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Amyloid beta peptide 1–40 enhances the action of Toll-like receptor-2 and -4 agonists but antagonizes Toll-like receptor-9-induced inflammation in primary mouse microglial cell cultures

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Abstract

The interaction of endogenous and exogenous stimulators of innate immunity was examined in primary cultures of mouse microglial cells and macrophages after application of defined Toll-like receptor (TLR) agonists [lipopolysaccharide (LPS) (TLR4), the synthetic lipopeptide Pam3Cys-Ser-Lys4 (Pam3-Cys) (TLR2) and single-stranded unmethylated CpG-DNA (CpG) (TLR9)] alone and in combination with amyloid beta peptide (Abeta) 1–40. Abeta 1–40 stimulated microglial cells and macrophages primed by interferon-γ in a dose-dependent manner. Co-administration of Abeta1–40 with LPS or Pam3-Cys led to an additive release of nitric oxide (NO) and tumour necrosis factor alpha (TNF-α). This may be one reason for the clinical deterioration frequently observed in patients with Alzheimer's disease during infections. In contrast, co-application of Abeta1–40 with CpG led to a substantial decrease of NO and TNF- α release compared with stimulation with CpG alone. Abeta 1–40 and CpG did not co-localize within the same subcellular compartment, making a direct physico-chemical interaction as the cause of the observed antagonism very unlikely. This suggests that not all TLR agonists enhance the stimulatory effect of Abeta on innate immunity.

Keywords: amyloid beta 1–40 peptide, cytosin-guanosin oligodesoxynucleotide, lipopolysaccharide, microglia, nitric oxide, tripalmytoyl-cysteinyl-seryl-(lysyl)3-lysine.

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A significant inflammatory component contributes to the pathology of Alzheimer's dementia (Bamberger and Landreth 2001). The clinical status of patients suffering from neurodegenerative diseases, in particular Alzheimer's and Parkinson's disease, frequently deteriorates during infections (Perry *et al*. 2003). A study of the course of Alzheimer's disease in monozygotic and dizygotic twins, in which one twin had suffered serious infection and developed Alzheimer's dementia, showed that persons who had not suffered from serious infections developed Alzheimer's dementia later or not at all, and also lived longer (Nee and Lippa 1999). Moreover, delirium is a frequent consequence of infection in the elderly, particularly prevalent in demented patients (Elie *et al*. 1998). It can occur in early stages of Alzheimer's dementia (Lerner *et al*. 1997). A strong association between delirium during the acute phase and functional decline up to six months after discharge from hospital has been noted, suggesting that an insult precipitating delirium accelerates long-term

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Abbreviations used: Abeta, amyloid beta peptide; BSA, bovine serum albumin; CFU/mL, colony-forming units/mL; CpG, cytosin-guanosin oligodesoxynucleotide; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; IL-10, interleukin 10; LPS, lipopolysaccharide; NO, nitric oxide; Pam3Cys, tripalmytoyl-cysteinyl-seryl-(lysyl)3-lysine; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PVDF, hydrophilized polyvinylidene fluoride; SDS, sodium dodecyl sulfate; TBS, Tris-buffered saline; TLR, Toll-like receptor; TNF-α, tumour necrosis factor alpha; WST-1, water-soluble tetratolium salt-1.

cognitive decline (Murray *et al*. 1993). Conversely, long-term treatment with non-steroidal anti-inflammatory drugs reduces the incidence of Alzheimer's disease and delays disease progression (Bamberger and Landreth 2001).

Toll-like receptors (TLR) play a key role in the recognition of products from virtually all classes of pathogenic organisms. Amyloid peptides also can stimulate the innate immune system (Bamberger *et al*. 2003; Guillemin *et al*. 2003; Fassbender *et al*. 2004; Sondag and Combs 2004; Verdier *et al*. 2004). For this reason, we hypothesized that bacterial compounds and amyloid peptides may jointly stimulate the innate immune system. This hypothesis was studied in primary mouse microglial and macrophage cultures.

Materials and methods

TLR agonists

N-palmitoyl-S-[2,3-bis(palmitoyloxy)-(2RS)-propyl]-[R]-cysteinyl-[S]-seryl-[S]-lysyl-[S]-lysyl-[S]-lysyl-[S]-lysyl-[S]-lysine × 3 HCl (Pam_3Cys-SKKKK × 3 HCl; EMC Microcollections, Tuebingen, Germany, molecular mass 1619.1 g/M according to the manufacturer's data) and heat-killed *Acholeplasma laidlawii* [equivalent of 2×10^6 colony-forming units (CFU)/mL; InvivoGen, San Diego, CA, USA] were used as specific agonists of TLR-2 (Aliprantis *et al*. 1999). Cytosin-guanosin (CpG) oligodesoxynucleotide 1668 (TCCATGACGTTCCTGATGCT) from TIB Molbiol (Berlin, Germany; molecular mass 6382.6 g/M according to the manufacturer's data) was used as specific ligand of TLR9 (Lipford *et al*. 1997; Dalpke *et al*. 2002). For activation of TLR4, microglial cells were exposed to endotoxin [lipopolysaccharide (LPS) from *Escherichia coli* Serotype 026:B6; Sigma, Taufkirchen, Germany; molecular mass approximately 10 000 g/M according to the manufacturer's data].

Amyloid beta peptide (Abeta 1–40)

The amyloid beta peptide 1–40 (Abeta 1–40) hydrochloride salt (Bachem, Bubendorf, Switzerland; molecular mass 4329.86 g/M according to the manufacturer's data) was used in these experiments because the hydrochloride (HCl) forms of amyloid beta peptides aggregate more readily than the trifluoroacetic acid forms of the peptides (Takata *et al*. 2003). The HCl form of Abeta 1–40 develops beta-sheet structures in phosphate-buffered saline (PBS) or culture medium within a few hours at 25°C, according to the manufacturer's instructions. Beta-structure formation is essential for amyloid peptide toxicity (Chalifour *et al*. 2003; Hwang *et al*. 2004). To prevent uncontrolled aggregation, lyophilized Abeta 1–40 was dissolved in distilled and sterilized water at a concentration of 1 mM/L, then 10 μL aliquots in LPS-free tubes were kept at − 80°C until use. For stimulation of microglia, these aliquots were diluted in cell culture medium. Dose–response curves were constructed with Abeta 1–40 doses from 0.1 to 100 μM/L. For co-stimulation experiments, Abeta 1–40 concentrations of 10 or 30 μM/L were used.

