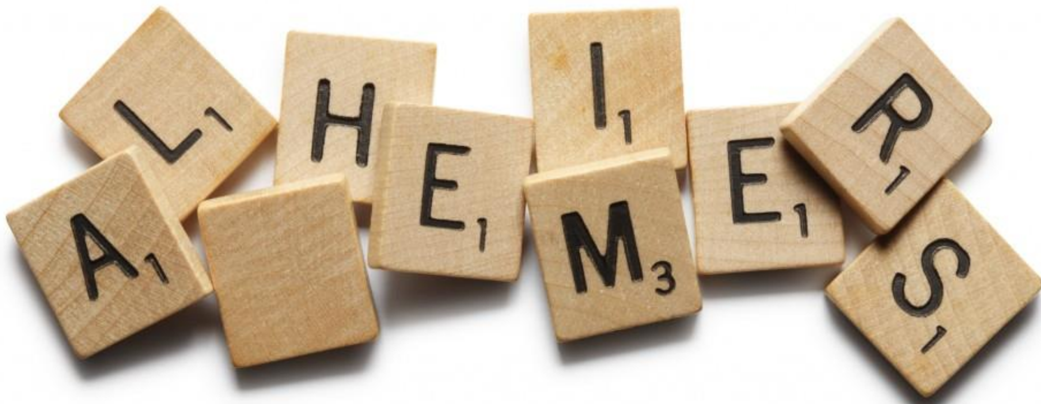
UNIVERSITAT ROVIRA I VIRGILI

FINAL DEGREE PROJECT

Guided by **Dr. Andrea Tenner**



Using primary mouse microglia to investigate the role of C5a in inducing an inflammatory response to Aβ.

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Biochemistry and Molecular Biology

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Abstract

The macrophages of the brain's innate immune system, microglia, represent around 10% of the total cells in the nervous system. Microglial cells suffer a rapid transformation from a quiescent to an activated phenotype to respond to pathological affairs, and this phenotype is characterized by increased cytotoxicity and motile activity. Complement activation products, including C5a, are thought to play a role in the pathogenesis of numerous neurological diseases. Primary neuronal and microglia cultures prepared from rodent nervous tissues represent a powerful tool not only to study the individual contribution of different cell types (such as neurons or glia) to disease progression, but also to investigate the role of neuron-glia interactions during development and pathogenesis of disease. It is known that fAβ has some toxic effects on neurons directly, but the strong presence of activated microglia at sites of fAβ deposits in AD suggest that these cells may also be contributing to the progression of the disease. In vitro cultured microglia release nitric oxide when fAβ and other co-stimulatory molecules such as IFN-γ or LPS are added. Primary neonatal microglia cultures were treated with or without C5a in the presence or absence of fAβ1-42 20µM in order to determine if C5a can synergistically enhance the production of NO elicited by fAβ. Although microglial cells are a heterogeneous and extremely sensitive population, in most of our experiments we found a synergistic response in cells treated with fAβ and C5a, thus supporting our hypothesis that C5a and fAβ synergize to enhance nitric oxide production. However, variability of the results obtained along the experiments performed can be explained by the fact that microglia are extremely sensitive to minor alterations on the CNS microenvironment.

Aims

This project has been done using the results obtained in the practicum performed at the University of California, Irvine, guided by Dr. Andrea Tenner. The aims of the study are:

1. Test the hypothesis that C5a ligation to CD88 (C5aR) additively or synergistically enhances the pro-inflammatory response initiated by fibrillar amyloid beta in vitro with primary neonatal microglia of wild type mice.
    - We tested the aim by evaluating the effect C5a has on NO generation and collecting media to test for production of pro-inflammatory cytokines, TNF-α, IL-1β and IL-6 in primary microglia from wild type mice.

2. Prove that the obtained results are caused by C5a ligation to CD88.
    - We tested that aim evaluating the effect C5a has on NO generation and production of pro-inflammatory cytokines, TNF-α, IL-1β and IL-6
        - In CD88 -/- mice
        - Adding PMX53, a C5a/CD88 receptor antagonist, to the cells

Background

It is normal that many older people forget someone's name or things from time to time, but getting confused in places a person knows well or asking questions over and over may be signs of a more serious problem: Alzheimer's disease.

Alzheimer's disease (AD) is the most common cause of dementia among older people. AD is a progressive and thus far irreversible brain disorder that begins slowly, destroying memory, thinking and language skills. Over time, symptoms get worse and people have trouble speaking, recognizing family members and even the ability to carry out the simplest of daily living tasks. In later stages, they may become aggressive, anxious and need total care. The causes of the initiation of Alzheimer's disease are still unknown but it seems likely that damage to the brain starts a decade or more before problems become evident. During the preclinical stage of Alzheimer's disease, people are free of symptoms, but toxic changes are taking place in the brain. Abnormal deposits of β-amyloid in plaques, neurofibrillary tangles (tau tangles throughout the brain made up of hyperphosphorylated tau) and neuronal loss are hallmarks of the disease. Neurons over time lose the ability to function and communicate with each other, and eventually they die. Additionally, a notable inflammatory reaction has been observed, characterized by the presence of reactive glia closely associated with the fibrillar plaques and upregulation of local synthesis of complement proteins. Furthermore, C1q is associated with fibrillar plaques along with tangles, and the presence of C5b-9 linked with dystrophic neurites in plaques and tangles points to that complement being completely activated in AD. The indicated *in vivo* observations are supported by *in vitro* studies confirming that fibrillar β-amyloid can activate both classical and alternative complement pathways and that complement component C5a activation is chemotactic for microglia.

<u>Fibrillar amyloid plaques, neurofibrillary tangles and AD</u>

Two characteristic lesions observed in Alzheimer's disease (AD) brains are fibrillar amyloid plaques and neurofibrillary tangles. In vitro studies have demonstrated the activation of both classical and alternative complement pathways by fibrillar Aβ (Fonseca et al., 2013).

Amyloid precursor protein (APP) is an integral membrane protein expressed in many tissues and concentrated in the synapses of neurons. It is best known as the precursor molecule whose proteolysis generates beta amyloid (Aβ) (Kang et al., 1987; Weldon et al., 1998). Primary function of this protein is not known, thought it has been involved as a regulator of synapse formation, neural plasticity and iron export. Aβ accumulation in the brain is one of the main pathological processes of AD patients. These deposits are the leaders of series of cellular events and also can elicit immunological responses where astrocytes and microglia could participate (Kitazawa et al., 2004). In addition, the mentioned accumulation in parenchyma and blood vessels generates motility in microglia and promotes acute and chronic inflammatory responses against the aggregates. Thus, production of nitric oxide (NO), reactive oxygen species (ROS), pro-inflammatory cytokines (TNFα, IL-1β and IL-6), and PGs (PGE2) is induced, which eventually could promote neuronal death (Figure 1) (Akiyama et al., 2000).

