Role of the Toll-Like Receptor 4 in Neuroinflammation in Alzheimer's Disease

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Key Words

Alzheimer's disease • Amyloid peptide • Microglia • TLR4 • CD14 • TLR2 • Neuroinflammation

Abstract

Microglial activation is a key feature in Alzheimer's disease and is considered to contribute to progressive neuronal injury by release of neurotoxic products. The innate immune receptor Toll-like-receptor 4 (TLR4), localized on the surface of microglia, is a first-line host defense receptor against invading microorganisms. Here, we show that a spontaneous loss-of-function mutation in the *Tlr4* gene strongly inhibits microglial and monocytic activation by aggregated Alzheimer amyloid peptide resulting in a significantly lower release of the inflammatory products IL-6, TNF α and nitric oxide. Treatment of primary murine neuronal cells with supernatant of amyloid peptide-stimulated microglia demonstrates that Tlr4 contributes to amyloid peptide-induced microglial neurotoxicity. In addition, stimulation experiments in transfected HEK293 cells allowed to define a tri-molecular receptor complex consisting of TLR4, MD-2 and CD14 necessary for full cellular activation by aggregated amyloid peptide. A clinical relevance of these findings

is supported by a marked upregulation of *Tlr4* mRNA in APP transgenic mice and by an increased expression of TLR4 in Alzheimer's disease brain tissue associated with amyloid plaque deposition. Together, these observations provide the first evidence for a role of the key innate immune receptor, TLR4, in neuroinflammation in Alzheimer's disease.

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Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disease characterized by neuronal loss, extracellular deposition of amyloid peptide (A β) and microglial accumulation and activation [1, 2].

Although microglial activation by aggregated A β [3, 4] may possess beneficial effects by phagocytic clearance of A β [5-7], it may also contribute to progressive neurodegeneration [8-10]. Neurotoxicity associated with microglial activation is considered to result from the production of neurotoxic mediators such as reactive oxygen and nitrogen species [11], proteolytic enzymes, glutamate [3], complement factors [12] or inflammatory cytokines [3, 13]. These products lead to neuronal cell death as

demonstrated in murine models [14, 15] and may contribute to chronic neurodegenerative conditions in AD [3, 16, 17].

Currently, there is increasing evidence that the innate immune system is involved in the neuroinflammatory process of neurodegenerative diseases such as AD [18–20]. Mononuclear phagocytes, such as microglial cells, are crucial components of the innate immune system. Cellular activation in response to pathogen-associated molecular patterns on microorganisms is mediated through interaction with innate immune receptors on the surfaces of mononuclear phagocytes, e.g., Toll-like receptors (TLRs) and the lipopolysaccharide receptor CD14 [21]. These receptors mediate activation of transcription factors such as NF- κ B, IRF3, IRF5 and IRF7 and subsequent production of inflammatory cytokines, interferon- α or - β [22, 23].

Lipopolysaccharide (LPS), a component of the cell wall of gram-negative bacteria, was first identified as the TLR ligand [24]. LPS is recognized by a receptor complex containing fully functional TLR4, myeloid differentiation protein 2 (MD-2), a TLR4-associated soluble factor, and CD14 [25, 26]. Apart from recognizing exogenous ligands, innate immune receptors can as well be activated by endogenous ligands, e.g. lipids, proteins or nucleic acids [27].

Recently, it has been demonstrated that Tlr4, that is mainly expressed on microglia, can mediate oligodendrocyte injury in mixed glial cultures [28]. More importantly, Tlr4 mediates extensive neuronal cell death upon LPS treatment in cell culture and in mice [29]. Tlr4 is also involved in both, brain inflammation and neural tissue injury in rat brains after intracerebroventricular administration of LPS [30]. Moreover, it has been demonstrated that the GPI-linked receptor, CD14, a co-receptor of Tlr4, is involved in A β -induced microglial activation [31].

These findings suggest a key role of microglial activation via TLR4 in neuronal cell damage. This, together with the increasing evidence for an involvement of the innate immune system in the pathophysiology of AD, prompted us to investigate the role of microglial TLR4 in AD pathophysiology.

Materials and Methods

Animals

C3H/HeN (wild-type) and C3H/HeJ mice were purchased from Charles River Laboratories (Sulzfeld, Germany). C3H/HeJ mice bear a spontaneous loss-of-function mutation in the *Tlr4*

gene, leading to a replacement of proline with histidine at position 712 in the intracellular part of the polypeptide chain and impeding LPS signal transduction [32–24]. TgCRND8 mice, which overexpress a double mutant form of human amyloid precursor protein (APP) 695 (KM670/671NL+V717F) under the control of hamster PrP promoter [35], were kindly provided by D. Westaway (University of Toronto, Canada). Non-transgenic littermates served as wild-type controls. All animals were maintained in an environmentally controlled facility.

