versus Aβ treatment)

and TNF-α (226±109 pg/ml). No stimulatory effects of LPS on IL-1α and IL-1β secretion were observed.

Effects of Aβ, C1q and SAP on the cytokine secretion by microglia in vitro

$A\beta_{1–42}$ (10 μM) had no stimulatory effects on the IL-6 release by adult human microglia in vitro. Although C1q on its own had no effect, addition of C1q (5 nM) to the Aβ significantly increased the IL-6 levels in microglial cell culture supernatants, compared to the IL-6 levels secreted by microglia exposed to either Aβ, or medium alone. Addition of SAP (85 nM) to the Aβ-C1q mixture led to a significant further increase in secreted IL-6 levels (Table 2). Except for SAP, the factors alone had no clear stimulatory effect on microglial IL-6 release. The stimulatory effect of SAP on microglial IL-6 secretion did not increase upon addition of C1q or Aβ (Fig. 3; Table 2).

Whereas addition of $A\beta_{1–42}$ (10 μM) to microglia had no effects on the secretion of IL-6, IL-1α and IL-1β, it clearly induced TNF-α secretion. Neither C1q (5 nM) or SAP (85 nM) alone, nor the combinations Aβ and C1q, Aβ and SAP as well as SAP and C1q influenced microglial TNF-α secretion. However, when added simultaneously, $A\beta_{1–42}$, SAP and C1q induced a significant increase in levels of secreted TNF-α compared to medium or $A\beta_{1–42}$ treated cells (Fig. 3; Table 2).

Effects of Aβ, C1q and SAP on microglia in vitro: cell-associated IL-1β or IL-1a

No effects on IL-1β or IL-1α secretion was observed when microglia were treated with Aβ, SAP and C1q, either alone or in combination, or with LPS for 24 h (Fig. 3; Table 2). Exposure to LPS did, however, induce the intracellular accumulation of IL-1α and IL-1β, as determined in a lysate of detergent (NP-40) solubilized microglia after removal of the supernatant (Fig. 4).

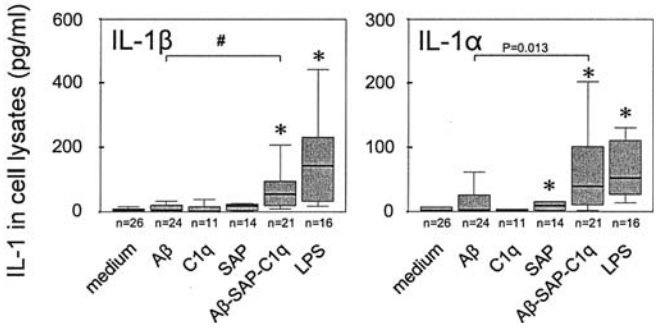

Fig. 4 Exposure of adult human microglia to a combination of Aβ (10 μM), SAP (85 nM) and C1q (5 nM) for 24 h leads to the intracellular accumulation of IL-1β (left panel) and IL-1α (right panel). The levels of IL-1β and IL-1α in NP-40 (0.5% v/v) lysates of microglia were determined by ELISA. Results are from nine independent experiments performed in triplicate, each with microglia from different cases. Values are depicted as boxplots (see legends of Fig. 3). ANOVA was performed to evaluate statistical significance (*P<0.01 versus medium; # P<0.01 versus Aβ treatment)

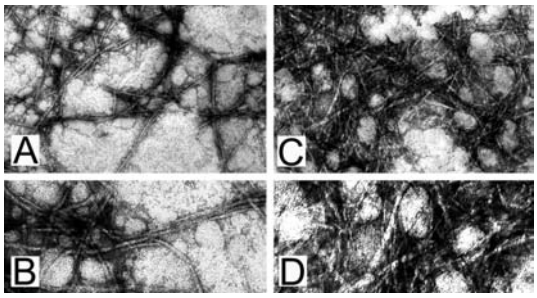

Fig. 5A–D Electron micrographs of synthetic $A\beta_{1-42}$ peptide fibrils. **A**, **B** Peptide (10 μM) alone after 1-h incubation in 50 mM TRIS-HCl pH 7.0 at 37°C; **C**, **D** peptide incubated at 37°C for 1 h in the presence of 5 nM C1q and 85 nM SAP. **A**, **C** ×54,000; **B**, **D** ×108,000

Exposure of microglia to either Aβ, SAP or C1q alone had no stimulatory effects on cell-associated IL-1β levels (Fig. 4). C1q added together with either Aβ or SAP induced the expression of cell associated IL-1β (Table 2). Exposure of the microglia to a mixture of all three factors resulted in cellular IL-1β levels that were significantly higher than those in either Aβ-, or Aβ + C1q-treated cells (Fig. 4; Table 2).

Similar to those of IL-1β, cellular IL-1α levels significantly increased upon exposure of the microglia to the combination of Aβ, SAP and C1q. Except for SAP alone, no stimulatory effects of added Aβ, C1q or double stimulations with combinations of Aβ and SAP or C1q on IL-1α expression were observed (Fig. 4; Table 2).