In order to exclude non-specific stimulation by peptide aggregates, microglial cells were incubated with the fragment 61–95 of α-synuclein (Bachem, Bubendorf, Switzerland; molecular mass 3260.65 g/M); α-synuclein represents the non-Abeta component of Alzheimer's disease amyloid (NAC) and was found to form amyloid fibrils (Iwai *et al*. 1995). To prevent uncontrolled aggregation, lyophilized α-synuclein 61–95 was dissolved in distilled and sterilized water at a concentration of 1 mM/L, and aliquots were kept in LPS-free tubes at − 80°C until use. For stimulation of microglia, these aliquots were diluted in cell culture medium to different final concentrations of 0.1, 0.3, 1.0, 3.3, 10 or 20 μM/L. A concentration of 10 μM α-synuclein 61–95 was used for co-stimulation experiments with the TLR-agonists LPS, CpG and Pam3Cys.

Primary mouse microglial cell culture

Primary cultures of microglial cells were established from brains of newborn C57BL/6 mice (1–3 days). After removal of the meninges, cells were mechanically dissociated and suspended in Dulbecco's modified Eagle's medium (DMEM) with Glutamax I (Gibco, Karlsruhe, Germany) supplemented with 10% fetal calf serum (FCS), 100 U/mL penicillin and 100 μg/mL streptomycin. Cells were plated at a density of two brains per T75 culture flask (Corning Costar, Wiesbaden, Germany) and incubated at 37°C in a humid atmosphere with 5% CO_2. Culture medium was changed twice a week. After 10–14 days, confluent mixed glial cultures were shaken 200 times per minute for 30 min. Microglial cells in the supernatant fluid were re-plated in 96-well cell culture plates at a density of 75 000 cells/well. After 3 h, microglial cultures were exposed to the different TLR agonists and Abeta 1–40 for 24 h in the presence of interferon-γ (100 U/mL). TLR agonists were used at a concentration inducing approximately 20% and 60% of the NO production achieved by stimulation with a high dose of LPS (100 nM/L) over 24 h (Ebert *et al*. 2005) [Pam_3Cys: 600 pM/L or 6 nM/L; heat-killed *Acholeplasma laidlawii* (2×10^6 CFU/mL); LPS: 10 pM/L or 100 pM/L; CpG: 5 nM/L or 50 nM/L]. Stimulation of microglial cells with TLR agonists for less than 24 h elicited minor or no responses. The Abeta 1–40 hydrochloride salt stock solution (1 mM/L) was diluted in cell culture medium (DMEM with Glutamax I, 10% FCS, penicillin 100 U/mL and 100 μg/mL streptomycin; Gibco-Invitrogen, Karlsruhe, Germany) to a final concentration of 10 or 30 μM/L. Control cultures were treated with medium and interferon-γ only.

Supernatant fluids from stimulated glial cultures and unstimulated controls were directly analysed for NO production, or stored frozen at − 80°C until measurement of tumour necrosis factor alpha (TNF-α) or interleukin 10 (IL-10). Microglial cells were assayed for cell viability or fixed in 4% formaldehyde.

For establishing TLR2- and TLR9-deficient microglial cells, brains of TLR2–/– and TLR9–/– (Takeuchi *et al*. 1999; Hemmi *et al*. 2000) mice were used for preparation. Mice were back-crossed at least seven times and were kindly provided by S. Akira and K. Takeda, Osaka, Japan, and H. Wagner and A. Heit, Munich, Germany.

Primary mouse macrophage cell culture

To ensure that immune cells from adult animals react to Abeta 1–40 and TLR agonists in a similar way to those from newborn mice, peritoneal macrophages were prepared from 10- to 12-month-old mice: 4 days after intraperitoneal injection of 0.5 mL thioglycolate (29 mg/L), C57BL/6 mice were anaesthetized with CO_2 and killed by decapitation. Peritoneal macrophages were harvested by peritoneal

lavage with pre-cooled PBS (2 mL). Cells were collected by centrifugation (1000 *g*, 10 min, 4°C) and the pellet was resuspended in DMEM with Glutamax I (Gibco) supplemented with 10% FCS, 100 U/mL penicillin and 100 μg/mL streptomycin. Cells were plated in 96-well cell culture plates at a density of 75 000 cells/well. After 6 h of culture, medium was changed completely. At 24 h later, macrophages were treated with different TLR agonists and Abeta 1–40 as described for microglial cells. Supernatant fluids were analysed for NO production and cells were assayed for cell viability or fixed in 4% formaldehyde.

Nitrite assay

NO release was quantified by measurement of nitrite, one of its stable reaction products, in the supernatant fluid of microglial cultures using Griess reagent. Aliquots (100 μL) of the supernatant fluid were mixed with 100 μL Griess reagent [equal volumes of 1% sulfonilamide in 30% acetate and 0.1% *N*-(1-naphthyl)ethylenediamine in 60% acetate] in a 96-well plate. After 10 min, the optical density at 570 nm was measured with a Genios multiplate reader (Tecan, Crailsheim, Germany). Concentrations were calculated by comparison of absorptions with a standard curve.

TNF-α, IL-1β and IL-10 enzyme immunoassay

The concentrations of TNF-α, IL-1β and IL-10 in the culture supernatant fluid were measured using commercial enzyme immunoassays (Quantikine M Mouse TNF-α, IL-1β and IL-10 Immunoassay; R & D Systems, Wiesbaden, Germany) according to the manufacturer's instructions.

Measurement of cell viability

Cell viability of microglial cells was determined using the WST-1 Cell Proliferation Reagent (Roche Applied Science, Mannheim, Germany). The assay is based on the cleavage of the tetrazolium salt WST-1 by active mitochondria producing a soluble formazan. This conversion only occurs in viable cells. Cells were incubated with WST-1 for 2 h. Then, the formazan dye formed was quantified by measuring the optical density at 490 nm using a Genios multiplate reader (Tecan). The absorbance directly correlated with the number of metabolically-active cells.

