MONOPHOSFORYL LIPID A - TLR4 AGONIST: A NEW PHARMACOLOGICAL STRATEGY FOR ALZHEIMER'S DISEASE?

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Key words: Agonist, Alzheimer's disease, monophosphoryl lipid A, neuroinflammation, therapeutic, toll like receptor 4.

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Abbreviations

Aβ - amyloid beta

AD - Alzheimer's disease

AGP - Aminoalkyl glucosaminide 4-phosphates

APOE - apolipoprotein E

APP - human amyloid precursor protein

AP-1 - activating protein-1

Cox-2 - cyclooxygenase 2

CSF - cerebrospinal fluid

CTB - cholera toxin B subunit

DAMPs - danger-associated molecular patterns (DAMPs)

EDA - extracellular domain A

ECx - entorhinal cortex

fAβ - fibrillar form of Aβ

GM-CSF-granulocyte macrophage-colony stimulating factor

GFAP - glial fibrillary acidic protein

HI - hippocampus

HIV - human immunodeficiency virus

HMGB1- high mobility group box 1 protein

HBV - hepatitis

HPV - papillomavirus

HSPs - heat shock proteins

IDO - indoleamine 2,3 dioxygenase

Iba-1 (Ionized calcium binding adaptor molecule 1)

i.n. - intranasal

iNOS - inducible Nitric oxide synthase

IRAK - interleucine receptor associated kinases

IRF - interferon regulatory factors

LPS - Salmonella minnesota R595 lipopolissharide

MAL - MyD88 adaptor-like

MAMPs - microbe-associated molecular patterns

MAPKs - MAP kinases

MHC - major histocompatibility complex

MPL - monophosphoryl lipid A

MRC1 - mannose receptor 1

MyD88 - myeloid differentiation primary-response gene 88

NSAIDs - Nonsteroidal anti-inflammatory drugs

NFTs - neurofibrillary tangles

NHP - non-human primates

PAMPs - pathogen-associated molecular patterns

PRRs- pattern recognition receptors

PTGS2 - prostaglandin-endoperoxide synthase 2

RAGE - receptor for advanced glycation end products

RIP1 receptor-interacting protein

ROS - reactive oxygen species

s.c. - subcutaneous

SPs - neuronal cells and senile plaques (SPs)

SRs - scavenger receptors

TIR Toll/interleukin (IL)-1 receptor

TIRAP - TIR domain-containing protein

TDM - trehalose dicorynomycolato

TLR - Toll like receptor

TNF-α - tumor necrosis factor-alpha

TRAF6 - TNF-receptor associated factor 6

TRAM - TRIF-related adaptor molecule

TRIF - TIR-domain-containing adapter-inducing interferon-β

TB - tuberculosis

White matter - WM

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Abstract

Neuroinflamation is a key process in Alzheimer's disease (AD). In one hand, overexpression of cytokines and inflammatory molecules sets a prejudicial environment that may lead to a great deal of amyloid deposition in the neurodegenerative process. On the other hand, certain degree of neuroinflammation plays a beneficial role in the clearance of Aβ by activated microglia. Notably, there is an increasing body of evidence suggesting that the innate immune system via Toll like receptor 4 (TLR4) finely orchestrates the well regulated inflammatory cascade that takes place in AD related pathology. The aim of this review was to present and discuss evidence showing that the TLR4 agonist – monophosphoryl lipid A (MPL) – may have beneficial effect on AD physiopathology. Pre-clinical (in vitro and in vivo approaches) as well as clinical studies were reviewed herein. The in vivo data elegantly showed that MPL enhanced Aβ plaque phagocytosis by decreasing number of Aβ deposits and size and also decreasing the quantity of soluble Aβ in the mice brain. Furthermore, MPL also improved cognition in mice. The mechanism gauging this MPL effect was proposed to be microglial activation by TLR4recruitment. Moreover it was demonstrated that MPL when used as a vaccine adjuvant increased the Aβ antibody titer and showed a safe profile in mice as well as in primate models. Clinical studies using MPL as an adjuvant in Aβ immunotherapy are currently ongoing and there are yet no conclusions. This review led one to conclude that TLR4 agonist MPL is a potentially safe and effective new pharmacological tool in AD.

Key words

Agonist, Alzheimer's Disease, monophosphoryl lipid A, neuroinflammation, therapeutic, toll like receptor 4.

Introduction

Nowadays, Alzheimer's Disease (AD) is a world's scourge affecting about 7,3 million people in Europe (1). Drugs that have been tested in AD can be organized in several categories including (2): 1 - drugs interacting with/or modulating: 1.1-receptors, 1.2- enzymes, 1.3- cytokines , 1.4- gene expression , 1.5- heat shock proteins, 1.6- hormones, 1.7- ion channels 1.8- nerve growth factors, 1.9- re-uptake transporters, 1.10- transcription factors; 2 - antioxidants , 3 - metal chelators , 4 - natural products, 5 - nontropics ("drugs without mechanism"), 6 - peptides, 7 - drugs preventing amyloid Aβ aggregation; 8 - drugs interacting with tau, and 9 - stem cells. However, none has yet proven to change AD natural history (3). Notably, there is increasing newly evidence pointing towards the beneficial pro-inflammatory state in Aβ clearance and AD pathological cascade while high inflammatory states facilitate both Aβ production and its deposition (4).

One of the most promising research avenues in AD is the cross-talk between inflammation and innate immunity led by Toll-like receptors (TLRs) (5). These receptors (TLR1 – TLR11) are evolutionary conserved type I transmembrane proteins expressed in both immune and non-immune cells (6). Relevantly, these are prototypical pattern recognition receptors (PRRs), which recognize conserved microbial signature molecules known as either pathogen-associated molecular patterns (PAMPs) or microbe-associated molecular patterns (MAMPs). Additionally, TLRs are also endowed with the capacity of recognizing an array of endogenous molecules termed danger-associated molecular patterns (DAMPs) (6). Their ability to initiate and propagate inflammation makes them attractive therapeutic targets in AD (5).

In fact, toll-like receptor 4 (TLR4) has been implicated in the pathogenesis of AD (7). Specifically, TLR4 agonist activity not only triggers inflammation pathways in several immune cells including microglia, monocytes and dentritic cells, thus creating a beneficial pro-inflammatory state, but also is implicated in Aβ clearance, by decreasing the number of Aβ oligomers, altering their constitution to smaller molecules and solubilizing them (8).

Monophosforyl lipid A (MPL) is a chemically detoxified lipid A moiety derived from *Salmonella minnesota* R595 lipopolisshariде (LPS). Similarly to LPS, MPL is a TLR4 agonist. However, this LPS-derived agent exhibits unique immunomodulatory properties at doses that are nonpyrogenic and show low toxicity (8,9). Therefore, MPL has been studied, both in vitro and in vivo on AD physiopathology (9). Additionally, MPL is already being commercialized as a vaccine adjuvant (10). In particular, it was recently reported that treating transgenic mice overexpressing human amyloid precursor protein (APP) with a liposomal vaccine containing MPL as an adjuvant (ACI-24; an anti-Aβ vaccine) restored their cognitive impairment (9). Remarkably, this vaccine entered a combined Phase I/II clinical trial in 2009 which is still ongoing (11).

Overall, our objective was to review the reports about MPL immunostimulatory properties via TLR4 receptors on AD natural history.

Methods

Herein we reviewed literature indexed in Pubmed as well as Medline databases offered by both “Biblioteca Central dos Serviços de Documentação dos Hospitais da Universidade de Coimbra” and “Biblioteca do Pólo de Ciências Médicas de Coimbra”. Publications held by the following organizations were also considered: European Collaboration on Dementia (12), Alzheimer Portugal (13) and World Health Organization (14).

In the present review, preclinical and clinical studies on MPL (by itself or as a vaccine adjuvant) as a TLR4 agonist on Alzheimer’s disease (AD) related pathology were used as inclusion criteria. Papers which were not written in English, or which were written before 1990 or which were about MPL as vaccine adjuvants without any relation to AD were excluded.

The following keywords were used: TLR4 (toll-like receptor 4), Alzheimer disease, immunotherapy, agonist, antagonist, LPS/lipopolysaccharide, MPL/monophosphoryl lipid A.

Results

I. Epidemiology and clinical characteristics of Alzheimer´s disease

Nowadays, the global prevalence of dementia is estimated to be higher than 24 million, and is predicted to double every 20 years up to 2040 (14). The most common form of dementia is Alzheimer´s disease (AD) which affects approximately 7,3 million people in Europe. This number is projected to reach 14-18 million by the year 2050 (1). In Portugal, the number of people affected by dementia is about 153 000, of those 90 000 people suffer from AD (13). Economically, AD is a major public health problem. In fact, the cost of health care, long-term care, and hospice services for people aged 65 years and older with AD and other dementias is expected to be over € 250 billion (14).

