UNIVERSITÀ DEGLI STUDI DI MILANO

SCUOLA DI DOTTORATO IN MEDICINA MOLECOLARE

CICLO XXVIII
Anno Accademico 2014/2015

TESI DI DOTTORATO DI RICERCA
MED/04

ROLE OF THE INFLAMMASOMES IN NEUROINFLAMMATION ASSOCIATED WITH ALZHEIEMER'S DISEASE

Dottoranda: Francesca La Rosa
Matricola N°: R10241

TUTORE: Prof. Mario Clerici
CO-TUTORE: Dott.ssa Marina Saresella
DIRETTORE DEL DOTTORATO: Prof. Mario Clerici

Immagination

is more important than

the knowledge…

A.Einstein

SOMMARIO

L'interleuchina-1 beta [IL1β] ed il complesso proteico "inflammasome", regolatore principale della produzione di IL1β sembrano svolgere un ruolo importante nella neuroinfiammazione osservata nella malattia di Alzheimer [AD]. L'AD è una patologia cerebrale a carattere neurodegenerativo, la cui prevalenza è stimata essere in aumento nel corso dei decenni; la caratteristica neuropatologica più comune e distintiva è rappresentata dalle placche di beta-amiloide [Aβ] che, con la loro deposizione, costituiscono l'elemento chiave per l'innesco di processi ossidativi e pro-infiammatori da parte della microglia attivata. L'interazione tra microglia attivata e placche di Aβ induce il rilascio di citochine pro-infiammatorie, come l'interleuchina-6 [IL6], o di fattori neurotossici, come il tumor necrosis factor-α [TNFα]. La produzione di placche amiloidee è dovuta da un lato all'iper-accumulo patologico degli oligomeri, dall'altro lato alla ridotta clearance extracellulare degli stessi. L'amiloide è fisiologicamente eliminata dal tessuto cerebrale attraverso i vasi sanguigni, verso i quali migra nel corso del suo tempo biologico, cioè anni. Il risultato è l'accumulo di amiloide dapprima attorno a tali vasi, poi nel tessuto cerebrale. L'amiloide insolubile può comportarsi da superantigene, attivando una risposta cronica infiammatoria sia umorale che cellulo-mediata. L'infiammazione tissutale è dapprima mediata dalla microglia attivata ma, e' stato dimostrato altresì il legame inscindibile tra infiammazione cerebrale e quella periferica. Oltre alla microglia, nuovi monociti sono continuamente reclutati dalla periferia verso il tessuto nervoso superando la barriera ematoencefalica [BBB] ed una volta arrivati, i monociti stimolati assumono il fenotipo macrofagico. La neuroinfiammazione è quindi un momento eziopatogenetico fondamentale nella genesi del deterioramento cognitivo e della demenza correlate alla patologia di Alzheimer. Sia studi post-mortem che su biopsie, tramite immunoistochimica, hanno rivelato la presenza di microglia attivata nelle placche amiloidee. Studi immunoistochimici inoltre, hanno rivelato la positività per l'IL1, marker di attivazione dell'immunità innata mediata da macrofagi e microglia. L'insulto infiammatorio è probabilmente il maggior responsabile della perdita neuronale. Ad oggi una diagnosi certa di AD è possibile solo mediante analisi post-mortem del tessuto cerebrale: per questo motivo negli ultimi anni sono stati ricercati ed individuati degli indicatori biologici ed ormonali di malattia, che potessero essere utili nella diagnosi precoce di AD. Tali indicatori sono espressi anche dai leucociti periferici, cellule di facile reperibilità che rappresentano un'impronta di ció che si verifica a livello di aree cerebrali inacessibili in vivo. Alla luce di ciò, i leucociti rappresenterebbero utili elementi per studiare le modificazioni che si instaurano a livello cerebrale; pertanto nella prima parte del mio progetto, per meglio investigare il possibile coinvolgimento dell' inflammosoma a livello periferico, cellule mononucleate dal sangue periferico [PBMC] sono state isolate da individui diagnosticati mediante la scala di valutazione Mini-Mental State Examination [MMSE] e classificati come segue: 1] individui che presentano cambiamenti lievi ma misurabili nelle capacità di pensiero definiti mild cognitive impairment [MCI] [MMSE 24-29]; 2] soggetti con patologia AD moderata [MILD] [MMSE 19-24]; 3] individui con patologia AD severa [MMSE <19]. Infine sono stati arruolati anche individui sani [HC] di pari sesso ed età come gruppo di controllo. PBMC sono stati stimolati con LPS e Aβ [1-42] per valutare l'

espressione dei geni e delle proteine coinvolte nella via dell' inflammasoma e di citochine proinfiammatorie. I risultati ottenuti suggeriscono un coinvolgimento di almeno due diversi complessi dell' inflammosoma nella neuroinfiammazione associata alla malattia di Alzheimer. Sulla base di questi risultati, la seconda parte del progetto vede come protagoniste cellule di Primary Microglia murina WT e knockout per i geni nlrp3 e asc, per completare il quadro di attivazione dell'inflammosoma in un contesto di neuroinfiammazione. I dati ottenuti confermano il coinvolgimento di NLRP3-inflammasoma nella produzione di citochine infiammatorie rendendo questo complesso proteico un potenziale bersaglio farmacologico per migliorare la terapia della Malattia di Alzheimer.

ABSTRACT

Interleukin-1 beta [IL1β] and the "inflammasome" protein complex, the main regulator of the IL1β production, plays an important role in neuroinflammation observed in Alzheimer's disease [AD]. AD is a neurodegenerative disease, whose prevalence is estimated to be increasing over the decades; the neuropathological characteristic most common are beta-amyloid [Aβ] plaques, whose deposition are the key element for the triggering of oxidative and pro-inflammatory processes by the activated microglia. The interaction between activated microglia and plaques Aβ induces the release of pro-inflammatory cytokines, such as interleukin-6 [IL6], or neurotoxic factors, such as tumor necrosis factor-α [TNFα]. The amyloid plaques production is due on one hand to the hyper-pathological accumulation of oligomers, on the other hand to the reduced extracellular clearance of the same. The amyloid is physiologically eliminated from the brain tissue through blood vessels, towards which migrates in the course of its biological time [years]. The result is the accumulation of Aβ at first around these vessels, and then into the brain tissue. The insoluble amyloid can act as superantigen, activating a chronic inflammatory response both humoral and cell-mediated activation. The inflammation tissue is initially mediated by activated microglia but, it was already shown a link between brain inflammation and that device. In addition to microglia, new monocytes are constantly recruited from the periphery to the nervous tissue overcoming the blood-brain barrier; stimulated monocytes show the same macrophage phenotype. Neuroinflammation is therefore a fundamental etiopathogenetic moment in the genesis of cognitive impairment and dementia related to Alzheimer's disease. Both post-mortem biopsies and immunohistochemistry, have revealed the presence of activated microglia in Aβ, plaques. Immunohistochemical studies have also revealed positivity for IL-1, markers of innate immunity activation mediated by macrophages and microglia. The inflammatory insult is probably most responsible for neuronal loss. Currently a diagnosis of AD is only possible through post-mortem analysis of the brain tissue, which is why in recent years have been researched and identified biological indicators and hormonal disease, which could be useful in the early diagnosis of AD. These indicators are also expressed by peripheral leukocytes, cells readily available, representing a footprint of that which occurs at the level of different brain areas in vivo. For this, the white blood cells represent useful elements to study the changes that develop in the brain. Therefore, in the second part of the project, to better investigate the possible involvement of inflammosome peripherally, peripheral blood mononuclear cells [PBMC] were isolated from individuals diagnosed with Mini-Mental State Examination [MMSE] and classified as follows: 1] individuals with mild but measurable changes in thinking skills defined mild cognitive impairment [MCI] [MMSE 24-29]; 2] patients with moderate disease AD [mild] [MMSE 19-24]; 3] individuals with severe AD pathology [MMSE <19]. Finally they were enrolled even healthy individuals [HC] of equal sex and age as a control group. PBMC were stimulated with LPS and Aβ [1-42] to evaluate the 'expression of the genes and the proteins involved in the inflammasome pathway and to evaluate proinflammatory cytokines production. The results suggest an involvement of at least two different inflammasomes-complexes in

neuroinflammation associated with Alzheimer's disease. Based on these results, the second part of the project aimed at completing the outline of inflammasome activation in neuroinflammation context by investigating WT or NLRP3 and Asc knockout mouse Primary Microglia cells . Data obtained confirm the NLRP3-inflammasome involvement in the inflammatory cytokines production making this complex a potential drug target for improving the therapy of Alzheimer's Disease.

INDEX

SYMBOLS LIST

AD: Alzheimer Disease ,
APA : American Psychiatric Association
ApoE: Apolipoprotein E
APP: β-amyloid precursor protein
ASC [PYCARD]: PYD and CARD domain containing
Aβ: Amyloid-beta
BACE1: Beta-secretase 1
BBB: blood–brain barrier
CARD: Caspase recruitment domain family
CNS: Central nervous system
DAMPs: Damage-associated molecular pattern molecules
DSM:Statitical Manual Mental Disorders
EAE: Autoimmune encephalitis
EOAD :early onset Alzheimer disease
FAD: Familial Alzheimer disease
HC: Health control
IL18: Interleukin 18
Il1β: Interleukin1, beta
IL33: Interleukin 33
IL37: Interleukin 37
IL6: Interleukin 6
IRAK: IL-1 receptor–associated kinases
IRF: interferon regulatory factor
KO: Knockout
LOAD: late onset Alzheimer disease
LPS: Lypopolisaccaride
LRRs: C-terminal leucine-rich repeats
MCI: Mild Cognitive Impairment
MMSE: Mini-Mental State Examination
MNR: Magnetic Resonance Imaging
NF-kB: Nuclear factor kappa-light-chain-enhancer of activated B cells
NFTs: Neurofibrillary tangles
NINCDS-ADRDA: Alzheimer Disease and Related Disorders Association
NLR: Nod-Like receptor
NLRP1: NLR family, pyrin domain containing1
NLRP3: NLR family, pyrin domain containing 3
NOD: Nucleotide-binding oligomerization domain containing 1
PAMPs: Pathogen-associated molecular patterns
PBMC: peripheral blood mononuclear cell
PET: Positron Emission Tomography
PRRs: Pattern recognition receptor
PS1,2: Presenilin 1,2
ROS: Reactive oxygen species
TLR: Toll-Like receptor
TNFα:Tumor necrosis factor a
WT: wild type
τ: tau-protein

1. INTRODUCTION

Transcription of pro-IL1β can be induced in monocytes, macrophages and dendritic cells upon Toll-Like receptor [TLR] or cytokine signaling [1]. Before IL1β can be secreted, it has to be translated and processed into mature IL1β by a macromolecular protein complex, the inflammasome. The most extensively studied inflammasome is the nucleotide-binding oligomerization domain [NOD] like receptor [NLR] family, pyrin domain containing 3 [NLRP3] inflammasome [2]. Upon inflammasome activation, the NLRP3 protein recruits the inflammasome adaptor protein apoptosis-associated speck-like protein containing C-terminal caspase recruitment domain [ASC], which in turn interacts with pro-caspase-1 leading to its cleavage and activation [3]. Finally, the active caspase-1 processes pro-IL1β into its mature form. A variety of different molecular structures, which can trigger inflammasome activation, have been identified, *e.g.* ATP, bacterial toxins or endogenous stimuli [4]. In addition to IL1β, the NLRP3 inflammasome also processes IL18, which is another pro-inflammatory cytokine. Inflammasome activation is crucial for host defense to pathogens, but recent studies have clearly identified a role for the inflammasome in the pathogenesis of several inflammatory diseases as well [1]. An increased NLRP3 inflammasome activity demonstrated by the pro-inflammatory cytokines production detected in AD subjects might be important to increase the inflammatory process responsible to the neurodegeneration in AD's pathology.

The role of myeloid immune cells, including microglia, macrophages and monocytes, in neural disease remains controversial. It is known as chemokine receptors and adhesion molecules are used selectively for the transmigration of leukocytes across the blood–brain barrier [BBB] during

neuroinflammation. Activated microglia appears to contribute to the development of several neurodegenerative disorders, such as Alzheimer's disease, but in the literature there are different results regarding PBMC infiltration in the CNS as a support to microglia. PBMC were implicated in experimental autoimmune encephalitis [EAE], the mouse models of multiple sclerosis, as blockage of their infiltration in the CNS ameliorated disease development [5,6]. Conversely, inhibition of PBMC infiltration in an Alzheimer's mouse model resulted in exacerbation of the disease [7]. Recruitment of PBMCs in the brain of an Alzheimer's disease mouse model appeared to mediate reduction of plaque formation in the brain. This study is among the few conducted on human PBMCs by means of which we validated inflammasomes-complex activation with the progression of the disease and we evaluated whether inflammation that follows its activation may be advantageous or not for AD.

1.1 ALZHEIMER'S DISEASE

AD, the most common form of dementia, is a degenerative disorder of the brain that leads to memory loss [22]. AD affects 5.3 million Americans and is the seventh leading cause of death in the United States. There are two main forms of the disease. Familial AD affects people younger than 65, accounting for nearly 500,000 AD cases in the United States alone. The remainder of AD cases occurs in adults aged 65 and older and is classified as sporadic AD. The prevalence of AD varies among many different factors, including age, co-morbidities, genetics, and education level. Damage to the entorhinal cortex, hippocampus and basal forebrain, leading to memory impairment, temporal and spatial disorientation and altered cognitive function, are pathological features of AD. Two histopathological lesions earmarked the disease: extracellular amyloid deposits [chiefly composed of

amyloid-β peptides] and intracellular neurofibrillary tangles [NFTs, made up primarily of abnormaly folded tau protein]. Over the last hundred years, intense focus has been directed toward understanding AD pathoetiology and potential treatment approaches aimed to controlling these pathology. The lengthening of life expectancy, especially in developed countries, the increased incidence of AD, requires a greater investment of financial resources. The diagnosis of AD requires a multidisciplinary approach, a combination of sensitive and specific diagnostic clinical criteria that allow the identification of the disease from the beginnings. Therapies strategies are prevent/delay the onset and progression but in several cases of dementia make the diagnosis is so difficult. There is no cure for AD, however promising research and development for early detection and treatment is underway.

1.1.1 HISTORY

Alzheimer's disease was discovered in 1906 by Alois Alzheimer, a German neurologist and psychiatrist. The disease was initially observed in a 51-year-old woman named Auguste D. Her family brought her to Dr. Alzheimer in 1901 after noticing changes in her personality and behavior. The family reported problems with memory, difficulty speaking, and impaired comprehension. Dr. Alzheimer later described Auguste as having an aggressive form of dementia, manifesting in memory, language and behavioral deficits [9]. Dr. Alzheimer noted many abnormal symptoms, including difficulty with speech, agitation, and confusion. He followed her care for five years, until her death in 1906. Following her death, Dr. Alzheimer performed an autopsy, during which he found dramatic shrinkage of the cerebral cortex, fatty deposits in blood vessels, and atrophied brain cells. He discovered neurofibrillary tangles and senile plaques, Bethune which has become indicative of AD. The condition was first discussed in medical literature in 1907 and named after Alzheimer in 1910.

In Alzheimer's disease, the first alterations in the cerebral cortex are found primarily in the entorhinal region, located in the anterior portions of the lap hippocampus of the temporal lobe [10]; these changes affect subsequently other areas of the cerebral cortex and subcortical nuclei of specific groups. Macroscopic examination show in AD patient a variable degree of cortical atrophy, decrease of the weight and volume of the organ, thinning of the layers of the neocortical convolutions in regions involved in the processes of memory and learning, as the temporal cortex, parietal, frontal, hippocampus and amygdale. Neuronal degeneration involves atrophy, which is followed by a compensatory ventricular enlargement, secondary to the loss of parenchyma. Normally all macroscopic changes observed in AD

patients are also found in healthy elderly subjects and physiological expression of brain aging. In Alzheimer's patients it is however characterized by their greater quantities and their peculiar distribution. At the histological level, it is possible to demonstrate the accumulation of two different proteins: the Aβ that is deposited extracellularly, and the protein phosphorylated τ which constitutes intracellular neurofibrillary tangles .

The Aβ is organized in different forms, the neuritic plaques [senile plaques], constituted by a central compact part of insoluble fibrils of Aβ protein, surrounded by an agglomerate with inflammatory degeneration of neurites [axons and dendrites], reactive astrocytes and activated microglia [monocytes or activated macrophages derived from reticuloendothelial system resident in CNS - SNC]; they are located mainly in hippocampus and limbic structures, the amygdala, the subiculum and entorhinal cortex. The tangles consist of compact bundles of filaments abnormal [paired helical filaments], mainly from protein τ; they affect mainly the soma, but may also extend to the dendrites. These filaments are the cells take a look "flame". The "tangles" are mainly located at the level of medium-sized pyramidal neurons of the entorhinal cortex, the limbic cortex, hippocampus, amygdala, neocortical layers of frontal and temporal lobes and the cholinergic system of the basal ganglia [10].

1.1.2 GENETICS OF AD

Alzheimer genetics is traditionally subdivided into early onset [EOAD] and late onset [LOAD]. EOAD has an onset before age 60–65 years and accounts for 1–5% of all cases. LOAD has an onset after age 60-65 years and is the predominant form of AD. Additionally family history may be consistent with familial, or sporadic AD [11]. A small number of families worldwide have a faulty gene on chromosome 21, the gene for amyloid

precursor protein [APP], which affects the production of the amyloid peptide. A slightly larger number of families lead to a failure in the gene presenilin 1 [PS1], localized on chromosome 14, and a very small number of families have a faulty gene presenilin 2 [PS2] in the chromosome 1 [12]. People with mutations in either gene tend to develop the disease between 30 and 40 years old and come from families in which several members also have early-onset Alzheimer's, so this form is called familial Alzheimer disease [FAD] [Table1]. Sporadic Alzheimer, late onset, is the most common form of the disease [Table 1]. The apolipoprotein E [ApoE], the effects are more subtle than those of the other genes of early onset and even individuals: Currently only one gene can exert great influence on the development of sporadic AD it is known two copies of the gene risky way not necessarily develop the disease [13].

1.1.3 FREQUENCY OF ALZHEIMER DISEASE

In 2005, Alzheimer Disease International commissioned an international group of experts to reach a consensus on dementia prevalence and estimated incidence in 14 World Health Organization regions, based on epidemiological data acquired over recent years. The results suggested that 24.2 million people lived with dementia at that time, with 4.6 million new cases arising every year. North America and Western Europe have at age 60 the highest prevalence of dementia [6.4 and 5.4% of the population at age 60], followed by Latin America [4.9%] and China and its developing western-Pacific neighbors [4.0%]. The annual incidence rates [per 1000] for these countries were estimated at 10.5 for North America, 8.8 for Western Europe, 9.2 for Latin America and 8.0 for China and its developing western-Pacific neighbors, increasing exponentially with age in all countries, especially through the seventh and eighth decades of life [14]. The prevalence rates for AD also rise exponentially with age, increasing

markedly after 65 years. There is almost a 15-fold increase in the prevalence of dementia, predominately Alzheimer disease, between the ages of 60 and 85 years. Compared with Africa, Asia and Europe, the prevalence of AD appears to be much higher in the US, which may relate to methods of ascertainment. The prevalence may be higher among African-American and Hispanic populations living in the US, but lower for Africans in their homelands, for reasons that remain uncertain [15].

1.2 RISK FACTORS THAT INCREASE ALZHEIMER'S DISEASE

The numerous risk factors identified to date, although not systematically confirmed, suggest that AD is a multifactorial etiology. The most important are describer in Table 2.

1.2.1 DIAGNOSTIC CRITERIA

Currently, the diagnosis of dementia is made according to the clinical criteria of Diagnostic and Statistical Manual Mental Disorders [DSM IV TR] published by American Psychiatric Association [APA]. Instead, in 1984, representatives from the National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer Disease and Related Disorders Association [NINCDS-ADRDA] developed a uniform set of criteria to enable clinicians and researchers to maintain consistency in the diagnosis and differentiation of the different types of dementia. According to these criteria, the clinical AD diagnosis is defined probable in the presence of cognitive impairment confirmed by neuropsychological tests. The definitive diagnosis is possible only through histopathology brain. Furthermore, research has made available biomarkers in serum and CSF, thorough genetic investigations and advanced neuroimaging techniques. In 2007, Dubois et al [16] have proposed a revision of the NINCDS-ADRDA,

suggesting integration of the clinical features of the disease [the presence of the disorder of memory] with the biological parameters in serum [mutations of APP, presenilin 1 and 2, ApoE ε4 allele] and CSF [β-amiloide1-42, tau protein] and structural neuroimaging data [atrophy of the medial temporal lobe structures detected with techniques *Magnetic Resonance Imaging* [MRI] and functional [alterations of cerebral metabolism detected with *Positron Emission Tomography* [PET]. All these factors would increase the specificity and sensitivity of AD diagnostic criteria and would identify individuals with at earlier stages of disease. The medical case is obtained by the assessment of the state of mind, made by administering cognitive tests, such as the Mini Mental State Examination [MMSE] [17] and neuropsychological testing, and by the assessment of functional independence of the subject, through the use of scales that assess basic activities instrumental of daily living, such as ADL [Activities of Daily Living, Katz index] [18] and IADL [Instrumental Activities of Daily Living, Lawton index] [19]. Aspects regarding the clinical features are then integrated with the structural and functional neuroimaging and the study of genetic and biochemical markers. As already mentioned above, there are three main categories of biomarkers currently identified for the diagnosis of Alzheimer's disease: genetic, biochemical and neuroimaging [Table3].

1.2.2 ETHIOLOGY

AD etiology is still unknown; currently accepted theory is the amyloid hypothesis which is supported by the observation of plaques containing this molecule in AD brain . However, the presence of amyloid plaques alone can not justify the onset of AD; amyloid plaques are developed in the brain tissue of elderly subjects, even cognitively intact, suggesting that the deposition of plaques is actually a paraphysiological phenomenon and the discriminating factors for the onset of AD may be the place where they are

deposited, as well as interaction with other co-factors, equally important for the development of the disease. Many of these have been extensively studied, in order to better define their role in the etiopathogenesis of AD; at present, the most important co-factors involved appear to be hyperphosphorylated τ protein, ApoE and other inflammatory mediators [20] [Figure1].

1.3 THE AMYLOID HYPHOTESIS

Senile plaques in AD brain contain Aβ, a peptide of 40-42 amino acids, derived from a larger precursor, APP [amyloid precursor protein] by a proteolytic process [35]. The APP is an integral transmembrane protein present in several isoforms, all encoded by a single gene located on human chromosome 21.

The APP is expressed in the CNS, but it is also ubiquitously expressed in different variations in peripheral tissues, such as muscle cells, epithelial and circulating cells (especially platelets) [21]. It has functions of adhesion to extracellular matrix receptors [average specific intracellular mechanisms] and modulation of gene expression. In addition, molecules secreted APP in forming synapses and probably play a role in the integrity of the mnemonic process. This protein has a massive N-terminal portion [4/5 of the total weight of the molecule], and a C-terminal portion of approximately 100 amino acids that constitutes the intracytoplasmic domain of the protein. In proximity to the C-terminal an amino acid sequence, referred to as Aβ, acts at the level of the three main enzymes involved in the metabolism of APP: α-secretase, β-secretase and γ -secretase. In relation to the activity of these enzymes, the APP can follow two different routes of processing [Figure2] [22].

No-amyloidogenic process is provided by dell'α- secretase [ADAM10],

which cleaves APP at residue 16 of the Aβ sequence and gives rise to a large fragment soluble N-terminal 83 aminoacids, called sAPPα, that is released into the circulation, and to another fragment of 83 aminoacids, at the level of the end C-terminal [CTF83], which remains attached to the cell membrane where, will encounter cleavage by the γ-secretase. The amyloidogenic pathway involves the action of β-secretase [BACE] and then a second cut by γ-secretase, with release of the Aβ peptide. The β-secretase performs the cleavage at the level of N-terminus of the Aβ sequence, releasing into the extracellular space a soluble fragment, called sAPPβ, and leaving attached to the cell membrane a C-terminal fragment of 99 amino acids [CTF99], which is then cleaved by γ-secretase exactly at the level of the remaining 40 or 42 of CTF99 [22]. Therefore, β and γ-secretase action results in the release of amyloidogenic Aβ fragments of 40 and 42 amino acids in the extracellular space, where they can accumulate; the $A\beta\text{-}_{40}$ is the form most produced, while $A\beta\text{-}_{42}$ is the main component of senile plaques since it has a high potential fibrillogenic and neurotoxic [23]. $A\beta_{40\text{-}42}$ fragments spontaneously aggregate to form a β-sheets structure which is toxic for neurons and synapses, both in its early form [monomeric, dimeric or oligomeric], both as deposit in aggregates, the senile plaques. It is believed that the toxic effects of Aβ can cause the development of dementia [24]. In healthy individuals the two ways of processing of APP are in balance with each other, while it is believed that in the individuals with Alzheimer's disease there is a state of imbalance, with respect to the prevalence of the amyloidogenic pathway via non amyloidogenic.

1.4 EVOLUTION OF THE DISEASE

The disease, as suggested by Braak and Braak [1995], evolves in a sequence of time and space through the structures of the medial temporal

cortex, subcortical nuclei and neocortical areas of the brain. It can be recognized six phases. In stage transentorinale [stages I and II], the signs of the disease are confined mainly to the entorhinal cortex and transentorinale, with lack of involvement of the hippocampus sections CA1 and CA2. The stadium limbic [stages III and IV] is characterized by the presence of neurofibrillary lesions in the entorhinal cortex, hippocampus moderates from tangles and their increasing use to amygdala, the thalamus and the hypothalamus. Finally, the neocortical stage [stage V and VI] is characterized by the progressive involvement of the neocortex [Figure3]. The clinical characterization suggested that the stadium transentorinale is the preclinical and asymptomatic period of the disease, while the stadium limbic represents the incipient stage of the disease characterized by the appearance of the first clinical symptoms. The stadium neocortical is characterized by the full-blown disease [25] The transition from one stage to another is gradual and sequential. Recent reports suggest that the presence of neurofibrillary tangles inside neurons does not necessarily mean the immediate cessation of cell function, but that these neurons can stay alive for many years after their appearance [26,27].