Bicine/Tris-Abeta-sodium dodecyl sulfate(SDS)-polyacrylamide gel electrophoresis (PAGE) and western blotting

To detect aggregation of Abeta 1–40 in the cell culture medium, each sample of Abeta 1–40 containing culture medium was diluted in sample buffer (0.36 M bis-Tris, 0.16 M bicine, 0.44 M sucrose, 1% SDS, 0.0075% bromphenol blue) to a calculated beta amyloid peptide (1–40) concentration of 200 pg/μL and boiled for 5 min at 95°C. A 5 μL aliquot (= 1000 pg) was mounted on each lane of a 12% polyacrylamide gel (Wiltfang *et al*. 1991). Gel thickness was 0.5 mm. The SDS concentration in the running buffer was 0.25%. Gels were run at room temperature for 1 h 20 min at a constant current of 15 mA per gel.

After the blotting step onto hydrophilized polyvinylidene fluoride (PVDF) membranes, immunostaining was performed with the monoclonal antibody 1E8 (Schering, Berlin, Germany) recognizing the amino terminal of Abeta 1–40. Next, membranes were washed and incubated for 1 h with an anti-mouse biotinylated antibody (Vector Laboratories, Peterborough, UK). Following a further washing step, horseradish peroxidase-coupled streptavidin was added for 1 h and chemiluminescence was visualized with ECL solution (Amersham Biosciences, Freiburg, Germany) according to the manufacturer's instructions, using the Fluor-S Max MultiImager (Bio-Rad, München, Germany).

Isolectin B4 immunocytochemistry

Isolectin B4 staining was used to assess the purity of microglial cultures and the morphology of microglial cells. For this purpose, microglial cells were plated on cover slips and fixed in 4% formaldehyde. Fixed cells were permeabilized with Triton X (0.1% in PBS) for 30 min and then incubated with biotinylated isolectin B4 (5 μg/mL, diluted in PBS + 1% BSA; Sigma) for 90 min. Thereafter, cells were treated with avidin-biotin complex (ABC, Vector, Burlingame, CA, USA) for 30 min; diaminobenzidine was used for visualization (5 min), resulting in a brown staining of the somata of microglial cells. Nuclei were counterstained with haemalum. The purity of microglia in the cultures was greater than 98%.

Amyloid Beta 1–40 immunocytochemistry and confocal laser imaging

Amyloid beta immunocytochemistry was performed to confirm Abeta 1–40 internalization by the microglial cells. Fixed cells were permeabilized with Triton X [0.1% in Tris-buffered saline (TBS)] for 30 min and then incubated with the mouse anti-Abeta antibody 1E8 (Schering, Berlin, Germany; dilution 1 : 20) for 90 min. Thereafter, cells were washed and incubated with a rabbit anti-mouse secondary antibody (Dako, Carpinteria, CA, USA; dilution 1 : 50) for 60 min. Detection was performed with the alkaline phosphatase/anti-alkaline phosphatase (APAAP) method and visualized with newfuchsin, yielding a red colour. Slides were evaluated with an imaging system (BX51, Olympus, Hamburg, Germany; software AnalySIS 3.2, Soft Imaging System GmbH, Münster, Germany).

Confocal imaging was performed on a Leica TCS SP2 confocal system fitted with an AOBS (Leica Microsystems Heidelberg GmbH, Mannheim, Germany). Cy2 was excited at 488 nm and Alexa 546 at 561 nm. Both channels were excited and detected separately to avoid spectral contamination. Each cell was scanned with a series of eight z-stacks, covering the thickness of the whole cell, and z-profiles were reconstructed using Leica Confocal Software. Amyloid-β protein was detected with the mouse monoclonal antibody 1E8 (Schering) and visualized by a biotinylated secondary sheep-anti-mouse polyclonal antibody (Amersham) and streptavidin-Cy2 (Jackson ImmunoResearch, Hamburg, Germany). The CpG was Alexa 546-conjugated (gift from PD Dr Stefan Bauer, Institute for Medical Microbiology, Klinikum rechts der Isar, Technische Universität München, Munich).

Statistics

GraphPad Prism Software (GraphPad Software, San Diego, CA, USA) was used to perform statistical analyses and graphical presentation. All data were expressed as means ± standard deviations (SD). Groups were compared by the two-tailed parametric one-way analysis of variance (ANOVA), and *p*-values were adjusted for repeated testing by Bonferroni's multiple comparison test; *p*-values < 0.05 were considered statistically significant.

Results

NO release upon stimulation with low concentrations of agonists of TLR2, -4 and -9, and Abeta 1–40

As expected from previously published data (Ebert *et al.* 2005), low concentrations of the TLR agonists Pam3Cys (TLR2, 600 pM/L), LPS (TLR4, 10 pM/L) and CpG (TLR9, 5 nM/L) alone induced an approximately equal release of NO (approximately 20–30% of the maximum released by high LPS concentrations) (Fig. 1a). Abeta 1–40 stimulated microglial cells from newborn mice and peritoneal macrophages from 10- to 12-month-old animals in a dose-dependent manner (Fig. 2). Abeta 1–40 (10 μM/L) alone elicited a similar microglial response as the TLR agonists in the concentrations mentioned above. Simultaneous treatment with LPS or Pam_3Cys in combination with Abeta 1–40 had an additive effect on NO release compared with treatment with LPS or Pam3Cys alone ($p < 0.001$; Fig. 1a).

Conversely, no additive effect was seen after combined treatment with Abeta 1–40 and CpG versus CpG alone. NO release was 21.7 ± 9.7% of maximum NO release in cultures treated with Abeta + CpG, and 24.5 ± 9.9% in cultures treated with CpG alone ($p > 0.05$). The combined treatment was approximately as potent as Abeta 1–40 alone (Fig. 1a). Cell viability measured by the water-soluble tetratolium salt-1 (WST-1) test showed no differences in all treatment groups.

Co-stimulation of peritoneal macrophages from adult mice also resulted in an additive effect of simultaneous treatment with LPS or Pam_3Cys in combination with Abeta 1–40, whereas no additive effect was seen after combined treatment with Abeta 1–40 and CpG (Fig. 2).

