1. TITLE OF PROJECT (Do not exceed 50 typewriter spaces)
Cell-specific inflammatory response to Aβ and small heat shock proteins

2. TYPE OF APPLICATION
_xNew__Competitive Renewal_x_Major Award__Standard Award__Pilot Award__Cross-border Project (ISAO/LECMA, Standard or Pilot Award only)

Yes, I received a grant from ISAO before, Grant No.: 07510 Grant Period: 2007-2008

3. PRINCIPAL INVESTIGATOR
a. NAME (Last, First): Verbeek, Marcel M.
b. DEGREE(S): PhD MSc
c. YEAR HIGHEST DEGREE EARNED: 1996
d. POSITION TITLE: Associate professor
e. COMPLETE ADDRESS: Radboud University Nijmegen Medical Centre Department of Neurology, 830 LGEM PO Box 9101, 6500 HB Nijmegen Telephone / Telefax: 024-3615192 / 024-3668754 E-Mail Address: marcel.verbeek@radboudumc.nl Website: www.neurochemistry.nl

4. PROJECT PERIOD
5. COSTS REQUESTED FOR PROJECT
150,000 €

6. VERTEBRATE ANIMALS
__Yes__x_No

7. HUMAN SUBJECTS
__Yes__x_No

8. NAME OF INSTITUTIONAL OFFICIAL / ADDRESS OF APPLICANT ORGANIZATION
Institution: Radboud University Nijmegen Medical Center Name of institutional official: Drs. W. de Vries Street: Department of Neurology, 935 Zip Code, City: PO Box 9101, 6500 HB Nijmegen Telephone: #31 24 3615388

9. TYPE OF ORGANIZATION
_x_Public__Private Non-Profit

I declare that to the best of my knowledge the statements and other information containing this application are truthful, complete, and accurate. I further understand that an incomplete application will not be reviewed. “Per” signatures are not acceptable.

10. PRINCIPAL INVESTIGATOR
(Signature of person named in 3)

11. INSTITUTIONAL OFFICIAL
(Signature of person named in 8)

Date, Signature Date, Signature
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F. Literature Cited

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Detailed Budget: Year 1

Detailed Budget: Year 2

Detailed Budget: Year 3 (for Major Applications only)

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Other Support

Consortium/Contractual Arrangements

Consultants/Collaborators Letters of Agreement

Include the following:

Summary of project in Non-Technical Terminology
Key Words
SUMMARY OF PROPOSED RESEARCH  State the objectives, hypothesis, and specific aims of the proposed research. Describe, briefly and concisely, the experimental design and methods for achieving these goals. The summary is meant to serve as a succinct and accurate description of the proposed work when separated from the application. Do not exceed the space provided.

Neuroinflammation is an important pathological process in the brains of Alzheimer disease (AD) patients, which is associated both with parenchymal and vascular extracellular accumulation of the amyloid β protein (Aβ). Neuroinflammation in AD is recognized by activation of microglia and astrocytes and by formation of a number of inflammatory factors, including cytokines, adhesion molecules, chemokines and is associated with cognitive decline. For long, it has been assumed that Aβ is primary responsible for inducing such neuroinflammatory reaction. Our preliminary studies, demonstrated that another class of molecules that is tightly associated with extracellular Aβ, i.e. the so-called small heat shock proteins (sHsps) can induce a strong pro-inflammatory response in cerebral cell cultures, and may be important as a co-stimulating signal for an Aβ-mediated pro-inflammatory response. In order to reduce neuroinflammation in AD by therapeutic intervention, it is important to delineate the mechanisms underlying these processes. Previous pharmacological attempts have focused on the role of Aβ in this process, with limited success. Our studies suggest that focusing therapeutic approaches on neuroinflammation evoked by sHsps may be an important alternative, and , given the amplitude of the inflammatory response induced by sHsps, a putative more promising strategy. In this project, we aim to elucidate the cellular processes that are responsible for the induction of a pro-inflammatory response by the potential synergistic action of Aβ and sHsps. We postulate (and will investigate) that for Aβ, to induce a strong inflammatory response, a second, co-stimulatory, factor needs to be present. Such co-factor may comprise (low levels) of sHsps. We will study, in various types of human cerebral cell cultures (cerebrovascular cells, astrocytes, microglia), the type of receptors (e.g. Toll-like receptors) and intracellular mechanisms (e.g. the inflammasome) involved in the sHsp- and Aβ-mediated pro-inflammatory response. Furthermore, we expect that we will be able to derive cell-specific receptors and mechanisms that mediate the induction by sHsps and/or Aβ of the neuro-inflammatory reaction. The results of our studies will allow for the future design of therapeutic interventions that aim to inhibit the pro-inflammatory reaction in AD patients and that may help to delay the progress of this devastating disease.

RELEVANCE OF PROPOSED RESEARCH TO ALZHEIMER’S DISEASE  State briefly and concisely how the proposed research is relevant to determining the cause of or to developing a treatment for Alzheimer’s disease. Do not exceed the space provided.