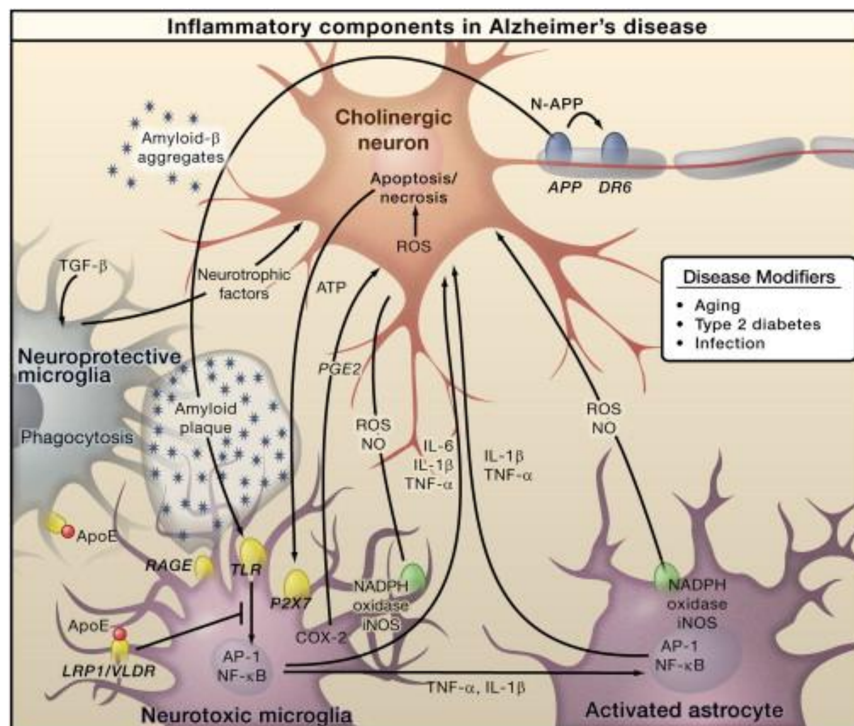

Figure 1. Inflammation in Alzheimer's disease. *The Aβ peptide produced by APP processing, form aggregates that activate microglia through TLRs and RAGE receptors. These receptors in turn, activate NF-κ B and AP-1 transcription factors, which induce the reactive oxygen species (ROS) production and the expression of inflammatory cytokines (IL-1, IL-6, TNF). These inflammatory factors directly act on the neurons and also stimulate the astrocytes, which amplify the pro-inflammatory signals, inducing neurotoxic effects. The inflammatory mediators generated by resident CNS cells, induce the production of adhesion molecules and chemokines, which recruit peripheral immune cell (Gate et al., 2010; Solito and Sastre, 2012). Source:(Glass et al., 2010)*

The complement system and AD

The complement system is composed of >30 small soluble and membrane-associated proteins present in the blood, normally circulating as inactive precursors and they have the capacity of identification, opsonization and lysis of pathogenic targets. When stimulated by a trigger, complement becomes activated and it leads to an enzymatic cascade whereby one protein promotes the sequential binding of the following protein (Forneris et al., 2012).

Complement activation can occur through three different pathways: classical, lectin and alternative. Each of these pathways hangs on different binding molecules for their induction, but eventually they all lead to the generation of Complement 3 (C3) convertase, which is responsible for the activity of complement (Figure 3). The initiation of the classical pathway involves the binding of C1q, the first protein in the complement cascade, to an antigen-bound antibody complex (IgG or IgM) to either the pathogen surface or to the C-reactive protein bound to the pathogen leading to the generation of the protease C3 convertase through C4 and C2 cleavage(Ricklin et al., 2010). In the lectin pathway, carbohydrate-binding proteins are involved to carbohydrate elements present on the membrane of pathogens and leading to the production of C3 convertase, as well. Finally, in the alternative pathway there is a natural hydrolysis of C3 to C3 (H_2O) and thus, a constant low level of activation. This hydrolysis forms C3 convertase as the other complement pathways and if the cleaved C3b binds to the pathogen surface it functions as a ligand for complement receptor 1 (CR1) (Liu and Niu, 2009).

Figure 2: Pathways activating and inhibiting complement. *The three complement activation pathways converge at the formation of the enzyme C3 convertase. This enzyme cleaves complement component C3 into the small soluble protein C3a and the larger component C3b, which becomes covalently bound to the pathogen surface. All pathways thus converge on the formation of C3b bound to a pathogen and lead to all of the effector activities of complement, shown in the bottom row. Source: Davidson College, North Carolina.*

The complement proteins interact in a carefully regulated manner to destroy invading bacteria and prevent the deposition of immune complexes in normal tissue. As previously commented, this system can be activated by diverse mechanisms proceeding through distinct pathways, yet all converge on a final common pathway in which five proteins assemble into a multimolecular complex, the membrane attack complex (MAC)(Xiong et al., 2003). The complement components of MAC are: C5b, C6, C7, C8, and several C9 molecules whose function is to permit the entrance of fluid (water) through its structure in targeted cells and thus it results in the lysis of the corresponding cell. The complement role in the pathogenic elimination by phagocytic recruitment and opsonization takes place through binding with complement receptors. To date, there are four known types of complement receptors: CR1 (CD35), CR2 (CD21), CR3 (CD11b/CD18), and CR4 (CD11c/CD18) (McGeer et al., 1989; Mevorach et al., 1998).

C3a and C5a are small complement fragments which act on specific receptors on endothelial cells and mast cells to produce local inflammatory responses. When injected systematically or produced in large amounts, they produce anaphylactic shock. In addition, C5a activates mast cells to release mediators such as TNF-α, which contributes to the inflammatory response by causing similar effects (Woodruff et al., 2010). C5a also acts directly on neutrophils and monocytes to release their granules adherence to vessel walls and, as a chemoattractant, to direct their migration towards sites of antigen deposition. It also increases their capacity for phagocytosis, as well as raising the expression of CR1 and CR3 on their surfaces (Fonseca et al., 2009).

- <u>C5a Receptors</u>

The C5a receptor, also known as CD88, is a G protein-coupled receptor with seven membrane-spanning domains. Its binding to C5a results in intracellular calcium mobilization and activation of several signaling pathways such as MAPK, ERK, DAG and PI3K. It has been shown that expression of CD88 in the periphery includes both myeloid and non myeloid cells such as endothelial and epithelial cells. A more recently described seven transmembrane receptor is C5L2 and it appears to be deficient in G protein coupling. The function of C5L2 is still not well defined, however, some evidence indicates that it can be anti-inflammatory and or modulator of the CD88 mediated signal transduction through the β arrestin pathway. C5L2 distribution is similarly extensive as CD88. In the central nervous system of human, rat and mouse, CD88 receptor protein and mRNA has been described to be expressed in astrocytes, microglia, subsets of neurons and neuronal progenitor cells. In the case of C52L expression there are more limited studies although there is indication for message in human brain and protein expression in rat astrocytes and subsets of neurons (Fonseca et al., 2009).

Reported studies with mouse models of AD showed the association of complement factors and receptors with amyloid plaques corroborating complement activation in those models. Recently, observations of a neurotoxic effect of a CD88 antagonist in two mouse models of AD supported that the consequences of C5a-CD88 interactions are unfavorable in the aging brain. It suggests that this interaction accelerates the disease and it may be a therapeutic target (Fonseca et al., 2009; Nolte et al., 1996).