NO release upon stimulation with higher concentrations of TLR agonists and Abeta 1–40

Upon stimulation with higher concentrations of Pam_3Cys (6 nM/L), heat-killed *Acholeplasma laidlawii* (TLR2 agonist, 2×10^6 CFU/mL), CpG (50 nM/L) or LPS (100 pM/L), leading to approximately 50–80% of the NO release of 100 nM/L LPS and using the threefold concentration of Abeta (30 μM/L) (NO release 33.3 ± 7.9% of maximum NO release), the NO release of microglial cells after combined treatment with Abeta 1–40 and CpG was significantly decreased compared with stimulation with CpG alone (CpG: 52.5 ± 4.7% vs. CpG + Abeta: 34.9 ± 5.5%; $p < 0.001$). The NO release after combined Abeta 1–40

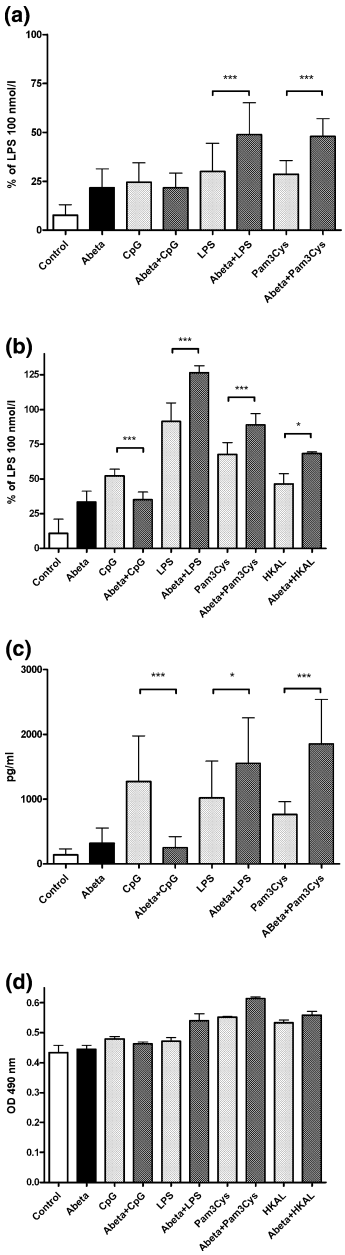

Fig. 1 NO release and TNF-α production in primary mouse microglial cells upon stimulation with low and higher concentrations of agonists of TLR2, -4, and -9 and amyloid beta peptide 1–40. Nitric oxide values are expressed as percentage of the NO release achieved after stimulation with a high dose of LPS (100 nM/L) over 24 h. TNF-α values are given in pg/mL. (a) NO release upon stimulation with low concentrations (approximately 20–30% of the maximum stimulation) of agonists of TLR2 (Pam3Cys 600 pM/L), TLR4 (LPS 10 pM/L) and TLR9 (CpG 5 nM/L) alone and in combination with 10 μM/L amyloid beta peptide 1–40. (b) NO release upon stimulation with higher concentrations (approximately 50–60% of the maximum stimulation) of agonists of TLR2 (Pam3Cys 6 nM/L; heat-killed *Acholeplasma laidlawii* 2×10^6 CFU/mL), TLR4 (LPS 100 pM/L) and TLR9 (CpG 50 nM/L) alone and in combination with amyloid beta peptide 1–40 (30 μM/L). (c) TNF-α release upon stimulation with low concentrations of agonists of TLR2, -4 and -9 and amyloid beta peptide 1–40 (10 μM/L). (d) Measurement of cell viability by WST-1 test after stimulation of the microglia for 24 h with Pam3Cys 6 nM/L, heat-killed *Acholeplasma laidlawii* 2×10^6 CFU/mL, LPS 100 pM/L, CpG 50 nM/L and amyloid beta peptide 1–40 (30 μM/L). Stimulation with high doses of TLR agonists and Abeta 1–40 did not lead to the death of microglial cells.

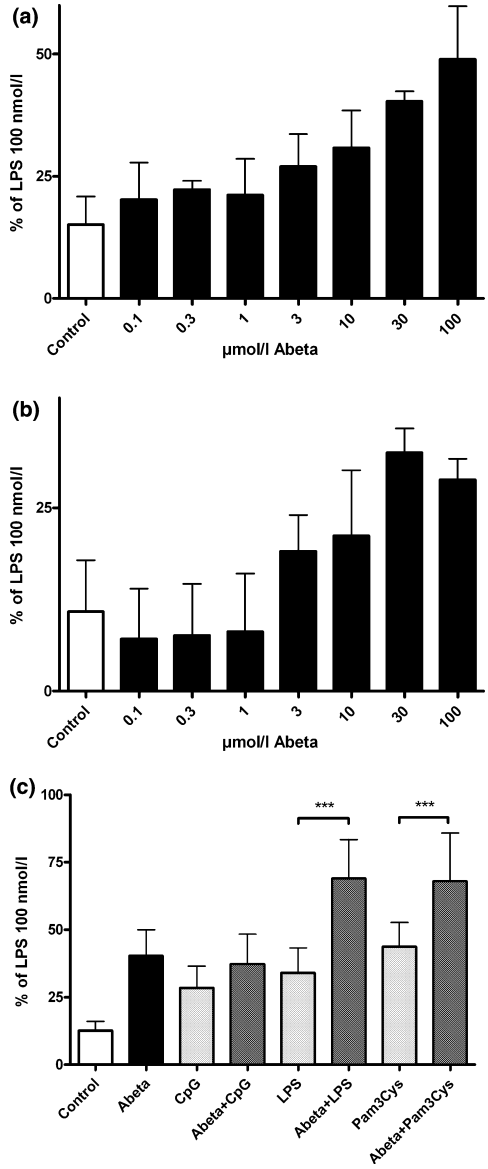

Fig. 2 Stimulation of peritoneal macrophages prepared from 10- to 12-month-old mice by Abeta 1–40 alone and in combination with TLR agonists. The stimulation was quantified by the respective NO release in comparison with the NO release caused by 100 nM/L LPS. (a) NO release from peritoneal macrophages from 10- to 12-month-old C57BL/6 mice elicited by different Abeta 1–40 concentrations. (b) NO release from cerebral microglial cells prepared from newborn C57BL/6 mice by different Abeta 1–40 concentrations. Note the similar dose–response curves in (a) and (b). (c) NO release from peritoneal macrophages from 10- to 12-month-old C57BL/6 mice upon stimulation with low concentrations (approximately 20–30% of the maximum stimulation) of agonists of TLR2, -4 and -9 and amyloid beta peptide 1–40 (concentrations identical to those in Fig. 1a). Note the similarities with the responses depicted in Fig. 1a (microglial cells from newborn mice).

and CpG treatment was equal to the release after exposure to Abeta 1–40 alone (Fig. 1b). As with low concentrations, Abeta 1–40 in combination with Pam3Cys, heat-killed *Acholeplasma laidlawii* (HKAL; TLR2) and LPS had an additive effect (Pam3Cys: 67.6 ± 8.6% vs. Pam3Cys + A-beta: 88.8 ± 8.2%; $p < 0.001$; HKAL: 46.43 ± 7.37% vs. HKAL + Abeta: 68.12 ± 1.41%; $p < 0.05$; LPS: 91.34 ± 13.47% vs. LPS + Abeta: 126.4 ± 5.08%; $p < 0.001$).