To assess possible effects on Aβ fibrillarity of C1q and SAP at concentrations used in the cell stimulation experiments, synthetic $A\beta_{1-42}$ diluted in 50 mM TRIS-HCl pH 7.0 (10 μM) was incubated either alone or in the presence of C1q and SAP at 5 nM and 85 nM concentrations, respectively at 37°C for 1 and 24 h. Electron microscopical examination indicated that C1q and SAP samples alone did not form fibrils, and that only a few electron-dense amorphous aggregates were detectable on grids. $A\beta_{1-42}$ generated long, straight or occasionally twisted fibrils, having a relatively regular diameter of 8.36±2.67 nm (median 8 nm). The fibrils were arranged to form a loose meshwork (Fig. 5A, B) and showed some tendency to lateral aggregation with formation of small bundles. The addition of C1q and SAP to the $A\beta_{1-42}$ peptide resulted in a striking increase in fibril density even after 1-h incubation, with generation of a large number of densely packed aggregates. Furthermore, the fibrillary structures differed from those formed by $A\beta_{1-42}$ alone in that they were shorter, less regular and more heterogeneous in size, with a diameter of 9.48±4.58 nm (median 10 nm) (Fig. 5C, D).

Discussion

The results of the immunohistochemical part of the present study indicate that the co-localization of SAP and C1q with cerebral Aβ deposits depends on the fibrillarity of the deposited Aβ. Strong immunostaining for C1q and SAP was seen in the majority of dense-cored and primitive neuritic plaques in the temporal cortex of AD cases. Weak to moderate immunostaining for C1q and SAP was observed in a variable number of circumscript diffuse plaques in AD and control temporal cortex specimens, but not in the irregular shaped diffuse Aβ plaques, in non-demented controls (Table 1). Besides to the temporal cortex, possible co-localization of C1q, SAP and activated microglia were also studied in caudate specimens from AD cases. No primitive or dense-cored neuritic plaques [36] are found in the caudate nucleus, only diffuse-type Aβ plaques, devoid of fibrillar amyloid, paired helical filaments and glial cells [18]. As described before [36], we observed two types of diffuse Aβ plaques in the caudate of AD cases upon Aβ immunostaining: irregular-shaped diffuse-type plaques and circumscript plaques. The circumscript plaques, but not the irregular-shaped, "true" diffuse plaques in the caudate nucleus of AD cases were found to be C1q and SAP positive (Fig. 2). These findings extend earlier reports that C1q [1, 14] and SAP [14, 53] do not bind in significant amounts to low-grade or non-fibrillar Aβ, and are in line with in vitro data, indicating dependence on the degree of fibrillarity of Aβ for binding of C1q and C1 activation [25, 43]. Whether direct C1 binding and activation by Aβ is the predominant pathway of classical complement activation in vivo remains to be investigated. Since SAP seems invariably co-localized with C1q-containing Aβ deposits in AD brain (Figs 1, 2), and SAP is known to interact with C1q [8] and to induce C1 activation when aggregated or bound to a solid phase in vitro [23, 52], it may at least contribute to the C1 activation in AD brain.

Clusters of activated microglia are observed in primitive and classic (dense-cored) plaques in the AD neocortex ([12, 21, 42] and this study). The activated, IL-1α-

positive microglia that are predominantly found in primitive, diffuse neuritic plaques may play an important role in the conversion of non-neuritic into neuritic Aβ plaques in AD [21]. Microglial activation is thought to occur during the transition of diffuse to primitive plaques, because no clusters of activated microglia are found in diffuse-type plaques [42]. In the present study, activated microglia were observed only in SAP- and C1q-immunoreactive Aβ plaques in AD and control cases. No clusters of activated (CD68 or HLA-DP-DQ-DR positive) microglia were observed in diffuse-type Aβ plaques in temporal cortex and caudate nucleus, or in the more circumscript Aβ plaques that are SAP and C1q positive. That circumscript, diffuse Aβ deposits in non-demented control temporal cortex and AD caudate nucleus are without glial activation, although they are to a certain extent decorated with SAP and C1q (Table 1), led us to suggest that binding of SAP and C1q to (low) fibrillar Aβ deposits precedes microglial attraction and activation, and may initiate microglial activation.

In vitro, SAP was shown to interact with aggregated Aβ [24]. Since SAP oligomers can bind and activate C1 [23, 52], the association of SAP with Aβ deposits could provide enough clustered SAP to bind and activate C1. The local misbalance in synthesis rates between C1 subcomponents and of the physiological inhibitor C1-Inh [47, 51] may allow C1 activation to escape from control in these AD-affected brain areas, which can result in the formation of complement activation products as well as of the lytic membrane attack complex. Complement activation products (e.g., C5a) can, in turn, attract microglia. In a recent study Aβ was shown to prime the complement C5a-induced cytokine release by THP1 cells, as a model for microglia [35]. This suggests that the combination of Aβ and complement activation products, which are present in AD amyloid plaques, may induce cytokine secretion by microglia attracted to the site. That Aβ-associated factors can modulate the Aβ-induced cytokine expression is an interesting finding, because up to then synergistic actions of Aβ and other stimuli like interferon-γ, LPS or phorbol myristate acetate (PMA) seemed needed for the secretion of cytokines [27], reactive nitrogen [32] or reactive oxygen intermediates [46] by rodent microglia or monocytes.