Studies of the expression of CD88 in neurons in AD are conflicting, likely due to nonspecific or cross reactivity of the antibodies used in the studies (Fonseca et al., 2009; O'Barr et al., 2001) observed comparable expression in neuron populations of AD and normal control brains, while Farkas and colleagues reported that the neuronal expression of CD88 is decreased in AD, although it was seen to be present in dystrophic neurites associated with plaques[14]. As for the C5L2, it is found associated with tangles in AD brain (Fonseca et al., 2009).

<u>Microglia and AD</u>

The macrophages of the brain's innate immune system, microglia, represent around 10% of the total cells in the nervous system. Microglial cells suffer a rapid transformation from a quiescent to an activated phenotype to respond to pathological affairs, and this phenotype is characterized by increased cytotoxicity and motile activity (Fonseca et al., 2013).

Specifically, Alzheimer's disease is characterized by an inflammatory response to Amyloid-β (Aβ), inducing the activation of microglia and the recruitment of astrocytes to the sites where Aβ deposits occur. Microglial phagocytic response is thus a fundamental part of the brain's defense mechanisms, and is a potent assistant to the systems in place that guarantee healthy neural function. When microglia become activated under pathological situations they surround dead and damaged cells, and analogous to phagocytic macrophages of the immune system they clear cellular debris from the area (Solito and Sastre, 2012). These cells also generate inflammatory mediators like cytokines, chemokines, prostaglandins, inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), free radicals, and stimulate the adaptive immune response. In other words, in response to pathological processes of the brain, microglial cells suffer a rapid transformation from a quiescent, ramified phenotype into an ameboid, activated

phenotype. This last conformation of microglia is characterized by up-regulation of surface receptors (like CR3, the receptor for complement component 3bi or major histocompatibility complex class I and II), secretion of immunoregulatory factors and phagocytic or cytotoxic activity (Nolte et al., 1996; Town et al., 2005).

Recently, *in vivo* studies presented evidence for enhanced motility of microglial cells after neurotoxic lesions or during degenerative diseases. The conditions that permit microglia to migrate towards sites of lesion inflammation are not well characterized (Nolte et al., 1996). Therefore, pharmacological tools that would specifically target the molecular mechanisms controlling the migration of microglia may be therapeutically useful by both enhancing the accumulation of beneficial microglia at lesion sites and preventing the accumulation of harmful microglia at these same sites (Kim et al., 2009). In addition, recent data demonstrates that the innate immune system capacity of response is higher in lineage with a parental history of late-onset AD pointing to heritable traits for AD that are related to the inflammatory processes. Furthermore, the association of high serum levels of certain acute-phase proteins with cognitive decline or dementia yields additional evidence for the early involvement of inflammation in AD pathogenesis (Ager et al., 2010; Ilschner et al., 1996).

Microglial activity is generally beneficial but the extended and progressive nature of its response in AD may foster neurodegeneration. Pathogenic triggers to microglia, including the increased deposition of Aβ peptides, can result in the production of excessive free radicals, proinflammatory cytokines, complement proteins and glutamate. Consequences of the attenuation of inflammation in AD are seen clearly in animal studies. Craft et al. showed reduced toxicity in an animal AD model with previous inhibition of glial inflammation. In AD, advanced glycation end product (AGE) accumulation is accelerated as it accumulates on plaques, and AGE-positive neurons and glia both increase with age and dramatically so with AD progression. Activation of AGE receptor (RAGE), by one of its ligands, such as AGE or Aβ, leads to liberation of proinflammatory mediators (free radicals and cytokines). A combination of both these ligands (AGE and Aβ) can lead to an enhanced microglial inflammatory response (Akiyama et al., 2000; Berbaum et al., 2008; Gasic-Milenkovic et al., 2003).

It is now known that two types of phagocytic cells, microglia and peripheral macrophages, within the CNS can induce the innate immune response. There are specific cytokines and chemokines, which are liberated during microglial and astrocytic activation and are able to signal across the blood-brain barrier, and recruit macrophages into the CNS (Rezai-Zadeh et al., 2009). Currently, there is a solid body of work implicating modifications and alterations in complement signaling in AD. Deregulation of the complement cascade, either by alterations in receptor expression, enhanced activation of different complement pathways or unevenness between complement factor levels, along with complement cascade inhibitors may all make a contribution to the association of complement in AD (Jacobs and Tavitian, 2012). Concerning to microglia and complement, there is evidence indicating that complement factors can manipulate microglia to adopt protective or harmful phenotypes. Consequently, microglia may be activated by detrimental complement signaling and the manifestation of Aβ plaques to enhance their secretion of cytokines in AD, which can stimulate secretion of additional complement factors, driving to a chronic inflammatory response. For the future, some work trying to figure out the complex interactions between AD, complement and microglia would be

a fundamental requirement to be able to manipulate the balance of complement activation and lead to an understanding for therapeutic advantage (Crehan et al., 2012).

Toll like receptors (TLRs) are single, membrane-spanning, non-catalytic receptors usually expressed in cells of the innate immune system such as macrophages and dendritic cells, and act to mobilize a sturdy immune reaction in response to pathogens. TLRs operate as dimers and often use co-receptors like CD14 to support in pathogen recognition. Particularly, CD14 interacts with TLR4 and TLR2-containing dimeric complexes to transduce the activation signals in response to bacterial pathogens. It has been reported in various studies a link between AD and TLRs, but the mechanistic link for how they participate in the microglial response to fAβ is still not defined (Zhang et al., 2007). Recent data from Case Western Reserve University showed that microglia cells lacking CD14, TLR4 and TLR2 were unable to initiate a Src-Vav-Rac signaling cascade, necessary for the induction of reactive oxygen species and phagocytosis. Moreover, in microglia deficient in CD14, TLR4, or TLR2 the fAβ-induced activation of p38 MAP kinase was revoked, impeding subsequent ROS and phagocytosis. Collectively, this study pointed out the functional interaction between CD14, TLR4 and TLR2 and other components of the microglial fAβ receptor complex to induce intracellular signaling cascades resulting in an activated phenotype. The authors reported that the participation of TLRs and the co-receptor CD14 is required in the response of microglial cells to fibrillar forms of Aβ. This response depends on CD14, which acts in connection with TLR4 and TLR2 to bind fAβ and activate intracellular signaling as well. All these data suggest that these innate immune receptors function as components of the microglial fAβ receptor complex and recognize the signaling mechanisms through which they contribute to microglial activation (Reed-Geaghan et al., 2009).