Preparation and characterization of aggregated peptides

Aggregated A β was obtained by dissolving synthetic human A β 1–42 (kindly provided by B. Penke, University of Szged, Hungary) in PBS buffer pH 7.4 (1.0 mg/ml), and subsequent incubation for 3 days at 37°C. Aggregation was prevented by diluting the stock solution to a final concentration of 62.5 μ g/ml and immediately freezing the solution. The peptide control, A β scrambled (randomized sequence), was prepared accordingly. The aggregated and non-aggregated conformation of each charge was confirmed by using electron microscopy. Mass spectroscopy of peptide preparations showed the absence of contamination, and endotoxin concentrations of A β samples were below detection limit (0.01 EU/ml) in the Limulus Amoebocyte assay (Whittaker Bioproducts, Walkersville, MD).

Cellular stimulation with A β

Murine microglia. Primary murine microglia of Tlr4-defective C3H/HeJ and wild-type C3H/HeN mice were prepared as described elsewhere [36]. Briefly, brains of newborn mice day 1–3 were isolated and meninges removed. After homogenisation and digestion with trypsin and DNase, cells were cultured in DMEM / 10% FCS / 2% Glutamax I / 100 U/ml penicillin and 100 μ g/ml streptomycin for at least 14 days, until development of complete microglia-astrocyte cell layer. After microglial harvest cells contained 95–98% microglia as identified by expression of the monocytic marker Mac-1 (rat anti-mouse Mac-1 PE-labelled antibody, Pharmingen, BD Biosciences, Heidelberg, Germany). For stimulation experiments 3×10^5 cells were incubated with aggregated, non-aggregated or scrambled A β 50 μ g/ml, respectively or with LPS 10 ng/ml (*S. abortus equi*, Sigma, Taufkirchen, Germany) as a positive control in 24-well-plates. Murine IFN γ 100 U/ml (Sigma) was administered as a co-stimulatory factor as previously described [37]. Inflammatory cytokines were quantified in the supernatant collected after 6 h by ELISA according to the manufacturer's instructions (R&D Systems, Wiesbaden, Germany). Detection levels were 15.6 pg/ml for IL-6 and 31.25 pg/ml for TNF α . Nitrite, the stable metabolite of NO, was measured by the Grieff assay [36] after 24 and 48 h. Detection level was 1.5 μ M.

Human monocytes. Peripheral human blood monocytes were isolated by double phase gradient using Histopaque 1077 and 1119 (Sigma) according to the manufacturer's instructions. Monocytes were stimulated as described above, but with or without 1 hour pre-incubation with the neutralizing anti-human TLR4 antibody (Biocarta, Hamburg, Germany) or isotype control antibody, each 20 μ g/ml. For quantification of inflammatory

cytokines (IL-6 and TNF α) by ELISA (R&D Systems) supernatants were collected after 12 h. Detection levels were 9.4 pg/ml for IL-6 and 15.6 pg/ml for TNF α . Treatment with peptides or antibodies did not affect cell viability as measured by MTT assays, performed according to the manufacturer's instructions (Promega, Mannheim, Germany). Every experiment was repeated 3-5 times.

Transfection and stimulation of human embryonic kidney cells 293

Human embryonic kidney cells (HEK) 293 cells were plated at a density of 5×10^4 /ml in 96 well plates in complete medium w/o G418. The following day, cells were transiently transfected using Polyfect (Qiagen) according to the manufacturer's protocol. An expression plasmid containing human *CD14* (kindly provided by D.T. Golenbock, University of Massachusetts Medical School, USA), and the Flag-tagged version of human *TLR4* (kindly provided by P. Nelson, University of Washington, USA) were subcloned into pREP9 (Invitrogen, Karlsruhe, Germany). The human *MD-2* expression plasmid was a kind gift from K. Miyake (University of Tokyo, Japan). The *CD14* and *MD-2* plasmids were used at 25 ng/transfection, and all other plasmids were used at 200 ng/transfection. The total DNA content was kept constant at 450 ng/transfection using pCDNA3 (Invitrogen). At 24 h post-transfection, cells were washed and stimulated (as described above) for another 18 h. Finally, supernatants were collected and the IL-8 content was quantified using a commercial ELISA (Biosource, Solingen, Germany). Detection level was 15.6 pg/ml. Every experiment was repeated 3 times.

Neurotoxicity of A β -activated C3H/HeJ and C3H/HeN microglia

Primary murine hippocampal neurons were isolated from E15 wt mice, seeded in 24-well plates at a density of 8×10^4 cells per well in neuron culture medium (BME supplemented with 2% B-27 and 1% FCS), and cultured for 7 days. Neurons were treated with supernatant from microglial cultures activated by 24 h stimulation with 50 μ g/ml aggregated or scrambled A β (as described above). Supernatants were centrifuged and diluted 1:2 with neuron culture medium. Neuronal death was detected after 24 h by MTT assay. Experiments were repeated 3 times.