1.4.1 CLINICAL EVOLUTION

The diagnosis of the disease and identification of its various stages of evolution is possible only using different evaluation criteria: tests to assess the cognitive condition of the patient, detailed clinical observations, interviews to the patient and a through family history. However no test pre-mortem is able to perform a certain clinical diagnosis of Alzheimer's disease. So, for every clinical diagnosis must follow a "post-mortem analysis". This means that during the course of the disease can only be done a diagnosis of Alzheimer's "possible" or "probable". In fact only the autopsy can give confirmation of the presence of typical lesions typical of

the disease. Currently, the term most used to describe the preclinical phase of dementia is Mild cognitive impairment [MCI]. People with MCI have memory loss greater than that which would be expected for the age, but they have a cognitive and functional impairment that they can be diagnosed as dementia. The most accepted diagnostic criteria for MCI are those proposed by [28]: a] subjective annoyance memory preferably confirmed by a person other than the individual; b] the presence of a memory deficits documented by a service in a test of episodic memory; c] absence of other cognitive deficits; d] normal abilities in activities of daily living; e] absence of dementia [Figure4]. From a review of major studies on the rate of conversion from MCI to dementia [29] it follows that in subjects with MCI this rate varies between 6 and 25% and is much higher than that observed in normal elderly subjects, where it ranges between 0.3% in individuals between 65 and 69 years and 5.3% in individuals between 85 and 89 years.

1.5 ALZHEIMER AND INFLAMMATION

The inflammatory reaction which characterizes the majority of neurodegenerative disorders, including AD, is often called "neuroinflammation" and involves mainly components of innate immunity. Microglial cells, the primary immune effector cells of the brain, play an integral role in maintaining brain homeostasis and protecting the brain from infections and insults. In the AD brain, microglial cells are observed to be phenotypically activated and form intimate associations with amyloid deposits, extending their processes into the plaque core [30,31]. Microglia are able to recognize and mount an immune response to Aβ peptides as well as migrate to areas of amyloid deposition, however, despite their ability to physically interact with these deposits they are unable to clear plaques from the brain. The role of microglia in the uptake and phagocytosis of amyloid in the brain was first noted in the early 90's by Wisniewski and Frackowiak who utilized electron microscopy to visualize amyloid internalization [32,33]. They postulated that resident microglial cells associated with plaques in the AD brain played a role in the formation of the amyloid fibrils in the brain, but not its phagocytosis [46]. However, in rare cases in which AD patients suffered from stroke they were able to visualize macrophages that had internalized and trafficked amyloid deposits into lysosomal compartments [34]. This is the first study that hypothesized that peripherally- derived myeloid cells were more competent than endogenous microglia in the clearance of amyloid in the brain. The deposition of amyloid in the parenchyma of the brain is associated with a robust inflammatory response [35]. It is thought that this chronic pro-inflammatory milieu produced by "activated" microglia is neurotoxic and facilitates neurodegeneration. It is now understood that the inflammatory response in AD is multifaceted with the role of microglia in disease progression being

both complex and quite controversial. The paradox over whether they play a neuroprotective role in the AD brain or facilitate and contribute to the neurotoxicity observed in AD is a puzzle that is currently under active investigation.

An extensive literature documents that inflammation plays an integral role in progression of AD, facilitating Aβ deposition, neuronal loss and cognitive deficits [36]. The appearance of amyloid plaques in the brain coincides with a dramatic phenotypic activation of the surrounding microglia which display increased immunoreactivity for CD11b, CD68, complement receptor 3 and CD45 [37]. Amyloid and activated microglia secreting cytokines usually precedes changes in neurodegenerative AD. Moreover, the polymorphisms present in the genes of some cytokines [IL1β, IL6, TNFα] and acute phase proteins [α1-antichymotrypsin] are associated with an increased risk of AD [38]. Accumulation of Aβ may be initially determined by defects in physiological mechanisms of its clearance and degradation in the brain. Aβ clearance physiological could happen thanks to the drainage to the perivascular spaces, or actively through the uptake and processing of the same by the astrocytes, the microglia or macrophages derived from monocytes: the latter would seem the only ones able to access deposits of fibrillar Aβ [39]**.** Currently, there is therefore a uniform consensus in accepting inflammation as key feature in the pathogenesis of AD [39,40]. Another demonstration of an activation of inflammatory processes in the early stages of disease would support the hypothesis of inflammation as the "prime mover", although the inflammation could play a key role in the etiopathogenesis of the disease.

The SNC can no longer be considered an organ isolated and protected from the action of the immune system. It is now known that T cells are able to cross the blood brain barrier [BBB] and to release mediators of inflammation in the course of neurodegenerative diseases. In addition, the

microglia expresses different molecules involved in the immune response including major histocompatibility antigens of type I and II that facilitate interaction with T lymphocytes, which, in turn, determine the production of inflammatory cytokines [41,42]. The plaques of Aβ induce the proliferation of astrocytes, which are also glial cells, which would participate in the Aβ clearance and degradation forming a protective barrier between the deposits of Aβ and neurons. However, the presence of activated astrocytes might also be justified for the neurotrophic activity of the substances produced. The neurons themselves, reacting to stimuli toxic, may directly contribute to the maintenance of the inflammatory response: traditionally believed as passive targets of inflammation, recent evidence suggests that they themselves are the source of complement factors and cytokines including IL1β, IL6 and TNFα.

1.6 INNATE IMMUNE RESPONCE IN ALZHEIMER'S DISEASE

1.6.1 MONOCYTE

Monocytes constitute a population of circulating leukocytes that are central cells of the innate immune system. They are part of the mononuclear phagocyte system that arises from the hematopoietic system, which is constituted by self-renewal hematopoietic stem cells and progenitor cells located in the bone marrow [BM] [43]. Monocytes come from the monocyte–macrophage dendritic cell progenitor and are incompletely differentiated cells that give rise to a heterogeneous mononuclear phagocyte lineage [56]. They express multiple clusters of differentiation [CD], namely CD115, CD11c, CD14 and CD16 in human or CD115, CD11b and Ly6C in mouse [44]. In parallel, both human and murine monocytes express different levels of chemokine receptors, among which are chemokine [C-X3-C motif] receptor 1 [CX3CR1] and chemokine [C-C motif] receptor 2 [CCR2] [45]. In human, monocytes are regrouped into three main subsets based on their CD14 and CD16 expression levels, which are the classical subset [$CD14^{++}CD16^{-}$], the intermediate subset [$CD14^{++}CD16^{+}$] and the nonclassical subset [$CD14^{+}CD16^{++}$] [46]. Monocytes are very potent phagocytic cells that respond to stress signals by expressing a variety of surface molecules, among which are scavenger receptors [for example, scavenger receptor SR-A, CD36], low-density lipoprotein receptors [for example, low-density lipoprotein receptor-related protein, LRP1], toll-like receptors [for example, TLR2, TLR4], chemokine receptors [for example, CCR2, CX3CR1], cytokine receptors [for example, macrophage colony-stimulating factor [M-CSF] receptor], Fcγ receptors and adhesion molecules [for example, leukocyte function-associated antigen, LFA-1], wherein the expression level of these molecules reflects their

respective functions [44]. Monocytes are involved in innate immunity by defending the organism against pathogens and toxins [44]. Little is known about monocyte interaction with the brain under physiological conditions. However, it has been proposed that circulating monocytes more precisely, the patrolling subset that has a long half-life [45] replenish the perivascular macrophage population in normal tissue, which is involved in maintenance of homeostasis of the perivascular space [45]. Under pathophysiological conditions, short-lived circulating proinflammatory monocytes are mobilized from the BM to the blood circulation in a CCR2-dependent manner [46,47]. These cells have been shown to possess the capacity to infiltrate inflamed tissues of several organs, including the brain [45]. The infiltration rate of monocytes increases in response to brain-derived inflammatory cues [37]. Following injured brain infiltration, monocytes can differentiate into activated macrophages that are involved in the production of various inflammatory molecules, such as IL1β and TNFα [44], and phagocytosis of toxic elements, including Aβ [48]. It is noteworthy to mention that morphologically these monocyte-derived macrophages are indistinguishable from brain resident microglial cells, but functionally they show a more efficacious phagocytic capacity [48]. The infiltration of monocyte subsets in the inflamed brain and their differentiation into macrophages totally depend on the inflammatory cues present within their microenvironment. Circulating monocytes are able to infiltrate the brain in AD. BM-derived macrophages, which originate essentially from infiltrated proinflammatory monocytes, have been shown to be more efficacious than resident microglia in clearing cerebral Aβ deposits in AD models [49].

1.6.2 MICROGLIA

Microglia are the resident macrophages of the brain, and constitute the main active immune cells in the brain. Although the origin of microglia is still

elusive, it is well accepted that these cells arise from myeloid precursors and constitute an ontogenically distinct population of mononuclear phagocytes [50]. As such, microglial cells arise from hematopoietic progenitors in the yolk sac during embryogenesis and are generated in the postnatal stage just after the formation of the BBB [51]. Microglia survey the brain and are actively involved in maintaining the brain's microenvironment by rapidly responding to pathogens and/or damage [Figure 4] [52,57]. Moreover, microglial cells adopt a special phenotype and cellular morphology that is characterized by high ramifications that constitute dynamic and motile sentinels, by which microglia sense any occurring change in their close microenvironment [47,53]. Under physiological conditions, recent reports show that microglia actively contribute to neuronal plasticity and circuit function [54].

Under pathophysiological conditions, microglial cells are activated and acquire a new morphology characterized by an amoeboid shape. Activated microglial cells are capable of performing several macrophage-like immune functions, such as cytokine release and phagocytosis [52,53]. In parallel with the newly acquired morphological shape, activated microglia upregulate several key surface markers involved in phagocytosis. Once activated, microglia can adopt diverse phenotypes ranging between two extremes: a classically activated M1 phenotype that is involved in proinflammatory actions, and an alternatively activated M2 phenotype that is mainly involved in anti-inflammatory actions and tissue repair [51]. The molecular cues present within the microglial microenvironment play a crucial role in mediating their activation phenotype. It is important to mention that, in the diseased brain tissue, both extremes cohabit within a spectrum of different intermediate phenotypes.

1.7 PATHOGEN RECOGNITION RECEPTORS [PRRS]

The immune response is divided into innate and adaptive. Innate immunity is by definition universal, rapid, highly conserved and present from birth; it is usually the first form of defense against an external insult. The adaptive immune response is highly specific and develops much more slowly upon exposure to toxins or pathogens. The innate immune response is involved in various inflammatory processes and has a particularly important role in the initial and final phases of bacterial and viral infections. Effectors of the innate immune system, the PRRs are activated immediately after exposure to infectious agents, limiting later replication. The PRRs are expressed by different cell types including macrophages, monocytes, dendritic cells, neutrophils, epithelial cells and, in general, in all cells of the adaptive immune response. The PRRs include membrane receptors Toll-like Receptors [TLRs] and C-type lectins [CTLs] that "control" the presence of PAMPs [pathogen-associated molecular patterns] extracellular and endosomal compartments [55,56]. The signal transduction of these receptors converges on a common set of signal modulators which often include the activation of the transcription factor NF-kB [nuclear factor kappa-light-chain-enhancer of activated B cells] and AP-1 [activator protein-1] that drive the production of pro-inflammatory cytokines, chemokines and the members of the family of transcription factors that mediate the response of IRF dependent antiviral interferon type 1 [IFN-dependent]. Another group of receptors are those intracellular PRRs denominated NOD-like receptors [NLRs] that recognize and bind to other molecules called PAMPs and danger-associated molecular patterns [DAMPs] [1].

1.7.1 TLR RECEPTORS: STRUCTURE AND LIGANDS

TLRs are divided into two subgroups depending on their cellular localization and their ligand [PAMP]. A group is composed of TLR1, TLR2, TLR4, TLR5, TLR6 and TLR11, expressed on the cell surface that recognize and bind to different substrates such as microbial lipids, lipoproteins and proteins; the other group includes TLR3, TLR7, TLR8 and TLR9, expressed exclusively in intracellular vesicles such as the endoplasmic reticulum [ER], endosomes, and lysosomes which recognize and bind to microbial nucleic acids. The TLRs have a transmembrane domain which binds an extracellular domain rich in leucine and an intracellular cytoplasmic domain [TIR]. The TLR4, one of the most studied and known TLR, has been identified as the receptor for the lipopolysaccharide [LPS], a component of the cell membrane of Gram-negative bacteria. TLR4 was the first TLR to be identified and whose property was crystallized making possible the computational simulation of the model that predicts the mechanism of interaction with the ligand [57]. The TLR4 forms a complex with myeloid differentiation factor 2 [MD2] on the cell surface, allowing the link with LPS. A structural study of the TLR4-MD2 complex with LPS showed that five or six lipid chains of LPS bind the hydrophobic part of MD2 and that the remaining lipid chains are associated with TLR4 [57,58]. The ligand binding to TLR4, leads to activation of the complex TLR-4/MD2 which culminates with the production of pro-inflammatory pathway through the myeloid differentiation primary response protein 88 [MyD88] employee, and with the production of interferon Type-1 through MyD88 independent pathway [Figure 7]. The MyD88-dependent signaling recruits IRAK1 [IL-1R-associated kinase] and IRAK4, and TRAF6 [TNF [tumor necrosis factor] - receptor-associated factor 6] making active the transcription factor NF-kB through dissociation of its inhibitor IkB. This process culminates with the

translocation of NF-kB in the nucleus and the subsequent activation of the transcription of immunoregulatory genes [59,60].

1.8 NOD-LIKE RECEPTORS [NLRS] AND INFLAMMASOMES

The NLRs are intracellular receptors belonging to the family of PRRs that recognize PAMPs and DAMPs. The family of NLRs is characterized the presence of a central domain [NACHT] which bind at the C-terminal leucine-rich repeats [LRRs] and N-terminal caspase recruitment domain [CARD]. The domain NACHT is the only common to all members of the family of NLRs [Figure 8]. NLR receptor forms a complex with the effector molecule, pro-caspase-1, with or without the help of an adapter molecule such as apoptosis-associated speck-CARD domain [ASC]. The complex so formed is called the "inflammasome" [Figure 9].

The concept of inflammasome was introduced for the first time by Tschopp and collaborators in 2002 that described it as a multiprotein complex capable, following an inflammatory insult, to control the production of pro-inflammatory cytokines important such as IL1β and IL18 [1]. IL1β is synthesized as an inactive precursor [pro-IL1β] in response to TLR ligands such as LPS. The crucial signal transduced by TLRs for the induction of pro-IL1β expression is likely activation of the transcription factor NF-kB downstream of the adapter MyD88. The cytokine IL18 shares many of the pro-inflammatory characteristics of IL1β and it is synthesized too as an inactive precursor [61]. Unlike pro-IL1β, however, pro-IL18 is expressed constitutively. Biologically active IL1β and IL-18 are generated from pro-IL1β and pro-IL18 by the aspartate-specific cysteine protease, IL1β-converting enzyme [ICE] e also known as caspase-1. The mechanism by which IL1β is secreted from the cell remains unclear. Pro-IL1β lacks a signal peptide and has a diffuse distribution in the cytoplasm. Caspase-1

itself is synthesized as an inactive 45-kDa zymogen [pro-caspase-1] that undergoes autocatalytic processing upon receipt of an appropriate stimulus. The active form of the enzyme comprises the subunits, p20 and p10, which assemble into a heterotetramer [61]. Although caspase-1 was the first mammalian caspase to be identified, how PRRs promote caspase-1 activation and subsequent processing of pro-IL1β remained elusive for many years. Activation of caspase-8 by death receptors and caspase-9 by Cytochrome c released from mitochondria had been shown to involve pro-caspase recruitment to the oligomerized adapters FADD and Apaf1, respectively. Tschopp and colleagues investigated whether caspase-1 also was activated within a multiple adapter complex. In vitro setting, they observed the formation of a complex composed of the intracellular adapters: ASC and NALP1 together with caspases-1 and 5. Like caspase-1, ASC and NALP1 contain a CARD domain [62].

In ASC-dependent inflammasomes, upstream sensor proteins, such as NLRP3 and AIM2, recruit ASC via interactions between their PYDs. ASC then interacts with caspase-1 via CARD/CARD interactions. Therefore, the bipartite nature of ASC represents the core structure of these inflammasomes. The NAIP/NLRC4/caspase 1 complex is a representative ASC-independent inflammasome, [Figure 10] in which NAIP is the sensor and NLRC4 is the adaptor, although both NAIP and NLRC4 belong to NLRs by domain definition . NLRC4 contains a CARD and can directly recruit and activate caspase-1 [62].

1.8.1 NLRP3-INFLAMMASOME

NLRP3 inflammasome was the first to be described by Hoffman. More recently Agostini et al. [63] demonstrated NLRP3-inflammasome ability to process the pro-IL-1β . The NLRP3 inflammasome is the most studied and

best characterized; is formed by the NOD-like receptor, the adapter molecule ASC and the effector molecule, pro-caspase 1. It is now generally accepted that activation and release of IL-1β requires two distinct signals. The first signal is triggered by TLR activation and leads to the synthesis of pro-IL-1β and other proteins required for inflammasome. The second signal that is required for caspase-1 activation and IL-1β secretion [64].

Signal 1: priming

Multiple signals, which are potentially provided in combination, can trigger the formation of an active inflammasome, which, in turn, leads to the cleavage and release of bioactive cytokines including IL-1β and IL-18. In general, priming stimuli can include any whose receptor signaling results in the activation of the transcription factor NF-κB, such as ligands for IL-1R1, TLRs, NLRs, and the cytokine receptors TNFR1 and TNFR2. The activation of NF-κB is critical for upregulating the transcription of both pro-IL-1β and NLRP3, as pro-IL-1β is not constitutively expressed and basal levels of NLRP3 are inadequate for efficient inflammasome formation. In contrast, transcriptional modulation is not required to license the inflammasome components, ASC and procaspase-1 [65].

Signal 2: activation

The second step in activation of the NLRP3 inflammasome is provided by one of a diverse group of agonists that triggers the specific activation of NLRP3, assembly of the inflammasome complex, and finally culminates in the activation of caspase-1. The activators include both exogenous and endogenous molecules such as crystalline molecules, that require phagocytosis for activation, ATP acting through its cell surface receptor P2X7R, lysosome-derived molecules and reactive oxygen species [ROS]

and pore-forming toxins such as nigericin [65] [Figure 11].

Recent results show, also, the NLRP3 inflammasome activation in the microglia by Aβ-triggers neuroinflammation [66,67] [Figure 11]. Within a short period of activation of the innate immune system, the acute inflammatory response is started by immune cells enabling secretion of various cytokines and chemokines in order to recruit immune cells to the site of infection. Neutrophils are the first to adhere to endothelial cells, and they begin to migrate across the vascular wall at the site of infection to engulf the invading pathogens and also secrete vasoactive and pro-inflammatory mediators [68]. Most of the early vascular changes observed in acute inflammation are due to inflammatory mediators that are released by inflammatory cells at the site of injury. These mediators, including histamine, platelet-activating factors [PAFs], bradykinin, and thrombin, increases vascular permeability followed by fluid accumulation [edema] and leukocyte extravasation. However, if the innate immune system exceeds its capacity or its defensive function becomes limited, it engages the adaptive immune system, activating specific T and B cells for pathogen clearance [69]. If this process is prolonged or inefficient, it progresses to the chronic state of inflammation that is associated with many diseases such as AD, Multiple Sclerosis, Chron's Disease.

1.8.2 NLRP1-INFLAMMASOME

NLRP1 was the first inflammasome to be characterized; however, studies of this inflammasome have been hampered by issues such as the uncertainty as to the identity of a specific ligand, the complexity of its domain structure, possible enzymatic modification and the substantial divergence between human and mouse NLRP1. Human NLRP1 is unique among inflammasome-forming NLRs as it possesses two protein–protein

interaction domains: an N-terminal PYD and a C-terminal CARD. Although ASC is not part of this inflammasome, it might enhance NLRP1 activation. Mice have three *Nlrp1* genes [*Nlrp1a*, *Nlrp1b* and *Nlrp1c*]. Instead of a PYD, murine NLRP1 proteins have a unique N-terminal domain for which there is no known homologue in humans. A distinct feature of NLRP1 in both humans and mice is the presence of a function-to-find domain [FIIND] at the C-terminal end. The activation of human NLRP1 seems to require auto-proteolysis at the FIIND. Interestingly, a naturally occurring splice variant of human NLRP1 fails to undergo auto-proteolysis at the FIIND region, which prevents subsequent maturation of IL-1β, whereas a single-nucleotide polymorphism [SNP] that alters this region enhances processing and activation of NLRP1 [1].

1.9 PERIPHERAL IMMUNE RESPONSE

Apart from brain-specific changes, abnormalities attributed to AD seen in many peripheral cell types such as erythrocytes, lymphocytes, platelets and fibroblasts [84,85,86] have led to the speculation that AD might be a systemic disorder, with the most prominent pathology in the cognitive functions of the central nervous system [CNS]. Indeed, a substantial body of data exists indicating that biochemical changes of brain cells in various neurobiological diseases with systemic manifestation are reflected in peripheral tissues, such as fibroblasts and leukocytes [70,71]. In the course of surveying a wide variety of these mirror changes, previous reports have proved that fibroblasts and lymphocytes can serve as models and diagnostic markers of several neurodegenerative disorders [71,72,73]. These peripheral tissues actually offer many advantages for the study of various patho-biochemical alterations in AD. One of the main risk factors in AD is age, and aging itself is a phenomenon which causes changes in the

immune system and in inflammatory parameters [74]. The term “inflammaging” characterizes a widely accepted paradigm that aging is accompanied by a low-grade chronic up-regulation of certain pro-inflammatory responses. This systemic immune alteration is evidenced by increased serum levels of pro-inflammatory cytokines, including IL-1β and TNFα, and other inflammatory biomarkers, such as coagulation factors [75]. This abnormal inflammatory state is thought to predispose older adults to several neuro-psychiatric disorders, including depression, Parkinson's disease [PD] and AD [92,93,94]. Aβ itself has the ability to alter the production of pro-inflammatory cytokines [95,96]; in turn, IL1β, particularly in combination with interferon [IFN]-γ, induces an over-expression of amyloid-precursor-protein [APP], with subsequent accumulation and aggregation of Aβ [79,80].

About 500 ml of cerebrospinal fluid are taken up every day in the blood and numerous evidence suggests that dysfunction of the BBB, typical of several neurodegenerative diseases that may affect the exchange between fluids protein [81]. As result, the spread of the metabolites present in the CSF, the peripheral system could reflect a neurodegeneration. In fact, changes were seen at the level of peripheral cells such as PBMCs [Peripheral Blood Mononuclear Cells] in patients with AD [99] and with dementia from multiple heart attacks [100]. Identifying the biochemical changes in the blood components of patients might allow to identify the "fingerprints" devices that reflect degenerative processes in the CNS [82].

As already mentioned, inflammation plays an important role in AD pathology and T cells frequently fall and accumulate in the Alzheimer brain. However, T cells may not need to infiltrate the brain to exert its effects [102]. In humans, native T cells typically express CD45RA ectopically. However, when these cells are in the context of antigen stimulation, become activated and then go into a resting phase, a change occurs in RA

isoform of CD45 RO [84]. This change of isoform can be taken as a marker of human memory T cell. Memory T cell is an important phenomenon because it allows a much faster response to reconnect with the native antigen cell that responds to it first. It has been shown that peripheral T cells in patients with Alzheimer present CD45RA change to RO isoform [85] and reduction in ectopic expression of receptors for IFNγ [86,87]. It is also difficult to understand how peripheral T cells may exert their effects without infiltrating the CNS. One possibility is that these activation could lead the secretion of cytokines such as IFNγ, and these proinflammatory cytokines enter the CNS and activate microglial and/or astrocytes. In another scenario, activated peripheral T cells can promote activation of myeloid cells such as monocytes, macrophages and/or dendritic cells that secrete proinflammatory cytokines such as TNFα, IL1β and IL6. Thus, there is evidence of elevated levels of IL6 in monocytes from patients with Alzheimer's [88]. These cytokines can promote brain inflammation and gliosis, once they have crossed the blood-brain barrier, and can exacerbate the inflammatory responses of the brain to amyloid plaques [89].

AIM

There is extensive evidence that accumulation of mononuclear phagocytes including microglial cells, monocytes, and macrophages at sites of β-amyloid deposition in the brain is an important pathological feature of Alzheimer's disease [AD] and related animal models, and the concentration of these cells clustered around Aβ deposits is several folds higher than in neighbouring areas of the brain [90-94]. Microglial cells phagocyte pathogens and toxins, but they can also be activated to produce inflammatory cytokines, chemokines, and neurotoxins [95]. Over the past decade, the roles of microglial cells in AD have begun to be clarified, and was proposed that these cells play a dichotomous role in the pathogenesis of AD [93, 95–100]. Microglial cells are able to clear soluble and fibrillar Aβ, but continued interactions of these cells with Aβ can lead to an inflammatory response resulting in neurotoxicity. Inflammasomes are inducible high molecular weight protein complexes that are involved in many inflammatory pathological processes. Recently, Aβ was found to activate the NLRP3 inflammasome in microglial cells in vitro and in vivo thereby defining a novel pathway that could lead to progression of AD [101–103]. In this work, we review possible LPS and Aβ-induced inflammasome activation in the periphery and discuss how this could contribute to the pathogenesis of AD. Also we investigated about inflammasome role in primary microglia Aβ- uptake .

The project is according to the following points:

- Analyze expression profile of genes involved in the pathway of the inflammasome and NLRP3 protein expression on PBMCs of subjects with different diagnosis of AD and healthy subjects.

- Analyze the level of pro-inflammatory cytokines produced downstream of inflammasome activation pathway on patients' PBMCs with Alzheimer's disease, mild cognitive impairment and healthy subjects.

- Analyze microglia Aβ-clearance in response to the production of inflammatory cytokines in WT, APP or NLRP3 and ASC knockout mice.