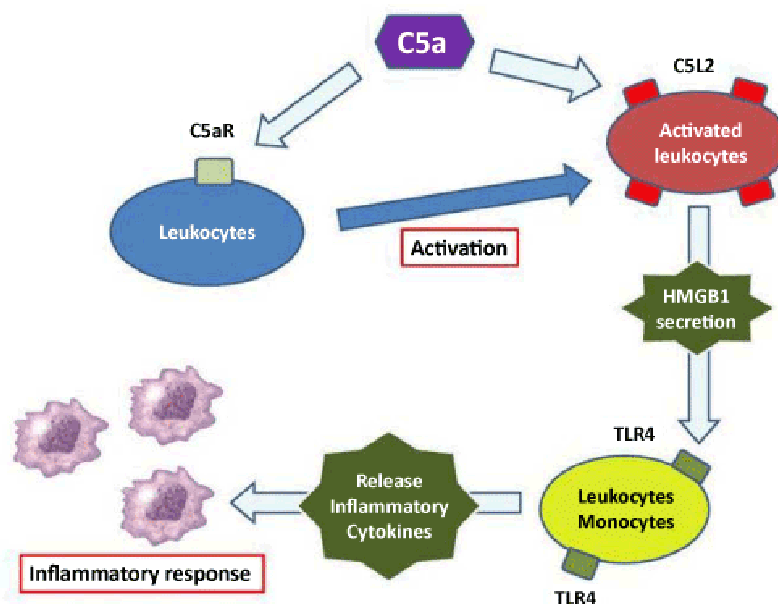

Figure 3: Role for C5a in a positive feedback inflammatory circuit. *When a bacterial infection is produced, LPS stimulates TLR4, and C5a generated during complement activation stimulates C5aR resulting in release of neutrophil granules and cytokine production by myeloid cells. These reactions create an inflammatory amplification circuit. Source:* Andrea's figure showing complement activation with TLR's?

Stimulants

- fAβ

Alzheimer's peptide Aβ is a natural cleavage product released within neuronal cells. If misfolded, Aβ forms distinct structures that are prone to aggregation, first into oligomers, then into progressively larger structures called amyloid plaques. It is now believed that Aβ oligomers are the toxic form of the peptide that may impair basic neuronal processes, such as the communication between the nerve cells, long before clinical symptoms become obvious. Although oligomeric forms of Aβ are quite harmful to neurons, it is fAβ that activates complement in the brain. When working properly, microglia may delay the progression of AD by contributing to the clearance of Aβ, due to its ability to phagocytose Aβ and release enzymes responsible for its degradation. Furthermore, microglia secrete anti-inflammatory cytokines and growth factors, which are neuroprotective. In addition, these macrophages also remove the damaged cells, that is a truly decisive step in the restoration of the natural brain environment, since if these cells remain in the brain, it can become a robust inflammatory stimuli, producing further tissue damage. However, as we age microglia become gradually less efficient and it tends to become over-activated. Thus microglia activation with aging may cause neuronal damage all alone (Miller and Stella, 2008).

- C5a

Several exogenous and endogenous factors recognized to be present in the CNS in the course of inflammation, such as cytokines, bacterial peptides or endotoxin and serum components like complement, might directly or indirectly influence microglial motility. Complement C5a, which is produced by enzymatic cleavage during complement activation in response to immunological and non-immunological events *in vivo*, is one candidate. This complement component performs various activating effects on monocytes and non-brain-derived macrophages, and confirmed to be a strong chemoattractant for microglial cells *in vitro*. The signaling pathways elicited by complement constituents in microglia have attracted moderate attention (Nolte et al., 1996). C5a receptor 1, also known as CD88, plays a role in calcium signaling, which is required in microglia for phagocytosis (Ager et al., 2010; Fonseca et al., 2009). Furthermore, C5a mediates intracellular calcium mobilization and increases microglial motility to damaged areas by a G-protein-dependent pathway. Recent studies suggest a correlation between microglial expression of C5aR/CD88 and Aβ deposition in murine transgenic models of AD, with C5aR/CD88 showing increased expression in microglia adjacent to Aβ plaques. It has been observed that C5a levels and CD88 (C5a Receptor) expression in microglia are enhanced in specific neuropathologies that implicate the complement cascade, and it supports the role of C5a in stimulating microglial cell migration (Fonseca et al., 2013; Nolte et al., 1996).

PMX53

As previously said, C5a is a powerful inflammatory mediator that can influence the development of many pathologies. Thus, an inhibitor able to block the effect of C5a would be helpful in medical applications. It has been reported that PMX53, a CD88 antagonist, significantly inhibited the induction of three cytokines (TNF, IL-1β and IL-17A) producing a repression of inflammation in human periodontitis (Abe et al., 2012; Schnatbaum et al., 2006).

These findings suggest that locally applied CD88 antagonists may potentially find application for the treatment of inflammatory diseases.

Positive controls

- LPS

Lipopolysaccharide (LPS) is a cell-wall component of Gram-negative bacteria such as *Salmonella* that has long been known to induce a reaction in the infected host. LPS varies in composition between different bacteria but essentially consists of a polysaccharide core attached to an amphipathic lipid, lipid A, with a variable number of fatty-acid chains per molecule (Akiyama et al., 2000; Solito and Sastre, 2012). As said above, LPS is an integral component of the outer membrane of Gram-negative bacteria, but during an infection it can become detached from the membrane and picked up by the LPS-binding protein present in the blood and in extracellular fluid in tissues. LPS is transferred from LPS-binding protein to a CD14, which is present on the surface of macrophages, neutrophils and dendritic cells (Ager et al., 2010).

- IFN-γ

The brain has lack of major histocompatibility complex (MHC) antigen expression on neural cells, lack of lymphoid drainage and presence of blood-brain barrier, which limits the invasion of immune cells and antibodies into the brain. That's why it has been considered an immunologically privileged site. However, some lymphokines, especially Interferon-γ (IFN-γ) is able to induce class I MHC antigen expression on neuronal and glial cells as well. In microglia, various populations of astrocytes and endothelial cells, IFN-γ can also induce MHC class II expression and this enables the brain cells to function as antigen presenting cells (APCs). In addition, in certain pathological conditions, this cytokine may induce active interaction with immune cells by altering the function of glial cells, and induce the production of a variety of cytokines. Moreover, the function of microglia as effector cells in either inflammatory demyelination or neuronal degeneration can be activated by IFN-γ. It enhances the production of TNF-α, nitric oxide (NO), and superoxide by microglia and astrocytes. Thus, activated microglia can kill neurons by secreting these soluble products (Kawanokuchi et al., 2006; Lue et al., 2001).

Cytokines

Cells from immune system are able to produce soluble proteins that mediate inflammation, called cytokines. They are produced by astrocytes and microglial cells in the CNS and play a fundamental role in its development during embryonic stages. In addition, cytokines are involved in the inflammatory processes of neurodegenerative diseases. Concretely, in AD these proteins are decisive in the development of the pathology. Elevated levels of pro-inflammatory cytokines such as IL-1β, IL-6, IL-10, TNF-α, and TGF-β (Jiang et al., 2011; Mrak and Griffin, 2005) have been shown in studies of cerebrospinal fluid and AD patient tissues and their increase is powerfully associated to microglia activation by exposure to Aβ aggregates.

- IL-1

This cytokine is recognized to induce APP-β mRNA expression in endothelial cells, suggesting a link between increased IL-1 and Aβ formation in AD patients. IL-1 is able to induce APP-β mRNA in endothelial cells, which suggest that there is a link between IL-1 increasing in AD

patients and Aβ formation. It has been discovered that IL-1 is a contributing factor to initiate dystrophic neurite formation in Aβ diffuse deposits. Furthermore, in AD patients, an increase in p38-MAP kinase activity may be promoted by an increase of IL-1, which could lead to tau hyperphosphorilation (Li et al., 2003; Sheng et al., 2000).