Neuropathological hallmarks of Alzheimer’s disease (AD) comprise senile plaques, cerebral amyloid angiopathy and neurofibrillary tangles. However, a prominent cerebral inflammatory reaction, recognized by activation of microglial cells and astrocytes and production of several inflammatory factors, is associated with these lesions. Many studies suggested that Aβ is the primary inducer of the cerebral inflammatory response. Our data however indicated that small heat shock proteins (sHsps) may be important (co-)stimulating factors for the inflammatory response in AD. The aim of this project is to study in vitro the exact mechanisms that lead to this neuro-inflammatory reaction. Identification of these pathways may help to design novel therapies aimed at inhibiting the inflammatory reactions, for instance focused on sHsps as major (co-)initiators of neuro-inflammation in AD.

PERSONNEL  (Includes P.I., key personnel and consultants)

<table>
<thead>
<tr>
<th>Name, Degree(s)</th>
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<th>Role in Project</th>
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<td>Vacancy</td>
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<td>Researcher</td>
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<td>Dr. H.B. Kuiperij</td>
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<tr>
<td>Dr. M. Netea</td>
<td>Post-doc</td>
<td>Co-PI</td>
<td>RUNMC</td>
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<tr>
<td>Dr. W. Boelens</td>
<td>Professor</td>
<td>advisor</td>
<td>RUNMC</td>
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<td>Dr. R. de Waal</td>
<td>Assoc. prof.</td>
<td>advisor</td>
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<td>Dr. MM Verbeek</td>
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<td>PI</td>
<td>RUNMC</td>
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</table>
BIOGRAPHICAL SKETCH  Give the following information for the Principal Investigator and key personnel listed on page 3. Begin with the Principal Investigator. Copy this page or use the same format for each person. Do not exceed one page per person.

Name: Marcel M. Verbeek  Position Title: Associate Professor, Neurochemist

Education  Begin with baccalaureate or other initial professional education and include postdoctoral training.

<table>
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<th>Institution and Location</th>
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<td>University of Delft</td>
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<td>1983</td>
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<tr>
<td>University of Delft</td>
<td>MSc</td>
<td>1998</td>
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<tr>
<td>University of Nijmegen</td>
<td>PhD</td>
<td>1996</td>
<td>Alzheimer’s disease</td>
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</table>

Research and Professional Experience  List in chronological order previous employment, experience, grants, and honors.

1988 - 1990  Research Scientist at the Gaubius Instituut TNO, Leiden, The Netherlands
1990 - 1995  Ph.D. scientist; Department of Pathology, RU Nijmegen, The Netherlands
1995 - 1999  Post-doc, Department of Pathology, RU Nijmegen
1999 - onwards Staff member, Neurochemist, Department of Neurology, Radboud University Nijmegen Medical Center

Nov 2007-onwards. Associate Professor, Neurochemistry of Neurodegeneration.

2008- onwards Principal investigator at the RUNMC, Donders Institute for Brain, Cognition and Behaviour, Centre for Neuroscience


Grants (selection):
2012: Internationale Stichting Alzheimer Onderzoek (ISAO): “Aβ43: an overlooked peptide associated with AD”. € 100,000
2011: Alzheimer’s Drug Discovery Foundation (USA): Cerebrospinal fluid biomarkers to discriminate frontotemporal dementia subtypes. $ 100,000
2010: Van Alkemade Keuls fonds: Een prospectief onderzoek naar de differentiaal diagnostische waarde van biomarkers in de liquor cerebrospinalis bij de ziekte van Parkinson en atypische parkinsonismen. € 41,250
2009: Continuous CSF Sampling Method To Characterize Amyloid Peptides, Tau Peptides And Other Potential Biomarkers in Healthy Subjects And Patients With Alzheimer’s Disease. A Phase 0 Study In A Specialized Unit For Neurodegenerative Disorders. Study sponsored by Schering Plough Research Institute. € 435,090
2009: Hersenstichting Nederland: Alternatieve metabole routes voor de biosynthese van dopamine; nieuwe behandelingsmogelijkheden van AADC deficiëntie? € 35,000.
2008: Center for Translational Molecular Medicine: In vivo molecular diagnostics in Alzheimer’s disease; the LeARN (Leiden-Alzheimer Research Nederland) project. € 1,556,000
2008: Stichting Internationaal Parkinson Fonds. Evaluation of a diagnostic test battery in Parkinson patients with 3 years follow-up. € 95,100;
2008: The AADC Research Trust. In search of an explanation for renal dopamine synthesis in AADC deficiency. € 32,000
2007: ISAO: “Small heat shock proteins and inflammation in Alzheimer’s disease”. € 80,000
2007: Hersenstichting Nederland: Brain specific proteins in CSF to estimate severity of cerebral damage after posthemorrhagic ventricular dilatation in preterm neonates; with Dr. D. Liem; € 25,000
PUBLICATIONS  List in chronological order the titles and complete references of publications in refereed journals during the past three years and to representative early publications pertinent to this application. Do not exceed two pages including this page. (selection of 140 publications)

4. de Bot ST, ..., Verbeek MM. CSF Studies Facilitate DNA Diagnosis in Familial Alzheimer's Disease Due to a Presenilin-1 Mutation. J. Alzh. Dis. 2009, 17(1), 53-57. IF 3.8

Older publications pertinent to this application:
**BIOGRAPHICAL SKETCH**  Give the following information for the Principal Investigator and key personnel listed on page 3. Begin with the Principal Investigator. Copy this page or use the same format for each person. Do not exceed one page per person.