Alzheimer´s disease is a neurodegenerative disorder encompassing cognitive and behavioral impairments that markedly interfere with social and occupational functioning. It is, yet, an incurable disease with a long and progressive course (1).

Currently, an autopsy or brain biopsy is the only way to make a definitive diagnosis of AD. In clinical practice, the diagnosis is usually made on the basis of the history and findings upon Mental Status Examination (4).

A patient with preclinical AD may appear completely normal on physical examination and mental status testing (15). Specific regions of the brain (eg, entorhinal cortex, hippocampus) probably begin to be affected 10-20 years before any visible symptoms appear (4). In light of this, the most predominant AD clinical signs include memory loss, confusion about the location of familiar places, trouble handling money and paying bills, compromised judgment and increased anxiety. While the disease

progresses other symptoms start to appear like problems recognizing friends and family members, difficulty with language and numbers, inability to learn new things or even hallucinations represent a mild status of the disease (15). Patients with severe AD cannot recognize family or loved ones and cannot communicate in any way. They are completely dependent on others for care, and all sense of self seems to vanish. In end-stage AD, patients may be in bed much or all of the time. Death is often the result of other illnesses, frequently aspiration pneumonia (1).

II. Pathogenesis of Alzheimer´s Disease

The etiology of AD, although being unknown, is considered multifactorial. In fact, several investigators now believe that converging environmental and genetic risk factors trigger a pathophysiologic cascade that, over decades, may lead to Alzheimer´s pathology and dementia (16). Some risk factors have already been identified, such as family history, the apolipoprotein E (*APOE*) 4 allele, obesity, insulin resistance, vascular factors, dyslipidemia, hypertension, inflammatory markers, Down Syndrome and traumatic brain injury (4).

There are two main AD hallmarks already identified: neurofibrillary tangles (NFTs) inside neuronal cells and senile plaques (SPs) (1). Neurofibrillary tangles are composed of an aberrantly-phosphorylated form of the microtubule-associated protein tau aggregating into paired helical filaments (1). Destabilization of the microtubular system triggered by NFT was proposed to induce abnormal protein processing and dystrophic neurites contributing to synaptic loss, increasing production of Aβ and, ultimately, neuronal death (4).

The hippocampus and medial temporal lobe are the initial sites of tangle deposition and atrophy (17). This can be seen on brain magnetic resonance imaging early in AD and helps to support a clinical diagnosis (18). In addition to NFTs, the anatomic pathology of AD includes senile plaques (also known as β-amyloid plaques) at the microscopic level and cerebrocortical atrophy at the macroscopic level. The amyloid substance is surrounded by a halo or layer of dystrophic neurites and reactive glia (both astrocytes and microglia) (19). Moreover, cerebrospinal fluid (CSF) biomarkers and positron emission tomography imaging in patients across the spectrum of AD show that Aβ is the first biomarker to accumulate, followed by the appearance of synaptic

dysfunction and increasing tau protein concentration (15). Additionally, the fibrillar form of Aβ (fAβ) has also been shown to alter the phosphorylation state of tau protein thus also contributing to NFTs. Notably, the accumulation of Aβ starts up to 20 years before any clinical symptoms are apparent (20).

III. Alzheimer´s Disease and Chronic Inflammation: a double edged sword.

The role of neuroinflammation and microgliosis in AD is attracting a great deal of attention as recent data suggests that both factors are early events in the pathogenesis of the disease. Particularly, a growing body of evidence has indicated the presence of pro-inflammatory mediators (complement, proteases, cytokines) in the brain and CSF of AD patients (21). In line with this information, it has been shown that microglial cells, which are the resident macrophages of the CNS, play a crucial role in the process of neuroinflammation. In response to cytokines and other signaling molecules from inflammation, microglia transform from a ramified, inactivated state to an activated phagocytic one, releasing pro-inflammatory mediators in the process. In terms of chronic neuroinflammation, these cells can remain activated for extended periods, releasing quantities of cytokines and neurotoxic molecules that contribute to long term neurodegeneration (22).

Astrocytes and microglia express a range of molecules known as "pattern recognition receptors" (PRRs) including TLRs, which are critical for eliciting innate immune responses and to initiate adaptive immunity that activate these cells and initiate a neuroinflammatory reaction (5). Microglia express the two classes of major histocompatibility complex, MHC class 1 and MHC class 2, and although these antigen presenters are mainly involved in the reaction to infectious disease, they are thought to play a role in the development of neuroinflammation (23).

It has been suggested that pro-inflammatory cytokine levels are related to the magnitude of the plaque burden in the AD brain (4). Besides, with the role of COX

(cyclooxygenase) pathways in neuroinflammation becoming better established, NSAIDs (Nonsteroidal anti-inflammatory drugs) have been identified as a class of drug with potential therapeutic effects (24). Indeed, aspirin (an irreversible COX-1 inhibitor) reduces neuroinflammatory and oxidative insults by reducing prostaglandins and increasing anti-inflammatory lipoxin (24). However evidence is lacking for clinical benefit of NSAIDs and selective COX-2 inhibitors in patients with neurodegenerative diseases. The most recent results of a large clinical trial, the Alzheimer disease anti-inflammatory prevention trial (ADAPT), suggest that certain NSAIDs may reduce the chances of an asymptomatic individual developing this disease, but these same drugs exacerbate later stage Alzheimer's (25).

In fact, during the development of AD pathology, microglia fails to restrict amyloid plaques and may contribute to neurotoxicity and cognitive deficits. Nevertheless, under specific conditions, microglia can participate in cerebral amyloid clearance. This complex relationship between microglia and Aβ pathology highlights both deleterious and beneficial roles of microglial activation in the context of AD (26). Although a cause and effect relationship between inflammation and AD is yet unveiled, it has been suggested that some components of this complex molecular and cellular machinery are most likely promoting pathological processes leading to AD, whereas other components engage in doing the opposite (2). Overall, inflammation behaves like a double-edged sword since on one hand the inflammatory response facilitates Aβ production and deposition and on the other hand it can facilitate Aβ clearance (8).

However, this apparently antagonistic strategy should not be rigidly attributed to specific cytokines under normal and pathological conditions. In fact, the effects of signaling molecules may differ, depending on its location within the CNS and on the context of disease (4).

IV – The importance of innate immune system in Alzheimer´s Disease related pathology.

The innate immune system that relies strongly on the microglia (resident macrophages in the brain) has a major role in Alzheimer's disease (27). While neglected for decades, neuroinflammatory processes coordinated by microglia are now accepted as etiologic events in AD evolution (26). Innate immune system implications are best described with the attraction process of microglia into diseased brain regions being often mediated by the interaction of chemokines and cytokines with their receptors, which are both expressed by microglial cells. In the context of AD, several lines of evidence support the notion that β-amyloid plaque-associated factors, including misfolded Aβ peptides themselves, act as microglial attractants (26). Cytokines have also been suggested to be involved in chemoattraction of microglia to amyloid lesions (4). Studies with Cx3cr1 knockout transgenic mice suggested that increased pro-inflammatory cytokines including tumor necrosis factor-alpha (TNF-α), IL-6, IL-1α and granulocyte macrophage-colony stimulating factor (GM-CSF) are related to microglia attraction (28). Once microglia moves toward Aβ deposits, these cells express various cell surface receptors allowing them to recognize and interact with misfolded Aβ peptides. In this context, the scavenger receptors (SRs) have garnered attention, as they can bind diverse ligands and affect the activation level, inflammatory status and phagocytic function of microglia (29). Bamberger and collaborators (30) described a receptor complex including CD36, the integrin-associated protein CD37 and the α6β1 integrin, which interacts with Aβ fibrils and activates microglial secretion of ROS (reactive oxygen species). Another key SR, the receptor for advanced glycation end

products (RAGE), has also been identified on CD68+ microglial cells close to senile plaques in AD patient brains and in cultured microglia from rat and mouse models of the disease(26). Remarkably, TLRs and associated receptors (e.g., CD14) are highly expressed by microglia in close proximity to plaques in AD patient brains and in mouse models of the disease (26).