- <u>IL-6</u>

IL-6 is a pro-inflammatory cytokine produced by microglial cells and involved in the immunoreactivity present in patient tissues with clinical dementia (Hull et al., 1996). Like IL-1, IL-6 induces APP-β expression. Since this molecule shows multifaceted immune functions and its regulation changes with age, it appears to be an interesting cytokine study for AD. In brain areas where amyloid deposition and microglia activation are prominent in AD patients, the expression of IL-6 mRNA is great increased. On the other hand, increased IL-6 levels in the brain have been implicated in early stages of plaque formation (Chakrabarty et al., 2010).

- <u>TNF-α</u>

The role of TNF-α can vary on neurons. It can have beneficial or harmful effects on different neurons depending on the concentration. One of its functions is to stimulate NF-κB transcription factor which, provokes the expression of pro-inflammatory molecules and promotes the synthesis of neural survivor factors such as manganese superoxide dismutase enzyme, calbindin and anti-apoptotic Bcl-2 protein (Wajant et al., 2003). The direct role of this cytokine in AD remains uncertain, however, TNF-α could be associated with an increased β - and γ –secretase enzyme expression. It has been shown in *in vitro* models that TNF-α stimulates BACE1 expression, an aspartic-acid protease important in the formation of myelin sheaths in peripheral nerve cells, thus, enhancing APP processing (Yamamoto et al., 2007). The use of TNF-α inhibitors decreases amyloid aggregate formation and attenuates the cognitive impairment, which means that is has a protective effect on APP deregulation. Nevertheless, a suppression of the microglial activity to efficiently remove Aβ aggregates is observed when suppression of the TNF-α receptor signaling pathway is long-term, thus provokes Aβ accumulation (Montgomery et al., 2011).

<u>Nitric Oxide</u>

Nitric Oxide (NO) is an important cellular signaling molecule involved in many physiological and pathological processes. L-arginine, in the presence of oxygen, is converted to L-citrulline and releases NO (Figure 6). The enzyme responsible for catalyzing this reaction is Nitric oxide synthase (NOS), and it occurs in three isoforms: the neuronal isoform (nNOS), distributed in neurons, astrocytes and blood vessels; the endothelial isoform (eNOS), located in endothelial cells, hippocampal pyramidal neurons, and some astrocytes; and the inducible isoform (iNOS), typically low but is increased in microglia and astrocytes during neuro-inflammation (Akiyama et al., 2000). It is hypothesized that under physiological conditions, NO regulates the delivery of neurotransmitters and hormones and promotes cell survival and long-term potentiation. However, in inflammatory conditions, high levels of NO are generated, thus it may contribute to synaptic transmission dysfunction, protein and lipid oxidative damage, excitotoxicity, and neuronal death (Liu et al., 2002). It has been revealed in tissue and neuronal analyses of AD patients that Aβ is associated with NO production in microglia cells and reactive astrocytes. NO formation causes protein and lipid modifications, mitochondrial damage, apoptosis and

promote Aβ formation, increasing the γ-secretase complex activity (Guix et al., 2012; Keil et al., 2004).

C5a has been reported to enhance IL-1β and IL-6 in Aβ primed human monocytes and fAβ is known to induce pro-inflammatory cytokines and nitric oxide (NO) from primary microglia[9]. It has also been reported that C5a Receptor, CD88, can synergize with TLRs (Toll Like Receptors) in the periphery to further enhance the pro-inflammatory cytokine response elicited by TLRs alone (Zhang et al., 2007). Since fAβ is a ligand for TLR2 and TLR4, we asked if C5a, acting on the CD88 on microglia, enhances the inflammatory response elicited by fibrillar Aβ.

The purpose of this report is first, to test the hypothesis that C5a ligation to CD88 (C5aR) additively or synergistically enhances the pro-inflammatory response initiated by fibrillar amyloid beta *in vitro* with primary neonatal microglia of both wild type and C5aR-/- mice. Secondly, to evaluate the effect C5a has on NO generation and production of pro-inflammatory cytokines, TNF-α, IL-1β and IL-6 that are induced by fibrillar Aβ in primary microglia of both wild type and C5aR-/- mice.

Materials and methods

Many experimental animal models of human neurodegenerative diseases have been developed to understand the events leading toward neuronal dysfunction and death. However, definitive comprehension of the molecular and cellular mechanisms in these animal models is problematic because of the complexity of the intact nervous tissue. Primary neuronal and microglia cultures prepared from rodent nervous tissues represent a powerful tool not only to study the individual contribution of different cell types (such as neurons or glia) to disease progression, but also to investigate the role of neuron-glia interactions during development and pathogenesis of disease (Kim and Magrane, 2011).

Reagents

Human Aβ (1–42) was provided by Dr. Charles Glabe from University of California at Irvine. Two common forms of amyloid-β peptides found in the AD brain are Aβ42 and Aβ40. Aβ42 is the predominant component in senile plaques and is considered to be more neurotoxic because it forms aggregates ans fibrils more quickly. A 250µg Aβ aliquot was slowly solubilized in 56.25µl filter sterilized MilliQ H_2O (MilliQ water is purified to remove cell activating LPS) and after let sit for 4-5 minutes, 56.25µl of 2x TBS was then slowly added, resulting in 112.5µl Aβ at 500µM. The reagent was then kept at 4ºC to induce fibril formation and after 20-24h, it was checked for β-sheet formation. The conformation of Aβ was determined by circular dichroism and showed that the peptide contained β-sheet fibrils (Figure 4) (Benoit et al., 2012; Kayed et al., 2003; Pisalyaput and Tenner, 2008). Recombinant human C5a was purchased from Sigma (Deisenhofen, Germany).

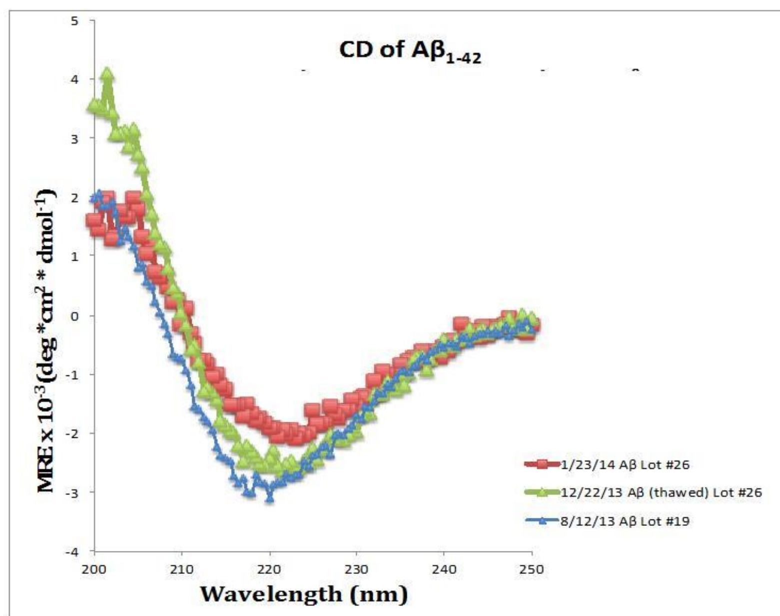

Figure 4. Determination of fAβ conformation by circular dichorism (CD). *fAβ, as prepared, has circular dichroism spectra with a minimal at 218nm, pointing to β-sheet present in the protein. β-sheet secondary structure is indicative of fibril conformation This data is representative of many Aβ preparations and did not change upon freeze-thawing of the aliquots. Source: Hernandez, MX.*

Mouse Interferon-γ recombinant protein was obtained from eBioscience, Inc. The $6.4 \cdot 10^6$U/ml stock was diluted in 1080µl PBS to achieve a final concentration of 10^6U/ml. Lipopolysaccharide from E. coli (LPS) was also purchased from Sigma (Deisenhofen, Germany) and dissolved in endotoxin-free sterile water at 1mg/ml. A specific C5aR (CD88) antagonist, PMX53, was received from Cephalon. A 10µl stock in sterile water was prepared.