Name: prof. M.G., Mihai Netea

**EDUCATION**  Begin with baccalaureate or other initial professional education and include postdoctoral training.

<table>
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<th>Institution and Location</th>
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<tr>
<td>University of Medicine and Pharmacy Cluj-Napoca</td>
<td>PhD</td>
<td>22-06-1998</td>
<td>Medicine; inf. diseases</td>
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<tr>
<td>Radboud University Nijmegen</td>
<td>PhD</td>
<td>31-08-1993</td>
<td>Medicine</td>
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**RESEARCH AND PROFESSIONAL EXPERIENCE**  List in chronological order previous employment, experience, and honors.

1998-2000, Post-doc (1 fte, fixed term), Department of Medicine, Radboud University Nijmegen
2000-2004, Resident/post-doc (1fte, fixed term), Jeroen Bosch Ziekenhuis 's-Hertogenbosch, and Radboud University Nijmegen Medical Center (RUNMC)
2006-2008 Infectious disease specialist/researcher (1fte, tenured), Department of Medicine, RUNMC since May 2008 Full Professor, Exp. Internal Medicine (1fte, tenured), Dept.Medicine, RUNMC since June 2008, Standing visiting professor, Department of Infectious Diseases, University of Medicine and Pharmacy, Cluj-Napoca, Romania

Grants (selection)
2004 Vidi Grant of the Netherlands Association for Scientific Research, for a 5-year project on “Pattern recognition of Candida albicans”.
2009 participation in CTMM grant “Molecular Diagnosis and Risk Stratification of Sepsis (MARS)” (2 PhD positions)
2010 Vici Grant of the Netherlands Organization for Scientific Research, on the project “Trained innate immunity: the role for host defence against fungal infections” (1.500.000 euro)

Recent publications
4. van de Veerendonk FL, ..., Netea MG. Reactive oxygen species-independent activation of the IL-1beta inflammasome in cells from patients with chronic granulomatous disease. Proc Natl Acad Sci U S A. 2010 Feb 16;107(7):3030-3. (9.3)
5. Ferwerda B, ..., Netea MG. Human dectin-1 deficiency and mucocutaneous fungal infections The New England Journal of Medicine, 2009, 361;1760-7 (47.0)
8. Ferwerda B, Netea MG. Functional and genetic evidence that the Mal/TIRAP allele variant 180L has been selected by providing protection against septic shock. Proc Natl Acad Sci U S A. 2009 Jun 23;106(25):10272-7 (9.6)
# BIOGRAPHICAL SKETCH

**Name**  
Robert M.W. de Waal  
Position Title  
Associate professor, Ph.D.

**EDUCATION:**

<table>
<thead>
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<tr>
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<td>drs.</td>
<td>1975</td>
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<td>University of Nijmegen</td>
<td>(Postdoc)</td>
<td>1991</td>
<td>Molecular biology</td>
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<tr>
<td>University of Wurzburg</td>
<td>(Postdoc)</td>
<td>2005</td>
<td>Multiphoton microscopy</td>
</tr>
</tbody>
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**RESEARCH AND PROFESSIONAL EXPERIENCE:**

1991:  
6 months practical training in recombinant DNA techniques at the department of Molecular Biology, Faculty of Science, University of Nijmegen. Supervisors: Prof. Dr. J. Schoenmakers and Dr. N. Lubsen

2005:  
4 months practical training in multiphoton microscopy for intravital imaging of tumors in experimental animals. Virchow Center, Department of Dermatology, University of Würzburg, Germany. Supervisor: Prof. Dr. P. Friedl.

1980-present:  
Staff member, associate professor (UHD), University Medical Centre St. Radboud, Nijmegen, NL: member of the daily board of the Department of Pathology, Research Coordinator of the department of Pathology.

**RECENT PUBLICATIONS:**

BIOGRAPHICAL SKETCH:

Name: Boelens, Wilbert C.
Position Title: (Bio) Chemist

EDUCATION

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<tr>
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<td>1992</td>
<td>Biochemistry</td>
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</table>

RESEARCH AND PROFESSIONAL EXPERIENCE:

1988 - 1992 Ph.D. Scientist at the Radboud University, The Netherlands
1992 - 1994 Post-doctoral research scientist at EMBL Heidelberg, Germany
1994 - onwards Staff member, Depart. of Biomolecular Chemistry, Radboud University, Nijmegen.

Honors:

Grants:

RECENT PUBLICATIONS:
RESEARCH PLAN  See instructions for details on how to complete this section. This section is limited to a total of SEVEN PAGES for Standard and THREE PAGES for Pilot Grant Awards. Applications that exceed page limitations or are not easily readable will not be reviewed.

Background

Neuropathology
The neuropathology of AD comprises intraneuronal neurofibrillary tangles, deposition of the amyloid β protein (Aβ) in senile plaques and cerebral amyloid angiopathy (CAA). These lesions are crucial to the development of AD and the mechanisms leading to their formation are among the most important open questions in today’s biomedical research on AD.