TLR4 expression experiments

Real-Time PCR. Quantitative expression of *Tlr4* in the brains of 6-month-old APP transgenic mice versus age-related littermate controls was determined by real-time PCR. RNA was isolated according to the manufacturer's instructions using an RNeasy kit (Qiagen, Hilden, Germany). Reverse transcriptase reactions were performed using 1 μ g of RNA, 1 μ M oligo-dT primer (Qiagen) and Omniscript Reverse Transcriptase (Qiagen) at a concentration of 4 units / 20 μ l reaction. Reactions were carried out at 37°C for 60 min followed by 93°C for 5 min to inactivate the enzyme.

For cDNA reactions 5 μ l of the reverse transcription product were added to an BioRad iCyclerTM PCR system containing 0.3 μ M of each primer, 1 x QuantiTect SYBR Green PCR Master Mix (includes 2.5 mM MgCl₂) as a fluorescent reporter

(QuantiTect SYBR Green PCR, Qiagen) and subjected to a 15 min initial hot start activation of the Taq polymerase at 95°C, followed by 50 cycles of amplification (94°C 30 sec, *Tlr4*: 55°C, GAPDH: 60°C each 30 sec, 72°C 30 sec). Primers were obtained from Invitrogen: *Tlr4*: sense TCT CCT CAG GTC CAA GTT GC, antisense CAG TGG TCA GTG TGA TTG TGG; GAPDH: sense: GGC ATG GAC TGT GGT CAT GA, antisense: TTC ACC ACC ATG GAG AAG GC. The correct size of the amplified PCR products was confirmed by 1.5% agarose gel electrophoresis and melting curve analysis. Samples were quantified by a standard curve generated by amplifying five serial dilutions in duplicate of *Tlr4* cDNA. All gene-specific mRNA expression values were normalized against expression values for the house-keeping gene GAPDH (*Tlr4*/GAPDH).

Reverse Transcriptase-PCR (RT-PCR). Relative levels of *Tlr4* mRNA in brains of 6-month-old APP-overexpressing mice versus age-related wild-type control mice were determined by RT-PCR. RNA was isolated according to the manufacturer's instructions with RNeasy kit (Qiagen, Hilden, Germany). cDNA as a template for RT-PCR was synthesized from total 0.063 μ g RNA with specific primers and Omniscript/Sensiscript reverse transcriptases (One Step PCR, Qiagen) at 50°C for 30 min. PCR amplification was performed in a 25- μ l reaction volume containing 1x PCR buffer, 0.4 mM dNTP's and 0.6 μ M of each primer. Thirty cycles were performed using recombinant Taq DNA polymerase (94°C 40 sec, 55°C 50 sec, 72°C 1 min) and a final 10 min 72°C extension. In all experiments amplification of GAPDH was run in parallel to normalize samples. Amplification of accurate targets was confirmed by sequence analysis. Gel electrophoresis on a 1.5% agarose gel was performed to separate PCR products. Primers were obtained from Invitrogen: *Tlr4*: sense TCT CCT CAG GTC CAA GTT GC, antisense CAG TGG TCA GTG TGA TTG TGG. Every experiments was repeated 3 times.

TLR4 expression in post-mortem brains of AD patients. Paraffin sections of human frontal, entorhinal and occipital cortex and from cerebellum from *post-mortem* autopsy brains with histologically confirmed AD and from age-matched non-demented control subjects were retrieved from the Department of Neuropathology, University of Goettingen, Germany. After deparaffinization, slides were pre-treated by heating in a microwave 7 times 5 min in citrate buffer 10 mM, pH 6.0, followed by formic acid for 1min and guanidinthiocyanat 4 M for 25 min, with washing steps in between. Endogenous peroxidase was then blocked by incubation in H₂O₂ 3% for 20min before blocking into TBST 1x containing FCS 10% for 15 min. Sections were incubated with goat-anti-human TLR4 Ab (1:100; Santa Cruz Biotechnology, Heidelberg, Germany, 1:100) and mouse anti-human A β Ab (clone 6E10, 1:2000; Chemicon, Hofheim, Germany, 1:5000) or their isotypes control in TBST 1X overnight at 4°C. After washing, slides were further incubated with biotinylated rabbit anti-goat Ab (Linearis GmbH, Wertheim-Bettingen, 1:200) in TBST 1x for 1 h followed by incubation with Cy3-labelled sheep anti-mouse (Jackson Immunoresearch Laboratories, Hamburg, Germany, 1:200) and Cy2-conjugated streptavidin (Jackson Immunoresearch Laboratories, 1:250) for another 1 h at room temperature. Slides were finally washed in TBS 1x, incubated into Soudan Black 0.5% for 1 min to reduce

the autofluorescence of the pigments. Slides were then mounted with mounting medium for fluorescence (Dako, Hamburg, Germany).

Results

Tlr4 mediates A β -induced activation of murine microglia

To investigate the role of Tlr4 in the microglial stimulation by aggregated A β , we stimulated microglia derived from *Tlr4* mutant C3H/HeJ mice and from wild-type (wt) control mice. LPS is the natural ligand of Tlr4 and was used as the positive control. As expected, LPS-induced cellular activation was significantly decreased in *Tlr4* mutant microglia compared to wt cells as assessed by quantification of nitrite, the stable metabolite of nitric oxide (*Tlr4* mutant: 25.7 μ M $\pm$ 8, wt: 74.4 μ M $\pm$ 10, p = 0.002, Fig. 1a) and IL-6 (*Tlr4* mutant: 879 pg/ml $\pm$ 1444, wt: 5642 pg/ml $\pm$ 1353, p = 0.02, Fig. 1b). Analysis of TNF α showed similar trends (data not shown).