2. MATERIALS AND METHODS

2.1 PATIENTS AND CONTROLS

One-hundred elderly Italian individuals were enrolled in the study: 20 individuals with a diagnosis of mild cognitive impairment [MCI] [MMSE score>24], 21 patients with moderate Alzheimer's disease [MMSE score 19-23], 19 patients with severe Alzheimer's disease [MMSE score<19] and 40 age-and sex-matched healthy controls [HC] were enrolled in the study. The clinical diagnosis of Alzheimer's disease was performed according to the NINCDS-ADRDA work group criteria [104] and the DMS IV-R [American Psychiatric Association,1994] [105]. All subjects underwent complete medical and neurological evaluation, as well as laboratory analysis, and CT scan or MRI. Additional investigations [e.g., EEG, SPECT scan, CSF examination, etc.] were performed in some cases to exclude reversible causes of dementia. Neuropsychological evaluation and psychometric assessment were performed with a neuropsychological battery that included the Mini- Mental State Examination [MMSE] [106]. Digit Span Forward and Backward, Logical Memory and Paired Associated Words Tests, Token Test, supra Span Corsi Block Tapping Test, Verbal Fluency Tasks, Raven Colored Matrices, the Rey Complex Figure, Clinical Dementia Rating Scale [CDR] [107], and the Hachinski Ischemic Scale. Individuals with a diagnosis of MCI were selected among subjects seen at our Memory Disorders Outpatients Service for the diagnostic evaluation of memory complaints without difficulties in daily activities. MCI diagnosis was based on Petersen's criteria [108] as follows: 1] reported cognitive decline, 2] impaired cognitive function, 3] essentially normal functional activities, and 4] exclusion of dementia. The healthy controls were selected according to the SENIEUR protocol for immuno-gerontological studies of European

Community's Control Action Program on Aging [109,110] and were unrelated healthy spouses of Alzheimer's disease and MCI patients. The cognitive status of HC was assessed by administration of MMSE [score for inclusion as normal control subjects > 28]. Written consent was obtained and ethical approval was granted by the Ethics Committee of the Don C Gnocchi Foundation in Milano, Italy.

2.2 BLOOD SAMPLE COLLECTION AND CELL SEPARATION

Whole blood was collected by venopuncture in Vacutainer tubes containing ethylenediaminetetraacetic acid [EDTA] [Becton Dickinson & Co., Rutherford, NJ, USA]. Peripheral blood mononuclear cells [PBMCs], consisting of T lymphocytes, B lymphocytes, monocytes and natural killer cells, were separated on lymphocyte separation medium [Fycoll-Hypaque, Organon Teknika Corp., Durham, NC, USA] and washed twice in PBS; viable leukocytes were determined by trypan blue exclusion.

2.3 CELL LINE

Microglia are key glial elements of the central nervous system [CNS] and considered the major immunocompetent cells in the brain. Microglia, along with neurons, have an important role in brains function and inflammatory neuropathology especially in all neurodegenerative disorders. They have been used in the different neurological disease models over the years. Microglial and neuronal primary cultures are being used widely to understand the diseases mechanisms in neurodegenerative research area. There are several microglia and neuronal like cell-lines are available to use as a preliminary source of information. These cell-lines have been shown high similarities in phenotype/morphology, cytokine profile and most importantly genomic resemblance.

2.4 ANIMAL

Wild type mice [Jackson Lab] and NLRP3, ASC knockout animals [kind gift from AG Latz] were used in the current experiment. Animal care and handling was performed according to the declaration of Helsinki and approved by the local ethical committee.

2.5 CELL CULTURES

- PBMC [$1x10^6$/ml] were cultured in RPMI 1640 supplemented with 10% human serum, 2 mM L- glutamine, and 1% penicillin [Invitrogen Ltd, Paisley, UK] alone or were primed with 2 µg/ml LPS for 2 hours [Sigma-Aldrich] before stimulation with 10 µg/ml of 1-42 amyloid-beta peptide soluble oligomer [$A\beta_{42}$] [Sigma-Aldrich, St. Luis, MO, USA] for either 4 [PCR], or 24 hours [FACS, confocal microscopy and ELISA] at 37°C in a humidified 5% CO_2 atmosphere.

- Primary Microglial Cell Culture were prepared from postnatal day 1 mouse pups were stripped of meninges. Primary microglia was prepared from wild type, NLRP3 and ASC knockout. Whole brains were dissected in HANKs [Hank's Balanced Salt Solution] salt solution [Biochrom, L2015], washed 3 times with HANK solution and trypsin was added and incubated at 37°C about 10 minutes. After mechanical disruption with a 10 mL sarstadt pipette the solution was centrifuged at 4°C at the speed of 1200 rpm for 10 minutes. Remaining brain pellet was mixed in the microglia complete Medium: DMEM [Dulbecco's Modified Eagle Medium] plus 10% FCS [Fetal Calf serum] and 5% Streptomycin/Penicillin. For each two brains one T/75 bottles was used and placed in unified medium containing

5% CO_2 at 37°C and mixed cultures were grown for almost 12-14 days until harvested. By tapping the flasks and collecting the microglia-enriched containing medium. Microglia were pelleted by centrifugation [1000 rpm, 5 min], resuspended in complete DMEM-medium and were plated 6-well plates at $1x10^6$ cells/well for the experiments that follow;

I experiment: cells were plated unstimulated and stimulated with LPS [200 ng/ml] for 2, 4, 6, 12 and 24 hours; cells were washed 2 times in PBS and supernatants were collected for ELISA analysis.
II experiment: cells were un-treated and stimulated with LPS [200 ng/ml] for 1 hour, washed 2 times in PBS and fresh medium was added; after 1, 5 and 11 hours the supernatant were collected.
III experiment: un-treatment condition and LPS [200 ng/ml] alone for 24 hours or plus ATP [1 uM] for 30 minutes or Nigericin [1.34 uM] for 2 hours treatment. Supernatants and cells were collected for ELISA and Western Blot analysis.
For Aβ- phagocytosis flow cytometry analysis cells were primed in the same condition with LPS, ATP and Nigericin; then the cells were washed two times in PBS and was added fresh conditioned medium with Beta-Amyloid [1-42] -Lys [Biotin], green fluorescent probe [FAM]-labeled [500 nM] for 4 hours.
Plated cells were grown in supplemented media 24 hours and then the medium was replaced with serum-free DMEM overnight.

2.6 RNA EXTRACTION AND REVERSE TRANSCRIPTION

RNA was extracted from un-stimulated, LPS-stimulated and LPS-primed and $A\beta_{1-42}$-stimulated-PBMC. RNA was extracted from cultured PBMCs by using the acid guanidium thiocyanate–phenol–chloroform method. The RNA was dissolved in RNase-free water and purified from genomic DNA

with RNase-free DNase [RQ1 DNase; Promega, Madison, WI]. One microgram of RNA was reverse transcribed into first-strand cDNA in a 20 µl final volume containing 1 µM random hexanucleotide primers, 1 µM oligo dT and 200 U Moloney murine leukemia virus reverse transcriptase [Clontech, Palo Alto, CA]. cDNA were evaluated for GAPDH expression by Real Time PCR to test the quality of RNA.

2.7 INFLAMMASOME SIGNALING PATHWAY

For Gene expression of inflammasome components and signaling pathways has been used the human Inflammasomes RT^2 Profiler PCR Array [Qiagen PAHS-097Z], which includes a set of optimized primer assays allowing the detection of mRNA transcripts of 84 genes related to the inflammasomes and their functions [Tab4], as well as five housekeeping genes, in a 96-well plate by real-time PCR. Individual specimens were pooled according to MMSE scores to create four groups of different disease states. Eight pooled samples, corresponding to treated and untreated cells from severe AD, moderate AD [MILD], MCI and HC, were subjected to the PCR arrays. Data was analyzed by the comparative Ct method using GAPDH as the reference gene. Results are expressed as the fold changes between each AD/MCI/MILD pool and HC treated and untreated cells from each disease state. Heat maps were generated and genes hierarchically clustered by Euclidean distance and single linkage using TIGR MultiExperiment Viewer [MeV] v4.9 [109].

2.8 REAL TIME QUANTITATIVE REVERSE TRANSCRIPTION

Real Time quantitative Reverse Transcription PCR [RQPCR] was performed on a ABI Prism 7000 instrument [PE Applied Biosystems, Foster City, CA, USA] with gene specific primers and the SybrGreen chemistry to

confirm the gene expression changes observed by arrays. All primers, casp1-5-8, IL1β, IL18, IL33, IL37, NLRP1 and NLRP3 used were cDNA specific and were purchased from Qiagen [Venlo, PB]. Amplification of specific PCR products was detected using the RT2 SYBR Green Fluor with a 25 µl final volume of 12.5 µl RT2 qPCR Mastermix [Qiagen] 10.5 µl H2O, 1.0 µl of either diluted template and 1.0 µl RT2 qPCR Primer Assay. Results were expressed as ΔΔCt and presented as ratios between the target gene and the GAPDH housekeeping mRNA.

2.9 FLOW CYTOMETRY IMMUNOFLUORESCENT STAINING

Unstimulated and LPS-primed and $A\beta_{1\text{-}42}$-stimulated-PBMC were stained with anti-CD14-PC7 [clone RMO52, isotype mouse IgG_{2a}, Beckman Coulter] mAb for 30 minutes at 4°. Cells were then washed, treated with FIX and PERM Cell kit [eBioscience, San Diego, CA, USA], and stained with anti- NLRP3-PE [clone 768319; isotype rat IgG_{2a} R&D], -NLRP1-APC [isotype rabbit polyclonal IgG Proteintech, Chicago, IL, USA], Caspase 5-FITC [isotype rabbit polyclonal; LSBioSciences,Inc, Seattle, WA USA], -ASC-FITC [clone HASC-71; isotype mouseIgG_{1k}, eBioLegend], IL-1β FITC [clone 8516; isotype mouse IgG_1; R&D] -IL33-APC [clone 39412 isotype rat IgG_{2B} R&D], or IL37-PE [clone 6A6, isotype mouse IgG_{1k} LSBioSciences, Inc] mAb for 30 minutes at 4°. FLICA staining of active caspase 1 and caspase 8 was performed using the green fluorescent probe FAM-YVAD-FMK caspase 1 and FAM-LETD-FMK caspase 8 [AM-FLICA, Immunochemistry, Bloomington, IN, USA]. NLRP1, Caspase5 and ASC were conjugated using the Lightning-Link APC or FITC conjugation kit [Innova Biosciences, Cambridge, UK].

For phagocytosis assay, stimulated and control mouse primary microglia WT, $NLRP3^{-/-}$ and $ASC^{-/-}$ cells were stained with 7-ADD mAB for

vitability. Cells were finally analyzed using a Beckman-Coulter GALLIOS flow cytometer equipped with a single 15 mW argon ion laser operating at 488 nm and interfaced with CXP Software 2.1. Two-hundred- thousand cells were acquired and gated on CD14 expression and side scatter properties. Isotype control or single fluorochrome-stained preparations were used for color compensation.

2.10 ELISA IMMUNOASSAYS

Human IL1β, IL18 and IL33 concentration was determined in unstimulated and in LPS-primed and $A\beta_{1-42}$-stimulated-PBMC supernatants. Levels of murine TNFα and IL-1β were determined in supernatant of LPS stimulated Primary Microglia cells and control. 96-well plates were coated overnight with monoclonal anti-human IL1β, IL18 or IL33 and anti-mouse TNFα or IL1β capture antibody [R&D Systems], washed with phosphate-buffered saline [PBS] containing 0.05% Tween-20 and blocked with PBS containing 1% bovine serum albumin [BSA], 5% sucrose and 0.05% NaN_3 following by a wash step. Successive treatments with washing in between were done with samples or standards, biotinylated polyclonal anti-human IL1β, IL18 or IL33 and anti-mouse TNFα or IL1β detection anti- body [R&D Systems] in 20 mM Tris with 150 mM NaCl and 0.1% BSA, streptavidin–horseradish peroxidase [HRP] conjugate, and equal volumes of HRP substrates 3,3',5,5'-tetramethylbenzidine and hydrogen peroxide. The reaction was stopped by the addition of 1% H_2SO_4 solution. The optical density of each sample was analyzed at 450 nm with a reference reading at 630 nm using a SpectraMax 340 absorbance plate reader [Molecular Devices, Union City, CA]. The cytokines concentration in the experimental samples was calculated from a mouse TNFα or IL1β and human IL1β standard curve of 15–2000 pg/ml; from human IL18 and IL33 standard curve of 15-1000

pg/ml. When necessary, samples were diluted to fall within the standard curve. All cytokine levels are reported as pg/mL and are based on the output from 10^6 cells in each well.

2.11 CONFOCAL MICROSCOPY ANALYSIS

Un-stimulated or LPS-primed or $A\beta_{1-42}$- or LPS-primed an $A\beta_{1-42}$ stimulated-PBMC were cultured on chamber slide [Lab Tek Nalge Nunc Intern. Naperville IL USA] for 24 h at 37°C. After washing off non adhering PBMC, monocytes grown on chamber slide were fixed in 4% paraformaldehyde in PBS for 15 min and treated for 1 hour at room temperature with FLICA staining of active caspase 1 or caspase 8 using green fluorescent FAM-YVAD-FMK probes, following the procedures suggested by the manufacturer [AM-FLICA]. Cells were then treated with FIX and PERM Cell kit [eBioscience], and stained with PE or APC or FITC conjugated -mAbs specific for NLRP3 or NLRP1 and ASC or caspase 5 for 24 hours at 4°C. Finally cells were fixed with paraformaldehyde 1% for 15 minutes, washed and mounted on slides using the Vectashield Mounting Medium [Vector Laboratories, Inc, Burlingame, CA, USA]. Fluorescent images were acquired on a Leica TCS DMRE spectral laser-scanning confocal microscope [Leica Microsystems, Wetzlar, Germany] with the appropriate filters and laser [488, 633] and a 63X objective lens. Image analysis was performed using the Leica Confocal Software and co-localization index with ImageJ Software. Co-localization indexes were calculated using the plug in JACoP [Justo Another Co-localization Plugin] [110]. The summarized colocalization efficiency data was expressed as Pearson correlation coefficient [PCC] as previously described [111,112,113]. Briefly this coefficient measure the significance of true colocalization. The significance test is derived to evaluate the probability that the measured value of r from

the two colors is significantly greater than values of r that would be calculated if there was only random overlap. This test is performed by randomly scrambling the blocks of pixels [instead of individual pixels, because each pixel's intensity is correlated with its neighboring pixels] in one image, and then measuring the correlation of this image with the other [unscrambled] image. The test produce values in the range −1 + 1, 0 indicating that there is no discernable correlation and −1 and +1 meaning strong negative and positive correlations, respectively.

2.12 WESTERN BLOTTING

Unstimulated and LPS-primed and $A\beta_{1-42}$ stimulated-PBMC were washed and lysed with M-PER Mammalian Protein Extraction Reagent with phosphatase and protease inhibitors [10 µl/ml, Halt™ Protease and Phosphatase Inhibitor Cocktail, [Thermo Scientific Inc], Rockford, IL, USA]. After protein estimation using the Bradford Assay reagent [Bio-Rad] 20 µg of cell lysate was resolved by electrophoresis on a 10% SDS-polyacrylamide gel under reducing conditions. Proteins were then transferred to PVDF membrane by electrophoretic blotting and blocked for 1 hour in blocking buffer [Blocker BLOTTO Blocking Buffer, Thermo Scientific]. Membranes were then incubated overnight with sheep anti-recombinant NALP3 [AF6789, R&D Systems] or for 1 hour with sheep anti beta-actin antibody [AF4000, R&D Systems], then diluted 1:1000 in blocking buffer followed by HRP-conjugated anti-sheep IgG [HAF016, R&D Systems]. Bound antibodies were visualized with a chemiluminescence development reagent [LiteAblot TURBO, Euroclone, Italy] according to the manufacturer's instructions. Bands were imaged in a Gel-Doc Cabinet and the density of the image was measured and normalized with beta-actin.

Mouse Primary Microglia: supernatants and extracts of control and treated

primary microglia cells were used. Cells were lysed with RIPA buffer [20 mM Tris pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% deoxycholic acid, and 0.1% SDS] supplemented with protease inhibitor cocktail [Sigma]. Collected lysates were centrifuged at 9000 ×g to remove cell debris and the supernatants were mixed 1:1 with Laemmli SDS sample buffer [Bio-Rad] containing 5% β-mercaptoethanol. Samples were denatured [95°C, 5 minutes] and separated on 15% Tris-HCl gels [Ready Gel, Bio-Rad] under denaturing conditions [25 mM Tris, 192 mM glycine, 0.1% SDS at pH 7.8] using a Mini Protean 3 Cell [Bio-Rad]. Gels were transferred to PVDF membrane [Milipore] in transfer buffer containing 25 mM Tris base, 192 mM glycine, and 10% methanol at pH 8.3 using a Tank VEP-2 electroblotting system [Owl Separation Systems]. Following protein transfer, the membrane was blocked for 1 hour at room temperature with TBST containing 5% BSA. Cell extracts supernatants were analyzed by immunoblot using goat anti-IL1β [R&D], mouse anti-NLRP3 [Cryo-2], rabbit anti-ASC [AL177], mouse anti-Caspase-1 [Casper-1] [all three from Adipogen], and mouse anti-alpha-tubulin [Abcam]. All first antibody were diluted into 1:1000 final concentration of TBST and 3% BSA and incubated over-night at 4°C; Secondary antibodies were used at 1:20000 as final concentration, incubated for 1 hour at room temperature. The membrane was washed 3 times 1X TBST for 5-10 minutes and developed with chemiluminescence agent ImmobilionTM [Milipore]. The protein bands were analyzed using analysis tools provided by Quantity One software. The optical intensity of the desired sized band was normalized with α- tubulin or β -actina from the respective well.

2.13 IMMUNOCYTOCHEMISTRY

The immunostaining experiment was performed in 24 well plates. 450.000 microglial cells/condition were cultured on poly-L-lysine coated coverslips and fixed with paraformaldehyde [4% in PBS] after LPS alone, LPS primed and Aβ–FAM-labeling stimulation and unstimulated condition. Cells were incubated overnight at 4°C with rabbit anti-Iba1 [1:100, Biocare Medical]. After washing with PBS, cells were incubated with anti-rabbit Alexa Fluor 594 secondary antibodies for 1-2 hours. Cells were then washed with PBS and mounted with DAPI-Fluoromount G [SouthernBiotech, USA].The imaging was performed with the help of fluorescence microscope from Olympus BX-61. For enhanced and congruous focusing, z-stack correction was employed. During every procedure, six images along z-axis spaced at distance of 1.6 µm were captured and then best suited image of the various channels were merged. Cell P Olympus software was utilized for imaging purpose.

2.14 STATISTICAL ANALYSES

Quantitative data were not normally distributed [Shapiro–Wilk test] and are thus summarized as median and Interquartile Range [IQR] [25° and 75° percentile]. Comparisons between groups were analyzed used a Kruskal-Wallis ANOVA for each variable. Comparisons among the different groups were made using a 2-tailed Mann-Whitney *U* test performed for independent samples. Data analysis was performed using the MedCalc statistical package [MedCalc Software bvba, Mariakerke, Belgium].

3. RESULTS

3.1 INFLAMMASOME PROTEINS ARE UP REGULATED IN LPS-PRIMED AND $A\beta_{1-42}$-STIMULATED-MONOCYTES OF AD PATIENTS

mRNA expression of 84 genes involved in the assembly of the inflammosome, its activation, and its down-stream signalling was quantified by qPCR in all patients and controls. Data obtained in LPS-primed and $A\beta_{1-42}$-stimulated-monocytes showed the presence in MILD and severe AD, as well as in MCI, of a significant up-regulation involving genes that codify for the proteins that form the inflammasome. Notably, though, whereas all the proteins necessary for the assembly of a functional inflammasome were up regulated in AD, NLRP 1 and 3 and caspase 8 alone were increased in MCI. To summarize: 1] the expression of NLRP1, NLRP3 and caspase 8 was greatly increased [nFold>10] in all groups of patients compared to HC, with no detectable differences being observed between individuals with a diagnosis of AD or MCI; 2] ASC [PYCARD], caspase 1 and caspase 5 expression was increased [nFold ≥10] in AD but not in MCI patients. Finally, minor differences were seen between patients and controls in the level of expression of these genes in monocytes-stimulated with either LPS or $A\beta_{1-42}$ alone, both stimuli, neverthertheless induced IL1β and IL6 up-regulation [nFold>10] in HC alone [Figure 12].

RT-PCR performed on each individual specimen was used to verify the expression of NLRP1, NLRP3, caspase 1, caspase 5 and caspase 8 in LPS and $A\beta_{1-42}$ –stimulated monocytes of patients and HC. Results of mRNA expression confirmed that: 1] NLRP1, NLRP3 and caspase 8 are significantly increased in AD and MCI individuals compared to HC [$p<0.05$], with the highest values observed in severe AD, and 2] caspase 1 and

caspase 5 are significantly increased in AD compared to MCI and HC [Figure 13A].

3.2 UP-REGULATION OF INFLAMMASOME-RELATED CYTOKINES IN LPS-PRIMED AND $A\beta_{1-42-}$ STIMULATED-MONOCYTES OF AD PATIENTS

Stimulation of monocytes with either LPS or $A\beta_{\mathbf{1-42}}$-alone resulted in the moderate increase of IL1β and IL6 expression; notably this was not sufficient to trigger inflammasome assembly as no differences were detect in these conditions either in NLRP3 or IL18 mRNA [Figure 1]. Once cells were activated with LPS and $A\beta_{\mathbf{1-42}}$, nevertheless, both IL1β [nFold>10] and IL18 [nFold ≥10] mRNA were significantly increased in severe and MILD AD compared to MCI and HC. Results obtained by single-PCR confirmed that in these experimental conditions IL1β and IL18 mRNA [$p<0.05$ *vs.* all other groups] is significantly increased in severe and MILD AD with the highest values in severe AD [Figure 13B].

IL33 and IL37, two relatively novel cytokines that are members of the IL-1 family, were analyzed as well in the study. We found that IL33 gene expression was greatly increased in MCI [nFold>10] compared to AD and HC; no significant differences, on the other hand, were observed in IL37 gene expression in any of the groups analyzed [Figure 1]. Single RT-PCR confirmed these results [Figure 13C].

3.3 NLRP3 PROTEIN IS SIGNIFICANTLY AUGMENTED IN LPS-PRIMED AND $A\beta_{1-42}$ STIMULATED- MONOCYTES OF AD AND MCI PATIENTS

The expression of NLRP3, the best-characterized protein within the inflammasome complex was investigated next by Western Blot analyses in LPS-primed and $A\beta_{\mathbf{1-42}}$-stimulated-PBMC of all patients and controls. Different NALP3 isoforms are known: the long form [118 KDa] is prevalent in cell lines [THP1 or Jurkat] while the short one [NLRP3s 75 KDa] is seen in primary immune cells [114]. Results of analyses performed on whole-cell lysates confirmed that NLRP3 expression is significantly increased in AD and MCI individuals with the highest amounts of protein being detected in patients with a diagnosis of severe AD [Figure 14].

3.4 LPS-PRIMED AND $A\beta_{1-42}$ STIMULATED MONOCYTES THAT EXPRESS INFLAMMASOME PROTEINS ARE SIGNIFICANTLY AUGMENTED IN AD

Peripheral CD14+ monocytes that express NLRP3 or co-express NLRP3 and ASC, NLRP3 and caspase 1, or NLRP3 and caspase 8 were augmented in LPS-primed and $A\beta_{\mathbf{1-42}}$-stimulated-cells of both groups of AD patients compared to MCI individuals and HC. The Fluorochrome Inhibitor of Caspases [FLICA] kit was used to analyze the presence in cells of active caspases; in this method, once inside the cell, the FLICA inhibitor probe binds covalently to active caspases [p20] alone. Staining of active caspase 1 and caspase 8 inhibitors was performed using the green fluorescent probe FAM-YVAD-FMK and FAM-LETD-FMK respectively. Results showed that 1] CD14+/NLRP3+, CD14+/NLRP3+/caspase1+, and CD14+/NLRP3+/Caspase8+ immune cells were significantly increased in

severe AD patients alone compared to MCI and HC [p<0.05] and 2] CD14+/NLRP3+/ASC+, cells were augmented both in severe and MILD AD compared to MCI and HC [MCI and HC *vs*. moderate AD p<0.05; *vs.* severe AD p<0.01] [Figure 15A].

Finally, LPS-primed and $A\beta_{\mathbf{1\text{-}42}}$ stimulated NLRP1-expressing CD14 cells were significantly increased as well in both groups of AD patients compared to MCI and HC [p<0.05] [Figure 15B]. These results confirm those obtained in PCR analyses and indicate that, even if some inflammasome components are up regulated in MCI, fully functional inflammasomes are not assembled in this situation.

3.5 CO-LOCALIZATION OF INFLAMMASOME PROTEINS IN LPS-PRIMED AND $A\beta_{1\text{-}42}$ STIMULATED MONOCYTES BY CONFOCAL MICROSCOPY ANALYSES

Aggregation and activation of inflammasome complexes was further analyzed by evaluating the co-localization of NLRP3 and NLRP1 with ASC, caspase 1 or caspase 8 by confocal microscopy. Co-localization efficiency was calculated using the Pearson co-localization coefficient [PCC]**.** Results showed a clear co-localization of NLRP3 with ASC, caspase 1 and caspase 8 in $A\beta_{\mathbf{1\text{-}42}}{}_{42\text{-}}$stimulated and LPS-primed**-** monocytes of AD patients alone; no co-localization could be detected in untreated cells [data not shown]. In particular: 1] NLRP3/ASC NLRP1/ASC and NLRP3/caspase1 co-localization was increased in both groups of AD patients compared to HC and MCI; 2], NLRP3/caspase8 and NLRP1/caspase5 co-localization was increased in severe AD alone; and 3] NLRP1/caspase1 co-localization was seen only in cells of MILD AD patients. In all cases, with the exception of

the NLRP1-caspase 1 complex, the highest PCC values were detected in cells of patients with severe AD disease [Figure 16 and Table I].

3.6 INFLAMMASOME-RELATED CYTOKINES PRODUCTION IN LPS-PRIMED AND $A\beta_{1-42}$ STIMULATED - MONOCYTES OF AD PATIENTS

Aggregation of the inflammasome results in the down-stream production of pro-inflammatory cytokines. Because FACS and confocal microscopy data indicated that the different subunits that compose the inflammasome do aggregate in LPS-primed and $A\beta_{1-42}$-stimulated- monocytes of AD patients, and PCR analyses showed that mRNA for these cytokines is increased in monocytes of AD patients, IL1β, IL-18, IL33 and IL37 were measured next. Results showed that the concentration of IL1β, IL-18 in supernatants was significantly increased in both groups of AD patients compared to MCI and HC [IL1β $p<0.05$; IL18 $p<0.01$] [Figures 17A and 17B]. Whereas lack of proper reagents prevented IL18-expressing cells to be analyzed by FACS, the percentage of CD14+/IL1β+ cells was significantly increased as well in MILD and severe AD compared to MCI and HC [$p<0.05$] [Figure 17A]. The fact that these cytokines were not increased in MCI is not surprising considering that in this condition NLRPs but not ASC expression was augmented, thus preventing the assembly of a functional inflammasome; these data confirm the findings obtained with FACS and confocal analyses.
IL33 could not be quantified in supernatants [data not shown], probably because the inflammatory mature form of this cytokines is not cleaved and secreted. Results obtained by flow-cytometry, nevertheless showed that CD14+/IL33+ cells were increased in MCI and in MILD AD, with the lowest percentages of these cells seen in severe AD [$p<0.01$] [Figure 17C]. Notably this cytokine was shown to have a multifaceted protective role

against Alzheimer's disease and to negatively modulate NF-kB activity, dampening inflammation. Finally, CD14+/IL37+ cells were marginally increased in AD compared to MCI and HC [Figure 17D].