The Aβ peptide is derived from the Aβ precursor protein (AβPP), a type I integral membrane protein, through sequential proteolytic processing mediated by β- and γ-secretase activities. The 4 kDa Aβ occurs in both non-fibrillar and fibrillar forms, both of which are found in AD brains. Diffuse senile plaques contain non-fibrillar Aβ, whereas in classic senile plaques also aggregated Aβ is found. CAA also consists predominantly of fibrillar Aβ. It is assumed that non-fibrillar Aβ of the diffuse senile plaques gradually transforms into the fibrils of classic senile plaques via step-wise formation of oligomers, protofibrils and mature fibrils (Walsh 1997; Lambert 1998). Aβ may be directly neurotoxic, but, currently, oligomers and protofibrils of Aβ are regarded as the most toxic species of Aβ (Lambert 1998), whereas Aβ in the form of mature fibrils (such as found in classic senile plaques) may be relatively inert. The most predominant species of Aβ are Aβ40 and Aβ42. The Aβ42 is the predominant species in diffuse senile plaques and is more prone to aggregation than Aβ40, which, in turn, is predominant in CAA.

Cellular degeneration in AD brains has been linked to the Aβ peptide; neurons in and around senile plaques as well as vascular cells in CAA degenerate, a process that is likely induced by Aβ.

Inflammation in AD brains

Neuroinflammation in AD comprises both activation of microglial cells and astrocytes in and around senile plaques and CAA (McGeer 1995; Rozemuller 2005; Simard 2006). Microglial cells are the brain’s counterpart of monocytes/macrophages and express elevated levels of interleukin (IL)–1β, produce reactive oxygen species and extracellular proteases, possibly in response to the presence of extracellular Aβ and as a consequence of phagocytosis of Aβ (Griffin 1995; Allan 2005). Other signs of a neuro-inflammatory reaction are activation of the complement system and co-deposition with Aβ of several complement factors (such as C1q, C3, C4 and the membrane attack complex), adhesion molecules (intercellular adhesion molecule-1, ICAM-1) (Verbeek 1994; 1997; Heneka 2010), and cytokines, such as IL-8, IL-6, IL-18, TGFβ1 (Ojala 2009; Wyss-Coray 1997), interleukin (IL)-1β, IL-8, monocyte chemoattractant protein 1 (MCP-1; Sokolova 2009) and CD40 ligand (Calingasan 2002). Moreover, cerebral microvascular amyloid in AD and familial CAA disorders is also associated with a localized neuroinflammatory reaction (Grabowski 2001; Vinters 1998; Yamada 1996). Inflammation, both directly via interaction of activated glial cells and indirectly via secreted neurotoxic mediators may compromise neuronal function. From epidemiologic studies it has become apparent that the chronic use of anti-inflammatory agents (especially non-steroid anti-inflammatory drugs (NSAIDs) such as indomethacin) reduces the risk of developing AD, suggesting that neuroinflammation plays an early role in AD development (McGeer 2007). Treatment of AD patients with NSAIDs, however, does not seem to be efficient (De Jong 2008).

Apart from its neurotoxic property, it has been demonstrated that Aβ may evoke a chronic, neuro-inflammatory reaction by inducing cytokine and ROS production (Combs 2001), which may be accelerated by factors such as C1q.

Small heat shock proteins, a class of Aβ-associated proteins in AD
Chaperones comprise a group of proteins that aid in the folding of other proteins preventing them from assuming improper conformations. Two classes of chaperones exist: high molecular weight heat shock proteins (Hsps) that have ATPase activity, such as Hsp70, and small Hsps (sHsps), that do not bind ATP (Horwitz 1992; van Montfort 2001). High molecular weight Hsps are directly involved in ATP-dependent protein folding. Small Hsps may interact with partially denatured proteins, prevent their aggregation and thereby assist in actual protein refolding by chaperones with ATPase activity. Based on
sequence similarities, ten human sHsps have been identified so far, including αB-crystallin and Hsp27, Hsp20, HspB2/B3 and HspB8 (Kappe 2003). Preceded by a less conserved, and in length more variable N-terminal domain, a conserved 80–100 amino acid long stretch in the C-terminal region called the α-crystallin domain hallmarks the sHsp family. In vitro, sHsps typically display chaperone-like activities, measured by their ability to protect model substrates from thermally or chemically induced aggregation. In previous studies we demonstrated that sHsps may interact with Aβ, modulate its aggregation behaviour and cytotoxic properties, but we also showed that sHsps on their own are capable of inducing a strong inflammatory response in cerebral cells (see “preliminary results”). For sHsps to exert their extracellular effects, active secretion from cells, or alternatively, release after cellular degeneration, is required. In two papers it was demonstrated that αB-crystallin may be secreted in the form of exosomes (Gangalum, 2011; Sreekumar, 2010).