V. Toll-like receptors: molecular structure, cell distribution and functions.

Toll-like receptors are evolutionarily well conserved type I transmembrane proteins on the surface of both immune and non-immune eukaryotic cells (5). These receptors comprise a N-terminal leucine-rich repeats which is the extracellular binding domain and a highly conserved C-terminal domain termed the TIR [Toll/interleukin (IL)-1 receptor] domain (31). Microglia express all TLRs identified to date, whereas astrocytes, oligodendrocytes and neurons express a more limited TLR repertoire (figure 1) (6).

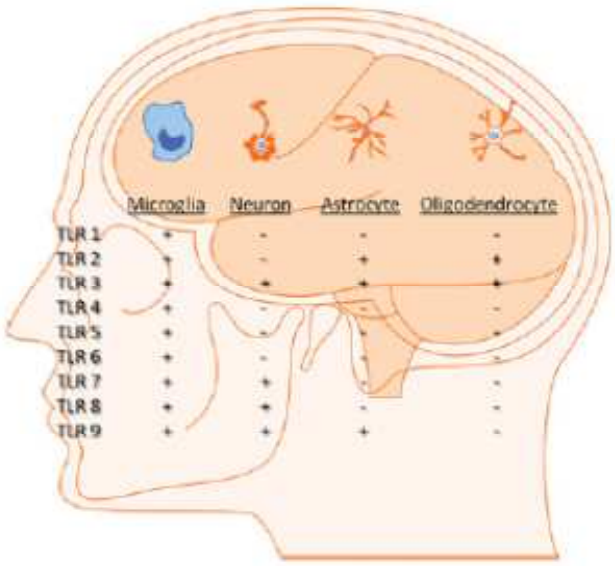


Figure 1: Expression of TLR family members in CNS cells. Microglia express all TLRs identified to date, whereas astrocytes, oligodendrocytes and neurons express a more limited TLR repertoire. Figure adadpted from Hanke *et al* (6).

The ligands for these "pattern recognition receptors" (TLRs 1 to 11) are components of pathogenic microbes and are often called "pathogen-associated molecular patterns" (PAMPs) (1), including bacterial cell wall components; bacterial genome DNA and viral, fungal and parasitic products. TLR immune system is also

concerned with damage signals from injured tissue. In fact, it is suggested that TLRs recognize not only PAMPs, but also stress/ damage or danger-associated molecular patterns (DAMPs) (7). In general, most DAMPs are the consequence of cell death, necrosis or tissue remodeling and include mammalian genomic DNA, high mobility group box 1 protein (HMGB1), heat shock proteins (HSPs: HSP22, HSP60, HSP70, HS96), extracellular matrix products (eg. hyaluronan, type III repeat extra domain of fibronectin), uric acid crystals, β-defensin and, finally, plant ligands (paclitaxel) are also known TLR ligands (32).

TLR signaling involving the transcription factors nuclear factor for the kappa light chain enhancer in B cells (NF-kappa B) and activating protein-1 (AP-1) plays a crucial role in inflammation trough encoding important pro-inflammatory cytokines, such as tumor necrosis factor (TNF), IL-1 beta, IL-6, IL-8, and IL-12 (22). TLR 3 and TLR 4 also activate the production of type 1 (α and β) interferons by inducing the transcription factors interferon regulatory factors 3 and 7 (IRF3 and IRF7) (5). Notably, it was demonstrated that fibrillar Aβ (1–42) may act as a PAMP-like infectious agent through TLR activation (33). This is highly suggestive that TLRs may be a potential therapeutic target in AD.

Besides inflammation, TLRs also exert determinant functions related to the processes of neurogenesis, learning and memory in the absence of any underlying inflammation/infectious aetiology (34).

VI. TLR4 modulation in AD

Activation of TLRs on microglia may modulate AD progression (21). On one hand, investigators observed that a deficiency of TLR2 and TLR4 in cultured microglia significantly reduced Aβ-triggered inflammation (7). Moreover, it was already demonstrated that Aβ triggers microglial inflammatory mediator production mainly via TLR4, but also via TLR5 and TLR6 (6).

On the other hand, stimulation of microglial cells with TLR2, TLR4 or TLR9 specific agonists boosts Aβ clearance both in vitro and in vivo (3, 6, 7). Additionally, microglia deficient in TLR2, TLR4, or the co-receptor CD14 are not activated by Aβ and do not exhibit a phagocytic response (5). Moreover, transgenic AD mice lacking TLR4 have markedly elevated levels of diffuse and fibrillar Aβ (3). Additionally, the TLR4-deficient mice also showed to have elevated TNF-α, IL1-β, IL-10, IL-17, CD11B and GFAP (glial fibrillary acidic protein) levels, further suggesting that TLR4 signaling is involved in AD progression (35). Altogether, TLR4 signaling shows a functional dichotomy: having a beneficial role in clearing the Aβ deposits or being prejudicial by triggering a high inflammatory cascade in the AD diseased brain as elicited before (7). The balance is tipped not only by the AD status but also by the TLR4 genetic background and by the level of inflammation produced by TLR4 agonism (36).

Upon ligand (e.g. LPS) binding there is the formation of a TLR4 complex with CD14 (37). In addition a small molecule, myeloid differentiation 2 receptor (MD-2), participates in this complex by associating with the TLR4 extracellular domain. Association of the *MD-2-ligand complex* to the ectodomain of the TLR4 finally transduces the signal through the association of intracellular Toll/Il-1R (TIR) domain, recruiting the adapter proteins thus triggering the signaling cascade. TLR4 uses TIR

domain-containing protein (TIRAP)/MyD88 adaptor-like (MAL) as a bridging adaptor to recruit the myeloid differentiation primary-response gene 88 (MyD88) to activate the NF-κB, p38 and JNK/MAPK pathways via TRAF6 (figure 2) (38).