Cell viability as indicated by the WST-1 test showed no decrease in viability, in all treatment groups, compared with the control group (Fig. 1d).

Cytokine release upon stimulation with TLR agonists and Abeta 1–40

Apart from NO, microglial cells produce a wide range of cytokines upon stimulation. In order to further characterize the action of different TLR agonists together with Abeta 1–40, the release of the pro-inflammatory cytokines, TNF-α and IL-1β, and of the anti-inflammatory cytokine, IL-10, were quantified.

Similar to their action on NO release, LPS and Pam3Cys in combination with Abeta 1–40 were more effective in stimulating the release of TNF-α than either compound alone. Moreover, the release of TNF-α was significantly lower after stimulation of the microglial cells with the combination of CpG and Abeta 1–40 compared to stimulation with CpG alone (CpG: 1272 ± 705.4 pg/mL vs. CpG + Abeta: 252.0 ± 169.2 pg/mL; $p < 0.001$) (Fig. 1c). The maximum IL-1β concentrations in the culture medium were more than one order of magnitude lower than the TNF-α concentrations. Upon stimulation with Pam_3Cys (600 pM/L), LPS (10 pM/L), CpG (5 nM/L) and Abeta 1–40 (10 µM/L) alone, and CpG (5 nM/L) plus Abeta 1–40 (10 µM/L), IL-1β levels in the cell culture supernatant fluids were below the detection limit. Co-stimulation with Pam_3Cys (600 pM/L) and Abeta 1–40 (10 µM/L), as well as LPS (10 pM/L) and Abeta 1–40 (10 µM/L), resulted in maximum IL-1β concentrations within the supernatant fluids of 16.9 and 22.6 pg/mL, respectively. Stimulation with LPS (100 nM/L) resulted in IL-1β concentrations from 81.6 to 110.2 pg/mL in the supernatant fluids. IL-10 release was only detectable in the supernatant fluid of CpG-stimulated microglial cells. The IL-10 concentrations in the supernatant fluids after exposure to 5 nM/L CpG were 27.1 ± 3.3 pg/mL, and after stimulation with 50 nM/L CpG, 100.0 ± 23.5 pg/mL. No detectable IL-10 concentrations (detection limit 4 pg/mL) were measured in supernatant fluids of cell cultures with the other stimulants of innate immunity applied, including cultures simultaneously stimulated with Abeta 1–40 and CpG.

NO release upon stimulation with Abeta 1–40, Pam3Cys and CpG in microglial cells deficient for TLR2 or TLR9

The NO release after stimulation of TLR2- and TLR9-deficient mouse microglial cells with Abeta 1–40 in the two different concentrations was similar to the NO release observed in wild-type mouse microglial cells (TLR2-deficient

microglia stimulated with 10 μM/L Abeta vs. unstimulated TLR-2 deficient microglia: 12.5 ± 3.2% vs. 3.8 ± 4.9% of the NO release by 100 nM/L LPS, $p < 0.01$; TLR9-deficient microglia stimulated with 10 and 30 μM Abeta vs. unstimulated TLR9-deficient microglia: 24.1 ± 1.8% and 41.4 ± 11.7% vs. 10.2 ± 3.9%; $p < 0.01$). Contrarily, virtually no NO was detectable after stimulation of TLR2-deficient microglia with Pam3Cys (Pam3Cys-stimulated 6.1 ± 5.3% vs. unstimulated microglia 3.8 ± 4.9% of NO release by 100 nM/L LPS, $p > 0.05$) and after challenge of TLR9-deficient microglia with CpG (CpG 1500 nM/L-stimulated 11.83 ± 2.83% vs. unstimulated microglia 16.5 ± 5.28% of NO release by 100 nM LPS; $p > 0.05$). The combination of Abeta 1–40 plus TLR agonist led to a similar NO release to that induced by Abeta 1–40 alone (TLR2-deficient mice: Abeta plus Pam3Cys 10.8 ± 4.8% vs. Abeta 12.5 ± 3.2% of NO release by 100 nM/L LPS, $p > 0.05$).

Detection of aggregation of Abeta 1–40 by Bicine/Tris-SDS-PAGE and western blot

To detect Abeta 1–40 aggregation within 24 h in the microglial cultures, culture supernatant fluids containing either Abeta 1–40 alone or Abeta in combination with TLR agonists (Abeta + CpG, Abeta + LPS and Abeta + Pam3Cys) were analysed by SDS-PAGE and western blotting in three different conditions: (i) before incubation with microglial cultures; (ii) after 24 h incubation in DMEM + 10% FCS without microglial cells at 37°C; and (iii) in the cell culture supernatant fluids after 24 h incubation at 37°C.

By comparison with pre-stained markers (molecular weights of 10, 15, 20, 25, 30, 40, 50, 60, 80 and 120 kDa), we estimated the apparent molecular weight of each Abeta 1–40 species. Before incubation the majority of amyloid beta was present in its monomeric (approximately 95%) and dimeric form (about 5%). The dimeric form with a size of approximately 10 kDa appears as a doublet (Walsh *et al*. 2000) (Fig. 3a).

After 24 h incubation of Abeta 1–40 in DMEM in the absence of microglial cells, the amount of the dimer increased and additional bands indicated the presence of larger Abeta 1–40 oligomers. These oligomers are probably more potent stimulants of microglial cells than large aggregates of Abeta (Gasic-Milenkovic *et al*. 2003). Pentamers (approximately 20 kDa), octamers (approximately 32 kDa) and decamers (approximately 40 kDa) were detected, and the trimer (approximately 12 kDa) and the hexamer could also be seen at low concentrations (Fig. 3b).