The inflammasome and Toll-like receptors
Interleukin-1β (IL-1β) is one of the key inflammatory products in AD brains. Both IL-1β and IL-18 are synthesized as inactive intracellular precursor proteins that are proteolytically processed by the cysteine protease caspase-1 into their biologically active form in response to pro-inflammatory stimuli. Caspase-1 activity is dependent on activation of a multi-protein caspase-1-activating complex, named the inflammasome (Chakraborty, 2010). The inflammasome is formed by members of the NAchT-Leucine-rich-repeat Proteins (NALP) family, such as NALP1, 2 or 3 or other related molecules. The NALP molecules form a complex with the adaptor protein ASC that connects the NALPs to caspase-1. Cells respond to a variety of Danger Associated Molecular Patterns (DAMPs) by activating the inflammasome. These DAMPs may, amongst others, include ds-RNA, heat shock proteins, reactive oxygen species, but also misfolded proteins such as Aβ (Halle 2008). Eventually, these events result in activation of caspase-1 that is responsible for proteolysis of pro-IL-1β /IL-18 into IL-1β/IL-18 (Keller 2008).

Other classes of so-called pattern recognition receptors (PRRs), to which the inflammasomes belong, comprise 1) lectin proteins that bind to microbial pathogens, and 2) the so-called Toll-like receptors (TLRs) that may bind microbial molecules, such as LPS, lipoproteins, double-stranded viral RNA, bacterial or viral CpG, but also host molecules such as heparan sulfate fragments and fibrinogen. Strikingly, also Hsp70 and HspB8 can bind to TLR2 and TLR4 (Roelofs 2009). TLRs depend on other co-receptors, such as CD14 and MD-2 for full ligand sensitivity. When activated, TLRs recruit cytoplasmic adapter molecules, such as MyD88, Tirap, Trif and Tram, in order to propagate a signal. The adapters activate protein kinases leading to the induction or suppression of genes that orchestrate the innate inflammatory response, e.g. production of IL-6, MCP-1 and IL-8 (Del Cornò 2009; Kurt-Jones 2002).

TLR activation may activate an intracellular signaling cascade leading to induction of pro-IL-1β, which is not constitutively expressed; then a second signal via stimulation of the inflammasome leads to activation of caspase-1, cleavage and maturation of IL-1β (Netea 2010). IL-1β, in turn, may induce the production of several downstream inflammatory products. In addition to these mechanisms it has been demonstrated that release of the lysosomal cysteine protease cathepsin B, induced by phagocytosis of aggregated Aβ or treatment with chromogranin A, can activate caspase-1 to process pro-IL-1β into the active IL-1β (Halle 2008, Terada 2010).

Preliminary studies
Small heat shock proteins in AD brains
Senile plaques (SPs), cerebral amyloid angiopathy (CAA) and neurofibrillary tangles are neuropathological hallmarks of Alzheimer’s disease (AD). We showed that several small heat shock proteins (sHsps) are associated with SPs and CAA, but with different expression patterns. Hsp20 was associated with several types of SPs (Wilhelmus 2006a,b) and with dyshoric angiopathy, i.e. Aβ deposits surrounding (capillary) CAA extending into the brain parenchyma but not with the (capillary) CAA itself. HspB8 was found in several types of SPs, in capillary CAA and in dyshoric angiopathy. HspB2 was demonstrated in both large-vessel and capillary CAA, classic SPs and dyshoric angiopathy; therefore it possibly binds to fibrillar Aβ, but not monomeric Aβ (Wilhelmus 2006a). Neither αB-crystallin nor Hsp27 was found in SPs, (capillary) CAA or dyshoric angiopathy, but they could be detected intracellularly, in activated astrocytes that surround senile plaques or CAA. None of the sHsps was present in tangles. Although αB-crystallin was not detectable in SPs/CAA we were able to detect its presence in
cerebrospinal fluid of unaffected individuals, confirming that αβ-crystallin may occur extracellularly, (unpublished data).

**Interaction of sHsps with Aβ in vitro (Wilhelmus 2006b,c)**

Binding between Aβ and sHsps was studied by surface plasmon resonance (SPR). αβ-crystallin demonstrated highest affinity for soluble wild-type Aβ_{1-42} compared to the other sHsps. Affinity of the sHsps for all forms of Aβ decreased in the following order: αβ-crystallin > Hsp20, Hsp27. HspB2 in complex with HspB3 (which is the natural partner of HspB2) failed to bind to Aβ_{1-42}. Aβ aggregates into fibrils via a process that initially involves the formation of oligomers and protofibrils. sHsps affect this aggregation process, as measured by a variety of techniques (including circular dichroism spectroscopy, Thioflavin T binding and electron microscopy). The strongest inhibitory effects were observed with αβ-crystallin, whereas HspB2/B3 was hardly active.

Degeneration of smooth muscle cells (SMCs) and pericytes is a prominent feature in CAA. We have studied Aβ-mediated cellular degeneration by using cultured human brain pericytes (HBPs), SMCs and astrocytes (Wilhelmus 2005, 2006a-c, Rensink 2004). In this model non-aggregated wild-type Aβ_{1-42}, but not Aβ_{3-40}, is cytotoxic for both cell types (Verbeek 1997). The cytotoxic effect of Aβ seems to be mediated by peptide accumulation and aggregation at the cell surface (Rensink 2004). sHsps inhibited Aβ-mediated toxicity towards cerebrovascular cells (Wilhelmus 2006b,c); the efficacy of the various sHsps again paralleled their Aβ-binding capacity.