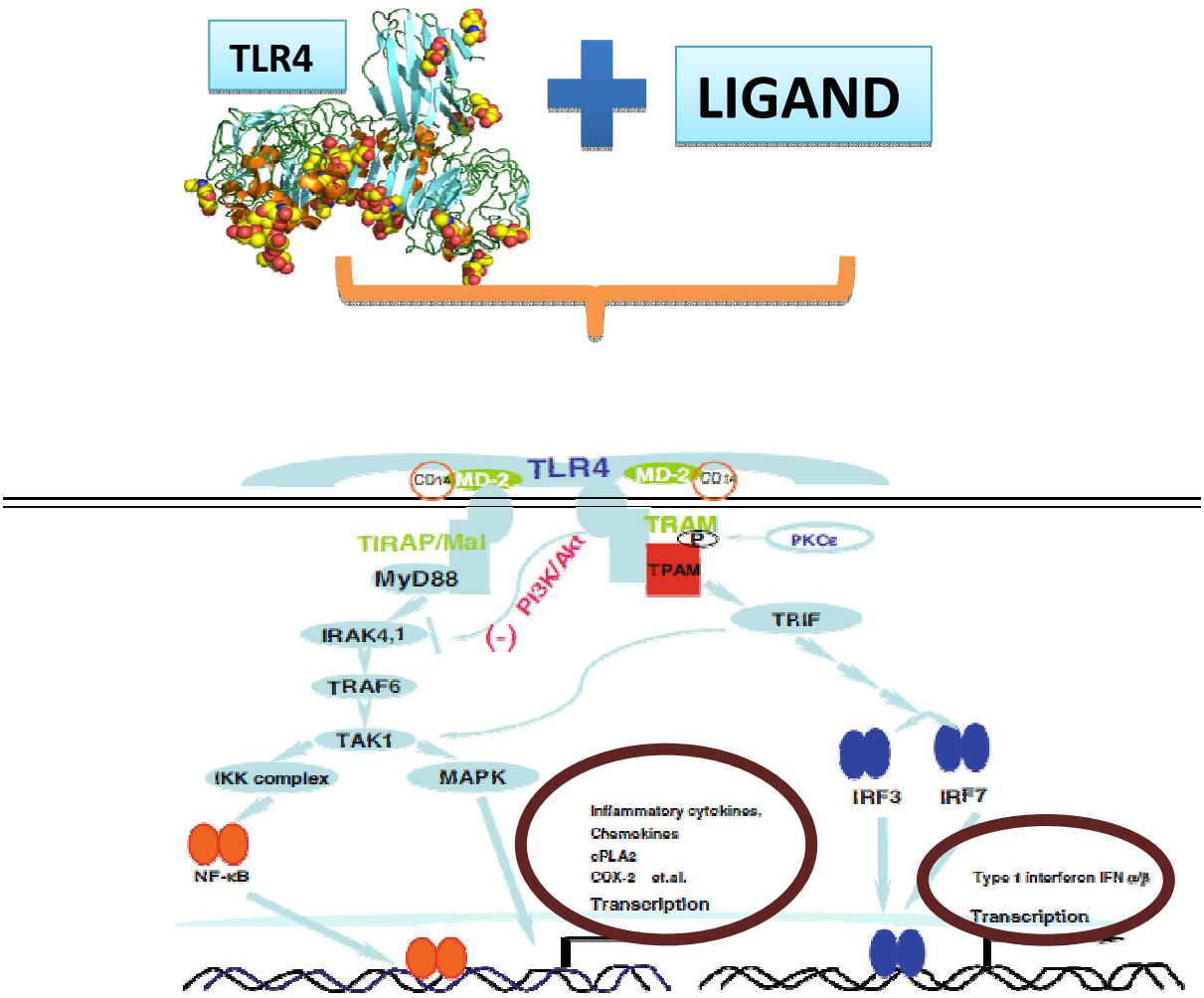


Figure 2: TLR4 signaling pathways in macrophages. Exposing macrophages to a TLR4 ligand, such as LPS, induces at least two pathways; 1-a MyD88 (myeloid differentiation factor 88) dependent cascade, recruiting the IRAK(interleucine receptor associated kinases) family of proteins (IRAK1, 4 are active isoforms, but IRAK2 and IRAK-M are negatively regulating isoforms controlled by PI3K/Akt (phosphoinositide 3-kinase/ protein kinase B) that regulate TRAF6 (TNF-receptor associated factor 6) and TAK1 (transforming growth factor b-activated kinases) leading to activation of NF-kB and MAPKs (MAP kinases) and transcription of a number of genes, including proinflammatory cytokines, chemokines, cytosolic PLA2 (phospholipase A2), Cox-2 (cyclooxygenase 2), iNOS (inducible Nitric oxide synthase). 2-a MyD88 independent pathway, through TRAM (TRIF-related adaptor molecule), TRIF (adaptor inducing interferon-β) TKB1/IKK (tyrosine kinase binding 1 / inducible IκB kinase), that activates IRF3 (interferon regulatory factors) and IRF7, which controls the gene expression of type I IFNs (interferons). In addition, TRIF (TIR-domain-containing adapter-inducing interferon-β) can also interact with RIP1 (receptor-interacting protein, not shown in this figure) and activate TAK1, inducing IKK complex and MAPK activation. Adapted from Chang *et al* (31).

Another pathway activated involves TRIF-related adaptor molecule (TRAM). Similar to MAL, TRAM is also membrane proximal and requires myristoylation to lodge into the membrane. TRAM recruits the TIR-domain-containing adaptor protein inducing interferon-β (TRIF) which activates the tumor-necrosis factor-receptor associated factor 3 (TRAF3), TRAF6 and receptor interacting protein 1 (RIP1)(39). Recent work with CD14 knockout mice suggested that TRL4 can function in two ways: one where full signaling occurs in the presence of CD14 and one limited to MyD88-dependent signaling (31). The outcome of appropriate or exagerated TLR4 activation is illustrated in figure 3. This illustration is suggestive that both TLR4 activation or TLR4 blocking can be used as new therapeutic targets on AD (37). In table 1 one can find a list of TLR4 agonists, antagonists and anti-TLR4 antibodies. Notably, amongst the most important TLR4 agonists developed so far with AD therapeutics implications ranks the well-studied LPS derivate, the MPL which is also listed in table 1 (40).

TLR4 Antagonist
CRX-526
E5531
E5564
TAK-242
Anti TLR 4 antibody
Anti-hTLR4-IgG
MAb2-hTLR4
MAb-hTLR4/MD2
PAb-hTLR4

TLR4 Agonist
LPS
MPL
E6020
AGP
HMGB1
EDA
Hyaluronic acid
Paclitaxel

Table 1: TLR4 agonists, antagonists and anti-TLR4 antibodies. AGP: Aminoalkyl glucosaminide 4-phosphates; HMGB1: high-mobility group box 1; EDA extracellular domain A.

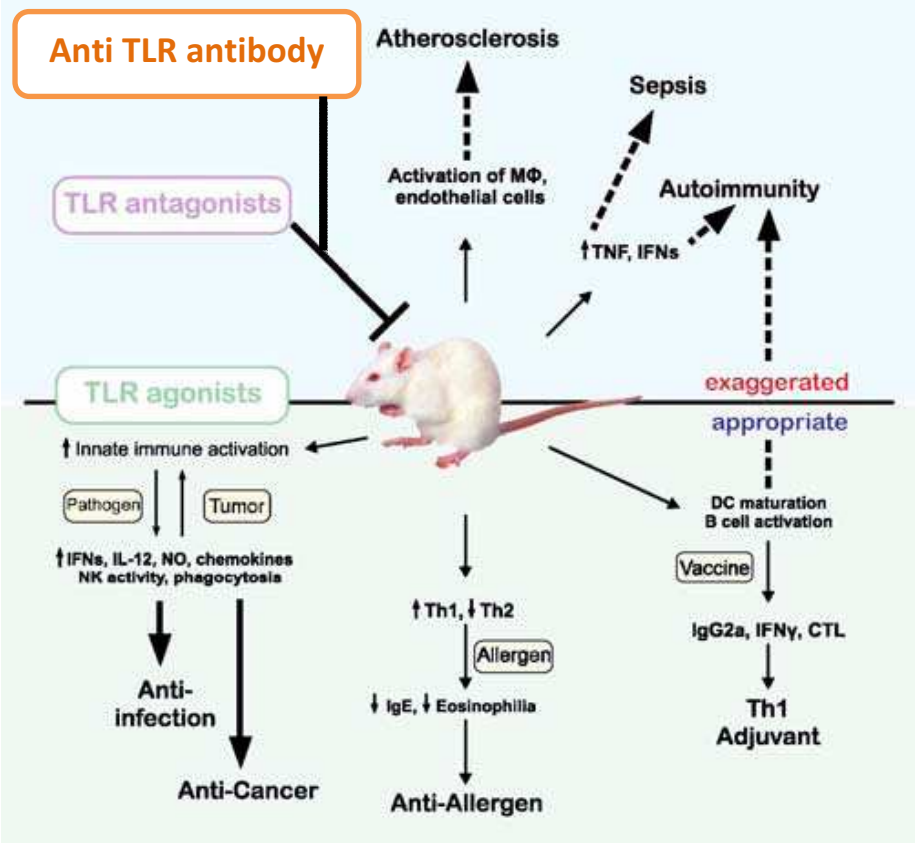


Figure 3: Outcome of appropriate and exaggerated TLR4 activation. TLR4 agonists as potential therapeutic tools on cancer, allergies, infections; TLR4 blocking (TLR4 antagonists or anti TLR antibodies) as potential therapeutic strategies on atherosclerosis, exaggerated inflammation (sepsis) or autoimmune processes; antagonists and TLR4 anti-antibodies. Adapted from Wasan *et al* (37).

VII. 3-*O*-desacyl-4'-monophosphoryl lipid A (MPL)

3-*O*-desacyl-4'-monophosphoryl lipid A (MPL) is obtained from *Salmonella Minnesota* R595 LPS (41) and is a LPS-derived TLR4 agonist that exhibits unique immunomodulatory properties at doses that are nonpyrogenic (5).

Thus, 3-*O*-desacyl-4'-monophosphoryl lipid A (MPL) acts as an immunostimulant and is part of already licensed or candidate vaccines (42). In particular, MPL directly affects the innate immune response, orchestrating the quality and intensity of the adaptive immune response to the vaccine antigen (10). The incorporation of MPL in different adjuvant formulations forms the basis of Adjuvant Systems (AS) that have been put in place since 1990 (10). For example MPL when combined with aluminum salts forms the basis for AS04(43). This aluminum salt - and TLR4 agonist - based adjuvant system retains its ability to activate TLR4 and stimulates most of the innate immune response (44). Furthermore the AS04-induced innate responses were primarily due to MPL.

By now, ACI -24 is the only vaccine being studied in the context of AD that utilizes MPL as adjuvant (2).