Mice

All animal experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee of University of California at Irvine. Non-transgenic (C57BL/6J) mice were used as controls. C5a receptor knockout mice were provided by Dr Rick Wetsel (Hollmann et al. 2008), generated by targeted deletion of the C5a receptor gene (CD88-/-) and backcrossed 10 generations to C57BL/6J mice.

Cell cultures

All the cultures were performed according to the "Neonatal Mice Dissection Protocol". Microglia cells were prepared from cortex of 1-3 days old mice. In brief, cortical tissue was carefully removed free of blood vessels and meninges in the dissection hood. Tissue was trypsinized with 1ml trypsin/EDTA 0.25% per brain previously warmed to 37ºC, cells were carefully dissociated with two fire-polished glass pipettes and washed twice by centrifugation 5 and 7minutes at 12000rpm. The cortical cells were cultured on T25 flasks which had been coated with 2.5ml mixture of 100µl of 10mg/ml poly-L-lysine (Sigma Aldrich, St Louis, MO, USA) in 50ml sterile ddH_2O, allowed to sit flat for 30minutes, aspirating the solution, leaving the flasks up right without lids to dry completely and finally washing with 10ml sterile ddH_2O and leaving it upright overnight. After the washes, cortical tissue was resuspended in 0.5ml of culture medium, which was Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 2% Penicillin Streptomycin (P/S).

Microglia were cultured for 12 to 14 days in vitro before treatment with change of 100% medium after 48h and 50% medium on days 7 and 10. Subsequently, microglia were separated

from the underlying astrocytic monolayer by agitation by agitation for 1:30h at 37ºC and $2.2 \cdot 10^5$ to $3.5 \cdot 10^5$ cells were harvested per flask and resuspended at 10^6 in HL-1 serum free medium. Cell concentration was determined using a hemocytometer prior to any experiments. A volume of 100µl and 100,000 cells were plated in each well in 96-well plates (Fisher). After 1 to 2h in the incubator at 37ºC in 5%CO_2, when the cells were adhered in the bottom of the plate, they were treated with fAβ 20µM, C5a 10nM, both fAβ 20µM and C5a 10nM, the positive control LPS 100ng/ml and INF-γ 150U/ml, or the C5a antagonist PMX53 100nM, and collected in a time course as exemplified in Figure 5. All the reagents concentrations were previously tested in order to identify the best concentrations that may produce an accurate stimulation to microglial cells.

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

Figure 5. Example of the distribution of the wells in a 96 –well plate. *The plated wells are the colored ones. 100µl of cells in HL-1 serum free medium were plated in each well. The given example is from 1 experiment out of 26. Legend: In light pink, untreated cells; in red, C5a 10nM; in green, fAβ 10µM; in orange, fAβ 10µM + C5a 10nM, in dark pink, fAβ 10µM + C5a 10nM + PMX53 100nM; in blue, IFN-γ 150U/ml + LPS 100ng/ml; yellow squares, wells collected at 1h, pink squares, wells collected at 3h; green squares, wells collected at 6h, blue squares, wells collected at 24h.*

Supernatants were accurately collected in 0.5ml tubes, spun in the microcentrifuge for 5min at max speed to remove any detached cells or cellular debris and kept at -80ºC for later Nitrite & Nitrate Oxide Assay. Cell lysates were collected using RA1 buffer from the RNA extraction kit (GE #25-0500-72) following the manufacturer's protocol and stored at -20˚C. Additionally, in some experiments, the supernatant was collected and kept at -80˚C for future cytokine analysis.

To assay for nitric oxide, a colorimetric assay was used using Griess reagents[34] (Figure 6). A Standard Curve from 0µM to 20µM was prepared in a 96-well plate with Assay Buffer diluted to 100ml MilliQ water (#780022 from Cayman Chemicals) and Nitrate Stock ($NaNO_3$), reconstituted powder in 1ml of Assay Buffer (#780014). A total volume of 80µl of each supernatant previously collected and frozen was added to the corresponding wells. All samples were assayed in triplicate. Immediately, 5µl of NADH, reconstituted with 3ml MilliQ water (part

of NaR-PkAt kit from NECi Co.), was added to all wells excluding blank. Subsequently, 10µl of Nitrate Reductase (NaR), reconstituted powder in 1ml of Enzyme Diluent (part of NaR-PkAt kit from NECi Co.), was also added to all wells excluding blank. The plate was covered with parafilm and incubated at 37ºC for 2 hours to allow Nitrate breackdown by the NaR with NADH cofactor into Nitrite. After 2h, 50µl Griess Reagent R1 (#780018 from Cayman Chemicals) and immediately 50µl Griess Reagent R2 (#780020) were added to all wells excluding blank, bringing a final volume of 200µl in all the wells with a dilution factor of 2.5. The plate was incubated at room temperature for 10 minutes and the Optical Density (OD) was read at 540nM using the SpectraMax spectrophotometer with plate reader. The blank consisted of 200µl of Assay Buffer.

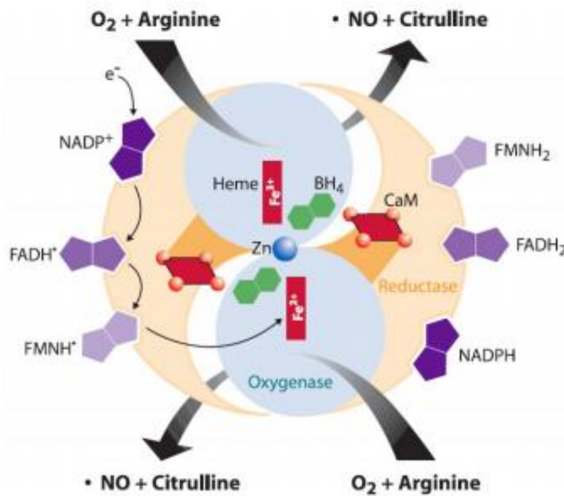

Figure 6. Nitric Oxide (NO) is synthesized in biological systems by the enzyme Nitric Oxide Synthase (NOS)*. NOS is a remarkably complex enzyme which acts on molecular oxygen, arginine, and NADPH to produce NO, citrulline, and NADP+. This process requires five additional cofactors (FMN, FAD, Heme, calmodulin andtetrahydrobiopterin) and two divalent cations (calcium and heme iron). Source: Nitrate/Nitrite Colorimetric Assay Kit (LDH method), Catalog Number KA1343, Abnova.*

Results

In vitro cultured microglia release nitric oxide when fAβ and other co-stimulatory molecules such as IFN-γ or LPS are added. Primary neonatal microglia cultures were treated with or without C5a in the presence or absence of fAβ1-42 20µM in order to determine if C5a can synergistically enhance the production of NO elicited by fAβ.