3.7 NLRP3 AND ASC REGULATE CYTOKINES PRODUCTION BY MICROGLIA UPON LPS EXPOSURE

Lipopolysaccharide [LPS] can trigger series of inflammatory reactions in phagocytes such as macrophages. LPS is a glycolipid derived from the membrane surface of gram negative bacteria [endotoxin]. In *in vitro* experiments using cultured microglia, LPS has generally been used for cell activation. Although the time course of LPS-induced activation of microglia is of interest, data reported in literature are contrasting. Thus in the first experiment we quantified cytokines production in LPS-stimulate cells for different times. As expected, first TNFα was release after 2h, second IL1β within 12 h increasing both over 24 h [Figure 18A]. Noteworthy: NLRP3 and ASC KO microglia displayed significantly attenuated levels of IL1β release in response to LPS; surprisingly TNFα level was less pronounced in NLRP3 KO compare to WT and ASC KO cells [2000pg/ml NLRP3vs 40000pg/ml for WT and ASC] [Figure 18A]. The same results were also obtained with indirect stimulation [Figure 18B]. In contrast IL18 level was not detectable in any condition. Collectively, these results demonstrate that as provided in WT microglia stimulated with TLR4 agonist, as LPS, is evident IL1β production for the alternative inflammasome pathway activation; also in NLRP3 and ASC KO microglia was not assessed any production IL1β confirming inflammasome function in the production of inflammatory cytokines.

3.8 THE NLRP3/ASC/CASPASE1 AXIS REGULATES IL1β PROCESSING IN MOUSE PRIMARY MICROGLIA

In order to better investigate IL1β production in inflammasome-manner activation, we stimulated LPS-primed murine microglia NLRP3 and ASC KO with ATP or Nigericin; as a result of the inflammasome activation IL1β, quantified on ELISA test, was very high in WT cells after Nigericin stimulation[Figure19]. The fact was confirmed by WB; Pro-IL1β was produced in the same way both in WT and KO conditions; in ATP treatment, pro-IL1β and pro-Casp1 increase compare to control, but only after inflammasome activation, cleaved IL1β and Caspase 1 [p20] were release-out of the cells [Figure 20]. Indeed in KO condition, microglia treated with ATP or Nigericin increase the production of inflammatory cytokines but cells can not release them. ASC protein was also evaluated; ASC, expressed constitutively in the cells, was greater produced in NLRP3 KO cells [Figure 20].

3.9 Aβ-PHAGOCYTOSIS AND NEUROINFLAMMATION IN MICROGLIA

Primary murine microglia were evaluated to determine whether proinflammatory environments could also regulate their capacity to stimulate phagocytosis. Microglia were treated with LPS for 24h to induce a pro-inflammatory environment as was demonstrated previously; by flow-cytometry analysis was not detected difference between LPS-primed and FAM-$A\beta_{1-42}$ stimulated cells compare to the control condition [FAM-$A\beta_{1-42}$ alone]. The same results was observed with indirect-LPS stimulated WT and KO cells [Figure 21].

LPS and inflammasome activators, such as ATP or Nigericin, treated- WT

cells were also evaluated in flow cytometry in order to asses if inflammasome activation can modify Aβ -uptake. Although inflammatory cytokines production has been well established, we didn't found significant statistical difference in $A\beta_{1-42}$-phagocytosis [Figure 22].The idea is that the FAM-Aβ can bind cells non-specifically; ICC experiment [Figure 23] on microglia confirmed that in all of the conditions $A\beta_{1-42}$ is in the proximity of the cells, as shown by the green background.

4. DISCUSSION

It is now widely recognized that the "inflammation" of nervous structures, or "neuroinflammation", is a common feature of all neurological disorders. For example, in multiple sclerosis the CNS appears infiltrated by various leukocyte subpopulations [115]. Moreover, even when an inflammatory infiltration is not obvious, as in Parkinson's disease or in Alzheimer's, there is still an intense microglial activation with a consequent increase in CNS of many inflammation mediators [116]. In other tissues, the inflammatory reaction is a natural healing process with respect to a damaging insult and therefore, if a direct and developed properly, a response advantageous for the organism. Not surprisingly, in neuroinflammation it is found beneficial effects, such as neuroprotection, the mobilization of neural precursor cells for repair of the damage, remyelination and axonal regeneration [115,116]. But in the other hand, a vast literature reports that neuroinflammation produces adverse and harmful consequences [117] and shows a close correlation between neuroinflammation and neurodegenerative disease, although it appears unclear whether the onset of inflammatory processes causing, or is the result of neurodegeneration [117].

As the idea of a possible association between Alzheimer's disease and inflammatory process it was initially strongly rejected by the scientific community, there is now a broad convergence in a state that the neuroinflammatory components play a crucial role in Alzheimer's disease [118,119,120]. Several scientific evidence reveal up-regulation of proinflammatory molecules such as cytokines or chemokines and complement factors in brains of AD individuals [121,122] and that activated microglia cells release ROS [123]. Furthermore, the presence of activated microglia, localized near the sites of deposit of amyloid peptide, has been demonstrated in murine models that over-express APP, either on tissue

from patients with AD [124]. It is widely accepted that the presence of damaged neurons, neurofibrillary tangles and deposits of amyloid peptide in the brain of AD are due to establishment of localized and chronic inflammatory processes.

However, these morphological characteristics [plaques, tangles and neuronal injury], the pathognomonic disease, are also found in patients in pre-clinical stages of the disease and yet it is not known when the inflammatory process develops and whether it is a primary or secondary event in the evolution towards the disease and its progression.

To better investigate this inflammatory process characteristic of AD subjects and to improve knowledge which will enable a greater understanding of the mechanisms underlying the disease, in the currently study we demonstrate the possible role of the inflammasomes in the neuroinflammation that accompanies Alzheimer's disease analyzing peripheral immune cells of patients with Alzheimer's disease and mouse primary microglia.

Inflammasomes are intracellular complexes formed by the assembly of multiple subunits that regulate the maturation and the secretion of pro-inflammatory cytokines [1]. At least four different inflammasomes are described; the NLRP3, in particular, is suspected to play a role in the pathogenesis of Alzheimer's disease. Thus, in animal models, NLRP3 upregulation was shown to induce the production of IFN1β by microglia [102]; and, on the other hand, its deficiency results in a decreased deposition of amyloid-β in the APP/PSI animal model of Alzheimer's disease [102,125,126]. Inflammasome complex activation requires the assembly of NLRP3 with ASC to produce active form of caspase1, IL1β and IL18. In our laboratory we have previously shown that peripheral monocytes in Alzheimer's disease are characterized by an inflammatory profile and are involved in the induction of both innate immune responses

via TLR stimulation and of acquired immunity, possibly secondarily to the presentation of Aβ -peptides in an MHC-restricted fashion [127,128]. So, these encouraging results obtained on PBMCs have provided the basis for the development of my research project aimed to extend the knowledge of the inflammatory component in the various stages of the development of AD pathology and population control with cognitive abilities in the normal range. The purpose is to identify the presence of indicator signals, changes of particular inflammatory molecules expression, which might be considered indicators of a possible pathological condition still symptomatologically silent or otherwise of a possible evolution in time to pathology.

In the first part of my research project, all experiments were performed on PBMC of AD, MILD, MCI patients and HC; instead in the second part of the project, developed at the research laboratories of the University of Bonn, the experiments were performed on mouse primary microglia cells.

I'll comment briefly achievements.

The NLRP3 is the best known inflammasome complex; its canonical-activation requires the assembly of NLRP3 with ASC to produce active form of caspase1, IL1β and IL18. We observed that the mRNA expression of these proteins was significantly increased in severe and moderate Alzheimer's disease patients. Inflammasome assembly leads to the production of proinflammatory cytokines; IL-1β and IL18 mRNA and secretion were indeed significantly increased in AD. Interestingly, stimulation of cells with $A\beta_{1-42}$ alone was sufficient to induce IL1β mRNA expression, but LPS priming resulted in a significantly increased production of IL1β and in the generation of IL18 in AD alone, indicating that these experimental conditions result in the assembly of fully functional inflammasomes in cells of AD patients alone. Notably, transfection of human PBMC with siRNA specific for NLRP3 and ASC greatly reduced

IL1β production, confirming that the production of this cytokine is dependent on inflammasome assembly [129]. That IL1β plays a role in the pathogenesis of AD has been repeatedly shown. Thus, this cytokine induces a loss of phagocytic activity by the microglia [129], stimulates the hyperphosphorylation of tau protein [130], affects synaptic plasticity, inhibits long-term potentiation [LTP] and, as a consequence, impairs learning and memory processes [131][132]. IL18 has been involved in AD as well, even if fewer data are available on this cytokine. Thus, increased amounts of IL18 were detected in the brain [133], plasma [134] , and peripheral blood lymphocytes of AD [135], and the expression of the IL-18R complex is greatly augmented in peripheral blood cells of MCI and AD individuals. [136]. Finally, recent data indicate that IL18 directly stimulates Aβ production by human neuron-like cells, suggesting a key role for this cytokine in the pathogenesis of AD [137].

NLRP1 and caspase8 mRNA were up-regulated in cells of severe Alzheimer's disease patients; CD14+/ NLRP3+/caspase8+ cells were also increased in these individuals in whom the colocalization of caspase8 with NLRP3 was also detected. Recent data showed that caspase8 is not only an inducer of cell apoptosis, but, together with the Fas-Associated protein with death domain [FADD], interacts with NLRP3 and is required for caspase1 activation, as well as for IL1β and IL18 secretion [138]. Confocal analyses confirmed that caspase8 is present in the NLRP3 inflammasome complex, where it is involved in the cleavage of pro-caspase1 and IL1β, suggesting a direct role for caspase8 in caspase1 processing [139]. Our data support these findings as well as recent results indicating that caspase8 contributes to both NF-kB–dependent priming and post-translational activation of the NLRP3 inflammasome [140]. Increased NLRP1 and caspase5 mRNA levels were detected as well in cells of

individuals with a diagnosis of severe Alzheimer's disease; these proteins colocalized in CD14 cells, and higher percentages of CD14+/NLRP1+/caspase 5+ immune cells were seen in these patients. These data indicate that the NLRP1 inflammasome complex is activated as well in severe Alzheimer's disease; notably, recent results indicate that SNPs in the NLRP1 gene are associated with Alzheimer's disease [141].
Two other novel cytokines, IL33 and IL37 were analyzed as well in this study; these cytokines are members of the IL1β family and are produced upon activation of the inflammasome. Whereas no significant differences were seen in IL37, mRNA levels of IL33 were increased both in MILD Alzheimer's disease and in MCI compared to the values observed in severe Alzheimer's disease. Notably, percentages of circulating CD14+/IL33+ cells confirmed this observation, as these cells were increased both in MCI and moderate Alzheimer's disease compared to severe Alzheimer's disease and HC. IL33 and IL37 are dual function proteins with both intra and extra-cellular mechanisms of action as, besides being able to bind to their cognate receptors on target cells, they can act intracellular as nuclear factor. IL33 was shown to be reduced in AD brains [142]. This cytokine is believed to have a neuroprotective role secondary to its ability to reduce Aβ peptides secretion [143] and to activate the phagocytosis of Aβ-amyloid peptide by the microglia [144]. Even more recently [145], IL33 was shown to interact intracellularly with the NF-kB p65 subunit. The resulting IL33/NF-kB p65 complex interferes with NF-kB–dependent transcription by impeding p65-mediated transactivation, thus playing a role as a negative modulator of NF-kB activity, with a dampening effect on inflammation. The observation that IL33 is increased in MCI and in MILD Alzheimer's disease can thus be seen as an attempt of the immune response to reduce neuroinflammation.

With regard to microglia, first we examined the cytokine production

implicated in mediating inflammasome activation in response to the largest array of PAMPs including LPS. Interestingly microglia from NLRP3 KO mice produced significantly less IL1β compared to WT cells, implicating a role for this cytoplasmatic receptor in this cytokine processing. We further studied the release of TNFα from microglia, as this is an another LPS-induced mediator of neurotoxicity and inflammation [146, 147]. Similarly, LPS-induced release of TNFα was attenuated in NLRP3 KO cells suggesting that NLPP3 inflammasome, through activation of caspase1 and subsequent IL1 signaling, contribute to the proinflammatory production mediated by microglia. As support of this result, TNFα quantification in caspase1 and IL1R- deficient microglia was much lower compare to the WT, indicating that TNFα depends on autocrine and paracrine effects of IL1β after caspase1 activation [101].

Next we analyzed the downstream inflammasome adaptor ASC. Specifically, ASC contains a C-terminal pyrin domain, which serves to bridge NLRP3 and other NLRs that lack a caspase-recruiting domain [CARD] to caspase1 [147]. Therefore, the absence of ASC would impact additional cytoplasmic NLR sensors besides NLRP3 and potentially identify alternative routes for IL1β activation [148], but in our results IL1β release was very less in ASC KO compared to WT microglia in response to LPS. This finding indicates that both NLRP3 and ASC, that bridge caspase1, are operative in microglia. To determine whether microglial cytokine production was dependent on NLRP3 inflammasome activity, Primary microglia isolated from WT, NLRP3 or ASC KO mice were primed with LPS and stimulated with classical inflammasome activators, such as ATP and Nigericin. Results showed the release of IL1β and caspase1 only after ATP or Nigericin stimulation. In addition we also tested both NLRP3 and ASC knockout microglia cells; the incapacity of these cells to release IL1β confirmed our results. Surprisingly in all cases, these potent microglial

inflammasome activators did not results in detectable IL18 production; in according with Prinz's work [149] IL18 is difficult to detect by western blot, specially in the supernatants.

The role of microglia in the uptake and phagocytosis of amyloid in the brain was first noted in the early 90's by Wisniewski and Frackowiak who utilized electron microscopy to visualize amyloid internalization [150-151]. They postulated that resident microglial cells associated with plaques in the AD brain played a role in the formation of the amyloid fibrils in the brain, but not its phagocytosis [151]. To date is known that sustained exposure to proinflammatory cytokines or some form of damage-associated molecular patterns could account for the attenuated microglial phagocytosis. That hypothesis is further supported by findings demonstrating that genetic deficiency in MRP14 increases microglial phagocytosis of Aβ [152]. Several surface receptors have been shown to mediate phagocytic clearance of Aβ, including TLR2, TLR4, TLR6, CD14 and CD36 [153]. A role for the tyrosine phosphatase CD45 has also been demonstrated [154]. However, the phagocytic uptake of Aβ maybe not only modulated by the receptors present on microglia but may also depend on how the Aβ aggregate is delivered. For example, neuronal exosomes bind Aβ and promote its phagocytic clearance in a phosphatidylserine-dependent way [153]. In this work we demostrated NLRP3 inflammasome importance in inflammatrory cytokines production, but the results obtained for Aβ-phagocytosis do not confirm our hypothesis. Anyway these results are not sufficient to establish whether the inflammation can be modify the Aβ clearance; FAM-Aβ is a protein with 5'-end-isomer [carbossifluorescein], therefore, the steric hindrance does not allow to generate Aβ aggregation.

Definitely further experiments are require for better realize the mechanism of Aβ-phagocytosis in neuroinflammation condition. Several hypotheses have been formed to explain this distinctive feature of microglia surrounding

Aβ plaques. The first initial hypothesis suggested that microglia are exclusively proinflammatory in AD and have a detrimental role in the disease's development [36,155]. Microglial cells lose their protective role, due to the persistent production and accumulation of proinflammatory cytokines within their microenvironment [79]. Under such conditions, microglial cells become hypersensitive and play a detrimental role through the excessive continuous production and secretion of proinflammatory and neurotoxic molecules [79]. Some studies indeed reported the regression of AD pathogenic features following non-steroidal anti-inflammatory drug treatment [156]. Identifying NLRP3 inflammasome as main responsible for the production of inflammatory cytokines both in central and peripheral level it can be useful certainly as future pharmacological target for the treatment of neurodegererative diseases, specially in AD.

5. CONCLUSIONS

NLRP3 up regulation in mice or human microglia cell line or in brain of AD patients has been previously reported [101, 102, 157], these are nevertheless the first data showing NLRP3 and NLRP1 inflammasome activation in Aβ stimulated peripheral monocyte of individuals with a diagnosis of AD. Migration of these cells across the blood-brain barrier would likely be an important factor in the neuroinflammation that accompanies Alzheimer's disease. The first results on mice primary microglia confirm the importance of NLRP3-inflammasome in CNS for pro-inflammatory cytokines production. Very recent results showed that nucleoside reverse transcriptase inhibitors, including stavudine, inhibit the activation of the inflammasome [158]. If neuroinflammation is deleterious in AD, these drugs could be an interesting tool in the treatment of this disease.

REFERENCES

1. Schroder K, Tschopp J, 2010. The inflammosomes. Cell. 140(6),821-832.

2. Strowig T, Henao-Mejia J, Elinav E, Flavell R. Inflammasomes in health and disease. Nature. 2012 Jan 18;481(7381):278-86.

3. Franchi L, Chen G, Marina-Garcia N, Abe A, Qu Y, Bao S, Shayman JA, Turk J, Dubyak GR, Núñez G. Calcium-independent phospholipase A2 beta is dispensable in inflammasome activation and its inhibition by bromoenol lactoneJ Innate Immun. 2009;1(6):607-17. doi: 10.1159/000227263.

4. Franchi L, Muñoz-Planillo R, Núñez G.Sensing and reacting to microbes through the inflammasomes. Nat Immunol. 2012 Mar 19;13(4):325-32. doi: 10.1038/ni.2231.

5. Izikson L, Klein RS, Charo IF, Weiner HL, Luster AD.Resistance to experimental autoimmune encephalomyelitis in mice lacking the CC chemokine receptor (CCR)2. J Exp Med. 2000 Oct 2;192(7):1075-80

6. Mahad D, Callahan MK, Williams KA, Ubogu EE, Kivisäkk P, Tucky B, Kidd G, Kingsbury GA, Chang A, Fox RJ, Mack M, Sniderman MB, Ravid R, Staugaitis SM, Stins MF, Ransohoff RM.Modulating CCR2 and CCL2 at the blood-brain barrier: relevance for multiple sclerosis pathogenesis. Brain. 2006 Jan;129(Pt 1):212-23. Epub 2005 Oct 17.

7. Kyrkanides S, Miller AW, Miller JN, Tallents RH, Brouxhon SM, Olschowka ME, O'BanionMK, OlschowkaJA.Peripheral blood mononuclear cell infiltration an d neuroinflammation in the HexBmouse model ofneurodegeneration. J Neuroimmunol. 2008 Oct 15;203(1):50-7.

8. 2010 Alzheimer's Disease Facts and Figures. Rep. Vol. 6. Chicago: Alzheimer's Association, 2010. Print. Alzheimer's and Dementia.

9. Khachaturian, Zaven S., and Teresa S. Radebaugh. Alzheimer's Disease: Cause(s), Diagnosis, Treatment, and Care. Boca Raton: CRC, 1996. Print.

10. Haroutunian, V. Neurofibrillary tangles in nondementedelderly subjects and mild Alzheimer disease. Arch Neurol, 1999.56(6): p. 713-8.

11. Goldman, J.S., et al., Genetic counseling and testing for Alzheimer disease: joint practice guidelines of the American College of Medical Genetics and the National Society of Genetic Counselors.Genet Med, 2011. 13(6): p. 597-605.

12. Selkoe DJ Alzheimer's disease results from the cerebral accumulation and cytotoxicity of amyloid beta-protein. (J Alzheimers Dis. 2001 Feb;3(1):75-80.

13. Cedazo-Mínguez A .Apolipoprotein E and Alzheimer's disease: molecular mechanisms and therapeutic opportunities. J Cell Mol Med. 2007 Nov-Dec;11(6):1227-38. doi: 10.1111/j.1582-4934.2007.00130.x.

14. Ferri CP, Prince M, Brayne C, Brodaty H, Fratiglioni L, Ganguli M, Hall K, Hasegawa K, Hendrie H, Huang Y, Jorm A, Mathers C, Menezes PR, Rimmer E, Scazufca M; Global prevalence of dementia: a Delphi consensus study. Alzheimer's Disease International. Lancet. 2005 Dec 17;366(9503):2112-7.

15. Epidemiology of Alzheimer Disease Richard Mayeux and Yaakov Stern.

16. Dubois, B., et al., Research criteria for the diagnosis of Alzheimer'sdisease: revising the NINCDS-ADRDA criteria. Lancet Neurol,2007. 6(8): p. 734-46.)

17. Folstein, M.F., S.E. Folstein, and P.R. McHugh, "Mini-menta lstate". A practical method for grading the cognitive state ofpatients for the clinician. J Psychiatr Res, 1975. 12(3): p. 189-98.9

18. Katz S., et al., Studies of Illness in the Aged. The Index of Adl: A Standardized Measure of Biological and Psychosocial Function.JAMA, 1963. 185: p. 914-9.

19. Lawton, M.P. and E.M. Brody, Assessment of older people: self-maintaining and instrumental activities of daily living.Gerontologist, 1969. 9(3): p. 179-86.

20. Tanzi R.E., Amyloid beta protein gene: cDNA, mRNAdistribution, and genetic linkage near the Alzheimer locus. Science,1987. 235(4791): p. 880-4.

21. Forlenza OV, Diniz BS, Gattaz WF.Diagnosis and biomarkers of predementia in Alzheimer's disease. BMC Med. 2010 Dec 22;8:89.

22. Bush A.I. The amyloid precursor protein of Alzheimer'sdisease is released by human platelets. J Biol Chem, 1990. 265(26):p. 15977-83.

23. Borroni B. Blood cell markers in Alzheimer Disease:Amyloid Precursor Protein form ratio in platelets. Exp Gerontol,2010. 45(1): p. 53-6.

24. Small S.A. and K. Duff, Linking Abeta and tau in late-onset Alzheimer's disease: a dual pathway hypothesis. Neuron, 2008.60(4): p. 534-42.

25. Struble R.G Is brain amyloid production a cause or a resultof dementia of the Alzheimer's type? J Alzheimers Dis, 2010. 22(2): p. 393-9.

26. Foster PP, Rosenblatt KP, Kuljiš RO. Exercise-induced cognitive plasticity, implications for mild cognitive impairment and Alzheimer's disease. Front Neurol. 2011 May 6;2:28.

27. Hatanpää K, Brady DR, Stoll J, Rapoport SI, Chandrasekaran K.Neuronal activity

and early neurofibrillary tangles in Alzheimer's disease. Ann Neurol. 1996 Sep;40(3):411-20.

28. Morsch R, Simon W, Coleman PDNeurons may live for decades with neurofibrillary tangles. J Neuropathol Exp Neurol. 1999 Feb;58(2):188-97.

29. Petersen RC, Smith GE, Waring SC, Ivnik RJ, Tangalos EG, Kokmen E. Mild cognitive impairment: clinical characterization and outcome. Arch Neurol. 1999 Mar;56(3):303-8.

30. Spalletta G, Baldinetti F, Buccione I, Fadda L, Perri R, Scalmana S, Serra L, Caltagirone C.Cognition and behaviour are independent and heterogeneous dimensions in Alzheimer's disease. J Neurol. 2004 Jun;251(6):688-95.

31. Perlmutter LS, Barron E, Chui HC. Morphologic association between microglia and senile plaque amyloid in Alzheimer's disease. Neurosci Lett. 1990;119(1):32–36.

32. Wisniewski HM, Wegiel J, Wang KC, Lach B. Ultrastructural studies of the cells forming amyloid in the cortical vessel wall in Alzheimer's disease. Acta Neuropathol. 1992;84(2):117–127.

33. Frackowiak J, Wisniewski HM, Wegiel J, Merz GS, Iqbal K, Wang KC. Ultrastructure of the microglia that phagocytose amyloid and the microglia that produce beta-amyloid fibrils. Acta Neuropathol. 1992;84(3):225–233.

34. Wisniewski HM, Barcikowska M, Kida E. Phagocytosis of beta/A4 amyloid fibrils of the neuritic neocortical plaques. Acta Neuropathol. 1991;81(5):588–590.

35. Akiyama H, Barger S, Barnum S, Bradt B, Bauer J, Cole GM, Cooper NR, Eikelenboom P, Emmerling M, Fiebich BL, Finch CE, Frautschy S, Griffin WS, Hampel H, Hull M, Landreth G, Lue L, Mrak R, Mackenzie IR, McGeer PL, O'Banion MK, Pachter J, Pasinetti G, Plata-Salaman C, Rogers J, Rydel R, Shen Y, Streit W, Strohmeyer R, Tooyoma I, Van Muiswinkel FL, Veerhuis R, Walker D, Webster S, Wegrzyniak B, Wenk G, Wyss-Coray T. Inflammation and Alzheimer's disease. Neurobiol Aging. 2000;21(3):383–421.

36. Patel NS, Paris D, Mathura V, Quadros AN, Crawford FC, Mullan MJ. Inflammatory cytokine levels correlate with amyloid load in transgenic mouse models of Alzheimer's disease. J Neuroinflamm. 2005;2(1):9.

37. Herber DL, Mercer M, Roth LM, Symmonds K, Maloney J, Wilson N, Freeman MJ, Morgan D, Gordon MN. Microglial activation is required for Abeta clearance after intracranial injection of lipopolysaccharide in APP transgenic mice. J Neuroimmune Pharmacol. 2007;2(2):222–231.

38. Akiyama , Cell mediators of inflammation in the Alzheimer disease brain. Alzheimer Dis Assoc Disord, 2000. 14Suppl 1: p. S47-53.

39. Fiala M. and R. Veerhuis, Biomarkers of inflammation and amyloid-beta phagocytosis in patients at risk of Alzheimer disease. Exp Gerontol, 2010. 45(1): p. 57-63.

40. Fassbender K., C. Masters, and K. Beyreuther, Alzheimer's disease: an inflammatory disease? Neurobiol Aging, 2000. 21(3): p.433-6; discussion 451-3

41. Chabot S. Cytokine production consequent to T cell--microglia interaction: the PMA/IFN gamma-treated U937 cellsdisplay similarities to human microglia. J Neurosci Methods, 2001.105(2): p. 111-20.

42. Block M.L. and J.S. Hong, Microglia and inflammation-mediatedneurodegeneration: multiple triggers with a common mechanism.Prog Neurobiol, 2005. 76(2): p. 77-98.