Recently, preaggregated, fibrillar $A\beta_{1-42}$ alone was shown to stimulate the secretion of pro-inflammatory cytokines by human microglia in vitro [28]. It remains to be determined, whether this effect is specific for human adult microglia as suggested [28], or is dependent on the aggregation state of the Aβ. In the present study, minor (IL-6) to significant (TNF-α) stimulatory effects of freshly solubilized $A\beta_{1-42}$, added at 10 μM concentrations, on the secretion of IL-6 and TNF-α by postmortem human microglia were observed in vitro (Fig. 3; Table 2), which suggests that indeed human microglia can respond to exposure to $A\beta_{1-42}$ with cytokine release. SAP and C1q that co-localize with Aβ deposits in AD brain ([14, 53] and this study) were found to significantly increase the $A\beta_{1-42}$-induced secretion of IL-6, as well as of TNF-α by microglia in vitro (Table 2; Fig. 3). Similar effects of SAP and C1q were seen on levels of cell-associated IL-1β and IL-1α. Low levels of IL-1 may have been released that were below the detection levels of the ELISA systems we used (0.4 and 3.8 pg/ml for IL-1β and IL-1α, respectively). In human fetal microglia LPS dose-dependently stimulates the intracellular accumulation of IL-1β, whereas only minor amounts can be detected in the culture supernatant [26]. Minute amounts of IL-1 may, however, already be sufficient to exert cellular effects. High levels of secreted prostaglandin E2 and IL-6 are reached when primary adult human astrocyte cultures are exposed to 250 U/ml, which equals 25 ng/ml, concentrations of IL-β, as a positive control [48].

Whether the effect of SAP and C1q on Aβ-induced microglial cytokine release is through enhancement of Aβ fibril formation, or whether the effects are mediated through cellular interactions of either C1q or SAP contained within the Aβ-SAP-C1q complexes, is subject of further investigation. The enhancement of aggregation and modification in fibril morphology upon addition of C1q and SAP to $A\beta_{1-42}$ as judged by electron microscopy (Fig. 5) are consistent with the view that C1q and SAP feature in Aβ fibrillogenesis [17, 49]. On the other hand, since microglia express C1q receptors that can enhance phagocytosis, and in addition can mediate cellular signaling leading to, e.g., enhanced CR1 activity [50], C1q-C1q receptor interactions may be involved in the cellular signaling.

The results from the immunohistochemical part of the present study, although studied in differently affected brain areas within AD cases, as well as in non-demented control and AD temporal cortex specimens, are related to hypothetical Aβ plaque type definitions and not to the progress of the disease (Table 1). Nevertheless, our findings that activated microglia accumulate in SAP- and C1q-immunoreactive fibrillar Aβ plaques, but not in non-fibrillar Aβ plaques, are comparable to results from longitudinal studies in transgenic mice that carry mutant amyloid precursor protein and presenilin-1 genes (PS/APP mice) [30] and in Down's syndrome (DS) cases [44]. In the PS/APP mice the number of fibrillar Aβ deposits in the frontal cortex increased with age, with a parallel increase in activated, CD11b immunoreactive microglia closely associated with the Aβ deposits. C1q co-localized with fibrillar Aβ plaques and, in addition, was found in plaque-associated, activated microglia [30].

When immunoreactivity for complement activation products and activated microglia in temporal cortex specimens were compared in young and old DS cases, teenage DS cases were found to have large numbers of diffuse plaques that are devoid of C1q and C3 and show no accumulation of activated microglia. In middle aged and old DS brain, on the other hand, C1q and C3 immunoreactivity were primarily confined to fibrillar Aβ plaques associated with activated microglia and dystrophic neurites [44]. Taken together, these findings suggest that a sequence of events including complement activation, activation of glial cells and neuritic changes is related to the progression of AD pathology.

Indeed, a parallel increase in numbers of congophilic Aβ plaques and of activated microglia was found to correlate with cognitive decline, when postmortem cerebral cortex specimens of AD cases with different degrees of cognitive status are compared [3]. Interestingly, the increase in numbers of clustered microglia and congophilic plaques levels off at a certain stage of the disease, whereas tau pathology further increases with ongoing cognitive decline. Recent in vivo imaging studies have shown that accumulation of activated microglia in AD pathology-prone areas occurs at a relatively early stage, probably before neurodegenerative changes occur [9]. Our present report indicates that around this relatively early stage of the AD disease process, when the numbers of activated microglia and congophilic plaques are high [3], the microglia may get triggered by plaque-associated factors C1q and SAP to secrete high levels of pro-inflammatory cytokines that can set off a process of accelerated neurodegeneration. Since the increase in numbers of activated microglia that express cytokines IL-1, IL-6 and TNF-α [4, 21] is seen at a stage of AD, when cognitive decline is still moderate and tau pathology not yet at its height [3], targeting microglial activation at this stage may provide therapeutic options for treatment of AD patients.

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