The microglial cell culture supernatant fluids after 24 h showed a substantially reduced amount of Abeta pentamers, hexamers, octamers and decamers (Fig. 3c). This observation suggests that Abeta 1–40 oligomers were bound/internalized by the microglial cells during the 24 h incubation at 37°C. Co-stimulation with Pam3Cys, LPS or CpG had no apparent influence on Abeta 1–40 aggregation and the binding/internalization of oligomers.

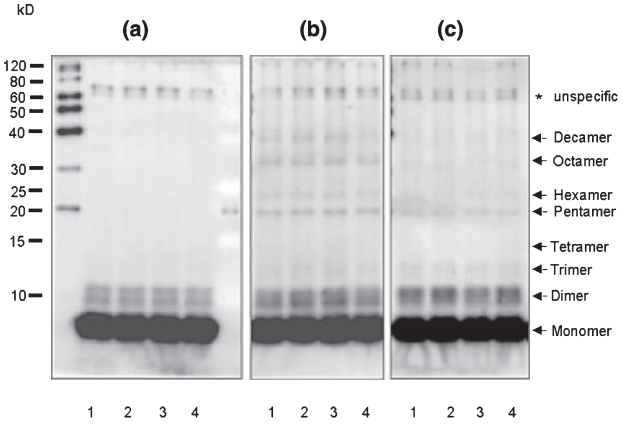

Fig. 3 Western blotting to quantify aggregation of amyloid beta peptide1–40 in the cell culture medium (a) directly after dilution in fresh DMEM culture medium, (b) after 24 h incubation without microglial cell cultures at 37°C and (c) in the cell culture supernatant fluid after 24 h incubation at 37°C. Lanes: 1 = Abeta; 2 = Abeta + CpG; 3 = Abeta + LPS; 4 = Abeta + Pam3Cys

Morphological changes of microglial cells upon stimulation with Abeta 1–40 and localization of Abeta 1–40 and CpG oligonucleotides within microglial cells

Microglial cells stimulated with Abeta 1–40 showed characteristic morphological changes of activation (rounding of the cells, loss of ramifications, formation of cytoplasmic vacuoles) (Fig. 4). Abeta 1–40 was detected by immunocytochemistry at the surface and within the cells, supporting the concept of binding and/or internalization of amyloid beta oligomers by the microglial cells (Fig. 5). After addition of Abeta 1–40 and Alexa 546-conjugated CpG to the medium, confocal laser microscopy revealed that Abeta and CpG did not co-localize within the microglial cells (Fig. 6). CpG-DNA was densely internalized in the vicinity of the nucleus in a compartment probably representing the endoplasmic reticulum (Latz *et al*. 2004). Contrarily, Abeta 1–40 remained on the surface of the cell or was internalized in a superficial compartment, probably corresponding to invaginations of the cell membrane, and some endosomes and lysosomes (Cole *et al*. 1999; Nagele *et al*. 2004) clearly distinct from the CpG-DNA-containing compartment.

Discussion

The brain in Alzheimer's disease contains activated microglia (McGeer and McGeer 2001; Perry *et al*. 2003). These microglia are primed by endogenous compounds, in particular fibrillar Abeta and post-translational sugar-derived modifications of plaque proteins (advanced glycation end products) (Gasic-Milenkovic *et al*. 2003). Aggregated forms

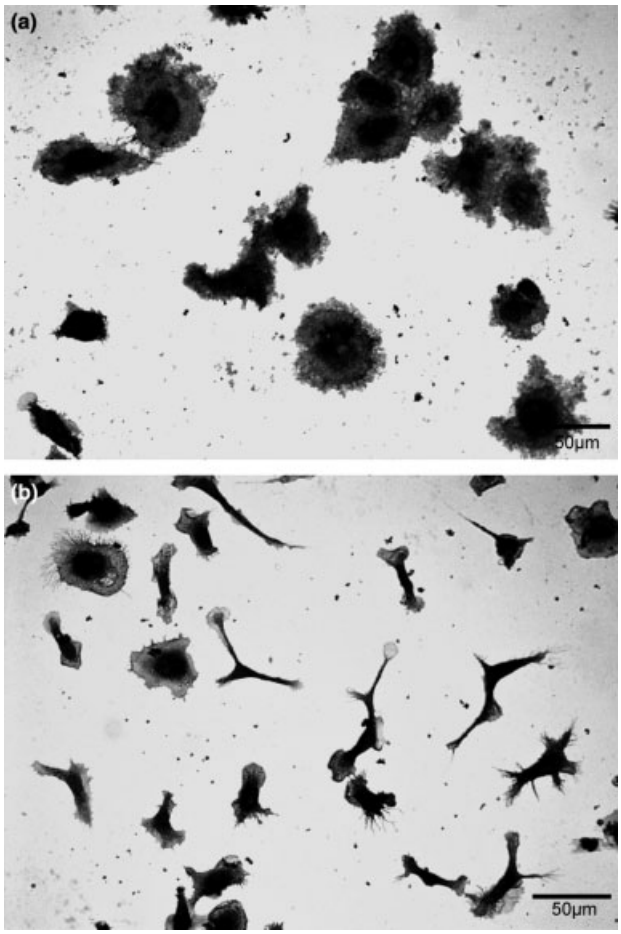

Fig. 4 Isolectin B4 immunocytochemistry of Abeta 1–40-stimulated primary mouse microglial cells (a) and unstimulated control cells (b). Note the rounding of the cells and loss of ramifications.