43. Geissmann F, Manz MG, Jung S, Sieweke MH, Merad M, Ley K. Development of monocytes, macrophages, and dendritic cells. Science.2010;327:656–61.

44. Ginhoux F, Jung S. Monocytes and macrophages: developmental pathways and tissue homeostasis. Nat Rev Immunol. 2014;14:392–404.

45. Auffray C, Sieweke MH, Geissmann F. Blood monocytes: development, heterogeneity, and relationship with dendritic cells. Annu Rev Immunol.2009;27:669–92.

46. Naert G, Rivest S. A deficiency in CCR2+ monocytes: the hidden side of Alzheimer's disease. J Mol Cell Biol. 2013;5:284–93.

47. Hanisch U-K, Kettenmann H. Microglia: active sensor and versatile effector cells in the normal and pathologic brain. Nat Neurosci.2007;10:1387–94.

48. Mildner A, Mack M, Schmidt H, Bruck W, Djukic M, Zabel MD, et al. CCR2+Ly-6Chi monocytes are crucial for the effector phase of autoimmunity in the central nervous system. Brain. 2009;132:2487–500.

49. Tsou C-L, Peters W, Si Y, Slaymaker S, Aslanian AM, Weisberg SP, et al. Critical roles for CCR2 and MCP-3 in monocyte mobilization from bone marrow and recruitment to inflammatory sites. J Clin Invest. 2007;117:902–9.

50. Malm T, Koistinaho M, Muona A, Magga J, Koistinaho J. The role and therapeutic potential of monocytic cells in Alzheimer's disease. Glia.2010;58:889–900.

51. Carlin LM, Stamatiades EG, Auffray C, Hanna RN, Glover L, Vizcay-Barrena G, et al. Nr4a1-dependent Ly6Clow monocytes monitor endothelial cells and orchestrate their disposal. Cell. 2013;153:362–75.

52. Ginhoux F, Greter M, Leboeuf M, Nandi S, See P, Gokhan S, et al. Fate mapping analysis reveals that adult microglia derive from primitive macrophages. Science. 2010;330:841–5.

53. Prinz M, Priller J. Microglia and brain macrophages in the molecular age: from origin to neuropsychiatric disease. Nat Rev Neurosci. 2014;15:300–12.

54. Block ML, Zecca L, Hong J-S. Microglia-mediated neurotoxicity: uncovering the molecular mechanisms. Nat Rev Neurosci. 2007;8:57–69.

55. Saijo K, Glass CK. Microglial cell origin and phenotypes in health and disease. Nat Rev Immunol. 2011;11:775–87.

56. Paolicelli RC, Bisht K, Tremblay M-È. Fractalkine regulation of microglial physiology and consequences on the brain and behavior. Front Cell Neurosci. 2014;8:129.

57. Aderem A, Ulevitch RJ. Toll-like receptors in the induction of the innate immune response. Nature 2000; 406(6797): 782-787.

58. Kesar V, Odin JA. Toll-like receptors and liver disease. Liver Int 2014; 34(2): 184-196.

59. Park BS, Song DH, Kim HM, Choi BS, Lee H, Lee JO. The structural basis of lipopolysaccharide recognition by the TLR4-MD-2 complex. Nature 2009; 458(7242): 1191-1195.

60. Kim HM, Park BS, Kim JI, Kim SE, Lee J, Oh SC, Enkhbayar P, et al. Crystal structure of the TLR4-MD-2 complex with bound endotoxin antagonist eritoran. Cell 2007; 130(5): 906-917.

61. Freeman LCTing JP.The pathogenic role of the inflammasome in neurodegenerative diseases. J Neurochem. 2015 Jun 27

62. NLRs structure Davies B. et al., 2011.The inflammasome NLRs in immunity, Martinon F, Burns K, Tschopp J. The inflammasome: A molecular platform triggering activation of inflammatory caspases and processing of proIL-beta. Mol Cell 2002; 10(2): 417-426.

63. Agostini L, Martinon F, Burns K, McDermott MF, Hawkins PN, Tschopp J. NALP3 forms an IL-1beta-processing inflammasome with increased activity in muckle-wells autoinflammatory disorder. Immunity 2004; 20(3): 319-325.

64. Fayyaz S. Sutterwala,Stefanie Haasken, and Suzanne L. Cassel. Mechanism of

NLRP3 inflammasome activation. Ann N Y Acad Sci. 2014 Jun;1319:82-95.

65. Kigerl KA[1], de Rivero Vaccari JP[2], Dietrich WD[2], Popovich PG[3], Keane RW. Pattern recognition receptors and central nervous system repair. .Exp Neurol. 2014 Aug;258:5-16.

66. Salminen A, Ojala J, Suuronen T, Kaarniranta K, Kauppinen A. Amyloid-beta oligomers set fire to inflammasomes and induce Alzheimer's pathology. J Cell Mol Med. 2008; 12(6A):2255-62

67. Masters SL, O'Neill LA. Disease-associated amyloid and misfolded protein aggregates activate the inflammasome. Trends Mol Med. 2011; 17(5):276-82.

68. Kolaczkowska E. and Kubes P. Neutrophil recruitment and function in health and inflammation. Nat Rev Immunol 13: 159–175, 2013.

69. Clark R. and Kupper T. Old meets new: the interaction between innate and adaptive immunity. J Invest Dermatol 125: 629–637, 2005.

70. Palotás A, Kálmán J, Laskay G, Juhász A, Janka Z, Penke B. Comparative studies on [Ca2+]i-level of fibroblasts from Alzheimer patients and control individuals. Neurochem Res. 2001 Jul;26(7):817-20.

71. Palotás A, Kálmán J, Palotás M, Juhász A, Janka Z, Penke B. Fibroblasts and lymphocytes from Alzheimer patients are resistant to beta-amyloid-induced increase in the intracellular calcium concentration. ProgNeuropsychopharmacol Biol Psychiatry. 2002 Jun;26(5):971-4.

72. Connolly GP. Fibroblast models of neurological disorders: fluorescence measurement studies.Trends PharmacoSci. 1998 May;19(5):171-7.

73. Idoate Gastearena MA, Vega Vázquez F.Diagnosis of neurometabolic and neurodegenerative diseases by cutaneous biopsy. Rev Neurol. 1997 Sep;25 Suppl 3:S269-80.

74. Eckert A, Förstl H, Zerfass R, Hartmann H, Müller WE. Lymphocytes and neutrophils as peripheral models to study the effect of beta-amyloid on cellular calcium signalling in Alzheimer's disease. Life Sci. 1996;59(5-6):499-510.

75. Dolman CL.Diagnosis of neurometabolic disorders by examination of skin biopsies and lymphocytes. Semin Diagn Pathol. 1984 May;1(2):82-97

76. Gerli R, Monti D, Bistoni O, Mazzone AM, Peri G, Cossarizza A, Di Gioacchino M, Cesarotti ME, Doni A, Mantovani A, Franceschi C, Paganelli R. Chemokines, sTNF-Rs and sCD30 serum levels in healthy aged people and centenarians. Mech Ageing Dev. 2000 Dec 20;121(1-3):37-46.

77. Giunta S. Exploring the complex relations between inflammation and aging (inflamm-aging): anti-inflamm-aging remodelling of inflamm- aging, from robustness to frailty. Inflamm Res. 2008 Dec;57(12):558-63.

78. Diniz BS, Teixeira AL, Ojopi EB, Talib LL, Mendonça VA, Gattaz WF, Forlenza OV. Higher serum sTNFR1 level predicts conversion from mild cognitive impairment to Alzheimer's disease. J Alzheimers Dis. 2010;22(4):1305-11.

79. Rogers J, Mastroeni D, Leonard B, Joyce J, Grover A.Neuroinflammation in Alzheimer's disease and Parkinson's disease: are microglia pathogenic in either disorder? Int Rev Neurobiol. 2007;82:235-46.

80. Scalzo P, Kümmer A, Cardoso F, Teixeira ALSerum levels of interleukin-6 are elevated in patients with Parkinson's disease and correlate with physical performance. Neurosci Lett. 2010 Jan 1;468(1):56-8.

81. Funderburg NT, Stubblefield Park SR, Sung HC, Hardy G, Clagett B, Ignatz-Hoover J, Harding CV, Fu P, Katz JA, Lederman MM, Levine AD. Circulating CD4(+) and CD8(+) T cells are activated in inflammatory bowel disease and are associated with plasma markers of inflammation. Immunology. 2013 Sep;140(1):87-97.

82. Hardy J, Selkoe DJThe amyloid hypothesis of Alzheimer's disease: progress and problems on the road to therapeutics. Science. 2002 Jul 19;297(5580):353-6.

83. Meda L, Cassatella MA, Szendrei GI, Otvos L Jr, Baron P, Villalba M, Ferrari D, Rossi F.Activation of microglial cells by beta-amyloid protein and interferon-gamma. Nature. 1995 Apr 13;374(6523):647-50.

84. Schmidt J, Barthel K, Wrede A, Salajegheh M, Bähr M, Dalakas MC. Interrelation of inflammation and APP in sIBM: IL-1 beta induces accumulation of beta-amyloid in skeletal muscle. Brain. 2008 May;131(Pt 5):1228-40.

85. Town T, Nikolic V, Tan J. The microglial "activation" continuum: from innate to adaptive responses. J Neuroinflammation. 2005 Oct 31;2:24.

86. Dutton RW, Bradley LM, Swain SL. Annu Rev Immunol. T cell memory 1998;16:201-23.

87. Tan J, Town T, Abdullah L, Wu Y, Placzek A, Small B, Kroeger J, Crawford F, Richards D, Mullan M. CD45 isoform alteration in CD4+ T cells as a potential diagnostic marker of Alzheimer's disease. J Neuroimmunol. 2002 Nov;132(1-2):164-72.

88. Shalit F, Sredni B, Stern L, Kott E, Huberman M. Elevated interleukin-6 secretion levels by mononuclear cells of Alzheimer's patients. Neurosci Lett. 1994 Jun 20;174(2):130-2.

89. Myagkova MA, Gavrilova SI, Lermontova NN, Kalyn YB, Selezneva ND, Zharikov GA, Kolykhalov IV, Abramenko TV, Serkova TP, Bachurin SO.Content of autoantibodies to bradykinin and beta-amyloid(1-42) as a criterion for biochemical differences between Alzheimer's dementias. Bull Exp Biol Med. 2003 Jul;136(1):49-52.

90. McGeer PL, Itagaki S, Tago H, McGeer EG. Reactive microglia in patients with senile dementia of the Alzheimer type are positive for the histocompatibility glycoprotein HLA-DR. Neurosci Lett. 1987 Aug 18;79(1-2):195-200.

91. Rozemuller JM, Eikelenboom P, Stam FC. Role of microglia in plaque formation in senile dementia of the Alzheimer type. An immunohistochemical study. Virchows Arch B Cell Pathol Incl Mol Pathol 1986;51(3):247–54

92. Frautschy SA, Yang F, Irrizarry M, Hyman B, Saido TC, Hsiao K, Cole GM. Microglial response to amyloid plaques in APPsw transgenic mice. Am J Pathol. 1998 Jan;152(1):307-17

93. Hickman SE, Allison EK, El Khoury J. Microglial dysfunction and defective beta-amyloid clearance pathways in aging Alzheimer's disease mice. J Neurosci. 2008 Aug 13;28(33):8354-60.

94. El Khoury J.Neurodegeneration and the neuroimmune system. Nat Med. 2010 Dec;16(12):1369-70.

95. Kettenmann H, Hanisch UK, Noda M, Verkhratsky A.Physiology of microglia. Physiol Rev. 2011 Apr;91(2):461-553.

96. El Khoury J, Luster AD. Mechanisms of microglia accumulation in Alzheimer's disease: therapeutic implications. Trends Pharmacol Sci. 2008 Dec;29(12):626-32

97. El Khoury J, Toft M, Hickman SE, Means TK, Terada K, Geula C, Luster AD. Ccr2 deficiency impairs microglial accumulation and accelerates progression of Alzheimer-like disease. Nat Med. 2007 Apr;13(4):432-8.

98. El Khoury JB, Moore KJ, Means TK, Leung J, Terada K, Toft M, Freeman MW, Luster ADCD36 mediates the innate host response to beta-amyloid. J Exp Med. 2003 Jun 16;197(12):1657-66.

99. Frenkel D, Wilkinson K, Zhao L, Hickman SE, Means TK, Puckett L, Farfara D, Kingery, ND, Weiner HL, El Khoury J.

Scara1 deficiency impairs clearance of soluble amyloidby mononuclear phagocyte and acceleratesAlzheimer's-like disease progression. Nat Commun. 2013;4:2030

100. Stewart CR, Stuart LM, Wilkinson K, van Gils JM, Deng J, Halle A, Rayner KJ, Boyer L, Zhong R, Frazier WA, Lacy-Hulbert A, El Khoury J, Golenbock DT,Moore KJ.CD36 ligands promote sterile inflammation through assembly of a Toll-like receptor 4 and 6 heterodimer. Nat Immunol. 2010 Feb;11(2):155-61.

101. Halle A, Hornung V, Petzold GC, Stewart CR, Monks BG, Reinheckel T, Fitzgerald KA, Latz E, Moore KJ, Golenbock DT.The NALP3 inflammasome is involved in the innate immune response to amyloid-beta. Nat Immunol. 2008 Aug;9(8):857-65.

102. Heneka MT, Kummer MP, Stutz A, Delekate A, Schwartz S, Vieira-Saecker A, Griep A, Axt D, Remus A, Tzeng TC, Gelpi E, Halle A, Korte M, Latz E, GolenbockDT.NLRP3 is activated in Alzheimer'sdisease and contributes to pathology in APP/PS1 mice.Nature. 2013 Jan 31;493(7434):674-8.

103. Sheedy FJ et al (2013) CD36 coordinates NLRP3 inflammasome activation by facilitating intracellular nucleation of soluble ligands into particulate ligands in sterile inflammation. Nat Immunol 14(8):812–20

104. McKhann G, Drachman D, Folstein M, Katzman R, Price D, Stadlan EM, 1984. Clinical diagnosis of Alzheimer's Disease: report of the NINCDS-ADRDA Work Group under the auspices of Department of Health and Human Service Task Force on Alzheimer's Disease. Neurology. 34(7), 939-944.

105. American Psychiatric Association, Diagnostic and Statistical Manual of Mental Disorders DSM-IV-R, 1994.

106. Folstein MF, Folstein SE, McHugh PR, 1975. Mini-mental state. A practical method for grading the cognitive state of patients for the clinicians. J Psychiat Res. 12 (3),189-198.

107. Hughes CP, Berg L, Danziger WL, Coben LA, Martin RL, 1982. A new clinical scale for staging of dementia. Br J Psychiatry. 140, 566-572.

108. Petersen RC, 2004. Mild cognitive impairment as a diagnostic entity. J Intern Med. 256(3), 183-194.

109. Saeed AI, Sharov V, White J, Li J, Liang W, Bhagabati N, Braisted J, Klapa M, Currier T, Thiagarajan M, Sturn A, Snuffin M, Rezantsev A, Popov D, Ryltsov A,Kostukovich E, Borisovsky I, Liu Z, Vinsavich A, Trush V, Quackenbush JTM4: a free, open-source system for microarray data management and analysis. Biotechniques. 2003 Feb;34(2):374-8.

110. Bolte S, Cordelières FP, 2006. A guided tour into subcellular colocalization analysis in light microscopy. J Microsc. 224, 213-232

111. Manders EMM. 1997. Chromatic shift in multicolour confocal microscopy. J Microsc. 185,321-328.

112. French AP, Mills S, Swarup R, Bennet MJ, Pridmore TP, 2008. Colocalization of fluorescent markers in confocal microscope images of plant cells, Nat Protoc. 4, 619-628.

113. Xiang Li, Zhang Y, Xia M, Gulbins E, Boini KM, Li PL, 2014 Activation of Nlrp3 Inflammasomes Enhances Macrophage Lipid-Deposition and Migration: Implicationof a Novel Role of Inflammasome in Atherogenesis PLOS ONE 9 (1), e87552.

114. Kummer JA, Broekhuizen R, Everett H, Agostini L, Kuijk L, Martinon F, van Bruggen R, Tschopp J. Inflammasome components NALP 1 and 3 show distinct but separate expression profiles in human tissues suggesting a site-specific role in the inflammatory response. J Histochem Cytochem. 2007 55(5):443-52.

115. De Santi L, Annunziata P, Sessa E, Bramanti P. Brain-derived neurotrophic factor and TrkB receptor in experimental autoimmune encephalomyelitis and multiple sclerosis. J Neurol Sci. 2009 Dec 15;287(1-2):17-26. doi: 10.1016/j.jns.2009.08.057. Epub 2009 Sep 16.

116. Block ML, Hong JS. Chronic microglial activation and progressive dopaminergic neurotoxicity. Biochem Soc Trans. 2007 Nov;35(Pt 5):1127-32.

117. Hirsch EC, Hunot S. Pathophysiological involvement of neuroinflammation in various neurological disorders. J Neural Transm. 2010 Aug;117(8):897-8.

118. Matzinger P. Science. The danger model: a renewed sense of self. 2002 Apr 12;296(5566):301-5.

119. McGeer EG, McGeer PL. Abeta immunotherapy and other means to remove amyloid. Curr Drug Targets CNS Neurol Disord. 2005 Oct;4(5):569-73.

120. Rojo LE, Fernández JA, Maccioni AA, Jimenez JM, Maccioni RB. Neuroinflammation: implications for the pathogenesis and molecular diagnosis of Alzheimer's disease. Arch Med Res. 2008 Jan;39(1):1-16.

121. Cuello AC, Ferretti MT, Leon WC, Iulita MF, Melis T, Ducatenzeiler A, Bruno MA, Canneva F. Early-stage inflammation and experimental therapy in transgenic models of the Alzheimer-like amyloid pathology. Neurodegener Dis. 2010;7(1-3):96-8..

122. Akiyama H, Barger S, Barnum S, Bradt B, Bauer J, Cole GM, Cooper NR, Eikelenboom P, Emmerling M, Fiebich BL, Finch CE, Frautschy S, Griffin WS, Hampel H, Hull M, Landreth G, Lue L, Mrak R, Mackenzie IR, McGeer PL, O'Banion MK, Pachter J, Pasinetti G, Plata-Salaman C, Rogers J, Rydel R, Shen Y, Streit W,Strohmeyer R, Tooyoma I, Van Muiswinkel FL, Veerhuis R, Walker D, Webster S, Wegrzyniak B, Wenk G, Wyss-Coray T. Inflammation and Alzheimer's disease. Neurobiol Aging. 2000 May-Jun;21(3):383-421.

123. Good TA, Murphy RM. Effect of beta-amyloid block of the fast-inactivating K+ channel on intracellular Ca2+ and excitability in a modeled neuron. Proc Natl Acad Sci U S A. 1996 Dec 24;93(26):15130-5.

124. Morgan D[1]Mechanisms of A beta plaque clearance following passive A beta immunization. Neurodegener Dis. 2005;2(5):261-6.

125. Heneka MT, Kummer MP, Latz E, 2014. Innate immune activation in neurodegenerative disease Nat Rev Immunol. 14(7), 463-77.

126. Heneka MT, Carson MJ, Khoury J, Landreth GE, Brosseron F, Feinstein DL, Jacobs AH, Wyss-Coray T, Vitorica J, Ransohoff RM, Herrup K, Frautschy SA, Finsen B, Brown GC, Verkhratsky A, Yamanaka K, Koistinaho J, Latz E, Halle A, Petzold GC, Town T, Morgan D, Shinohara ML, Perry VH, Holmes C, Bazan NG, Brooks DJ, Hunot S, Joseph B, Deigendesch N, Garaschuk O, Boddeke E, Dinarello CA, Breitner JCCole GM, Golenbock DT, Kummer MP, 2015a. Neuroinflammation in Alzheimer's disease. Lancet Neurol. 14, 388-405.

127. Saresella M, Calabrese E, Marventano I, Piancone F, Gatti A, Alberoni M, Nemni R, Clerici M, 2011.Increased activity of Th-17 and Th-9 lymphocytes and a skewing of the post-thymic differentiation pathway are seen in Alzheimer's disease. Brain Behav Immun., 25(3),539-47.

128. Saresella M, Marventano I, Calabrese E, Piancone F, Rainone V, Gatti A, et al. A complex proinflammatory role for peripheral monocytes in Alzheimer's disease. J Alzheimers Dis. 2014;38:403–13.

129. Darisipudi MN, Thomasova D, Mulay SR, Brech D, Noessner E, Liapis H, Anders HJ Uromodulin triggers IL-1β-dependent innate immunity via the NLRP3 inflammasome J Am Soc Nephrol. 2012 Nov;23(11):1783-9.

130. Ni J, Wang P, Zhang J, Chen W, Gu L, 2013. Silencing of the P2X7 receptor enhances amyloid-b phagocytosis by microglia Biochemical and Biophysical Research Communications. 434, 363–369

131. Griffin WS, Liu L, Li Y, Mrak RE, Barger SW, 2006. Interleukin-1 mediates Alzheimer and Lewy body pathologies. Journal of Neuroinflammation. 3,5-9.

132. Pickering M, O'Connor JJ, 2007. Pro-inflammatory cytokines and their effects in the dentate gyrus. Prog Brain Res.163,339–354.

133. Murray CA, Lynch MA, 1998. Evidence that increased hippocampal expression of the cytokine interleukin-1 beta is a common trigger for age- and stress-induced impairments in long-term potentiation. J Neurosci: Off J Soc Neurosci. 18(8),2974–2981.

134. Ojala J, Alafuzoff I, Herukka SK, van Groen T, Tanila H, Pirttila T, 2009. Expression of interleukin-18 is increased in the brains of Alzheimer's disease patients. Neurobiol Aging. 30(2),198–209.

135. Malaguarnera L, Motta M, Di Rosa M, Anzaldi M, Malaguarnera M, 2006. Interleukin-18 and transforming growth factor-beta 1 plasma levels in Alzheimer's disease and vascular dementia. Neuropathology 26(4), 307-12.

136. Bossù P, Ciaramella A, Salani F, Vanni D, Palladino I, Caltagirone C, Scapigliati G, 2010. Interleukin-18, from neuroinflammation to Alzheimer's disease. Curr Pharm Des. 16(38), 4213-24.

137. Salani F, Ciaramella A, Bizzoni F, Assogna F, Caltagirone C, Spalletta G, Bossù P, 2013. Increased expression of interleukin-18 receptor in blood cells of subjects with mild cognitive impairment and Alzheimer's disease. Cytokine. 61(2),360-3.

138. Sutinen EM, Pirttilä T, Anderson G, Salminen A, Ojala JO, 2012. Pro-inflammatory interleukin-18 increases Alzheimer's disease-associated amyloid-β production in human neuron-like cells. J Neuroinflammation. 9,199.

139. Gurung P, Anand PK, Malireddi RK, Vande Walle L, Van Opdenbosch N, Dillon CP, et al. FADD and caspase-8 mediate priming and activation of the canonical and noncanonical Nlrp3 inflammasomes. J Immunol 2014; 192(4):1835-46.

140. Gurung P, Kanneganti TD. Novel roles for caspase-8 in IL-1β and inflammasome regulation. Am J Pathol. 2015; 185(1):17-25.

141. Pontillo A, Catamo E, Arosio B, Mari D, Crovella S. NALP1/NLRP1 genetic variants are associated with Alzheimer disease. Alzheimer Dis Assoc Disord. 2012; 26(3):277-81.

142. Chapuis J, Hot D, Hansmannel F, Kerdraon O, Ferreira S, Hubans C, Maurage CA, Huot L, Bensemain F, Laumet G, Ayral AM, Fievet N, Hauw JJ, DeKosky ST, Lemoine Y, Iwatsubo T, Wavrant-Devrièze F, Dartigues JF, Tzourio C, Buée L, Pasquier F, Berr C, Mann D, Lendon C, Alpérovitch A, Kamboh MI, Amouyel P, Lambert JC, 2009. Transcriptomic and genetic studies identify IL-33 as a candidate gene for Alzheimer’s disease. Molecular Psychiatry. 14,1004-1016.

143. Fryer JD, Taylor JW, DeMattos RB, Bales KR, Paul SM, Parsadanian M, Holtzman DM, 2003. Apolipoprotein E markedly facilitates age-dependent cerebral amyloid angiopathy and spontaneous hemorrhage in amyloid precursor protein transgenic mice. J Neurosci. 23,7889–7896.

144. Lee DCY, Landreth GE, 2010. The role of microglia in amyloid clearance from the AD brain J Neural Transm. 117, 949–960.

145. Ali S, Mohs A, Thomas M, Klare J, Ross R, Schmitz ML, Martin MU, 2011. The dual function cytokine IL-33 interacts with the transcription factor NF-κB to dampen NF-κB-stimulated gene transcription. J Immunol.187,1609-16.

146. Xie Z, et al. Peroxynitrite mediates neurotoxicity of amyloid β-peptide1–42- and lipopolysaccharide-activated microglia. J. Neurosci. 2002;22:3484–3492. 22.

147. Combs CK, Karlo JC, Kao SC, Landreth GE. β-Amyloid stimulation of microglia and monocytes results in TNFα-dependent expression of inducible nitric oxide synthase and neuronal apoptosis. J. Neurosci. 2001;21:1179–1188.

148. Mariathasan S1. ASC, Ipaf and Cryopyrin/Nalp3: bona fide intracellular adapters of the caspase-1 inflammasome Microbes Infect. 2007 Apr; 9(5):664-71.

149. Marco Prinz and Uwe-Karsten Hanisch; Murine Microglial cells produce and respond to Interleukin-18; Journal of Neurochemistry, 1999.

150. Frackowiak J, Wisniewski HM, Wegiel J, Merz GS, Iqbal K, Wang KC. Ultrastructure of the microglia that phagocytose amyloid and the microglia that produce beta-amyloid fibrils. Acta Neuropathol. 1992;84(3):225–233.

151. Wisniewski HM, Barcikowska M, Kida E. Phagocytosis of beta/A4 amyloid fibrils of the neuritic neocortical plaques. Acta Neuropathol. 1991;81(5):588–590.

152. Kummer, M.P.. Mrp14 deficiency ameliorates amyloid b burden by increasing microglial phagocytosis and modulation of amyloid precursor protein processing. J. Neurosci. 32, 17824–17829 (2012).

153. Grommes, C. et al. Regulation of microglial phagocytosis and inflammatory gene expression by Gas6 acting on the Axl/Mer family of tyrosine kinases. J. Neuroimmune Pharmacol. 3, 130–140 (2008).