Similarly, upon challenge with aggregated A β Tlr4-defective microglia produced significantly less nitrite (*Tlr4* mutant: 24.8 μ M $\pm$ 7, wt: 38.7 μ M $\pm$ 5, p = 0.0004, Fig. 1a), IL-6 (*Tlr4* mutant: 666 pg/ml $\pm$ 221, wt: 1384 pg/ml $\pm$ 129, p = 0.0006, Fig. 1b) and TNF- α (*Tlr4* mutant: 1309 pg/ml $\pm$ 569, wt: 2069 pg/ml $\pm$ 207, p = 0.018, data not shown) than did wt microglia. In contrast, scrambled A β , used as a peptide control, stimulated neither wt nor *Tlr4* mutant microglia (nitrite: *Tlr4* mutant: 6 μ M $\pm$ 3 wt: 6.8 μ M $\pm$ 4, p = 0.25; IL-6: *Tlr4* mutant: 218 pg/ml $\pm$ 107, wt: 325 pg/ml $\pm$ 33, p = 0.27, Fig. 1a,b).

TLR4 mediates A β -induced activation of human monocytes

An anti-human TLR4 neutralizing antibody was used to investigate the role of TLR4 in the activation of human monocytes by aggregated A β . Isolated human blood monocytes were pre-treated with a neutralizing anti-TLR4 antibody or isotype control antibody for 1 hour followed by stimulation with LPS (positive control) or aggregated or scrambled A β . Cellular activation after LPS stimulation was significantly reduced in cells pre-treated with the blocking anti-TLR4 antibody, as assessed by IL-6 release (anti-TLR4: 38 % $\pm$ 4.5, isotype control: 100 %, p < 0.001, Fig. 1c) and TNF α release (not shown). Similarly, TLR4 neutralization significantly inhibited IL-6 release (anti-TLR4: 5 % $\pm$ 2.9, isotype control: 100 %, p < 0.0001, Fig. 1c) and TNF α release (not shown) in cells stimulated with aggregated A β . In contrast TLR4 neutralization

Fig. 1. Tlr4 mediates A β -induced activation of murine microglia and human monocytes: Stimulation of murine Tlr4 mutant or wt microglia (a,b) or human monocytes (c) with 50 μ g/ml aggregated A β or scrambled A β and LPS 10 ng/ml (a,b,c) as positive control. Nitrite release (Griess reaction) (a) and IL-6 release (ELISA) (b) are shown as cellular activation markers; black and white bars correspond to wt and Tlr4 mutant mice, respectively. (c) Human monocytes were treated with (white bar) or without (black bar) anti-TLR4 neutralizing antibody and IL-6 release was quantified by ELISA. Data are expressed as mean of absolute values $\pm$ SD (a,b) and as mean % of isotype control $\pm$ SD (c). * = p < 0.05, student T-test.

inhibited neither IL-6 release (anti-TLR4: 105 % $\pm$ 1.2, isotype control: 100 %, Fig. 1c) nor TNF α release (not

Fig. 2. A β -induced activation depends on the tri-molecular receptor complex, TLR4-MD-2-CD14: Stimulation of HEK293 cells after challenge with 50 μ g/ml aggregated, non-aggregated or scrambled A β , respectively, using LPS as the positive control. Cells were transfected with TLR4-MD-2 or TLR4-MD-2+CD14. Release of the cellular activation marker IL-8 was measured by ELISA. Note that aggregated A β can only stimulate TLR4-MD-2-CD14⁺ HEK293 cells. Data are expressed as mean $\pm$ SD.

shown) in cells stimulated with scrambled A β . Due to immense interindividual differences already in the baseline of cytokine production in the different subjects examined, the results of TLR4 receptor neutralization are presented as percentage of the cytokine production after treatment with the isotype control antibody, which was defined to be 100%.

A β -induced activation depends on the integrity of the tri-molecular receptor complex, TLR4, MD-2 and CD14

We next investigated the role of the integrity of the receptor complex TLR4, CD14 and MD-2 in A β -induced cellular activation in HEK293 cells, which are completely unresponsive to LPS before *CD14/TLR4* and *MD-2* transfection [38]. HEK293 cells were transfected with *TLR4* and *MD-2*, both, with and without *CD14*. Only cells transfected with the tri-molecular receptor complex readily produced IL-8 after stimulation with low amounts LPS, used as a positive control (Fig. 2). Analogously, HEK293 cells transfected with *TLR4* and *MD-2* alone were not activated by exposure to aggregated A β . Only HEK293 cells transfected with all three molecules (*TLR4*, *MD-2* and *CD14*) produced IL-8 in a dose-dependent fashion following challenge with aggregated A β (Fig. 2). Both, treatment with non-aggregated A β or with scrambled A β failed to trigger IL-8 production (Fig. 2), suggesting that a aggregated conformation is a structural pre-requisite for cellular activation. As earlier reported,