VIII. MPL as a disease modifying agent in Alzheimer's disease natural history: a review of the studies

Paper's Title	***Authors and publication year***	**Type of study**	**MPL as adjuvant or MPL as single compound vaccination**
Modulation of the humoral and cellular immune response in Aβ immunotherapy by the adjuvants monophosphoryl lipid A (MPL), cholera toxin B subunit (CTB) and *E.coli* enterotoxin LT (R192G)	*Maier et al.* 2005 (45)	Pre-clinical: *in vivo + in vitro*	Aβ1 40/42 vaccination + **MPL as adjuvant**
Preventive immunization of aged and juvenile non-human primates to beta-amyloid	*Kofler et al.* 2012 (33)	Pre-clinical: *in vivo*	Aβ42 vaccination+ **MPL as adjuvant**
Toll-like receptor 4 stimulation with the detoxified ligand monophosphoryl lipid A improves Alzheimer's disease-related pathology	*Michaud et al.* 2012 (41)	Pre-clinical: *in vitro and in vivo*	**MPL as a single compound (in vitro and vaccination strategy)**
The safety profile of aci-24, an oligo-specific amyloid beta vaccine, demonstrated decrease of large microbleedings in brain of aged Alzheimer's disease mouse model	*Stanco-Piorko et al.* 2009 (46)	Pre-clinical: in vivo	**ACI - 24** (a liposome-based vaccine with tetra-palmitoylated amyloid beta 1-15 peptide)
Double-Blind, Randomised, Placebo-Controlled, Adaptive Design Study of the Safety, Tolerability, Immunogenicity and Efficacy of ACI-24 in Patients with Mild to Moderate Alzheimer's Disease	started on 2011 and is currently ongoing (11)	Clinical: Phase I/II	**ACI-24**

Table 2: Pre-clinical and clinical studies with the detoxified ligand monophosphoryl lipid A in the improvement of Alzheimer's disease-related pathology

Pre-clinical and clinical studies have been carried out to test the Toll-like receptor 4 stimulation with the detoxified ligand monophosphoryl lipid A (MPL) in the improvement of Alzheimer's disease-related pathology. This molecule was used either as a vaccine adjuvant to Alzheimer's immunotherapy with active Aβ vaccination or by itself as a single compound (table 2).

Aβ vaccination is an approach under investigation to prevent and/or treat Alzheimer's disease. Maier *et al*. (2005) affirmed that successful active immunization with A-β 1 40/42 vaccination requires a strong and safe adjuvant to induce anti-Aβ antibody formation (45). In particular, MPL is a potent adjuvant, being useful in breaking tolerance to self-antigens such as Aβ. Herein, MPL was used in combination with another immunostimulatory agent - the trehalose dicorynomycolato from the cord factor of the tubercle baccilus (TDM). These authors used 6 to 8 – week-old male B6D2F1 animals for the in *vivo studies* and splenocyte isolated from these mice and cultured for the *in vitro* studies. The main aim of the study was to establish the humoral as well as the cellular immune reaction triggered by the adjuvant MPL/TDM following Aβ 40/42 immunization when compared to other adjuvants, namely cholera toxin B subunit (CTB) and *E. coli* enterotoxin LT (R192G). Brains and snouts of immunized mice were also analyzed.

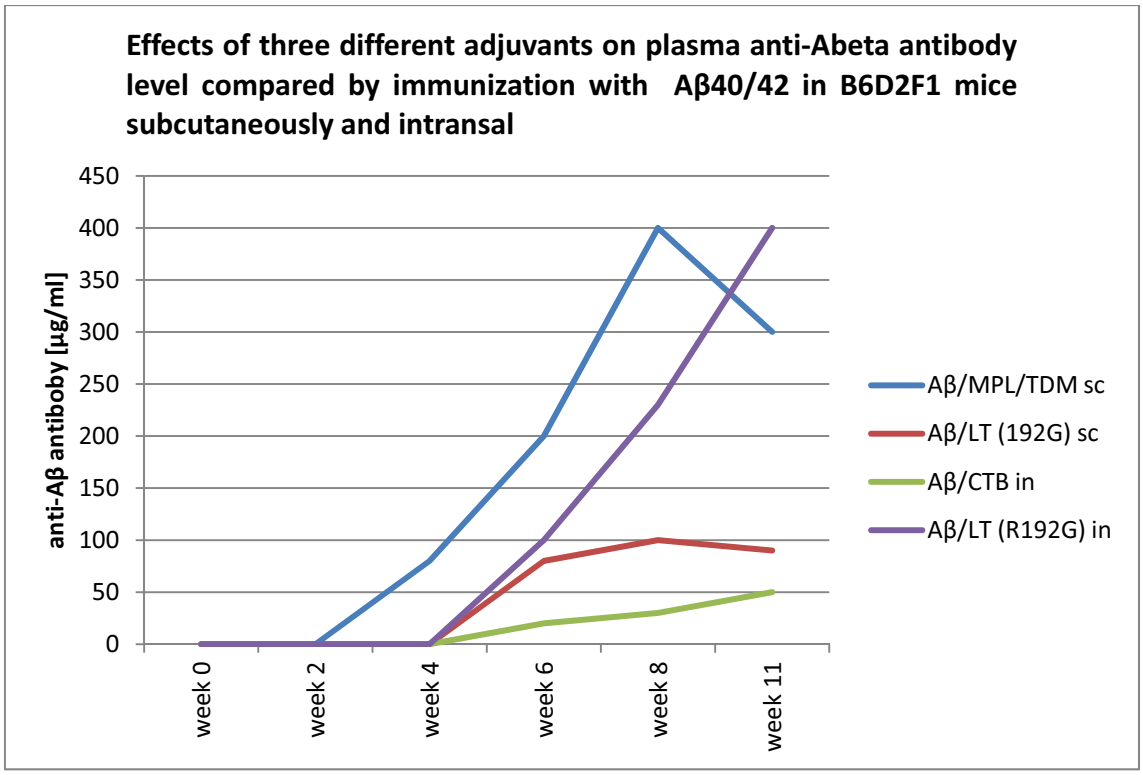
In the first set of experiments two of the three adjuvants plus Aβ40/42 peptide were tested by subcutaneous (s.c.) injection. After the initial immunization, mice received a boost injection of 25 µg MPL/25 µg TDM or 5µg of LT(R192G) combined with 50µg of Aβ40/42 at weeks 2, 5 and 10 (5 days prior to the termination of the experiment). All s.c. immunizations were given as a single injection at the base of the tail. In the second set of experiments all three adjuvants were tested in intranasal immunization studies. Both mice nostrils received a total of 5µg LT (R192G) or 10µg

CTB or 10µg MPL/10µg TDM emulsion combined with 50 µg Aβ40/42 daily for 11 weeks. Aβ40/42 was replaced by distilled water in all vehicle control groups. Each of the treatment and control groups consisted of four mice.

Every 2 weeks blood plasma was collected and anti-Aβ antibody levels were determined by ELISA. Additionally, anti-Aβ antibodies were not detected in pre-immune plasma from any mouse included in these studies.

In the first set of experiments, low levels of anti-Aβ antibodies could be detected in three out of four animals given s.c. Aβ + MPL/TDM two weeks after the prime injection. In the following weeks, antibody levels increased up to of 419±135µg/ml at week 8 (3 weeks after the second boost). Thereafter, Aβ levels slightly decreased as no additional injection was given until 5 days before final analysis at week 11 (graphic 1).

In mice immunized s.c. with Aβ + LT (R192G), antibodies were detected no sooner than at week 4 in all mice and increased thereafter up to 129±55µg/ml at week 8. Overall, the whole experiment antibody titers of Aβ + MPL/TCDM immunized mice were 2 to 4-fold higher compared to Aβ + LT(R192G) animals at weeks 6 and 8, respectively ($p < 0.05$) as shown in graphic 1. Finally, anti- Aβ antibodies were not detected in plasma from vehicle control mice.



Graphic 1: The effects of three different adjuvants on plasma anti-Aβ antibody levels were compared following B6D2F1 mice immunization with Aβ40/42 administered subcutaneously or intranasaly.

In the second set of experiments, weekly intranasal (i.n.) treatment with Aβ 40/42 + CTB induced detectable but low levels of antibody by 4 weeks in all immunized mice. Thereafter, antibody titers increased slowly up to an average of 63±11µg/ml at week 11. On the other hand, i.n. immunization with Aβ + LT (R192G) resulted in faster generation and higher levels of anti-Aβ antibodies with a final concentration of 402±156µg/ml at week 11. On the contrary, the weekly intranasal application of Aβ with the MPL/TCDM emulsion did not produce significant amount of antibodies. However, at the final bleed, low levels of anti -bodies with an average value of 6±8µg/ml were detected. Consequently, antibodies of this group were not further characterized. Again, anti-Aβ antibodies were not detected in plasma from vehicle control mice.

To further characterize the humoral and cellular profile of the elicited response to the immunization protocol, both in vitro and in vivo approaches were used as indicated in table 3. The humoral response was profiled by Ig isotyping the plasmatic anti-Aβ antibodies collected from the four groups which produced robust antibody titers at week 11 using ELISA.