154. Zhu, Y. et al. CD45 deficiency drives amyloid-β peptide oligomers and neuronal loss in Alzheimer's disease mice. J. Neurosci. 31, 1355–1365 (2011).

155. Yuyama, K., Sun, H., Mitsutake, S. & Igarashi, Y. Sphingolipid-modulated exosome secretion promotes clearance of amyloid-β by microglia. J. Biol. Chem. 287, 10977–10989 (2012)

156. McGeer PL, McGeer EG. NSAIDs and Alzheimer disease: epidemiological, animal model and clinical studies. Neurobiol Aging. 2007; 28:639-47.

157. Pan XD, Zhu YG, Lin N, Zhang J, Ye QY, Huang HP, Chen XC, 2011. Microglial phagocytosis induced by fibrillar b-amyloid is attenuated by oligomeric b-amyloid: implications for Alzheimer's disease. Molecular Neurodegeneration, 6, 45

158. Fowler BJ, Gelfand BD, Kim Y, Kerur NM, Tarallo V, Hirano Y, Amarnath S, Fowler DH, Radwan M, Young MT, Pittman K, Kubes P, Agarwal HK, Parang K, Hinton DR, Bastos-Carvalho A, Li S, Yasuma T, Mizutani T, Yasuma R, Wright C, Ambati J, 2014. Nucleoside reverse transcriptase inhibitors possess intrinsic anti-inflammatory activity. Science. 346,1000-3.

FIGURES AND TABLES

AD	Predispoding factors	Onset-age	Incidence
Familial	APP and PS mutation	Early < 65 age	<5%
Sporadic	Elderly, physiological and environmental factors risk, ApoE4, Down syndrome	Late, > 65 age and most common after age 80	> 95%

Table 1. Main differences between familial and sporadic AD

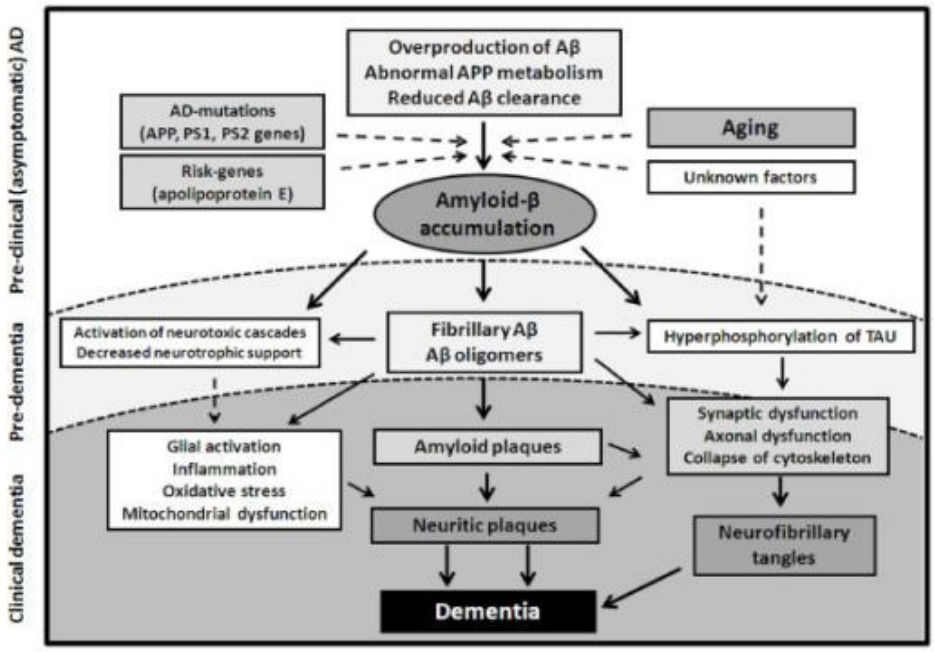
Age	The main risk factor	5% of the subjects 60-75 years <20% subjects over 80 years
Sex	Greater susceptibility for women than men	2:1 ratio
Genetic factors	Mutations responsible for the FAD. ApoE ε4 [especially homozygous]; Family history of dementia	<5% 44% of AD cases In 40-50% of patients with AD no familial incidence
Enviroment	Alcohol- smock- diet-	

Table2. Risk factors for AD

Genetic Markers	**ApoE ε4**	located on chromosome 19; 50% of cases of late-onset AD is homo- or heterozygous for this character
	APP	located on chromosome 21; example of familial AD; early onset <65 years
	PS 1, 2	localized on chromosome 14 and 1 respectively; example of familial AD; early onset 30-40 years
Biochemical Markers	**Specific**	CSF Aβ1-42, p-tau and τ-Tau concentrations
	No-Specific	inflammatory markers and oxidative stress associated with the primary disease process
Neuroimaging	**NMR**	volumetric atrophy of the entorhinal cortex and trans-entorhinal, hippocampus and neocortex;
	PET	Aβ deposits and distribution on the AD brain;

Table 3. Diagnostic Criteria for AD diagnosis

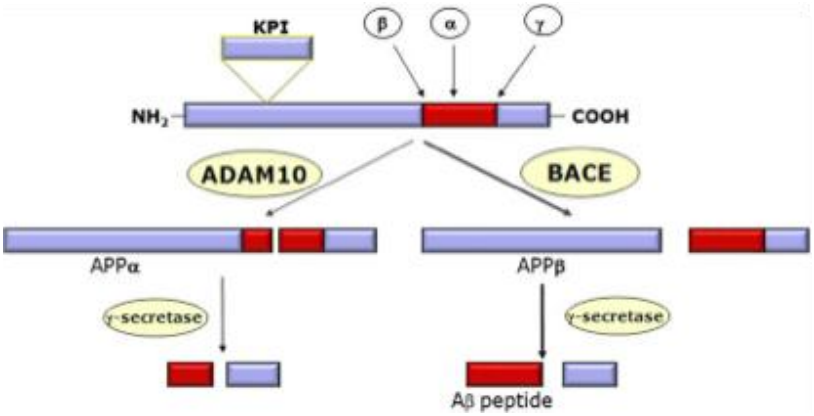
Figure 1. Hypothetical model of the pathological processes in Alzheimer's disease [AD], focusing on the amyloid β peptide [Aβ] cascade.



Dotted arrows indicate possible or secondary mechanisms affecting core pathological processes within the amyloid cascade. Background shades of gray separated by dotted lines are a schematic representation to integrate the progression of pathological events along with the development of the cognitive syndrome of AD [these thresholds are arbitrary and not experimentally validated, and represent the authors' point of view of the disease process]. Three clinical phases of the disease are defined: presymptomatic [or preclinical] AD may last for several years or decades until the overproduction and accumulation of Aβ in the brain reaches a critical level that triggers the amyloid cascade; in the predementia phase, compatible with the definition of mild cognitive impairment secondary to AD, early stage pathology is present in varying degrees, from mild neuronal dystrophy to early stage Braak pathology, according to individual resilience and brain reserve. Finally, in the clinically defined dementia phase, there is a progressive accumulation of the classical pathological hallmarks of AD

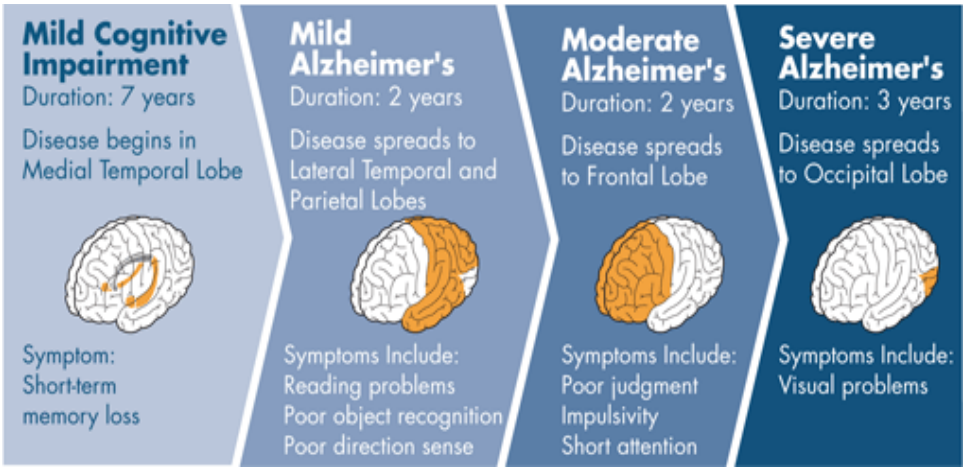
[that is, neuritic plaques and neurofibrillary tangles], bearing relationship with the progression of cognitive deficits and the magnitude of functional impairment. APP = amyloid precursor protein; PS1/2 = presenilin 1/2; TAU = microtubule-associated protein Tau [34].

Figure 2. APP- processing.



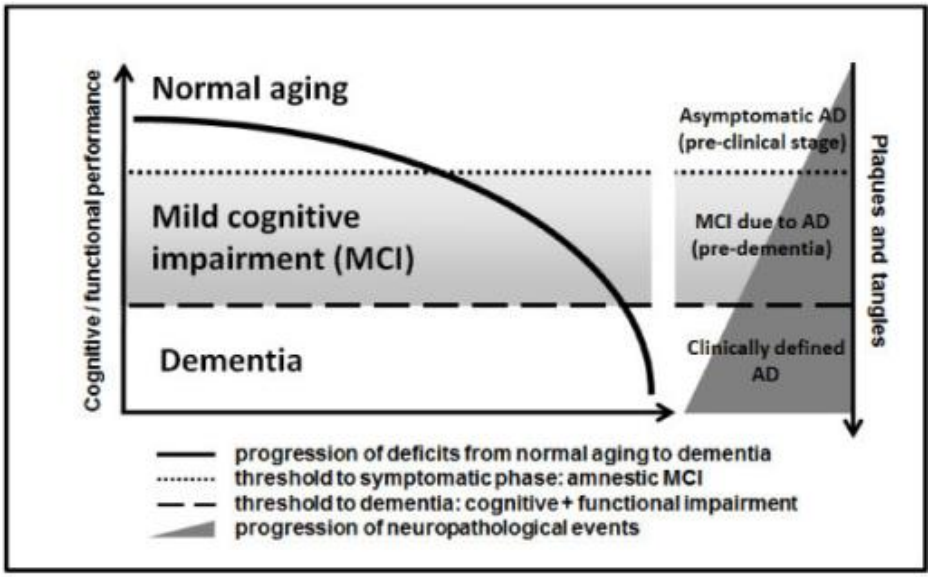
Amyloid Precursor Protein [APP], which contains Kunitz Proteinase Inhibitor [KPI], might be processed by alpha-secretase [ADAM10], which precludes formation of amyloid deposits, or by beta-secretase [BACE], leading to release of Abeta peptide [22].

Figure 3. Pathological evolution characterized by a gradual spread of amyloid deposits and neurofibrillary tangles, which are distributed from the entorhinal cortex and the limbic and neocortical area:



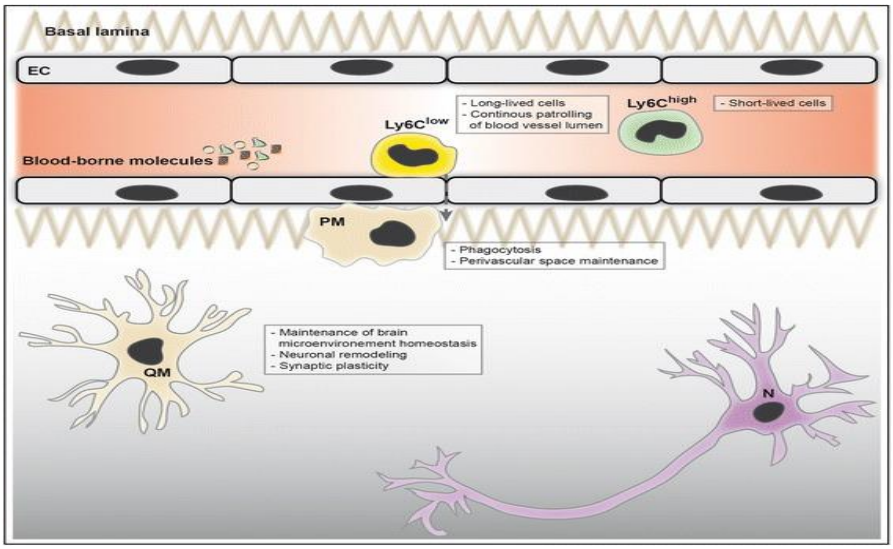
Relationship between the progression of cognitive and functional symptoms and the neuropathological events in the transition from asymptomatic Alzheimer's disease [AD] to mild cognitive impairment due to AD and clinically manifest dementia of the AD type.

Figure 4. Alzheimer's disease evolution.



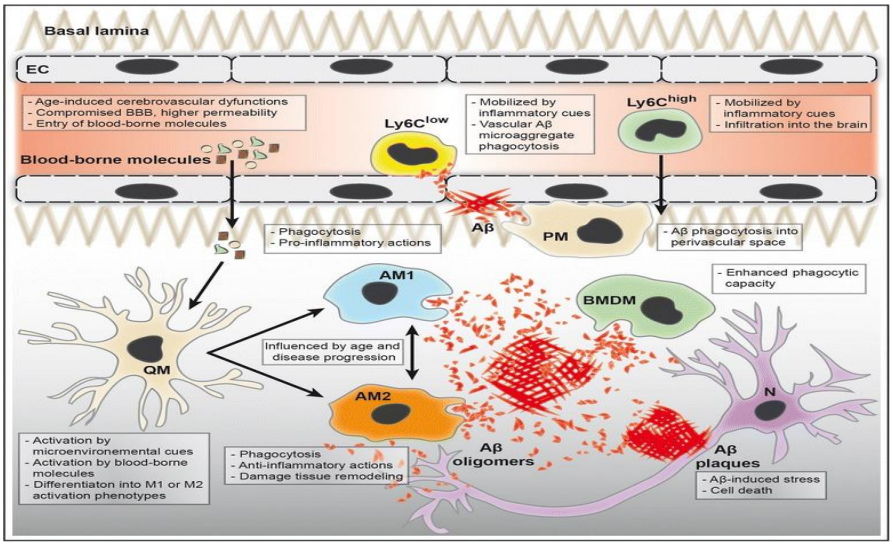
Correlation between the progression of cognitive symptoms and functional and neuropathological events in the transition from pre-clinical disease and AD [34].

Figure 5. Innate immunity profile in the healthy brain.



Intact blood–brain barrier [BBB] restricts entry into the brain of pathogens, toxins and blood-borne molecules, such as immunoglobulin, albumin, thrombin, plasmin, fibrin and laminin. Bone marrow-derived circulating monocytes are divided in two main subsets, which are the patrolling anti-inflammatory [Ly6C^{low}] monocytes and the circulating proinflammatory [Ly6C^{high}] monocytes. Ly6C^{low} monocytes are long-lived cells that ensure continuous surveillance by crawling on blood vessel lumen. Ly6C^{high} monocytes are short-lived cells that are present in blood circulation. Perivascular macrophages [PM] probably arise from Ly6C^{low} monocytes and contribute to the maintenance of homeostasis of the perivascular space, mainly via its phagocytic activity. Quiescent microglia [QM] maintain a healthy brain microenvironment suitable for neurons [N], by continuously sensing any occurring changes via their high ramifications, secreting neurotrophic factors, namely brain-derived neurotrophic factor, and promoting neuronal remodeling and synaptic plasticity.

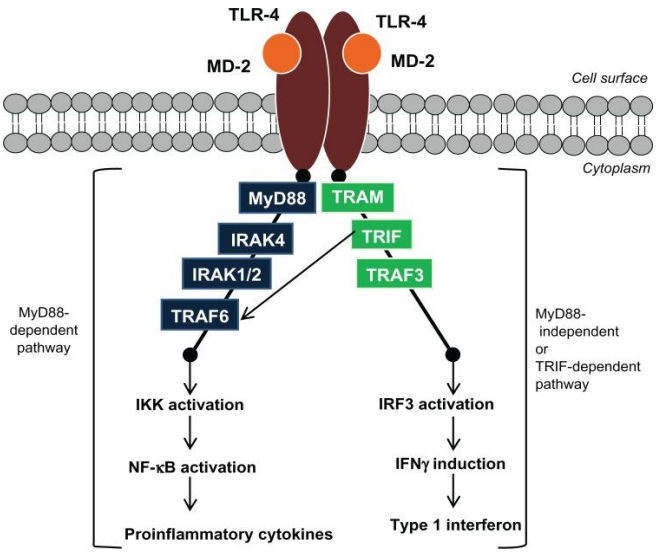
Figure 6. Innate immune responses in the Alzheimer's disease brain.



Age-induced cerebrovascular dysfunction induces deregulation of tight junction protein expression, which compromises the integrity of the blood–brain barrier [BBB]. A compromised BBB promotes the entry of blood-borne molecules within the perivascular space and brain parenchyma. Patrolling [Ly6C^{low}] monocytes are mobilized by inflammatory cues triggered by vascular amyloid-beta [Aβ] microaggregates, contributing to their phagocytosis. Circulating proinflammatory [Ly6C^{high}] monocytes are also mobilized by brain-derived inflammatory cues, adhere to brain endothelium and consequently infiltrate brain parenchyma. Aβ-induced inflammatory conditions promote the differentiation of Ly6C^{high} monocytes into bone marrow-derived macrophages [BMDM] that exhibit enhanced Aβ phagocytic activity. Perivascular macrophages [PM] could contribute to parenchymal Aβ deposit elimination via an efficient Aβ species clearance at the BBB. In an Aβ-induced inflammatory microenvironment, neurons [N] become stressed leading to their dysfunction and ultimately their death. Taken together, the presence of Aβ plaques, soluble Aβ species,

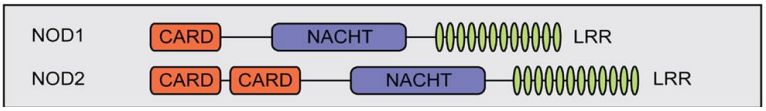
proinflammatory molecules and blood-borne molecules constitute a stressful microenvironment that activates the quiescent microglia [QM]. Amoeboid activated microglial cells can adopt two main phenotypes that coexist in Alzheimer's disease brain: M1 classically activated microglia [AM1] and M2 alternatively activated microglia [AM2]. The switch between these two extreme phenotypes is influenced by age and disease progression. The AM1 phenotype is involved in Aβ phagocytosis and proinflammatory actions, such as secretion of cytokines/chemokines within the brain parenchyma. The AM2 phenotype is also involved in Aβ phagocytosis, but in contrast they have anti-inflammatory actions, including damaged tissue repair and remodeling, and cytokine/chemokine production. EC, endothelial cells.

Figure 7. TLR4 signaling.



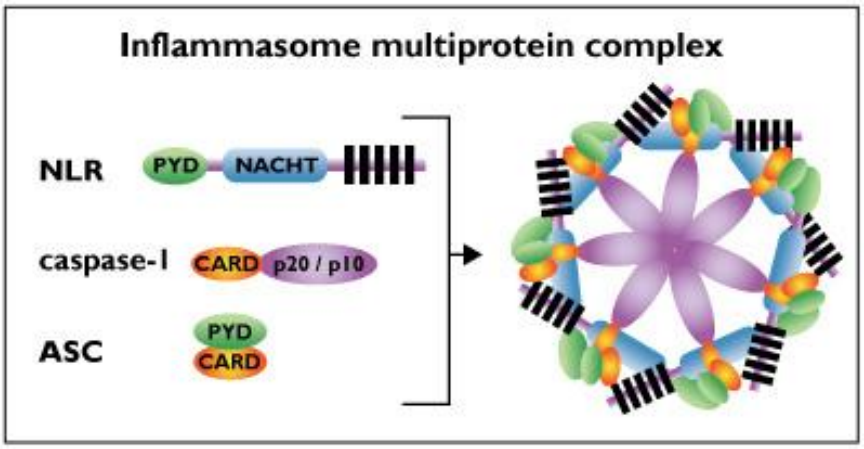
The lipopolysaccharide [LPS] of Gram negative bacteria is a well-known inducer of the innate immune response. Toll-like receptor [TLR] 4 and myeloid differentiation factor 2 [MD-2] form a heterodimer that recognizes a common 'pattern' in structurally diverse LPS molecules. Based on the signaling adaptors, TLR signaling can be classified into two major pathways: the MyD88- and TRIF-dependent pathways. TLR3 is the only TLR that exclusively signals through TRIF [IFN-production], whereas TLR4 signals through both TRIF and MyD88 [73].

Figure 8. Nod1,2 receptors structure.



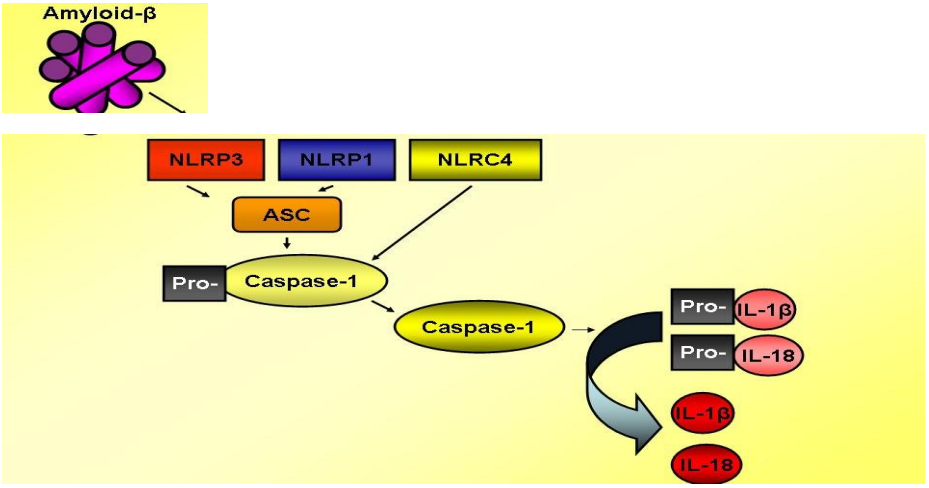
NLRs contain 3 domains: central NACHT [or NOD] domain, which is common to all NLRs, most of NLRs have also c-terminal leucine-rich repeat [LRR] and variable N-terminal interaction domain. NACHT domain mediates ATP-dependent self-oligomerization and LRR senses the presence of ligand. N-terminal domain is responsible for homotypic protein-protein interaction and it can consist of caspase recruitment domain [CARD] or pyrin domain [PYD] [74].

Figure 9. Inflammasome structure.



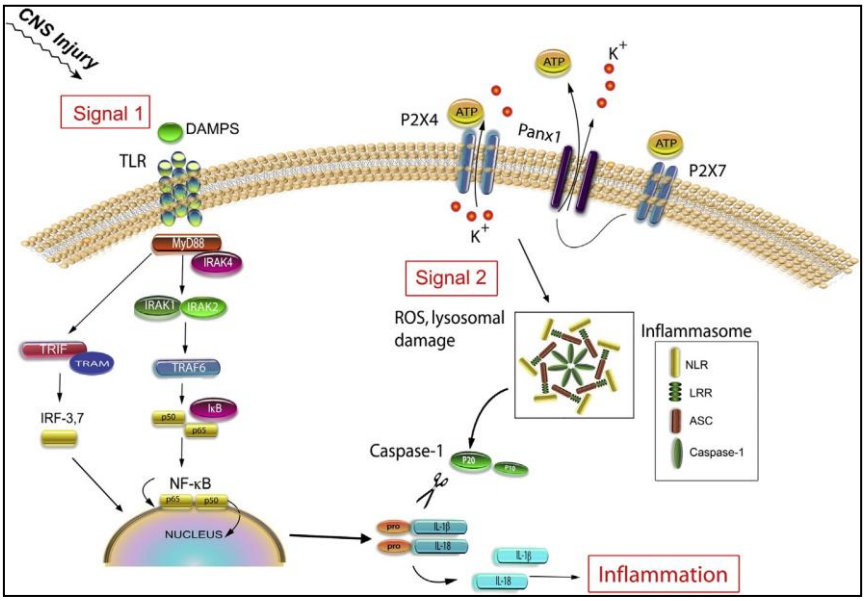
Caspase-1 is activated within the inflammasome multiprotein complex through interaction with ASC [apoptosis-associated speck-like protein containing a carboxy-terminal CARD], a bipartite adapter protein that bridges NLRs and caspase-1 [74].

Figure 10. ASC-dependent and independent inflammasome activation. The pathogenic role of the inflammasome in neurodegenerative diseases [76].



Amyloid-β is known to trigger the activation of NLRP3. The exact trigger for NLRP1 in AD is not known but it may possible that potassium effluxes which trigger NLRP1 [as well as NLRP3] could occur during AD pathogenesis and in the process trigger NLRP1 activation. Recently it was suggested that palmitate, a saturated fatty acid, may play a role in activating NLRC4 during AD. These potential triggers may possibly activate NLRP3, NLRP1 and NLRC4 during AD but have yet to be confirmed [dashed lines]. NLRC4 can associate with pro-caspase-1. NLRP1 and NLRP3 associate with the adaptor protein ASC. ASC initiates the cleavage of pro-caspase-1 into the mature form of caspase-1, which cleaves pro-IL-1β and pro-IL-18 into their mature forms of IL-1β and IL-18 which have been implicated in the pathogenesis of Alzheimer's disease [76].

Figure 11. Inflammasome activation-pathway.