stimulation with high amounts of LPS (10ng/ml) also resulted in a CD14-independent cellular activation [39-41]. Our further preliminary experiments showed that also HEK293 cells transfected with *TLR2*, a further innate immune receptor can be activated by aggregated A β (A β 20 μ g/ml: 784 $\pm$ 1.4 pg/ml; positive control 10 μ g/ml Pam3CSK4: 1609 $\pm$ 237 pg/ml), which was even more pronounced after co-transfection of *TLR2* with *CD14* (20 μ g/ml A β : 1267 $\pm$ 72 pg/ml; 10 μ g/ml Pam3CSK4 1451 $\pm$ 102 pg/ml).

Tlr4 mediates A β -induced microglial neurotoxicity

To assess the role of microglial Tlr4 in neurotoxicity, we treated hippocampal neurons with supernatants from scrambled or aggregated A β -stimulated microglia derived from *Tlr4* mutant mice and wt controls. Neuronal cell death was assessed by MTT assay. Supernatant of LPS-stimulated microglia, containing neurotoxic products, is known to induce neuronal cell death and was, therefore, used as a positive control for detection of neuronal toxicity. Cell death was calculated as percentage of the untreated control neurons, which were defined to be 100% viable. Indeed, hippocampal neurons showed significantly more neuronal cell death after exposure to supernatant from LPS-stimulated microglia from wt control mice compared to supernatant from LPS-stimulated microglia from *Tlr4* mutant mice (*Tlr4* mutant: 58 % $\pm$ 3.5, wt: 18 % $\pm$ 3, p = 0.003, Fig. 3). Similarly, neuronal cell death was significantly less pronounced after exposure to supernatant

Fig. 3. TLR4 mediates A β -induced microglial neurotoxicity: Primary hippocampal wt neurons were exposed to supernatants of aggregated or scrambled A β -stimulated Tlr4 mutant or wt microglia. Neuronal cell death was detected by the MTT assay. Note that neurons treated with supernatant derived from C3H/HeJ (Tlr4 mutant) aggregated A β -stimulated microglia were less susceptible for cell death. Data are expressed as mean % of untreated control $\pm$ SD. * p <0.05, student T-test.

Fig. 4. Overexpression of Tlr4 in APP transgenic mice: (a) Tlr4mRNA expression by iCycler real-time PCR in the brains of independent 6-month-old APP transgenic mice versus wt controls. Tlr4 expression is shown as mean of relative units normalized to GAPDH expression $\pm$ SD. Black and white bars correspond to APP and non-transgenic littermates, respectively. (b) RT-PCR for Tlr4mRNA expression and expression of the housekeeping gene GAPDH in 6-month-old APP mice and age-related controls. Shown is one representative experiment. In both experiments APP animals showed an increased Tlr4mRNA expression compared to control mice.

from aggregated A β -treated Tlr4-defective cells (*Tlr4* mutant: 77 % $\pm$ 9; wt: 20 % $\pm$ 3.5, p = 0.007, Fig. 3). After treatment with the peptide control (scrambled A β) neither supernatant from wt nor from Tlr4-defective microglia induced neuronal cell death (*Tlr4* mutant: 97 % $\pm$ 4; wt: 73 % $\pm$ 2, Fig. 3).

Tlr4mRNA is upregulated in APP overexpressing mice

To analyze Tlr4 in experimental AD, we measured *Tlr4* mRNA expression levels in brains of 6-month-old, APP-overexpressing mice and their age-matched non-transgenic littermates using both real-time PCR (Fig.

Fig. 5. Overexpression of TLR4 in AD brains: TLR4 and A β deposits were immunofluorescently stained in brains of AD patients. Shown is the staining of the entorhinal cortex (a-l). TLR4 reactivity is stained in green, A β depositions are stained in red. Increased TLR4 staining can be detected in glial cells surrounding plaques in AD patients (a-i). TLR4 isotype control antibody staining is shown in (j-l). Scale bar is 100 μ m.

4a) and reverse transcriptase PCR (Fig. 4b). *Tlr4* mRNA exhibited a trend towards higher expression in APP overexpressing mice (real time PCR: expression as mean of relative units normalized to GAPDH expression: APP: 0.23 ± 0.13 ; wt: 0.1 ± 0.03 , $p = 0.2$).

TLR4 expression is markedly increased in AD brains

To extend the investigation to human disease, we analyzed post-mortem brains of AD patients for TLR4 expression levels and potential correlation to amyloid plaques by immunofluorescence. Brains of AD patients showed a pronounced TLR4 expression, which was associated with A β plaque deposition (entorhinal cortex: Fig. 5).