I. <u>Establishment of NO assay.</u>

Colorimetric Nitrite and Nitrate Oxide Assays were performed since such assay permitted quantification by reference of included standard curves generated with Assay Buffer and $NaNO_3$. The standard curve was done in technical duplicates and NO generation was detectable from 0 to 20µM, as described in Materials and Methods.

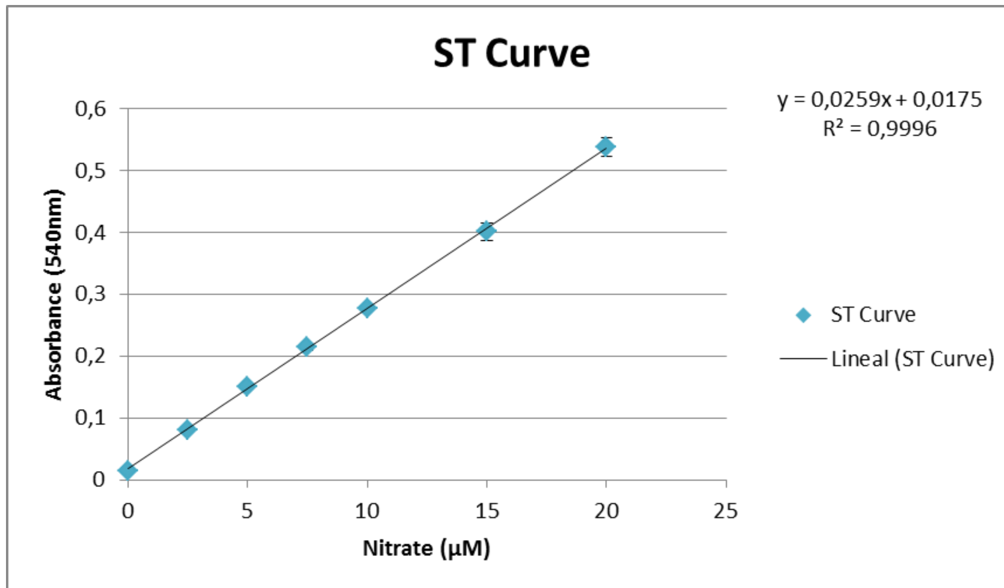

Figure 7. Representation of Standard Curve for the assays. *This NO Assay standard curve of concentration (µM) versus absorbance (at 540nm) was used to determine the concentration (in µM) of Nitrite Oxide, using the equation y = 0.023 + 0.026x with an R^2 value of 0.999, where y is concentration and x is absorbance. This Standard Curve is representative of 13 assays performed.*

II. Establishment of conditions to trigger NO secretion by microglia.

To determine if C5a can synergistically or additively enhance the production and release of NO elicited by fAβ, primary microglia cultures were treated with or without C5a (CompTech) in the presence or absence of 20µM fAβ1-42. We previously determined the concentration of fAβ to use by performing a dose response curve and selecting a concentration that limits NO production above basal levels but also maximizes response upon addition of C5a. Based on the results obtained, we used 10nM C5a to test for synergy, a concentration that does not induce much NO production on its own (Figure 8), but increases the release of NO (after 24 hours) when added with fAβ 20µM in preliminary results.

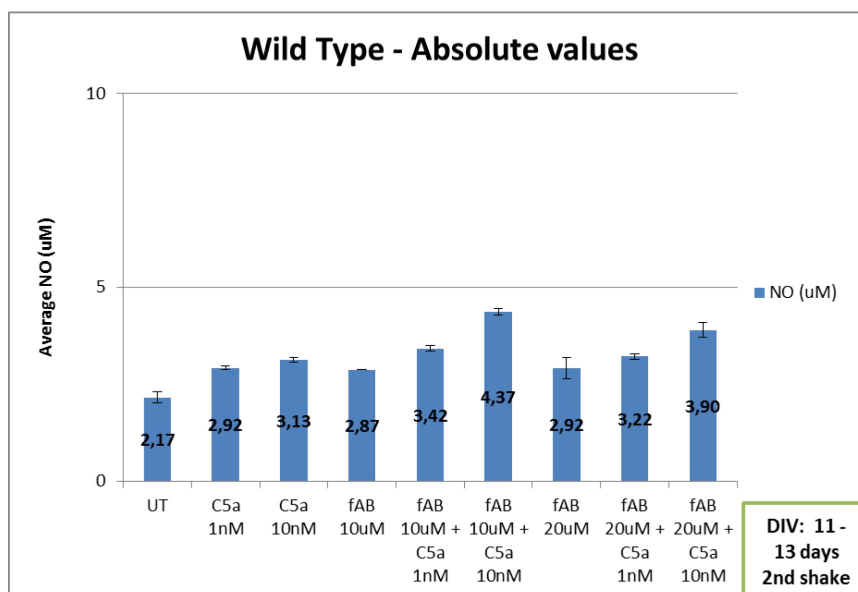

Figure 8. Dose response graphical representation of C5a and fAβ. *Some assays were performed with different concentrations of C5a (1nM, 10nM and 100nM) to settle which one did not induce a response on its own but increases the release of NO when added with fAβ (10 µM and 20µM). Points are averages of triplicates from a one experiment representative of at least 7. Source: Hernandez, MX.*

III. Kinetics of generation of NO by stimulated microglia.

NO Assay of primary microglia isolated from neonates of non-transgenic mice (C57BL/6J) was performed in order to see the synergistic response of the stimulants in a time course study. The quantification of the colorimetric assay showed a synergistic reaction on cells treated with fAβ 20µM + C5a 10nM, most of them at 24h (Figure 9). However, in some of the experiments we observed an enhancement of NO production at 6hrs and no enhancement at all in a few experiments.

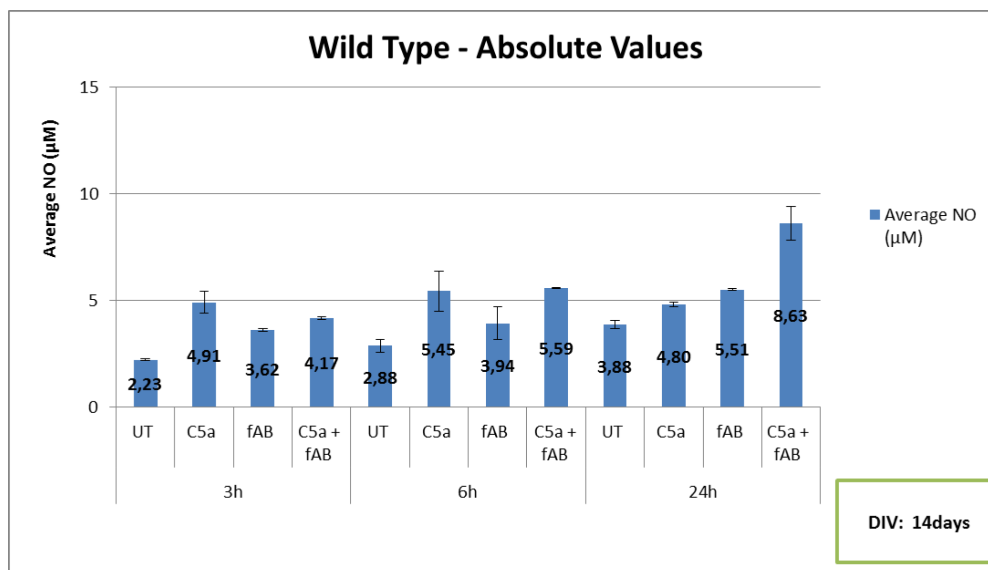

Figure 9. Graphical representation of the increase of the NO production by stimulants in wild type neonatal primary microglia. *These results are representative of 5 experiments. As we can appreciate, both fAβ 20µM and C5a 10nM are not producing a response at 3 and 6h. However, at 24h we can observe a huge synergistic response in wells stimulated by both fAβ 20µM and C5a 10nM. In all the experiments, LPS 100ng/ml + IFN-γ 150U/ml was used as the positive control and it had a good response in all of them. DIV: Days in vitro.*