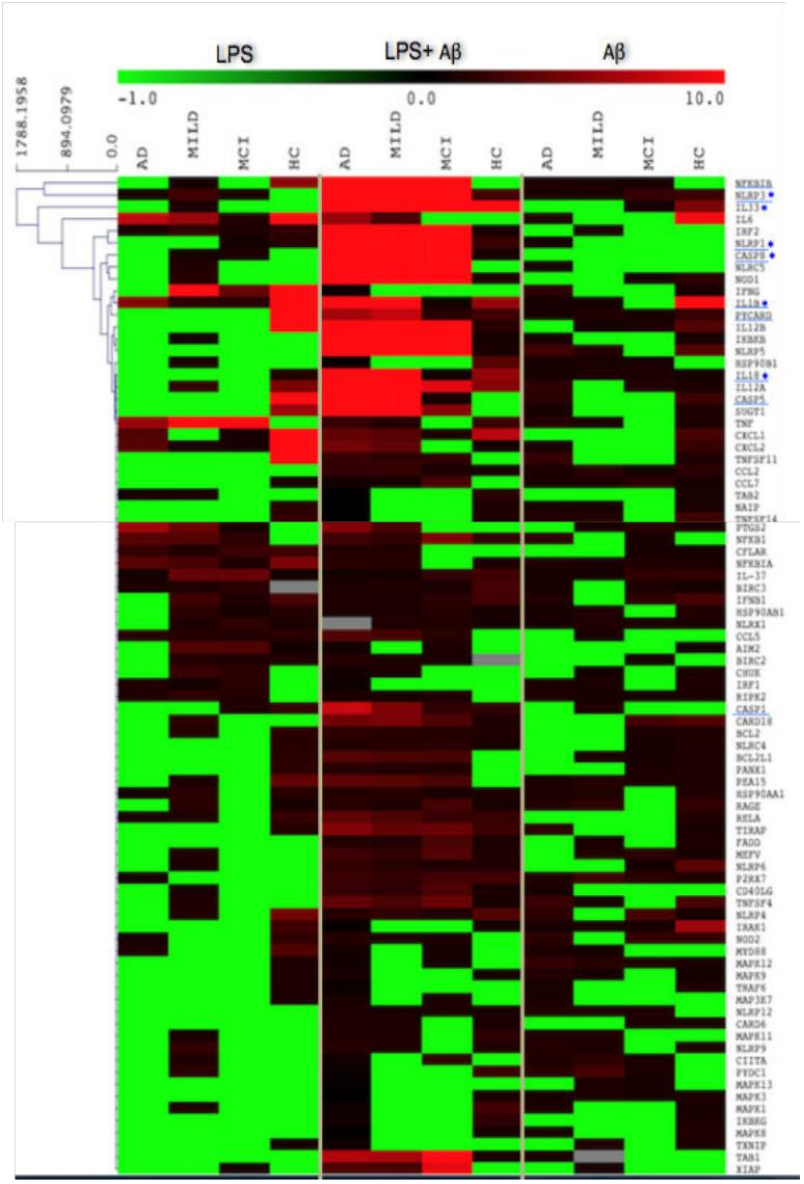
Two-signal model of innate immunity. TLRs and NLRs cooperate to orchestrate the innate immune response to injury. Activation of TLRs [via DAMPs released from CNS injury] leads to nuclear translocation of NFκB and transcription of pro-inflammatory cytokines, such as pro-IL-1β. Activation of NLRs [via a second signal] triggers inflammasome formation, caspase-1 activation, and cleavage of pro-IL-1β into its active form [79].

Inflammasomes	AIM2: AIM2, CASP1 [ICE], PYCARD [TMS1, ASC]. IPAF: CASP1 [ICE], NAIP [BIRC1], NLRC4 [IPAF], PYCARD [TMS1, ASC]. NLRP1: CASP1 [ICE], CASP5, NLRP1. NLRP3: CASP1 [ICE], NLRP3, PYCARD [TMS1, ASC].
Negative Regulation of Inflammasomes	BCL2, BCL2L1 [BCLXL], CARD18 [ICEBERG], CD40LG [TNFSF5], CTSB, HSP90AA1, HSP90AB1 [HSPCB], HSP90B1 [TRA1], MEFV, PSTPIP1, PYDC1 [POP1], SUGT1, TNF, TNFSF11 [RANKL], TNFSF14, TNFSF4 [OX40L].
Signaling Downstream of Inflammasomes	IFNG, IL12A, IL12B, IL18, IL1B, IL33, IRAK1, IRF1, MYD88, P2RX7, PANX1, PTGS2 [COX2], MOK, RIPK2, TIRAP, TXNIP. MYD88, P2RX7, PANX1, PTGS2 [COX2], MOK, RIPK2, TIRAP, TXNIP
NOD-Like Receptors	CIITA, NAIP [BIRC1], NLRC4 [IPAF], NLRC5, NLRP1, NLRP12 [NALP12], NLRP3, NLRP4, NLRP5, NLRP6, NLRP9, NLRX1, NOD1 [CARD4], NOD2
Signaling Downstream of NOD-Like Receptors	BIRC2 [c-IAP1], BIRC3 [c-IAP2], CARD6, CASP8 [FLICE], CCL2 [MCP-1], CCL5 [RANTES], CCL7 [MCP-3], CFLAR [Casper], CHUK [IKKα], CXCL1 [GRO1, GROa, SCYB1], CXCL2 [GRO2, GROb, SCYB2], FADD, IFNB1, IKBKB [IKKβ], IKBKG, IL6, IRF1, IRF2, MAP3K7 [TAK1], MAPK1 [ERK2], MAPK11 [p38Beta], MAPK12 [P38GAMMA], MAPK13 [p38delta], MAPK3 [ERK1], MAPK8 [JNK1], MAPK9 [JNK2], NFKB1, NFKBIA [IκBα, MAD3], NFKBIB [TRIP9], PEA15, RELA, RIPK2, SUGT1, TAB1 [MAP3K7IP1],
Pro-Inflammatory Caspases	CASP1 [ICE], CASP5.

Table 4 : 84 genes involved in the Inflammasome pathway activation.

Figure 12. Messenger RNA expression levels of genes within the inflammasome pathway.

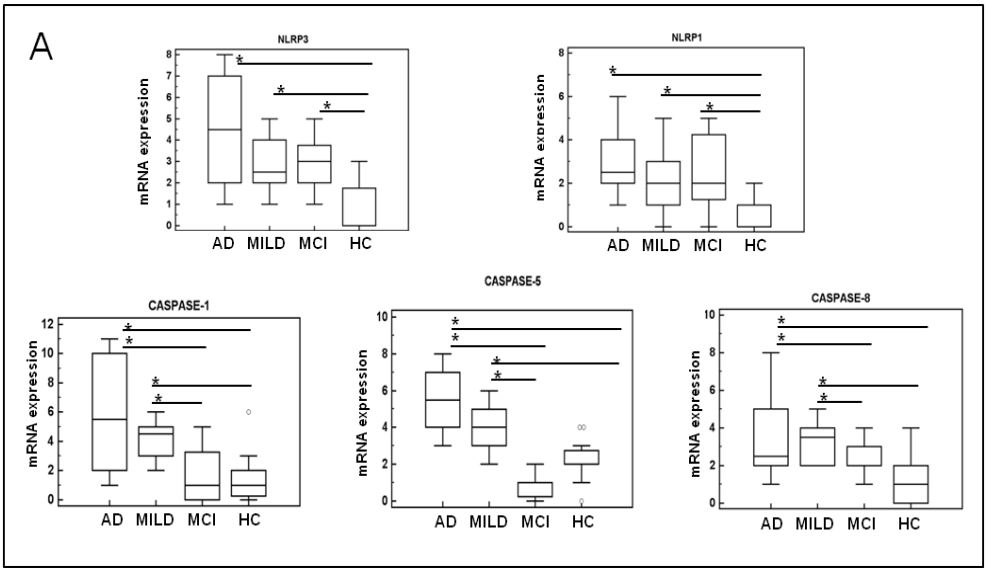
Expression of 84 genes involved in the inflammasome pathway assessed by real-time quantitative RT-PCR-array in monocytes of individuals with a diagnosis of either severe Alzheimer's disease [AD], moderate Alzheimer's disease [MILD] or Mild Cognitive Impairment [MCI] and of age- and sex-matched Healthy Controls [HC]. Heat maps of Log_2 Fold changes are presented. Results obtained upon stimulating cells with LPS or $A\beta_{1-42}$ alone, or upon LPS-priming followed by $A\beta_{1-42}$ stimulation are presented. Fold-changes >10 of the genes of importance are summarized in the Table.

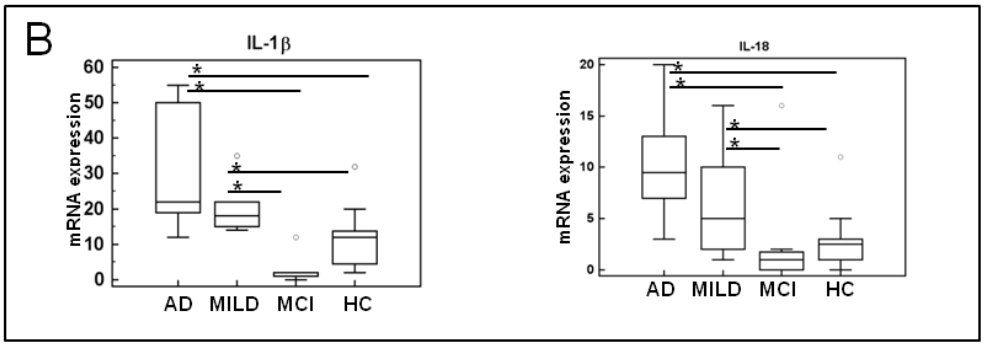


Genes	AD	MILD	MCI	HC
NLRP1	437,2	385,2	119,3	2,3
NLRP3	1334,4	1007,1	309,7	2,9
ASC	28,0	19,0	3,0	2,0
CASP1	20,0	15,0	4,0	6,0
CASP5	16,0	17,0	1,2	-1,2
CASP 8	138,3	129,7	275,9	1,0
IL1β	37,0	19,0	1,0	5,9
IL18	20,0	10,0	3,0	6,0
IL33	365,9	404,3	1259,0	42,6

Figure 13. mRNA expression by Real-Time PCR.

Single Real-Time PCR results obtained in LPS and $A\beta_{1-42}$ -stimulated monocytes of individuals with a diagnosis of either severe Alzheimer's disease [AD], moderate Alzheimer's disease [MILD] or Mild Cognitive Impairment [MCI] and of age- and sex-matched Healthy Controls [HC]. NLRP3, NLRP1, caspase 1, caspase 5 and caspase 8 are shown in panel A; IL1β and IL18 in panel B and IL33 and IL37 in panel C. The results are shown as fold-change expression from the unstimulated samples. Gene expression was calculated relative to GAPDH housekeeping gene. Summary results are shown in the bar graphs. The boxes stretch from the 25 to the 75 percentile; the line across the boxes indicates the median values; the lines stretching from the boxes indicate extreme values. Outside values are displayed as separate points. Statistical significance is shown *[$p<0.05$],**[$p<0.01$].


A
NLRP3
NLRP1
CASPASE-1
CASPASE-5
CASPASE-8
mRNA expression
AD MILD MCI HC


B
IL-1β
IL-18
mRNA expression
AD MILD MCI HC

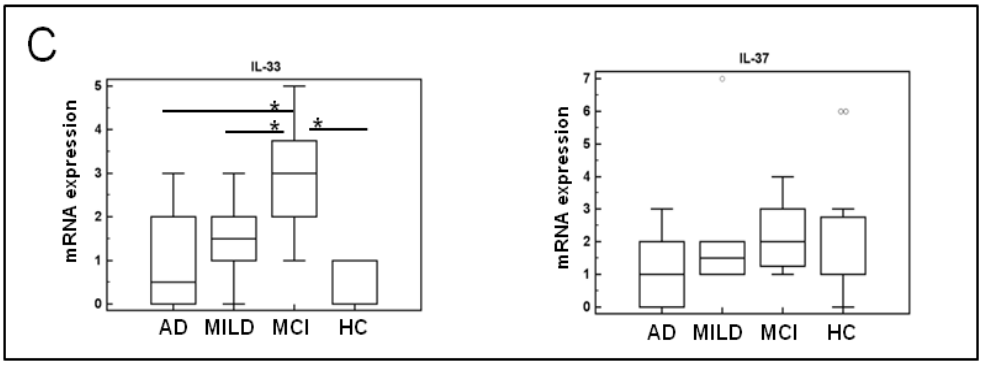
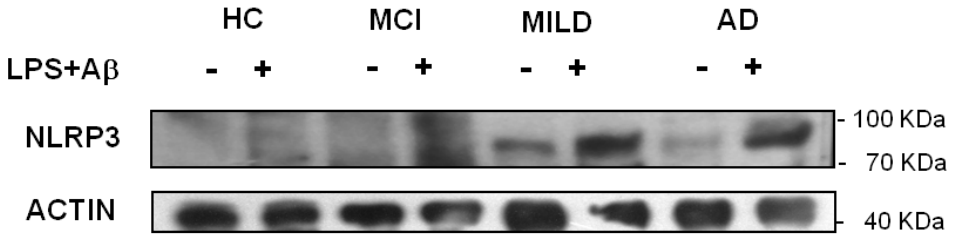

C
IL-33
IL-37
mRNA expression
AD MILD MCI HC

Figure 14. NLRP3 expression in Monocyte by Western blot.

NLRP3 protein expression assessed by western blotting in monocyte of individuals with a diagnosis of either severe Alzheimer's disease [AD], moderate Alzheimer's disease [MILD] or Mild Cognitive Impairment [MCI] and of age- and sex-matched Healthy Controls [HC]. The same protein concentration of whole-cells lysates was loaded into the gel, as confirmed by actin. Representative results obtained in un-stimulated or in LPS-primed and $A\beta_{1\text{-}42}$-stimulated PBMC are presented in the upper panel. Quantitative evaluation [arbitrary unit] of NLRP3 expression obtained comparing band density in un-stimulated or in LPS-primed $A\beta_{1\text{-}42}$-stimulated PBMC is shown in the lower panel. Mean values of 5 experiments and standard error are presented.


HC
MCI
MILD
AD
LPS+Aβ
- + - + - + - +
NLRP3
ACTIN
100 KDa
70 KDa
40 KDa

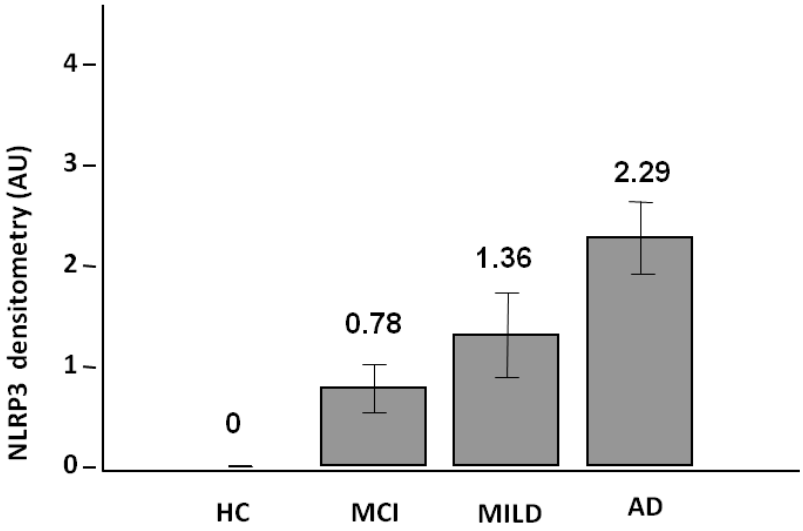

NLRP3 densitometry (AU)
4
3
2
1
0
0
0.78
1.36
2.29
HC
MCI
MILD
AD

Figure 15. FACS analyses: co-expression of inflammasome proteins in CD14+ cells.

Co-expression of inflammasome proteins in LPS-primed and $A\beta_{1\text{-}42}$-stimulated CD14+ of individuals with a diagnosis of either severe Alzheimer's disease [AD], moderate Alzheimer's disease [MILD] or Mild Cognitive Impairment [MCI] and of age- and sex-matched Healthy Controls [HC]. Two-hundred-thousand cells were acquired and gated on CD14 expression and side scatter properties. CD14+ cells co-expressing NLRP3-associated inflammasome components are shown in panel A; CD14+ cells co-expressing NLRP1-associated inflammasome components are shown in panel B. Summary results are shown in the bar graphs. The boxes stretch from the 25 to the 75 percentile; the line across the boxes indicates the median values; the lines stretching from the boxes indicate extreme values. Outside values are displayed as separate points. Statistical significance is shown *[$p<0.05$], **[$p<0.01$].

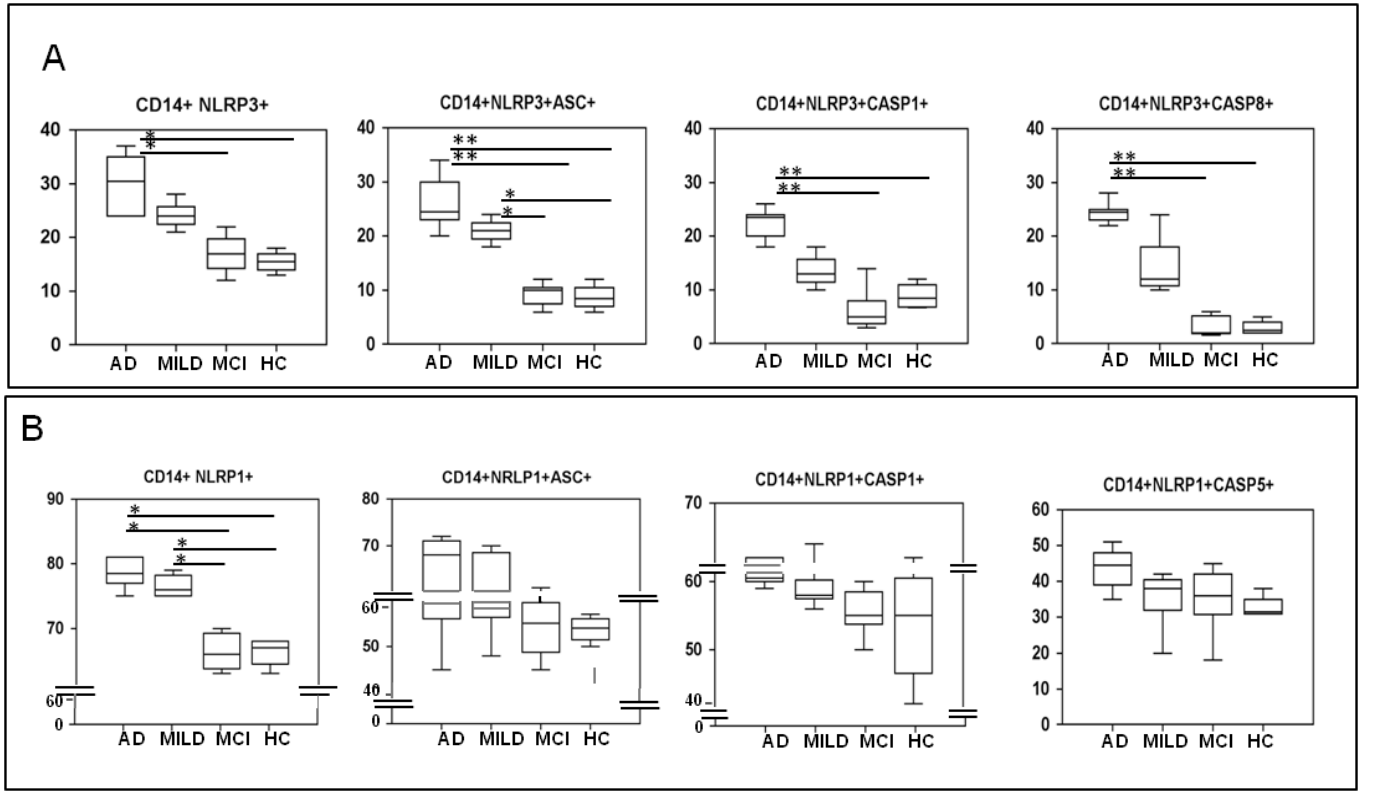

A
CD14+ NLRP3+
CD14+NLRP3+ASC+
CD14+NLRP3+CASP1+
CD14+NLRP3+CASP8+
AD MILD MCI HC
B
CD14+ NLRP1+
CD14+NRLP1+ASC+
CD14+NLRP1+CASP1+
CD14+NLRP1+CASP5+
AD MILD MCI HC

Figure 16. Confocal microscopy: co localization of inflammasome proteins in CD14+ cells.

Representative confocal fluorescence images [left part of the figure] data summarizing the results of 5 experiments [right part of the figure] showing the effect of LPS-priming and $A\beta_{1-42}$-stimulation on the co-localization between NLRP3 and ASC [Upper panels] and of NLRP1 and Caspase 1 [lower panels]. Monocytes of individuals with a diagnosis of either severe Alzheimer's disease [AD], moderate Alzheimer's disease [MILD] or Mild Cognitive Impairment [MCI] and of age- and sex-matched Healthy Controls [HC] are shown. Summary results of PCC [Pearson co localization efficiency] are presented in the bar graphs. The boxes stretch from the 25 to the 75 percentile; the line across the boxes indicates the median values; the lines stretching from the boxes indicate extreme values. Outside values are displayed as separate points. Statistical significance is shown *[p<0.05], **[p<0.01].

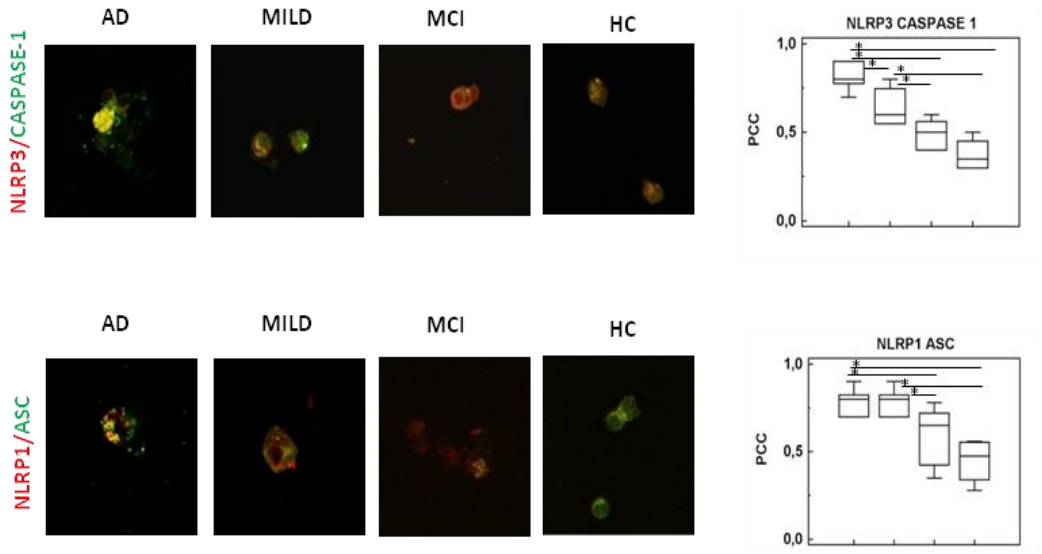


Table 5. Co-localization coefficient of inflammasome proteins.

Pearson's co-localization coefficient [PCC] of NLRP1 and NLRP3 with ASC, caspase-1, caspase-5 and caspase-8, in LPS-primed and $A\beta_{1\text{-}42}$-stimulated CD14+ monocyte of patients with a diagnosis of either severe or moderate [MILD] Alzheimer's Disease [AD] and mild cognitive impairment [MCI]. Result obtained in and in age-and sex-matched healthy controls [HC] are also shown. Index reference values: PC range= -1 and +1.

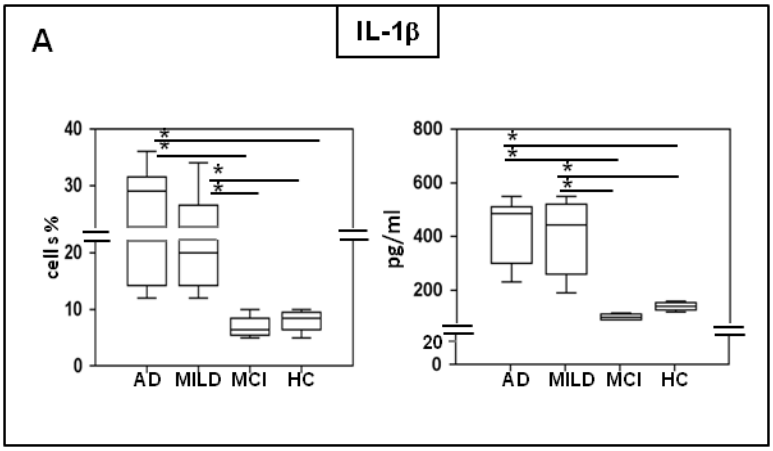
PCC	severe AD	MILD AD	MCI	HC
NLRP1+ ASC+	0.8 *° [0.7-0.82]	0.8 §^ [0.7-0.82]	0.5 *§ [0.3-0.6]	0.4 °^ [0.3-0.5]
NLRP1+ CASPASE-1+	0.6 [0.4-0.7]	0.8 [0.7-0.84]	0.6 [0.4-0.65]	0.5 [0.5-0.7]
NLRP1+ CASPASE-5+	0.7 #*° [0.5-0.8]	0.4 # [0.23-0.5]	0.3 * [0.1-0.32]	0.3 ° [0.2-0.5]
NLRP3+ ASC+	0.8 *° [0.6-0.82]	0.8 §^ [0.7-0.82]	0.6 *§ [0.5-0.7]	0.5 °^ [0.4-0.6]
NLRP3+ CASPASE-1+	0.9 #*° [0.8-0.9]	0.8 #§^ [0.5-0.8]	0.5 *§ [0.4-0.56]	0.3 °^ [0.3-0.4]
NLRP3+ CASPASE-8+	0.8 *° [0.7-0.85]	0.6 [0.5-0.72]	0.5 * [0.3-0.55]	0.5 ° [0.3-0.6]

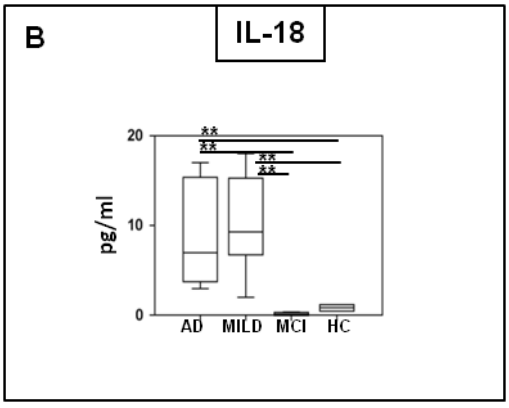
Median values, interquartile range and statistical significances are presented [p<0.05]

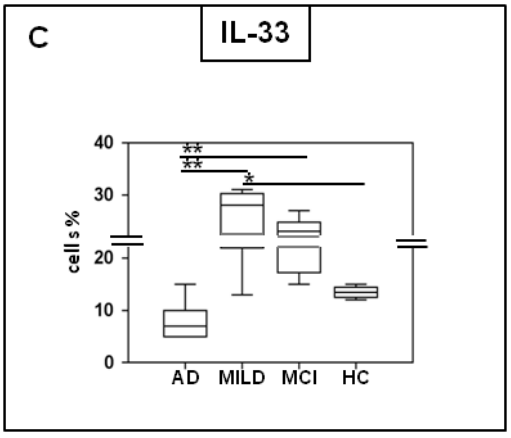
* AD vs. MCI; # AD vs. MILD AD; ° AD vs. HC; § MILD AD vs. MCI; ^ MILD AD vs. HC.