Discussion

Understanding the effects of the innate immunity in the context of neurodegenerative diseases could lead to the development of novel therapeutic strategies. Our findings indicate that TLR4, a receptor so far associated with defence against microorganisms, may be relevant in chronic neuroinflammation in AD. We showed that A β -induced activation of murine microglia and human monocytes depends on functional active TLR4 and that this Tlr4-dependent activation is important for neurotoxicity by microglia. This is demonstrated by the increased neuronal death after addition of toxic product-containing supernatant of Tlr4 positive microglia to neurons. Production of toxic products such as proinflammatory

cytokines by TLR4 is triggered either using a MyD88-dependent or a MyD88-independent signalling pathway, resulting in the activation of transcription factors, e.g., NF- κ B, IRF3, 5 or MAPKs, such as p38, JNK [23].

The study showed that that a tri-molecular complex consisting of TLR4, MD-2 and CD14 has to be complete for full cellular stimulation by aggregated A β , as we observed that markers of activation were only released after HEK293 cells were competent for all of the receptors TLR4, MD-2 and CD14. Earlier studies demonstrated that treatment with LPS results in a strong cellular activation after the combined transfection with *TLR4*, *MD-2* and *CD14* [25, 42]. Analogously, we found a strong cellular activation upon treatment with low amounts of LPS only in HEK293 cells transfected with the combination of *TLR4*, *MD-2* and *CD14*. In accordance with earlier reports [39-41], stimulation with high amounts of LPS resulted in a CD14-independent IL-8 release. The reason how TLR4 becomes activated in the pathophysiology of AD is unclear. An autocrine upregulation might be one possibility.

Our additional preliminary experiments suggest also a role of other related innate immune receptors, i.e., TLR2 in A β -mediated cellular activation and strengthen the need for their further investigation in this condition.

We observed expression of *Tlr4* mRNA at low levels in wild-type mice, and at high levels in APP-overexpressing mice at 6 months of age, when the clinical and histopathological disease features manifest [35]. Similarly, we observed increased TLR4 expression in the human AD brains in close correlation with characteristic AD plaques. These findings further support a role of TLR4 in neuroinflammation in AD.

Microglial activation in AD pathophysiology is discussed as a two-edged sword. Recently, Tahara et al. have reported that *Tlr4* loss-of-function mutation in APP transgenic mice increase diffuse and fibrillar A β deposition and that uptake of A β is reduced in *Tlr4* mutant microglia [43]. This together with our results, demonstrating the reduced activation of *Tlr4* mutant

microglia upon A β challenge, leads to the hypothesis that in some disease stages microglial activation with subsequent cytokine release can counterpart A β plaque development as it can be protective due to the increased phagocytotic clearance of A β [44].

On the other hand, release of highly neurotoxic products by microglia can be detrimental. Thus, TLR4 mediates release of microglial inflammatory products that are toxic for neurons. This is supported by a recent report that describes a crucial role of microglial Tlr4 in LPS-induced neuronal injury [29]. A further indication for a disease-promoting role of TLR4 in neurodegeneration in AD is the observed association between the human *TLR4* Asp299Gly polymorphism (which results in an attenuated efficacy of LPS signalling and a reduced capacity to elicit inflammation) and the reduced prevalence of late-onset AD [45].

In conclusion, our results provide the first evidence for a role of the key innate immune receptor, TLR4, in neuroinflammation in AD. We have shown that microglial activation by A β requires a fully functional receptor complex of TLR4, MD-2 and CD14. A clinical relevance of these findings is suggested not only by the observation that *Tlr4* is upregulated in brains of APP transgenic animals, but also by the demonstration that TLR4 is clustered around senile plaques in post-mortem AD brains. These findings contribute to the understanding of the innate immune response in AD, which may lead to the identification of novel therapeutic targets.

Acknowledgements

We thank D. Westaway and A. Chishti (University of Toronto) for providing TgCRND8 mice, D.T. Golenbock (University of Massachusetts), P. Nelson (University of Washington) and K. Miyake (University of Tokyo) for providing expression plasmids.

References

- 1 Braak H, de Vos RA, Jansen EN, Bratzke H, Braak E: Neuropathological hallmarks of Alzheimer's and Parkinson's disease. *Prog Brain Res* 1998;117:267-285.
- 2 Thal DR, Capetillo-Zarate E, Del Tredici K, Braak H: The development of amyloid beta protein deposits in the aged brain. *Sci Aging Knowledge Environ* 2006;Mar 8 (6):re 1.
- 3 Akiyama H, Arai T, Kondo H, Tanno E, Haga C, Ikeda K: Cell mediators of inflammation in the Alzheimer disease brain. *Alzheimer Dis Assoc Disord* 2000;14:S47-S53.