**Extracellular sHsps induce cytokine production**

We studied the potential of sHsps to induce an inflammatory response in various cultured cerebral cell types in comparison to equimolar amounts of Aβ. First, since it is assumed that Aβ is directly responsible for the observed inflammatory reaction in AD, we investigated if equimolar amounts of sHsps could induce the production of inflammatory factors to the same extent as Aβ. In fact, our published results (Wilhelmus 2009) show that Aβ only had a very marginal effect on IL-6 production by smooth muscle cells (SMCs), astrocytes and microglial cells, while in contrast various sHsps strongly induced IL-6 production. We recently extended these observations by studying the production of a panel of inflammatory proteins in response to either Aβ or sHsps (Bruinsma, 2011). While Aβ_{1-40} and Aβ_{1-42} (12.5 μM) did not induce production of IL-8, ICAM-1, MCP-1 or CD40 ligand by SMCs or astrocytes above control levels (Figure 1A-C), incubation of SMCs with 12.5 μM Hsp20, HspB8 or HspB2B3 resulted in a strong and significant induction of IL-8 (Figure 1A), ICAM-1 (Figure 1B) and MCP-1 (Figure 1C) secretion. Similarly, incubation of astrocytes with 12.5 μM Hsp20, HspB8 or HspB2B3 also resulted in a strong and significant induction of IL-8, ICAM-1 and MCP-1 secretion, but only 12.5 μM HspB8 and HspB2B3 induced CD40 ligand production by astrocytes (not shown). In addition, Hsp27 (Figure 1A-C) and αβ-crystallin (not shown) did not, or only to a limited extent, affect production of these inflammatory factors. Interestingly, our preliminary results show that stimulation of microglial cells with Aβ_{1-42} in combination with Hsp20 induces IL-6 more strongly than either Aβ_{1-42} or Hsp20 alone (Figure 2), indicating that sHsps may act as a co-stimulatory signal to induce a strong inflammatory response in AD.

To exclude LPS contamination, potentially present in the purified sHsp samples, we co-incubated cell samples with PMB and sHsps to inhibit LPS-mediated inflammation. PMB had no, or only a minimal effect, on the production levels of IL-8 by SMCs and astrocytes induced by Hsp27, Hsp20, HspB8 and HspB2B3 (Figure 1D), confirming the strong inducing effect, independent of LPS, of these sHsps on cytokine production.

Second, we investigated if there was a possible relation between the expression pattern of sHsps (i.e. intracellular vs. extracellular) and their capacity to induce an inflammatory response. As described above, we published that HspB2/B3, Hsp20 and HspB8, but neither αβ-crystallin nor Hsp27, strongly induced IL-6 production by cultured cerebrovascular cells and astrocytes. A largely similar pattern of induction of inflammatory factors was observed with regard to ICAM-1, CD40L and IL-8: these were all induced by HspB2/B3, Hsp20 and HspB8, but not by αβ-crystallin and only to a limited extent by Hsp27. Thus, interestingly, predominantly those sHsps that are observed with extracellular Aβ deposits in AD brains (i.e. HspB2/B3, Hsp20 and HspB8) are capable of inducing an inflammatory response in SMCs and astrocytes, whereas those sHsps that are only expressed intracellularly (i.e. αβ-crystallin and Hsp27) do not initiate an inflammatory response or only to a limited extent if added to cultured cells.
Third, in contrast to the effects observed in cerebrovascular cells and astrocytes, we observed that all sHsps, including aβ-crystallin and Hsp27, induced microglial IL-6 production. These observations indicate that the regulation of an inflammatory response to Aβ and/ or sHsps is cell-specific and, as such, coordinated by cell-specific mechanisms, involving various cell-surface receptors and intracellular mediators. Furthermore, we observed that neither Aβ nor any of the sHsps induced IL-1β production in microglial cells. This suggests that probably two independent signals are necessary for the induction of pro-IL-1β levels and activation of caspase-1, respectively.

**Relevance for AD.** In conclusion, since inflammation, i.e. microglial and astrocyte activation and production of inflammatory factors, is intimately associated with the pathophysiology of AD and contributes to local cell damage, the identification of sHsps as strong potential inducers of this inflammatory response in the brain warrants identification of the underlying mechanisms. Such knowledge may elucidate new, potential targets at which therapeutic strategies may be aimed at to inhibit this adverse event.

**Hypothesis.** We hypothesize that 1) sHsps are capable of inducing a pro-inflammatory response by cell-specific expression of receptors and intracellular mediators of an inflammatory reaction that may comprise pattern recognition receptors such as the various TLRs; 2) The pro-inflammatory effects of Aβ requires interaction with the Nalp3-inflammasome or Aβ-receptors such as LRP-1 as well as orchestration with a co-stimulatory signal, e.g. provided by (low levels) of extracellular sHsps or other co-stimulatory agents to induce a robust inflammatory response.