Figure 17. IL1β, IL18, IL33 and IL37 production.

IL1β, [panel A], IL18 [panel B], IL33 [panel C] and IL37 [panel D]. Interleukin-1β and IL-18 production was assessed by multiplex ELISA in supernatants. CD14+/IL1β+, CD14+/IL33 and CD14+/IL37+ cells were analyzed by flow-cytometry. In both cases, LPS-primed and $A\beta_{1\text{-}42}$-stimulated monocytes of individuals with a diagnosis of either severe Alzheimer's disease [AD], moderate Alzheimer's disease [MILD] or Mild Cognitive Impairment [MCI] and of age- and sex-matched Healthy Controls [HC] were analyzed. Summary results are shown in the bar graphs. The boxes stretch from the 25 to the 75 percentile; the line across the boxes indicates the median values; the lines stretching from the boxes indicate extreme values. Outside values are displayed as separate points. Statistical significance is shown *[$p<0.05$], **[$p<0.01$].


A
IL-1β
cells %
pg/ml
AD MILD MCI HC
AD MILD MCI HC


B
IL-18
pg/ml
AD MILD MCI HC


C
IL-33
cells %
AD MILD MCI HC

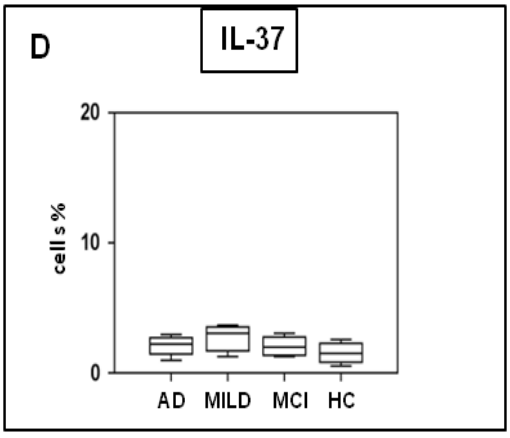

D
IL-37
cells %
AD MILD MCI HC

Figure 18. TNFα and IL1β release was evaluated in supernatants of WT and Knockout microglia cells collected after 2, 4, 6, 12, 24 hours with direct LPS-stimulation [A] and 1, 5, 11 hours with LPS-undirect stimulation [B]. Statistical differences are express between WT *vs* NLRP3 KO condition. [*=pvalue<0.05].

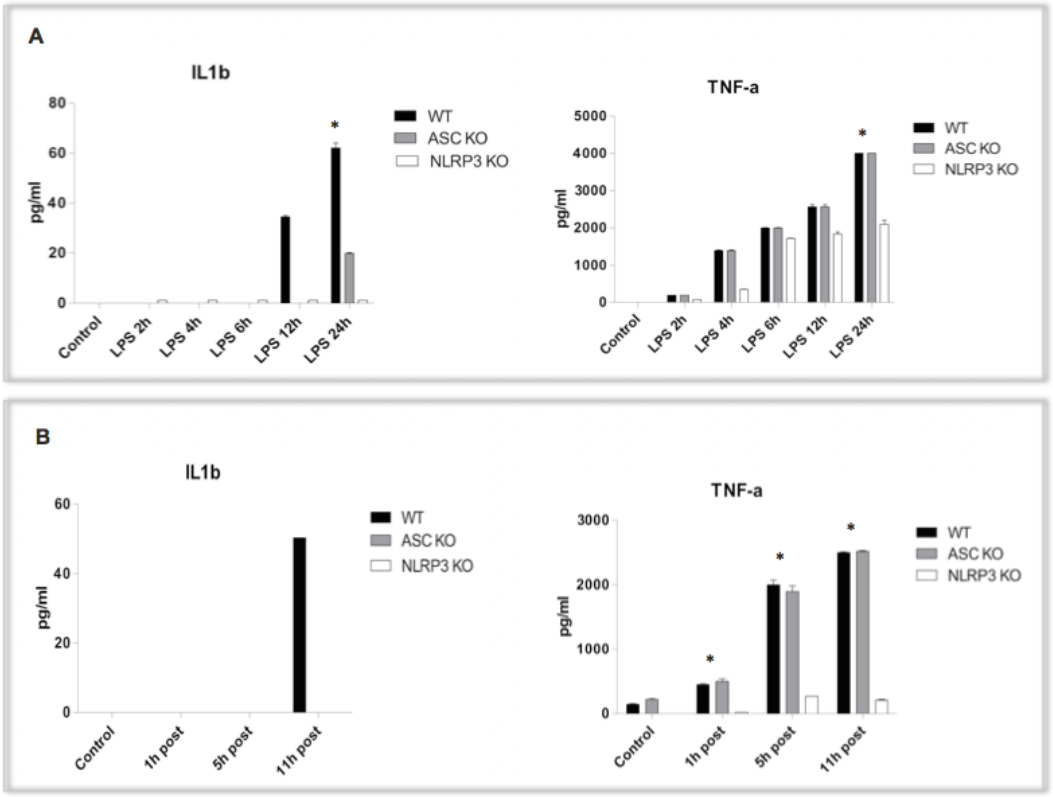


Figure 19. Mature IL1β level was evaluated by ELISA on the supernatants of WT and KO cells upon inflammasome activation. Production was increase in the presence of ATP [1μM for 30'] and Nigericin [1.34 mM for 2 hours] confirming that the release of IL1β depends on NLRP3-inflammasome activation. Statistical differences are express between WT *vs* NLRP3 and ASC KO condition. [*=pvalue<0.05]

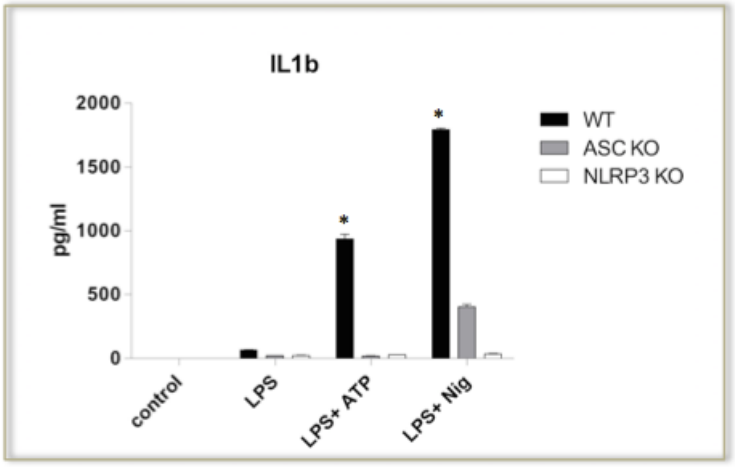
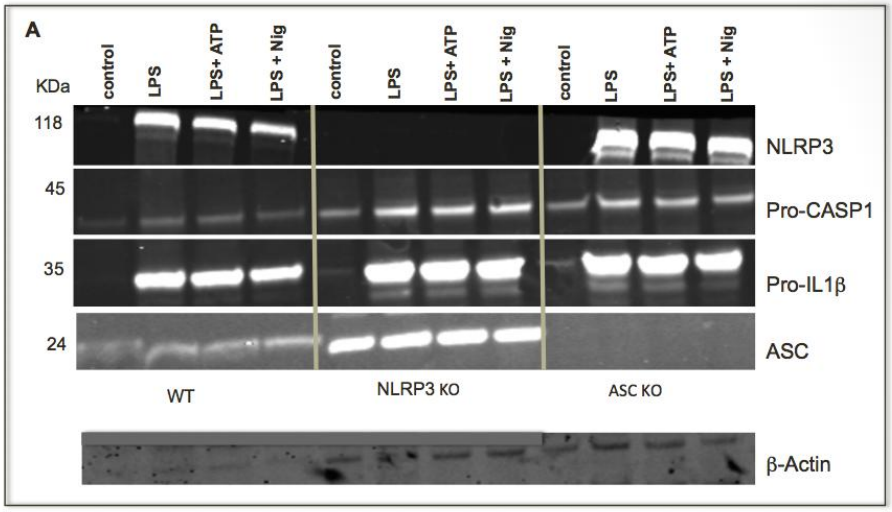
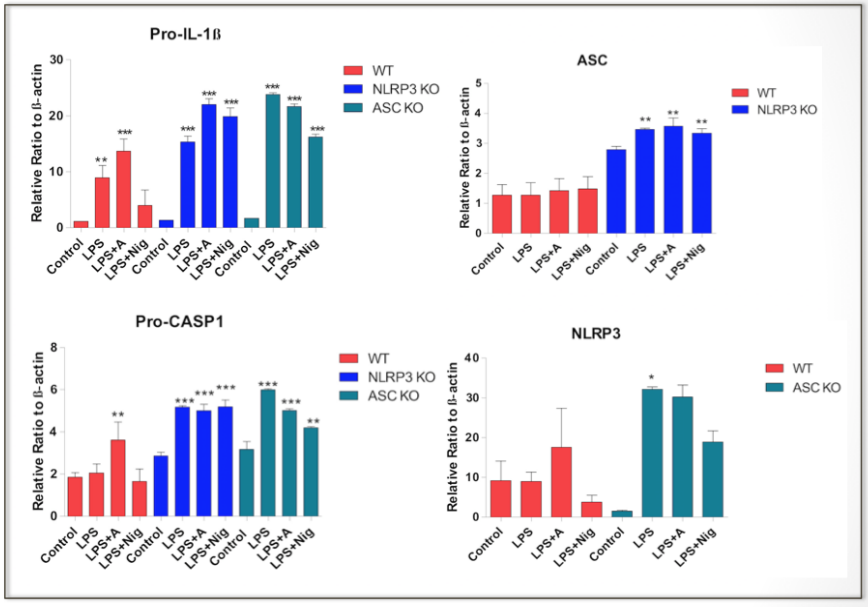


Figure 20. Western Blot analysis of NLRP3-inflammasome complex activation was performed by loading equal amount of RIPA fraction in each lane. LPS primed microglia [1x10^6/condition] were stimulate with ATP [1mM, 30 minutes] or Nigericin [Nig, 1.34 µM for 2 hours]. The panel A represents bands for NLRP3, pro-Casp1, pro-IL1β and ASC in WT and KO lysate cells. The results were normalized to β-actin for total amount of protein loaded in the respective lane (Relative Ratio to β-actin). The panel B represents IL1β and Casp1 on supernatants of WT microglia.


A
KDa
control
LPS
LPS+ ATP
LPS + Nig
118
45
35
24
NLRP3
Pro-CASP1
Pro-IL1β
ASC
WT
NLRP3 KO
ASC KO
β-Actin


Pro-IL-1ß
Relative Ratio to ß-actin
WT
NLRP3 KO
ASC KO
Control
LPS
LPS+A
LPS+Nig
ASC
Pro-CASP1
NLRP3

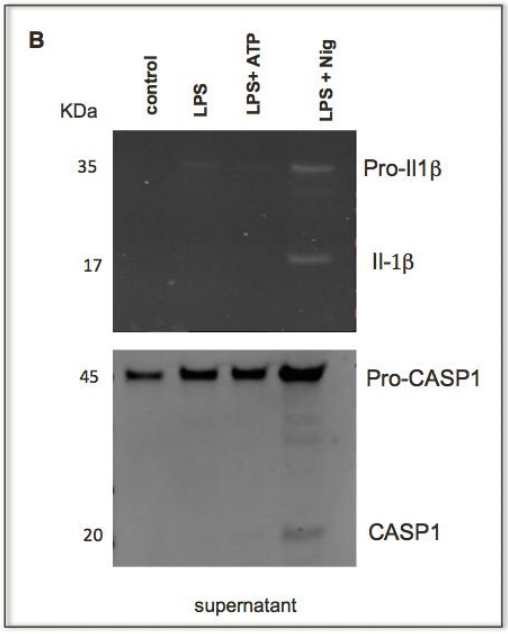

B
control
LPS
LPS+ ATP
LPS + Nig
KDa
35
17
45
20
Pro-Il1β
Il-1β
Pro-CASP1
CASP1
supernatant

Figure 21. Primary microglia WT and KO Aβ-phagocytosis was evaluated by Flow Cytometry analysis. The panel A represents LPS-direct stimulation for 2, 4, 6, 12 and 24 hours; the panel B represents the effect of indirect-LPS stimulation after 1, 5, 11 hours. The results are express as percentage of FAM-Aβ positive cells [LPS 200ng/ml; FAM-Aβ 500nM/ml].

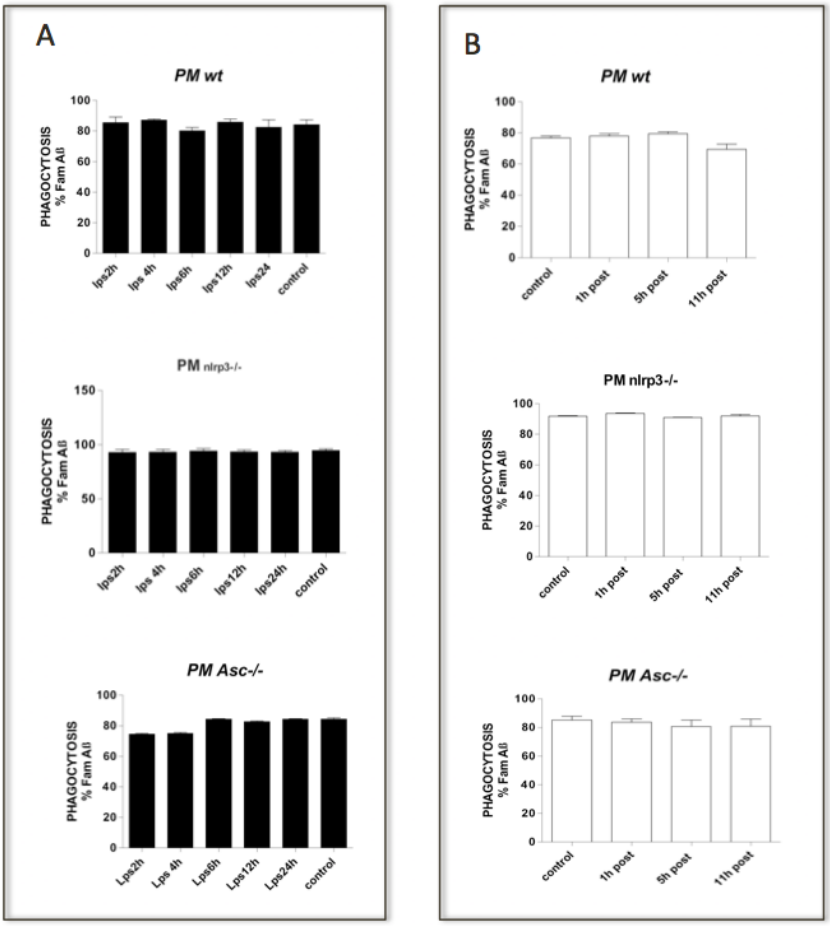


Figure 22. The panel A shows vitality [7-AAD] and Aβ-phagocytosis [FAM-Aβ-FITC] by flow cytometry analysis upon inflammasome activation condition; LPS-primed and ATP or Nigericin stimulated WT microglia cells were evaluated. The panel B represents results express as percentage of FAM-Aβ positive cells.

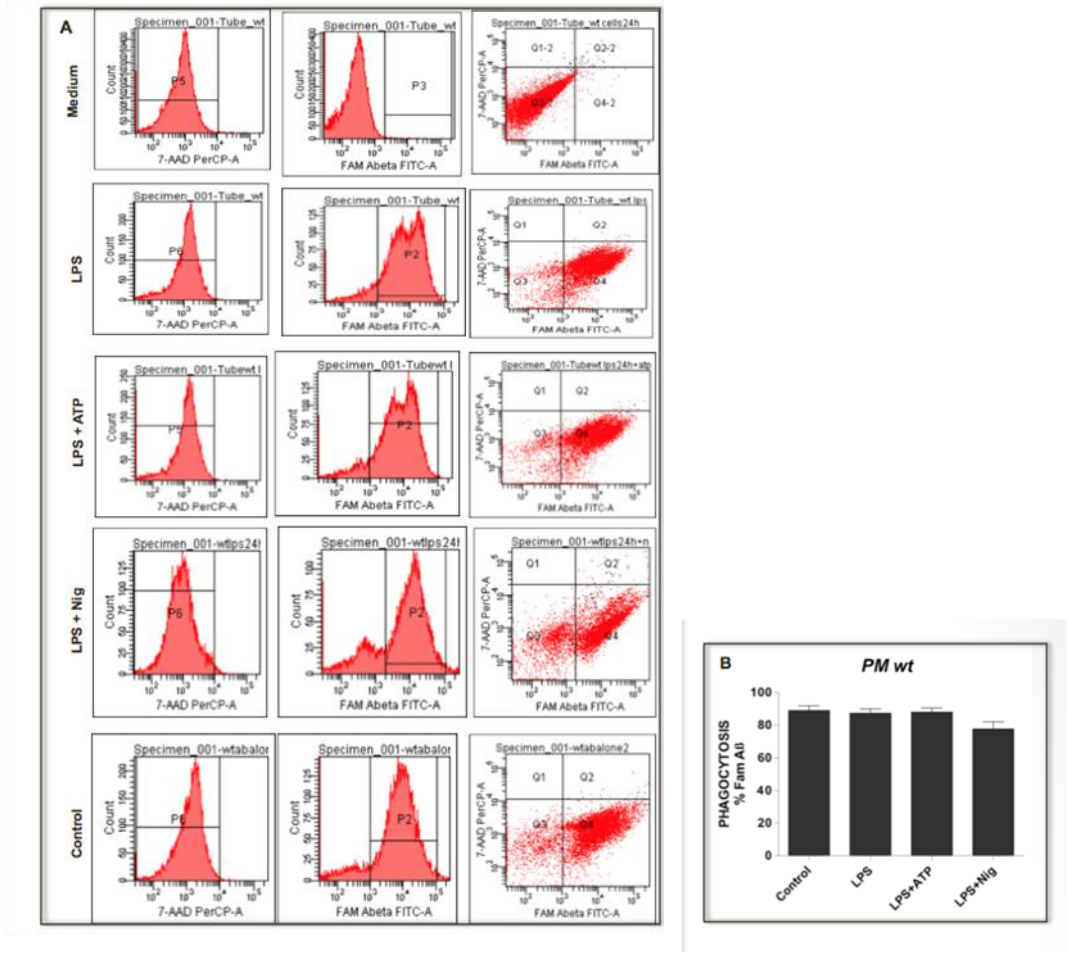
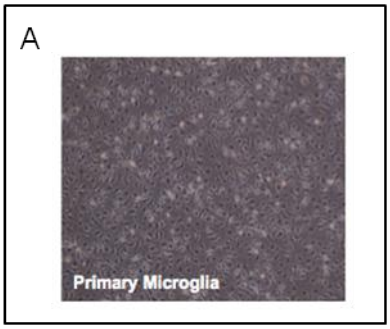
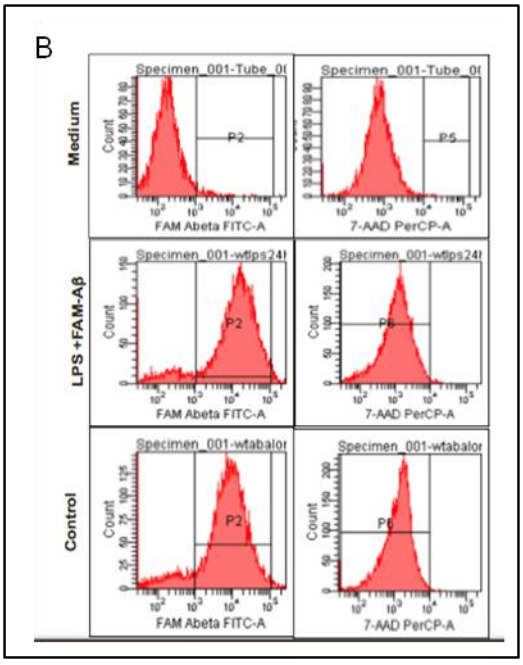
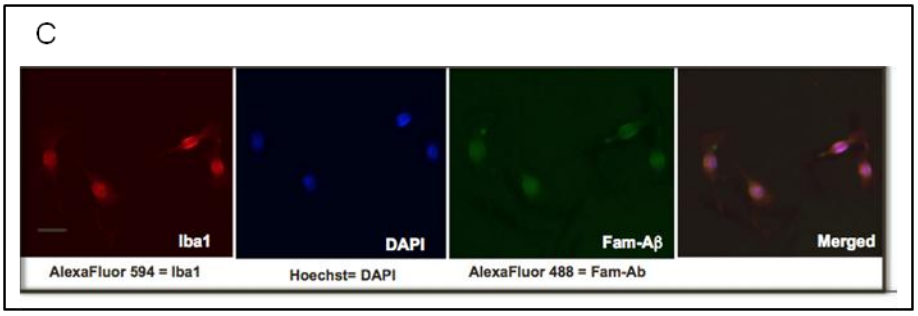


Figure 23. Primary Microglia cells morphology [A]; Flow cytometry Aβ-phagocytosis on WT cells [Medium], LPS-primed and FAM-Aβ stimulation and FAM-Aβ alone treated microglia [Control] [B]; Immunohistochemistry of FAM-Aβ in microglia WT [C]. Immunostaining experiment demonstrated what expected from flow cytometry results; the presence of Aβ appears diffuse between cells and not only localized within the cell. [LPS 200 ng/ml; FAM-Aβ 500nM/ml]. Scale bar represents 20 Um.


A
Primary Microglia


B
Medium
LPS +FAM-Aβ
Control
Specimen_001-Tube_0(
Specimen_001-wtlps24(
Specimen_001-wtabalor
Count
P2
P5
P6
FAM Abeta FITC-A
7-AAD PerCP-A


C
Iba1
DAPI
Fam-Aβ
Merged
AlexaFluor 594 = Iba1
Hoechst= DAPI
AlexaFluor 488 = Fam-Ab

SCIENTIFIC PRODUCTIONS

Publications:

- ✓ Biasin M, Sironi M, Saulle I, de Luca M, La Rosa F, Cagliani R, Forni D, Agliardi C, lo Caputo S, Mazzotta F, Trabattoni D, Macias J, Pineda JA, Caruz A, Clerici M. Endoplasmic reticulum aminopeptidase 2 haplotypes play a role in modulating susceptibility to HIV infection. *AIDS*. 2013 Jul 17;27(11):1697-706.
- ✓ Saresella M, Piancone F, Marventano I, Rosa FL, Tortorella P, Caputo D, Rovaris M, Clerici M. A role for the TIM-3/GAL-9/BAT3 pathway in determining the clinical phenotype of multiple sclerosis. FASEB J. 2014 Aug 4.
- ✓ Saresella M, Gatti A, Tortorella P, Marventano I, Piancone, F. La Rosa F, Caputo D, Rovaris M, Biasin M, Clerici M. Toll-like receptor 3 differently modulates inflammation in progressive or benign multiple sclerosis. Clin Immunol. 2014 Jan;150(1):109-20.

Abstracts of international and national congresses:

- ✓ Francesca La Rosa Marina Saresella, Elena Calabrese, Ivana Marventano, Federica Piancone, Daria Trabattoni , Mara Biasin Mario Clerici. “Activation of NLRP3 inflammasome is associated with the progression of Alzheimer’s disease “ 15° ICI (International Congress of Immunology) Milan, Italy August 22-27 /2013.

- ✓ *Educational Grant to attend the 2014 Joint ACTRIMS-ECTRIMS Meeting* for Poster Presentation on 10-13 September 2014 Boston, Massachusetts,

  USA Francesca La Rosa’ Marina Saresella, Ivana Marventano, Federica Piancone,Laura Mendozzi, Domenico Caputo, Marco Rovaris, Mario Clerici. "UP-regulation of Nod-like receptor signaling in multiple sclerosis”.

- ✓ Francesca La Rosa Marina Saresella, Ivana Marventano, Federica Piancone,Laura Mendozzi, Domenico Caputo, Marco Rovaris, Mario Clerici. "UP-regulation of Nod- like receptor signaling in multiple sclerosis” 12th International Congress of NeuroImmunology (ISNI)14th Esni Course - European School of Neuroimmunology - 9 -13 November 2014 | MAINZ, Germany.

- ✓ Francesca La Rosa, Michael T. Heneka. “The Role of Inflammasomes for Ab Microglia Phagocytosis in Alzheimer’s Disease“. 4th Venusberg-Meeting on Neuroinflammation. May 7th to 9th, 2015 Bonn, Germany.

- ✓ *Educational Grant* to attend the 15th ESNI Course -European School of

Neuroimmunology - 1-4 June 2015 Praghe Czech Republic.
Francesca La Rosa, Michael T. Heneka. “The Role of Inflammasomes for Ab Microglia Phagocytosis in Alzheimer’s Disease“.

- ✓ 17th International Symposium on DAMPs and HMGB1- September 10-12 2015 Bonn, Germany

- ✓ 17th International Conference of Imuunology (ICI) - 25-27 September 2015 London- United Kindom. Francesca La Rosa, Michael T. Heneka. “The Role of Inflammasomes for Ab Microglia Phagocytosis in Alzheimer’s Disease“.

Acknowledgments

...Lassù dittu Carmini Caruni, rè di la muntagna: ognuno a ntressi so 'mpara e 'nsigna...

Cioè ognuno a spese sue impara dall'esperienza, ed oggi che volge al termine, non è facile citare e ringraziare, in poche righe, tutte le persone che hanno contribuito alla nascita e allo sviluppo di questa tesi : chi con una collaborazione costante, chi con un supporto morale o materiale, chi con consigli e suggerimenti o solo con parole di incoraggiamento, sono stati in tanti a dare il proprio apporto alla mia carriera universitaria e a questo lavoro.

La mia gratitudine va a tutti i miei colleghi della Fondazione Don Carlo Gnocchi per la disponibilità e il calore che hanno mostrato in questi tre anni e per l'atmosfera serena e piacevole che ha accompagnato la mia esperienza di "dottoranda". Un grazie speciale va alla mie compagne di scrivania, Chicca ed Ivana, da ciascuna di esse ho avuto qualcosa da imparare.

In particolare, voglio rivolgere un ringraziamento alla dott.ssa Marina Saresella ed al prof. Mario Clerici, relatore della tesi, per avermi aiutato ad indirizzare il mio lavoro di progetto e di tesi, e per fornirmi insegnamenti preziosi per il mio futuro professionale.

Ringrazio inoltre il prof. Michael Heneka dell'università di Bonn e tutto il suo fantastico teem per la cordialità con cui sono stata accolta nel loro laboratorio e per i preziosi insegnamenti.