- 4 Walker DG, Link J, Lue LF, Dalsing-Hernandez JE, Boyes BE: Gene expression changes by Amyloid beta peptide-stimulated human postmortem brain microglia identify activation of multiple inflammatory processes. *J Leukoc Biol* 2006;79:596-610.
- 5 Rogers J, Strohmeyer R, Kovelowski CJ, Li R: Microglia and inflammatory mechanisms in the clearance of amyloid beta peptide. *Glia* 2002;40:260-269.
- 6 Streit WJ: Microglia as neuroprotective, immunocompetent cells of the CNS. *Glia* 2002;40:133-139.
- 7 Koenigsknecht J and Landreth G: Microglial phagocytosis of fibrillar beta-amyloid through a beta1 integrin-dependent mechanism. *J Neurosci* 2004;24:9838-9846.
- 8 Eikelenboom P, Bate C, Van Gool WA, Hoozemans JJ, Rozemuller JM, Veerhuis R, Williams A: Neuroinflammation in Alzheimer's disease and prion disease. *Glia* 2002;40:232-239.
- 9 McGeer PL and McGeer EG: Inflammation and the degenerative disease of aging. *Ann N Y Acad Sci* 2004;1035:104-116.
- 10 Blasko I, Stampfer-Kountchev M, Robatscher P, Verhuis R, Eikelenboom P, Grubeck-Loebenstien B: How chronic inflammation can affect the brain and support the development of Alzheimer's disease in old age: the role of microglia and astrocytes. *Aging Cell* 2004;3:169-176.
- 11 El Khoury J, Hickman SE, Thomas CA, Loike JD, Silverstein SC: Microglia, scavenger receptors, and the pathogenesis of Alzheimer's disease. *Neurobiol Aging* 1998;19:S81-S84.
- 12 Hoozemans JJ, Veerhuis R, Rozemuller AJ, Eikelenboom P: The pathological cascade of Alzheimer's disease: the role of inflammation and its therapeutic implications. *Drugs Today* 2002;38:429-443.
- 13 Broytman O, Malter JS: Anti-Abeta: The good, the bad, and the unforeseen. *J Neurosci Res* 2004;75:301-306.
- 14 Cunningham C, Wilcockson DC, Champion S, Lunnon K, Perry VH: Central and systemic endotoxin challenges exacerbate the local inflammatory response and increase neuronal death during chronic neurodegeneration. *J Neurosci* 2005;25:9275-9284.
- 15 Floden AM, Li Shanshan Combe CK: β -Amyloid-stimulated microglia induce neuron death via synergistic stimulation of tumor necrosis factor α and NMDA receptors. *J Neurosci* 2005;25:2566-2575.
- 16 Eikelenboom P, Roremuller JM, van Muiswinkel FL: Inflammation and Alzheimer's disease: relationships between pathogenic mechanisms and clinical expression. *Exp Neurol* 1998;154:89-98.
- 17 McGeer EG and McGeer PL: Innate immunity in Alzheimer's disease: a model for local inflammatory reactions. *Mol Interv* 2001;1:22-29.
- 18 McGeer PL and McGeer EG: Local neuroinflammation and the progression of Alzheimer's disease. *J Neurovirol* 2002;8:529-538.
- 19 Nguyen MD, Julien JP, Rivest R: Innate immunity: the missing links in neuroprotection and neurodegeneration? *Nat Rev Neurosci* 2002;3:216-227.
- 20 Simard AR and Rivest S: Neuroprotective properties of innate immune system and bone marrow stem cells in Alzheimer's disease. *Mol Psych* 2006;11:327-335.
- 21 Ulevitch RJ and Tobias PS: Receptor-dependent mechanisms of cell stimulation by bacterial endotoxin. *Annu Rev Immunol* 1995;13:437-457.
- 22 Kawai T, Takeuchi O, Fujita T, Inoue J, Muhlradt PF, Sato S, Hoshino K, Akira S: Lipopolysaccharide stimulates the MyD88-independent pathway and results in activation of IFN-regulatory factor 3 and the expression of a subset of lipopolysaccharide-inducible genes. *J Immunol* 2001;167:5887-5894.
- 23 Kaisho T and Akira S: Toll-like receptor function and signalling. *J Allergy Clin Immunol* 2006;117:979-987.
- 24 Beutler B: TLR4: central component of the sole mammalian LPS sensor. *Curr Opin Immunol* 2000;12:20-26.
- 25 Jiang Q, Akashi S, Miyake K, Petty HR: Lipopolysaccharide induces physical proximity between CD14 and toll-like receptor 4 (TLR4) prior to nuclear translocation of NF-kappa B. *J Immunol* 2000;165:3541-3544.
- 26 Da Silva Correia J, Ulevitch RJ: MD-2 and TLR4 N-linked glycosylations are important for a functional lipopolysaccharide receptor. *J Biol Chem* 2002;277:1845-1854.
- 27 Ishii KJ, Coban C, Akira S: Manifold mechanisms of toll-like receptor-ligand recognition. *J Clin Immunol* 2005;25:511-521.
- 28 Lehnardt S, Lachance C, Patrizi S, Lefebvre S, Follett PL, Jensen FE, Rosenberg PA, Volpe JJ, Vartanian T: The toll-like receptor TLR4 is necessary for lipopolysaccharide-induced oligodendrocyte injury in the CNS. *J Neurosci* 2002;22:2478-2486.
- 29 Lehnardt S, Massillon L, Follett P, Jensen FE, Ratan R, Rosenberg PA, Volpe JJ, Vartanian T: Activation of innate immunity in the CNS triggers neurodegeneration through a Toll-like receptor 4-dependent pathway. *Proc Natl Acad Sci USA* 2003;100:8514-8519.
- 30 Xia Y, Yamagata K, Krukoff TL: Differential expression of the CD14/TLR4 complex and inflammatory signaling molecules following i.c.v. administration of LPS. *Brain Res* 2006;1095:85-95.
- 31 Fassbender K, Walter S, Kuhl S, Landmann R, Ishi K, Bertsch T, Stalder AK, Muehlhauser F, Liu Y, Ulmer AJ, Rivest S, Lentsch A, Gulbins E, Jucker M, Staufenbiel M, Brechtel K, Walter J, Multhaup G, Penke B, Adachi Y, Hartmann T, Beyreuther K: The LPS Receptor (CD14) links Innate Immunity with Alzheimer's Disease. *FASEB J* 2004;18:203-205.
- 32 Poltorak A, He X, Smirnova I, Liu MY, Van Huffel C, Du X, Birdwell D, Alejos E, Silva M, Galanos C, Freudenberg M, Ricciardi-Castagnoli P, Layton B, Beutler B: Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. *Science* 1998;282:2085-2088.
- 33 Hoshino K, Takeuchi O, Kawai T, Sanjo H, Ogawa T, Takeda Y, Takeda K, Akira S: Cutting edge: Toll-like receptor 4 (TLR4)-deficient mice are hyporesponsive to lipopolysaccharide: evidence for TLR4 as the Lps gene product. *J Immunol* 1999;162:3749-3752.
- 34 Qureshi ST, Lariviere L, Leveque G, Clermont S, Moore KJ, Gros P, Malo D: Endotoxin-tolerant mice have mutations in Toll-like receptor 4 (Tlr4). *J Exp Med* 1999;189:615-625.
- 35 Chishti MA, Yang DS, Janus C, Phinney AL, Horne P, Pearson J, Strome R, Zuker N, Loukides J, French J, Turner S, Lozza G, Grilli M, Kunicki S, Morissette C, Paquette J, Gervais F, Bergeron C, Fraser PE, Carlson GA, St. George-Hyslop P, Westaway D: Early-onset amyloid deposition and cognitive deficits in transgenic mice expressing a double mutant form of amyloid precursor protein 695. *J Biol Chem* 2001;276:21562-21570.
- 36 Ishii K, Muehlhauser F, Liebl U, Picard M, Kuhl S, Penke B, Bayer T, Wiessler M, Hennerici M, Beyreuther K, Hartmann T, Fassbender K: Subacute NO generation induced by Alzheimer's β -amyloid in the living brain: reversal by inhibition of the inducible NO synthase. *FASEB J* 2000;14:1485-1489.
- 37 Meda L, Cassatella MA, Szendrei GI, Otvos L Jr, Baron P, Villalba M, Ferrari D, Rossi F: Activation of microglial cells by amyloid protein and interferon- γ . *Nature* 1995;374:647-649.
- 38 Medvedev AE and Vogel SN: Overexpression of CD14, TLR4 and MD-2 in HEK293T cells does not prevent induction of in vitro endotoxin tolerance. *J Endotoxin Res* 2003;9:60-64.