The subclass of immunoglobulin that is induced after immunization is an indirect measure of the relative contribution of Th2-type cytokines versus Th1-type cytokines. For example, the production of IgG1-type antibodies is primarily induced by Th2 cytokines, whereas production of IgG2a-type antibodies reflects the involvement of Th1-type cytokines.

Mice s.c. immunized with Aβ + MPL/TDM produced high levels of IgG2b and IgG1, with lower levels of IgG2a, IgG2c and IgM Aβ antibodies. While mice receiving Aβ+ LT(R192G) s.c. made mainly IgG2b and IgG1 anti-Aβ antibodies, i.n. immunized mice with the same combination showed mainly IgG2a and IgG2c, besides IgG2b antibodies. Mice i.n. immunized with Aβ+ CTB exhibited moderate levels of IgG2b Aβ antibodies. Overall, IgG2b anti-Aβ antibodies were present in relatively high levels in all four groups.

The average IgG1/IgG2a ratio for the Aβ+MPL/TDM s.c. was 1 suggesting a mixed Th1/Th2 immune response. However, the average IgG1/IgG2a ratio was 2.4±0.6 in s.c. Aβ+ LT (R192G) immunized animals thus suggesting a trend towards more a Th2 response. In contrast, the IgG1/IgG2a ratio in Aβ+ CTB and Aβ+ LT(R192G) i.n. immunized animals was less than one, suggesting a trend towards more a Th1 response.

To further characterize the cellular immune response induced by different adjuvants against Aβ40/42, splenocyte cultures were established from individual

animals included in the same 4 experimental groups, 5 days after the final boost and re-stimulated with different concentrations of Aβ40/42. The highest stimulation index was observed in mice immunized i.n. with Aβ+LT (R192G), followed by those immunized by i.n. Aβ+CTB and s.c. Aβ+ MPL/TCDM. On the contrary, splenocytes isolated from s.c. Aβ+ LT(R192G) immunized animals did not show any significant proliferation even after re-stimulation with the highest concentration of Aβ40/42. To determine whether Th1 or Th2 T cell responses were induced, the authors analyzed the production of IFN-ɤ (Th1), IL-4 and IL-10 (Th2) cytokines in splenocytes culture supernatant from individual mice re-stimulated with 50 µg/ml Aβ40/42. The average levels for IFN-ɤ were *circa* 1084pg/ml in cultures of i.n. Aβ+ LT (R192G) immunized animals, *circa* 490pg/ml in the s.c. Aβ+MPL/TCDM group and *circa* 160 pg/ml in cultures of s.c. Aβ+ LT (R192G) and i.n. Aβ+ CTB immunized animals. No IFN-ɤ could be detected in supernatants of adjuvant control animals or in non-restimulated cells. Moreover, no IL-4 and IL-10 could be detected in supernatants of any of the splenocyte cultures from animals re-stimulated with Aβ40/42 (table 3).

	In vivo: plasma at 11 week		**In vitro: splenocytes at 5 days after final boost and re-stimulation**	
Groups	**Ig subclass**	**IgG1/IgG2 ratio**	**Stimulation index***	**IFN-ɤ (Th1) IL-4/IL-10 (Th2)**
Aβ40/42 + MPL/TCDM s.c	IgG1 high IgG2 a low IgG2 b high IgG2 c low IgM low	1 Mixed Th1/Th2	++	IL-4/IL-10 = 0 IFN-ɤ 490pg/ml
Aβ + LT(R192G) s.c	IgG1 low IgG2 b high	2.4±0.6 Mixed Th1/Th2; sligthy deviated to Th2	+	IL-4/IL-10 = 0 IFN-ɤ 160 pg/ml
Aβ + LT(R192G i.n	IgG1 low IgG2 a high IgG2 b high IgG2 c high	<1 Mixed Th1/Th2; sligthy deviated to Th1	++++	IL-4/IL-10 = 0 IFN-ɤ 1084 pg/ml
Aβ 40/42 + CTB i.n	IgG1 low IgG2 b moderate levels	<1 Mixed Th1/Th2; sligthy deviated to Th2	+++	IL-4/IL-10 = 0 IFN-ɤ 160 pg/ml
Aβ40/42 + MPL/TCDM i.n → **not considered as it showed no evidence of anti-** Aβ antibodies				

* ++++ meaning highest (320) stimulation and + the lowest (180).

Table 3: Humoral and cellular profile of the elicited response to the immunization protocol using in vitro and in vivo approaches. Data extracted from Maier et al. 2005 (45).

Finaly, Maier *et al.* (2005) showed no signs of inflammation or toxicity of immunized mice and also showed that the generated antibodies specifically bind to Aβ diffuse and compacted plaques on AD human brain sections by using plasma from each of the immunized mice to immunostain paraffin cortical brain sections from AD patients; the monoclonal anti Aβ antibody 6E10 was used as a positive control (figure 4).

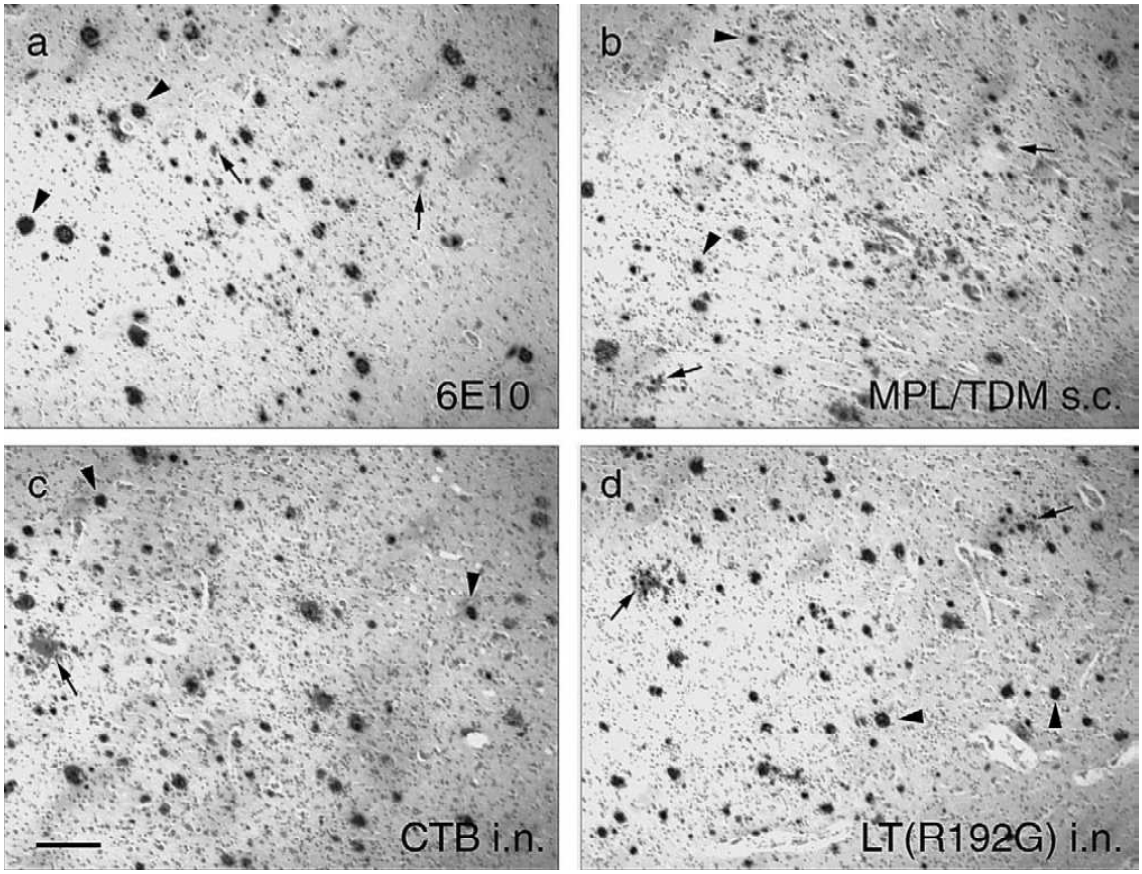


Figure 4: Anti-Aβ antibodies generated with the different adjuvants bind specifically to diffuse (arrows) and compact (arrowheads) plaques on AD cortical brain sections. Anti- Aβ antibody 6E10 (a) or a 1:1000 dilutions of mouse serum from mice immunized with s.c. Aβ + MPL/TDM(b), i.n. Aβ + CTB(c) and i.n. Aβ + LT(R192G) (d) were used for staining of formalin fixed, paraffin embedded brain tissue from AD patients. A representative example is shown for each adjuvant. Hematoxylin counterstain. Magnification bar, 200 μm. Figure extracted from Maier et al. 2005 (45).