**Aims.** 1) To study cell-specific expression of receptors that may mediate the pro-inflammatory response to sHsps and/or Aβ; 2) To study the possible involvement of TLRs in the induction of an inflammatory response to sHsps; 3) To study the role of inflammasome activation and other “Aβ-receptors” in Aβ-mediated inflammation; 4) To study the possibility that an Aβ-mediated pro-inflammatory response requires a co-stimulatory signal e.g. via TLR activation and signalling; 5) To derive and identify, from the experiments in 1-4, a cell-specific inflammatory response of cerebral cell types (SMCs, astrocytes and microglial cells) to Aβ and sHsps with regard to receptors, intracellular activation mechanisms and production of inflammatory products relevant to AD.

**Workplan.**

Ad 1) Our preliminary data indicate that various cerebral cell types (smooth muscle cells, astrocytes, microglial cells) do not respond identically to stimulation by sHsps and Aβ. This indicates that various cell-specific receptors may mediate initiation of an inflammatory response in these cerebral cell types. Therefore, we aim to identify by immunocytochemistry and western blotting receptors that are potentially involved in inducing a pro-inflammatory response in SMCs / astrocytes / microglia after treatment with either Aβ or sHsps. Several classes of receptors may be involved in the recognition of Aβ / sHsps that mediate the inflammatory response, including: (A) Toll-like receptors (TLR), such as TLR2, TLR4 and their co-receptors CD14, MD-2, MyD88; (B) lipoprotein receptors such as LRP-1, VLDLR, ABCA and LDLR that are known to interact with Aβ (expression of these receptors in SMCs and astrocytes has already been studied by us and will be extended to microglial cells); (C) Other Aβ-binding receptors such as RAGE, CD36, ABCA and p-glycoprotein; (D) inflammasome proteins, i.e. activated caspase-1, Nalp3, ASC.

Ad 2) The involvement of TLRs in mediating an inflammatory response evoked by sHsps will be performed as follows: Cultured cells will be incubated with various sHsps (HspB2/B3, HspB8, Hsp20; produced recombinantly) as previously described (12.5 μM) and production and secretion of a variety of inflammatory factors (IL-1β, IL-18, IL-8, MCP-1, IL-6, TNFα, soluble ICAM-1 and CD40 ligand) will be studied by ELISA quantification (all operational; R&D DuoSet format). All incubations will be performed in the presence of polymyxin B (PMB) to suppress potential LPS-mediated effects. In situations of a positive inflammatory response to either sHsp treatment, the involvement of specific TLRs, especially TLR2 and TLR4, will be studied by co-incubation with anti-TLR antibodies and TLR signaling inhibitors (Invivogen). Additional evidence for a potential involvement of TLRs will be obtained by incubation of either CHO cells transfected with TLR2 / TLR4 or TLR-deficient CHO cells (available via prof. Netea) to demonstrate specific interaction of sHsps with TLRs.
Ad 3) Caspase-1 has been identified as the main mediator of IL-1β production. Activated caspase-1 cleaves pro-IL-1β and pro-IL-18 to release the active cytokines IL-1β and IL-18. Activation of caspase-1 may occur via at least two pathways: recognition of signalling molecules by the inflammasome, such as the NALP3 –ASC inflammasome and via cathepsin B activation. Cathepsin B may be released by lysosomal activation after Aβ phagocytosis. We aim to study the involvement of caspase-1-mediated production of inflammatory factors after induction with Aβ as follows. Cells will be incubated with synthetic Aβ40 or Aβ42. Activation of caspase-1 will be followed by labelling cells with the fluorescent probe FLICA that selectively binds to activated caspase-1 and by studying the formation of activated caspase-1 cleavage product p10 by Western blotting. Additional evidence for the involvement of caspase-1 will be derived from co-incubation experiments with the specific inhibitor of caspase-1, Z-YVAD-fmk. The role of cathepsin B will be studied by using CA-074-Me, an inhibitor of cathepsin B (Calbiochem), whereas inhibitors of other cathepsins will serve as controls for specificity. To demonstrate a potential role for lipoprotein receptors in the Aβ-mediated inflammatory reaction, cells will be co-incubated with receptor-associated protein (RAP) or soluble LRP which is known to block interaction with these lipoprotein receptors. The interaction with ABCA1 will be blocked by the inhibitors 4,4-diisothiocyanostilbene-2,2′-disulfonicacid (DIDS), ABCA1 transport proteins and cyclosporine A.

Since it has been demonstrated in multiple studies that the biological effect of Aβ may strongly depend on its aggregation state, we will study the pro-inflammatory effect of various conformational forms of Aβ. Oligomers of Aβ will be formed by incubation of 100 μM Aβ in PBS for 24 hrs at 4 °C. Presence of oligomers will be checked by using an in-house developed oligomer-specific Aβ ELISA and Western blotting. Similarly, absence of such oligomers in monomer preparations will be verified. Fibrils of Aβ will be prepared by incubation of 100 μM Aβ in 10 mM HCl for 1-5 days at 37 °C and checked by Western blotting. Equimolar amounts of monomeric, oligomeric or fibrillar Aβ will be incubated as above and secretion of inflammatory factors will be quantified by ELISA as above. The involvement of the various receptors will be studied as above, under the conditions (with various Aβ preparations) that induce an inflammatory response.