- 39 Sweet MJ and Humes DA: Endotoxin signal transduction in macrophages. *J Leukocyte Biol* 1996;60:8-26.
- 40 Kirschning C, Wesche H, Ayres TM, Rothe M: Human Toll-like receptor 2 confers responsiveness to bacterial lipopolysaccharide. *J Exp Med* 1998;191:2091-2097.
- 41 Latz E, Visintin A, Lien E, Fitzgerald KA, Monks BG, Kurt-Jones EA, Golenbock DT, Espevik T: Lipopolysaccharide rapidly traffics to and from the Golgi apparatus with the Toll-like receptor 4-MD-2-CD14 complex in a process that is distinct from the initiation of signal transduction. *J Biol Chem* 2002;277:47834-47843.
- 42 Da Silva Correia J, Soldau K, Christen U, Tobias PS, Ulevitch RJ: Lipopolysaccharide is in close proximity to each of the proteins in its membrane receptor complex. *J Biol Chem* 2001;276:21129-21135.
- 43 Tahara K, Kim HD, Jin JJ, Maxwell JA, Li L, Fuuchi K: Role of toll-like receptor signalling in A β uptake and clearance. *Brain* 2006;Sept 19:1-14.
- 44 Nicoll JA, Wilkinson D, Holmes C, Steart P, Markham H, Weller RO: Neuropathology of human Alzheimer disease after immunization with amyloid-beta peptide: a case report. *Nat Med* 2003;9:448-452.
- 45 Minoretti P, Gazzaruso C, DiVito C, Emanuele E, Bianchi M, Coen E, Reino M, Geroldi D: Effect of functional toll-like receptor 4 Asp299Gly polymorphism on susceptibility to late-onset Alzheimer's disease. *Neurosci Lett* 2006;391:147-149.