Along the premises already held by Maier *et al.* (2005) that immunization against Aβ is a promising approach for the treatment of AD before its clinical onset, Kofler *et al (2012)* immunized non-human primates with the combination of Aβ42 admixed with MPL.

In detail, ten non-human primates (NHP) of advanced age (18–26 years) and eight 2-year-old juvenile NHPs were immunized at 0, 2, 6, 10 and 14 weeks with aggregated Aβ42 admixed with MPL as adjuvant, and monitored for up to 6 months. Anti-Aβ antibody levels and immune activation markers were assessed in plasma and cerebrospinal fluid samples before and at several time-points after immunization. Microglial activity was determined by [^{11}C] PK11195 (a selective radioligand of the peripheral benzodiazepine receptor) PET scans acquired before and after immunization, and by post-mortem immunohistochemical and real-time PCR evaluation. Cerebral cortex (combined region covering the frontal, lateral temporal, and parietal cortices), thalamus, cerebellum, and subcortical white matter were PET scanned. Regarding, immunohistochemical assessment of microglial density, mid-frontal cortex (MFCx), hippocampus (HI), entorhinal cortex (ECx) and white matter (WM) were probed for Iba-1 (Ionized calcium binding adaptor molecule 1) levels. Finally, quantitative RT-PCR analysis of expression of microglial (CD68 and Iba-1) and astrocytic (GFAP) markers as well as indicators of classical [CXCL10, CXCL9, indoleamine 2,3 dioxygenase (IDO), CCR7, prostaglandin-endoperoxide synthase 2 (PTGS2)] and alternative [CCL17, mannose receptor 1 (MRC1)] microglia activation were performed in cortical samples.

Aβ oligomer composition was assessed by immunoblot analysis in the frontal cortex of aged immunized and non-immunized control animals.

Kofler *et al* (2012) concluded that all juvenile animals developed a strong and sustained serum anti-Aβ IgG antibody response, whereas only 60-80 % of aged animals developed detectable antibodies as detailed in table 4. Moreover, the immune response in aged monkeys was more variable between animals, delayed and significantly weaker when compared with the juvenile group.

Group	**Anti-Aβ antibody in plasma measured every two weeks up to 16 week after immunization**	**Anti-Aβ antibody in CSF measured on the 16th week after immunization**
Juvenile (n=8)	-100% showed strong response against both Aβ40 and Aβ42 after 1st or 2nd week after immunization	-100% showed light response against Aβ40 and Aβ42
Aged (n=10)	-80% showed response against Aβ42 -60% showed response against Aβ40 -5/8 aged animal responder showed Ig levels similar to juveniles -Anti- Aβ antibodies appeared after 3rd week of immunization	-44% showed light response against Aβ42 -22% showed light response against Aβ40 One animal was euthanized due to colon carcinoma at week 6

Table 4: Anti-Aβ antibody in plasma and CSF after immunization from juveniles and aged monkeys.

Anti-Aβ antibody levels in the CSF were determined at week 16, when serum antibody concentrations were at peak levels. All juvenile animals contained anti-Aβ antibodies in the CSF, although the levels were much lower than in the serum. By contrast, anti-Aβ antibodies were only seen in 22- 44% of the aged animals (table 4).

Kofler *et al* (2012) also concluded that no significant changes were seen in any brain region between pre and post immunization scans, although there was a slight trend towards increased [^{11}C] PK11195 retention over time in the aged but not in the juvenile group. Post-mortem analysis further failed to show differences between immunized and

non-immunized animals in aged macaques. The juvenile immunized animals were not euthanized. Taken together, the PET, immunohistochemistry and PCR data indicate that vaccination against Aβ was not associated with significant microglial activation of these animals. Additionally, the immunization protocol did not induce increased inflammation, neither of the autoimmune/ encephalitic type nor of the innate arm of the immune system.

Moreover, average Aβ40, Aβ42 and tau plasma and CSF levels were not significantly different between pre- and post-immunization time points for either aged or juveniles (table 5). Moreover pre-immunization baseline for plasma and CSF levels were not significantly different between juvenile and aged animals (table 5).

Notably, although no effect on overall brain Aβ levels were seen, a shift in the composition of soluble oligomers towards smaller species was observed in the aged animals.

Group	*Plasma*			*CSF*		
	Pre-imunization Baseline		**Post-immunization**	**Pre-imunization baseline**		**Post-immunization**
	Juvenile (n=19)	Aged* (n=16)	No differences were seen between baseline and post-immunization	Juvenile (n=19)	Aged* (n=16)	No differences were seen between baseline and post-immunization
Aβ 40 (pg/ml)	16.0 (15.1-21.0)	15.1 (11.4-18.8)		1494 (923-2077)	1514 (971-2145)	
Aβ 42 (pg/ml)	ND	ND		78.4(65.3-118.8)	107.4 (59.8-162.1)	
Tau (pg/ml)	--	--		131.4(89.4-168.2)	345.2)249.0(94.2-	

***No significant differences between pre-immunixzation baseline levels from juvenile and aged groups.**

ND: not detected

Table 5: Average Aβ40, Aβ42 and tau plasma and CSF levels following immunization were not significantly different from baseline levels for either aged or juvenile groups.

Finaly, Kofler *et al (2012)* concluded that preventive Aβ immunization is a safe therapeutic approach without serious side-effects in both aged and juvenile NHPs.

Recently Michaud *et al* (2012) tested MPL, not as a vaccine adjuvant (as in the previous studies), but as a therapeutic molecule by itself in Alzheimer's disease-related pathology and produced an exciting and promising study.

These authors conducted both *in vitro* and *in vivo* studies. Microglia dosed with 50 μg/mL MPL and 20 μg/mL LPS showed cytoskeletal remodeling and also a significant increase in the internalization of Aβ1-42 oligomers, although at very different levels: while MPL only induced a moderate pro-inflammatory response in microglia but a strong microglia phagocytic response, LPS exhibited a profound alteration in cell morphology and cytoskeletal remodeling. Moreover, MPL did not increase cyclooxygenase-2 (COX-2) and nitrite levels, nor triggered the expression of cytokine-inducible NOS (iNOS). However it induced both TNFα and CCL2 mRNA expression, although at lower levels when compared to LPS. No differences between both drugs were noticed in cell migration.

On the other hand, to characterize the ligand–receptor interaction of MPL with the TLR4 receptor complex these authors used the HEK293 cell line transfected with TLR4, MD2, and CD14 genes, as well as an NF-κB and AP-1 reporter system. At the highest concentration of MPL tested (20 μg/mL), the activation of NF-κB and AP-1 was at a level comparable to a 200-fold lower concentration of LPS (0.1 μg/mL). Incubating TLR2-transfected HEK293 cells with up to 2.5 μg/mL of MPL did not induce any activation of NF-κB and AP-1.

Michaud *et al* (2012) also found that there was a smaller increase in the levels of cytokines (TNF-a and IL6) and chemokines (CCCL3, CCLX-1 and IP-10) measured in the sera of wild-type C57BL/6 mice 2 and 6 h following a single intra-peritoneal (i.p) injection of 50 μg/mL MPL when compared to 20 μg/mL LPS-injected mice. This moderate inflammatory response is consistent with the *in vitro* data. Moreover, these authors showed that both MPL and LPS triggered a strong and similar monocytopoiesis 24h following i.p. administration when compared with controls as measured by flow cytometry.

To investigate whether treatment with MPL might affect AD-related pathology in vivo, Michaud *et al* (2012) administered MPL(50 μg/mL) , LPS (3 μg/mL) or PBS (controls) weekly by i.p. injection to APPswe/PS1 mice for 12 consecutive weeks beginning when the mice were 3 month old.

Cognitive function and Aβ deposition were assessed for each mouse 2 and 3 week after the final injection, respectively. Using a T water maze behavioral test to assess hippocampus-based spatial learning and memory, experiments were conducted to determine whether clearance of Aβ correlated with improved cognitive functions in these mice. Compared with the PBS-treated control group, the MPL-treated APP_{swe}/PS1 mice showed significant improvement in cognitive functions ($p<0.05$ vs controls).