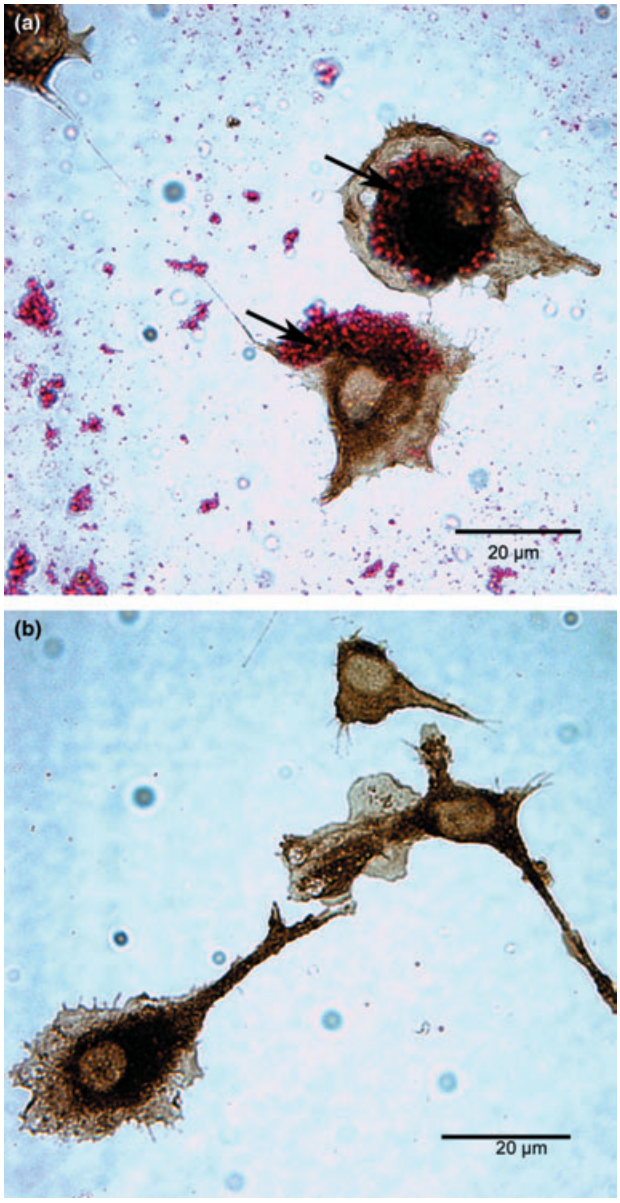

Fig. 5 Amyloid beta immunocytochemistry of Abeta 1–40-stimulated primary mouse microglial cells (a) and unstimulated control cells (b). Note the accumulation of amyloid beta on the surface and/or within the cells (arrows).

of amyloid beta that accumulate in the central nervous system in Alzheimer's disease are internalized by microglial cells, implicating particular scavenging receptors (El Khoury *et al*. 1996; Paresce *et al*. 1996; Cole *et al*. 1999). Several membrane proteins have been implicated in binding Abeta either in its monomeric or its fibrillary form. The serpin-enzyme complex receptor and the insulin receptor can bind the monomeric form of Abeta. The alpha$_7$-nicotinic acetylcholine receptor, the receptor for advanced glycosylation end-products (RAGE), and the formyl peptide receptor like-1 receptor are able to bind the monomeric and fibrillar forms of Abeta. Amyloid precursor protein, the *N*-methyl-D-aspartate receptor, the P75 neurotrophin receptor, the collagen-like Alzheimer amyloid plaque component precursor/collagen XXV, the scavenger receptors A and BI, the LPS receptors CD14 and TLR4, and a multireceptor complex including the B-class scavenger receptor CD36, the integrin-associated protein/CD47 and the $\alpha_6\beta_1$H-integrin, have been reported to bind the fibrillar form of Abeta (Bamberger *et al*. 2003; Guillemin *et al*. 2003; Fassbender *et al*. 2004; Sondag and Combs 2004; Verdier *et al*. 2004). The contribution of each mechanism to the microglial activation by amyloid beta aggregates is not clear. Peritoneal macrophages prepared from RAGE-deficient mice, however, responded to Abeta 1–40 in a similar manner to macrophages from wild-type mice (S. Ebert and P. Teismann, unpublished observation).

Primary microglial cell cultures are activated by bacterial agonists of TLR2, -4 and -9 in a dose-dependent manner (Ebert *et al*. 2005). Here, we demonstrate that combined treatment with Abeta and the bacterial TLR agonists