Ad 4) Our preliminary experiments suggested that Aβ induced only a limited inflammatory response in microglial cells compared to the strong response initiated by sHsps, and we identified a lack of response to Aβ in SMCs / astrocytes. In addition, our preliminary experiments suggest that, for Aβ to elicit a full-blown inflammatory response, a co-stimulatory signal, like extracellular Hsp20, is required. We aim to extend these preliminary studies by treating microglial cells with Aβ in combination with varying concentrations of the available sHsp preparations, followed by analysis of secreted inflammatory factors as described above in part 2. Co-stimulation of these cells with sHsps (in the presence of PMB) will reveal a potential co-stimulatory pathway of these compounds via a combination of the above-studied receptors (TLRs, inflammasome, Aβ receptors). To confirm potential co-stimulatory pathways, co-stimulation with stimulators of TLRs (such as LPS, CpG) or of the inflammasome (such as chromogranin A), will be combined with Aβ treatment. We will select the most appropriate form of Aβ aggregation (monomers, oligomers, or fibrils; results from part 3) in these studies.

Ad 5) By extending the knowledge obtained in our preliminary experiments to the currently proposed studies, it can be expected that these studies will yield novel insights to reveal the presence of a cell-specific response of various cell types to sHsps and Aβ treatment. Despite the large degree of homology between the various sHsps, cell-specific regulatory mechanisms are to be expected. By analysis of the experiments in parts 1-4, we will be able to derive whether such cell-specific mechanisms truly exist; in that case, no additional experiments will be required within this aim 5.

Expected results. Identification of a (cell-specific) repertoire of receptors involved in generating a pro-inflammatory response to Aβ or sHsps. Identification of a co-stimulatory signalling pathway that explains a pro-inflammatory response to Aβ. These novel insights may guide the design of novel therapies aimed at attenuation of AD-associated inflammation.

Time planning
Months 1-4: expression profiling of receptors in cultured cells (aim 1); Months 4-15: establishment of receptors involved in sHsp-mediated production of inflammatory factors (aim 2); Months 4-15: establishment of receptors involved in Aβ-mediated production of inflammatory factors and mechanisms of caspase-1 activation (aim 3a); Months 12-18: Study of effects of various Aβ aggregates
(aim 3b); Months 19-30: Study of co-stimulatory signals in Aβ-mediated pro-inflammatory response (aim 4); Months 25-30: Analysis of cell specificity of events (aim 5); Months 30-36: data analysis, manuscript preparation.

References
BUDGET JUSTIFICATION  Describe the specific functions of the Principal Investigator, key personnel, and consultants.

We mainly request salary for a junior investigator (PhD student) for a period of four years.

A Ph.D student with experience in biochemistry and cell biology is requested. He/she will be responsible for the cell biological, biochemical and immunohistochemical studies. Salary and appointment will be according to the current RUNMC regulations for Ph.D. students.

Supervision of the biochemical and cell biological studies will be by dr. Verbeek and dr. de Waal (both paid by the Radboud University Nijmegen Medical Centre (RUNMC)). Prof. Netea (paid by RUNMC) will give advise and will participate in discussions concerning inflammatory pathways. Dr. Boelens (paid by RUNMC) will advise on the biology of small heat shock proteins.

Synthetic Aβ peptides used in this study are already available.

OTHER SUPPORT  List all current and pending research support. See instructions for details on how to complete this section. Use continuation pages if necessary. ISAO will not duplicate funding from other sources.

Alzheimer's Drug Discovery Foundation (USA): Cerebrospinal fluid biomarkers to discriminate frontotemporal dementia subtypes. $ 100,000.

American Alzheimer Association, Investigator-Initiated Grant: ApoE and ApoJ in the cerebrovascular clearance of amyloid beta protein; $ 200,000.

Van Alkemade Keuls fonds: Een prospectief onderzoek naar de differentiaal diagnostische waarde van biomarkers in de liquor cerebrospinalis bij de ziekte van Parkinson en atypische parkinsonismen. € 41,250

Hersenstichting Nederland: Alternatieve metabole routes voor de biosynthese van dopamine; nieuwe behandelingsmogelijkheden van AADC deficiëntie? € 35,000.

Continuous CSF Sampling Method To Characterize Amyloid Peptides, Tau Peptides And Other Potential Biomarkers in Healthy Subjects And Patients With Alzheimer’s Disease. A Phase 0 Study In A Specialized Unit For Neurodegenerative Disorders. € 435,090. Study sponsored by Schering Plough Research Institute.

Stichting Internationaal Parkinson Fonds. Evaluation of a diagnostic test battery in Parkinson patients with 3 years follow-up. € 95,100

The AADC Research Trust. In search of an explanation for renal dopamine synthesis in AADC deficiency. € 32,000

Center for Translational Molecular Medicine: In vivo molecular diagnostics in Alzheimer’s disease; the LeARN (Leiden-Alzheimer Research Nederland) project. Project leader of work package “Development and validation of analytical techniques for novel biochemical markers”. € 1,556,000