Administration of MPL caused a significant reduction in the number and size of Aβ deposits, as well as the quantity of soluble Aβ in the brain (P = 0.0020, 0.0018, and 0.0027, respectively, versus control group). Compared with controls, the size and number of Aβ plaques were considerably greater in LPS treated animals, whereas the level of soluble Aβ monomers was equivalent. The percentage of CD45+ brain cells that contained Aβ after i.p. injections of MPL was determined by Michaud *et al.* (2012) to investigate whether MPL treatment led to lower Aβ levels by promoting phagocytosis

of Aβ. The brains of mice treated with MPL had significantly higher percentages of Aβ-positive cells than those of the control group ($p < 0,001$ vs controls).

ACI-24 (AC Immune®, Lausanne, Switzerland) is an Aβ1-15 peptide to which two lysines that are tetrapalmitoylated on the ε-nitrogens were attached to both ends. The antigen is embedded in a liposome membrane. A mixture of the lipids DMPC (1,2-dimyristoyl-sn-glycero-3-phosphocholine), DMPG (1,2-Dimyristoyl-sn-Glycero-3-PhosphoGlycerol), cholesterol, and MPL in a ratio of 9 : 1 : 7 : 0.06, respectively was used as adjuvant (2). Pihlgren *et al.* (2009) showed that ACI-24 vaccine induced anti-aβ antibody titers in the APPxPS1 transgenic mice with high specificity for oligomeric Aβ species (46). These antibodies were of IgG2b and IgG3 isotypes, indicating a preferential Th2 vaccine response. The brain tissues showed no evidence of microglia activation nor astrogliosis and no increase in the levels of the pro-inflammatory cytokines IL-1β, IL-6, TNF-α, or IFN-ɤ (46). The histological staining with Perl's Iron revealed that the number of sections per mouse containing large hemorrhages was significantly lower in mice treated with ACI-24 compared to the vehicle-treated controls (46). Additionally, ACI-24 immunization did not induce infiltration of peripheral monocytes (MHC-II), or of peripheral T- and B-cells (CD4 or CD45) into the brains of the treated transgenic mice (46). The results show that immunization of old transgenic mice with ACI-24 induces oligo-specific anti-Aβ antibodies and decreases the number of large micro-hemorrhages without inducing micro-hemorrhages (46). Furthermore, ACI-24 immunization does neither cause cellular brain inflammation nor enhance the release of pro-inflammatory cytokines, nor does it result in the penetration of peripheral monocytes into the brain. These results, together with the preferentially Th2 associated

antibody isotype profile, indicate a low risk of encephalitis and thus demonstrate a positive safety profile for ACI-24 in a relevant AD animal model (46).

ACI-24 is currently being evaluated in a Phase I/II clinical trial in Denmark, Finland, and Sweden (2). The phase I/II Double-Blind, Randomized, Placebo-Controlled, Adaptive Design Study of the Safety, Tolerability, Immunogenicity and Efficacy of ACI-24 in Patients with Mild to Moderate Alzheimer's Disease started on 2011 and is currently ongoing (11).

The overall study objective is to assess the safety, immunogenicity and efficacy of repeated doses of ACI-24 at 3 different dose levels administered to patients with mild to moderate Alzheimer's disease patients (11).

The secondary objectives of this trial are to explore the following parameters: the efficacy of ACI-24 in reducing Aβ level in the brain of patients with mild to moderate Alzheimer's disease; the effect of ACI-24 on whole brain and hippocampal volume; the effect of ACI-24 on T cell activation; the effects of ACI-24 on putative biomarkers of the progression of Alzheimer's disease including total tau and phosphorylated tau protein (phosphotau) and Aβ levels (Aβ1-42 and Aβ1-40) in blood and CSF; the efficacy of ACI-24 on clinical endpoints in patients with mild to moderate Alzheimer's disease (11).

Primary end points refer to ***(1)*** safety and tolerability assessments: adverse events; global assessment of tolerability; physical, neurological and psychiatric examination; vital signs; MRI imaging; electrocardiogram; routine hematology and biochemistry evaluation in blood and urine; inflammatory markers in blood and cerebrospinal fluid; ***(2)*** biological assessments: immune response titer (serum anti-Aβ1-

42 IgG titer); and finally, ***(3)*** efficacy assessments: change from baseline over 1 year of total cognitive score (Neuropsychological Test Battery) (11).

The subjects will be treated for approximately 12 months (treatment period) and will enter a follow-up of 24 months (follow-up period).There will be 186 individuals studied, 56 from ages 18 to 65, and 130 above 65 years old, both male and female (11).

There are no conclusions from the study available yet.

Discussion and Conclusion

Herein we review evidence suggesting that finely tuning the innate immune system may be instrumental in AD therapeutics (49). In fact, Michaud *et al* (2012) showed that whereas chronic systemic LPS administration in APP_{swe}/PS1 mice exacerbated the Aβ plaque load due to its strong TLR4 agonism and pro-inflammatory response, MPL - a derivative of the lipid moiety of lipopolysaccharide, which also targets TLR4 - decreased Aβ load including decreasing the quantity of soluble Aβ in the brain and improved cognition in this AD mouse model while triggering a modest inflammatory response (41). These authors suggested that the underlying mechanism could be microglial activation through TLR4 triggering. Moreover the authors further concluded that compounds that sparingly stimulate innate immune system may have a great AD therapeutical potential. Two other temptative explanations for this Aβ brain burden reduction seen in this AD mice model could be the following: 1 - the Aβ peripheral clearance by dendritic cells, lymphocytes, and monocytes could have triggered brain Aβ redistribution to blood (47); 2 - MPL may promote mobilization of microglial precursors from bone marrow into the brain perivascular spaces thus directly phagocyting Aβ from brain circulation (48).

Specifically Aβ modifications are clearly related to AD pathology since soluble oligomers are increased in human AD brain tissue. Moreover Aβ soluble oligomers are more strongly associated with synaptotoxicity and cognitive impairment than fibrillar Aβ (49). Notably, toxicity has been attributed to dimers, but also to trimers, pentamers, and dodecamers (50).

Aging is concurrent with diminished function of both the adaptive and innate arms of the immune system (51). This includes an increased threshold for immune

response induction, which may be overcomed by supplementing a vaccine with an appropriate immunostimulatory adjuvant (52). Interestingly, MPL is being used in humans safely as a vaccine adjuvant in approved vaccines against human papillomavirus (HPV) and hepatitis B (HBV); and is being evaluated in candidate vaccines against malaria, HIV (human immunodeficiency virus), TB (tuberculosis). HPV and HBV vaccines have reached licensure worldwide and in Europe, respectively (10).

Notably there is a growing amount of studies confirming the immunostimulatory vaccine adjuvant characteristics of MPL when using active anti-Aβ immunization strategies, in AD related pathology (33).

In particular, the vaccination route (subcutaneous or intra-nasal) had impact on anti-Aβ antibodies production in immunized mice with Aβ peptides+MPL. (53). In fact, the MPL oil-in-water emulsion used for subcutaneous immunizations did not generate a significant amount of antibody titer when used intranasally (33). This might be due to less absorption of this emulsion by the mucosal epithelia and/or to differences in the antigen processing immune cells at the two locations (e.g., dendritic cells for *subcutaneous* immunization versus M cells in the nasal epithelium and lymphocytes and dendritic cells in the nasopharyngeal-associated lymphoreticular tissue (NALT) for *intranasal* immunization) (33). In this review it is also highlighted that the number of immunizations may also influence the immune response triggered by Aβ+MPL strategy, besides route of administration. Therefore all these parameters must be taken in consideration when designing clinical trials using MPL as an adjuvant when combined with Aβ.

MPL was also tested as adjuvant in Aβ preventive immunotherapy in non-human primates (NHP) (35). This model has close genetic relationship to humans, identical APP amino acid sequence and natural development of neuritic amyloid plaques and amyloid angiopathy with age. However, there are also differences between AD patients and NHP models, such as less severe tau pathology and significant differences in cerebrospinal fluid biomarkers. Nonetheless, it was demonstrated that this preventive Aβ immunization triggered a significant shift in oligomer size with an increase in in the dimer/pentamer ratio in aged animals compared with non-immunized controls. This might facilitate removal of toxic Aβ species from the brain, thus tipping the balance between Aβ accumulation and clearance towards the latter.

Altogether this study strongly suggested that preventive Aβ immunization using MPL as an adjuvant is a safe therapeutic approach lacking adverse CNS immune system activation. Along these lines, a vaccine using MPL as an adjuvant - ACI-24 - is currently undergoing a phase I/II clinical study in patients with mild to moderate Alzheimer's disease.

Overall, this review stresses that MPL holds a great potential as safe and effective treatment for the most common neurodegenerative disease world-wide – Alzheimer disease.

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