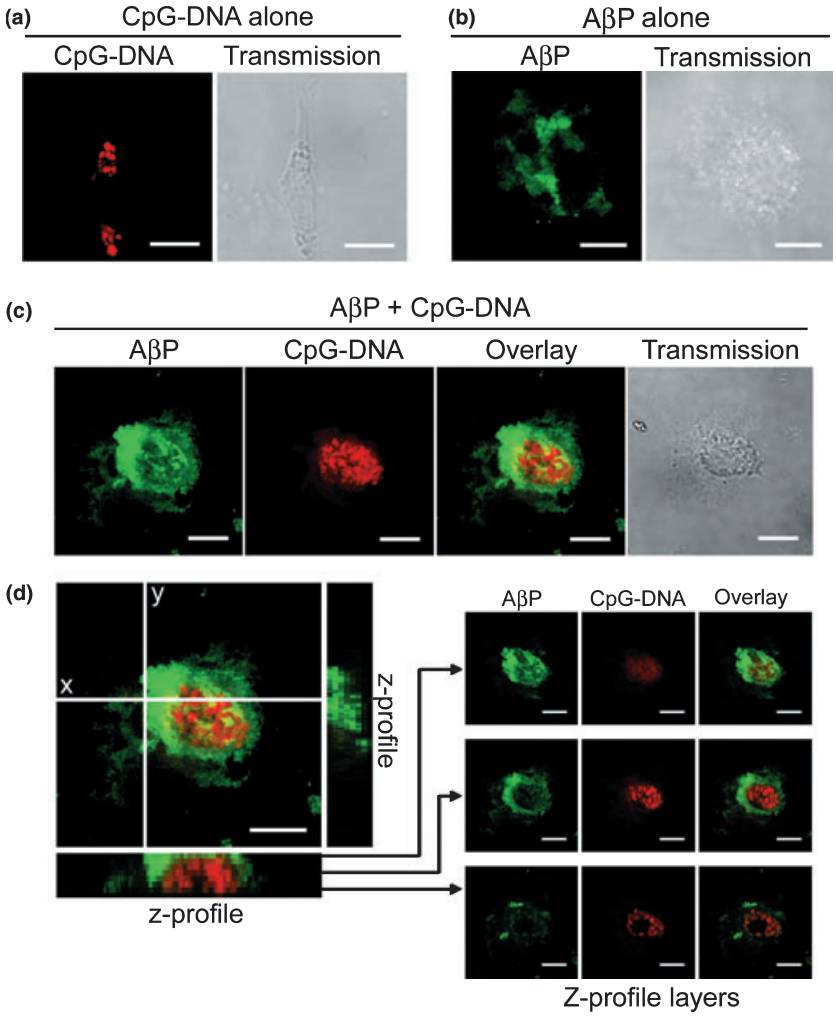

Fig. 6 Confocal microscopy co-localization analysis of Abeta 1–40 and CpG-DNA. (a) Alexa 546-conjugated CpG-DNA (red) was internalized in the cytosol of microglia. Scale bar: 8 μm. (b) Abeta 1–40 detection by immunocytochemistry (Cy2-labelling, green) demonstrated labelling on the surface and in the superficial cytoplasm of microglia. Scale bar: 8 μm. (c) Co-incubation of microglia with CpG-DNA (red) and Abeta 1–40 (green) revealed lack of overlay and co-localization between both components. Scale bar: 8 μm. (d) Detailed confocal microscopy reconstruction in the z-axis revealed nearly complete separation between Abeta 1–40 and CpG-DNA [the z-profile in two perpendicular section lines (*x* and *y*) also is shown]. While the CpG-DNA was densely internalized in the perinuclear region, Abeta 1–40 remained on the surface of the cell or was internalized in a compartment probably corresponding to invaginations and superficial endosomes or lysosomes. Several sections from the z-stack of a microglial cell (no. 1, 4 and 7 of 8 z-stacks) are shown in the right panel of (d) (z-profile layers). Minimal amyloid signal was present in the CpG-DNA-containing perinuclear compartments. Abeta 1–40 immunoreactivity on the surface of the cell did not contain any CpG-DNA signal, clearly demonstrating a lack of physicochemical interaction between Abeta 1–40 and CpG-DNA in the medium or within the cells. Scale bar: 8 μm.

Pam3Cys, heat-killed *Acholeplasma laidlawii* or LPS (ligands of TLR2 and -4, respectively) causes greater activation of microglia primed with interferon-γ than with either compound alone. Until now, this phenomenon had only been shown for the TLR4 agonist endotoxin: pre-incubation with β-amyloid peptide increased the sensitivity of microglial cells to stimulation by bacterial LPS (Gasic-Milenkovic *et al.* 2003). Similarly, systemic endotoxin challenge in preclinical murine prion disease resulted in greater IL-1β synthesis within the brain and in a quicker loss of motor activity than in animals not challenged by endotoxin (Combrinck *et al.* 2002). The additive effect of endogenous and exogenous stimuli renders cerebral microglial cells sensitive to low concentrations of bacterial products, i.e. intercurrent systemic infections can lead to acute exacerbations of neurodegenerative disorders and can probably speed up disease progression through the further activation of already primed microglial cells (Perry *et al.* 2003). For this reason, an approach aiming at the reduction of proinflammatory bacterial products in bacterial infections of patients with

Alzheimer's dementia and other neurodegenerative diseases appears promising. Bactericidal antibiotics which inhibit bacterial protein synthesis release smaller quantities of proinflammatory/toxic bacterial compounds compared with β-lactams and other cell-wall active drugs (Stuertz *et al*. 1998; Nau and Eiffert 2002). In several *in vitro* and *in vivo* systems, bacteria treated with bacterial protein synthesis inhibitors induce less inflammation than bacteria treated with β-lactam antibiotics. In mouse models of *Staphylococcus aureus* peritonitis/sepsis (Azeh *et al*. 2002) and of *Streptococcus pneumoniae* meningitis (Nau *et al*. 1999), a lower release of proinflammatory bacterial compounds was associated with a reduced mortality. In a rabbit model of *S. pneumoniae* meningitis, the reduction of the release of proinflammatory/directly toxic bacterial products was associated with an attenuated neuronal injury (Böttcher *et al*. 2000; Gerber *et al*. 2003; Böttcher *et al*. 2004). Data from humans suffering neurodegenerative diseases or in animal models mimicking neurodegenerative diseases are presently lacking.

In this study, co-administration of Abeta 1–40 and CpG, an analogue of bacterial DNA, unexpectedly had no additive effect upon microglial activation. During exposure to the combination, less NO and TNF-α were released than during treatment with CpG alone, but not less than during the sole administration of Abeta 1–40. Apparently, microglial cells do not react uniformly to stimulation with various TLR agonists; CpG was the only compound investigated that not only induced the release of NO and TNF-α, but also of the anti-inflammatory IL-10. The cause of the divergent behaviour after activation of TLR9 versus stimulation of TLR2 and TLR4 probably lies in the different location of the receptors (TLR2 and -4: cell surface; TLR9: endoplasmic reticulum) (Latz *et al*. 2004) and dissimilarities of the signalling cascade (Wagner 2004). Whereas TLR9 signalling is strictly dependent on the adaptor molecule MyD88 and is initiated at the endosome, TLR2 and -4 signalling occurs at the cell surface and involves MyD88-dependent and -independent mechanisms including the adaptor molecules TIRAP, TRIF and TRAM (Wagner 2004). It has been noted in other conditions that activation of TLR9 by CpG can have anti-inflammatory properties; CpG can prevent the induction of allergic responses and reverse established immune responses either by the induction of a T_H1 response opposing the T_H2 allergic response, or by an as yet unknown mechanism (Kline *et al*. 1998; Krieg 2003). Moreover, treatment of mice with CpG after infection with prion protein resulted in substantially longer survival times (Sethi *et al*. 2002). However, prion pathogenesis is independent of MyD88 signalling (Prinz *et al*. 2003) and high dose CpG treatment was shown to result in a destruction of lymphoid follicles, thereby inhibiting prion neuroinvasion (Heikenwalder *et al*. 2004). The different location of Abeta 1–40 and CpG in the microglial cell demonstrated by confocal laser microscopy makes a physicochemical interaction as the cause of the antagonism between CpG and Abeta 1–40 extremely unlikely.

In conclusion, persons with prevalent inflammation in the central nervous system caused by endogenous stimuli such as Abeta may experience aggravation of disease during systemic infections by further activation of already primed microglial cells by low concentrations of microbial ligands of TLRs. CpG did not enhance microglial stimulation by Abeta1–40, suggesting that this TLR agonist does not aggravate Abeta-induced microglial activation. In patients with Alzheimer's dementia, bacterial infections should be treated rapidly, and an antibiotic regimen aimed at minimizing the release of proinflammatory bacterial products may be beneficial.

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