IV. Generation of NO by CD88 knockout.

NO Assay quantification in neonatal primary microglia from CD88 knockout mice showed sinergistic response in cells collected at 6h (Figure 10). In the totality of the experiments there was a high variability in the untreated cells response, probably caused by noise in the system. Furthermore, an unexpected nitric oxide generation by C5a was produced. Since these microglia lacked CD88, we did not expect C5a to have any effect. Further experiments will be performed in order to find out the reason we observed the release of NO due to C5a.

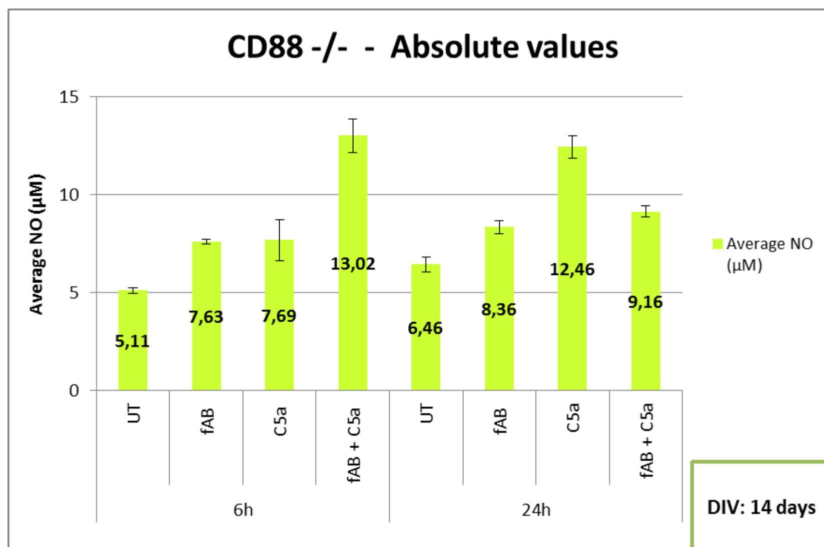

Figure 10. Graphical representation of the increase of the NO production by stimulants in CD88-/- neonatal primary microglia. *These results are representative of 3. At 24h there is a high NO production in cells treated by C5a (10nM), which goes in line with previous experiments. As in wild type, LPS 100ng/ml + IFN-γ 150U/ml was used as the positive control and it had a good response in all of them. DIV: Days in vitro.*

V. Effect of CD88 antagonist PMX53.

We also tested the effect CD88 antagonist PMX53 100nM had on primary neonatal microglia from wild type. In microglia treated by PMX53 100nM at 24h, no NO values were expected since this drug binds to the C5a receptor (CD88) but does not activate the receptor. In the case where there is a competition between C5a and PMX53 to bind in CD88, i.e. when we added PMX53 to fAβ 20µM + C5a 10nM we saw essentially no decrease compared with the cells treated with fAβ 20µM + C5a 10nM. One possible explanation could be that the antagonist is not interacting with CD88, thus permitting the ligation of C5a to its receptor. It is known that PMX53 binds substantially with less affinity to the murine CD88 compared to that of the human CD88. Reproducible results with the antagonist were not observed in the experiments performed.

Table 1. Summary of the results obtained. *This table contains the information about the results of the synergy obtained when C5a and fAß were added together in all experiments, the genotype of the mice, number of flasks used in each experiment, days the cells stayed in vitro, number of cells harvested in the total of flasks and number of cells in each flask.*

<table>
<tr><th>Genotype</th><th>Nº flasks</th><th>Days in culture</th><th>nº cells</th><th>Ratio cells/flask</th><th>Time collected / Treatment</th><th>Synergy</th><th>PMX53</th></tr>
<tr><td rowspan="2">CD88 -/-</td><td rowspan="2">17</td><td rowspan="2">14</td><td rowspan="2">3,00E+06</td><td rowspan="2">1,76E+05</td><td>6h; fAB 20uM & C5a 10nM</td><td>Yes</td><td rowspan="2">Alone, values similar to UT</td></tr>
<tr><td>24h; fAB 20uM & C5a 10nM</td><td>No</td></tr>
<tr><td>CD88 -/-</td><td>15</td><td>14</td><td>3,21E+06</td><td>2,14E+05</td><td>6h: fAB 20uM & C5a 10nM</td><td>Yes</td><td></td></tr>
</table>

					24h; fAB 20uM & C5a 10nM	No	Alone, values similar to UT; with fAB + C5a, increase NO production
CD88 -/-	29	13 & 14	7,44E+06	2,57E+05	1h; fAB 20uM & C5a 10nM	Yes	Alone, values similar to UT
					3h; fAB 20uM & C5a 10nM	No	Alone & with fAB + C5a, alues similar to UT
					6h; fAB 20uM & C5a 10nM	Yes	Alone, values similar to UT; with fAB + C5a, increase NO production
					24h; fAB 20uM & C5a 10nM	No	Alone & with fAB + C5a, alues similar to UT
wt	20	14	5,00E+06	2,50E+05	3h; fAB 20uM & C5a 10nM	No	
					6h; fAB 20uM & C5a 10nM	Yes	Alone, values similar to UT
					24h; fAB 20uM & C5a 10nM	No	Alone, values similar to UT; with fAB + C5a, increase NO production
wt	17	13 & 14	3,42E+06	2,01E+05	3h; fAB 20uM & C5a 10nM	No	
					6h; fAB 20uM & C5a 10nM	No	
					24h; fAB 20uM & C5a 10nM	Yes	
wt	18	13 & 11	5,84E+06	3,24E+05	1h; fAB 20uM & C5a 10nM	No	
					3h; fAB 20uM & C5a 10nM	Yes	
					6h; fAB 20uM & C5a 10nM	No	
					24h; fAB 20uM & C5a 10nM	No	Alone, values similar to UT; with C5a, decrease NO production

wt	20	14 & 13	1,08E+06	5,40E+04	24h; fAB 20uM & C5a 10nM	No
wt	21	14	3,60E+06	1,71E+05	3h; fAB 20uM & C5a 10nM	No
					6h; fAB 20uM & C5a 10nM	No
					24h; fAB 20uM & C5a 10nM	No
wt	15	14	3,90E+06	2,60E+05		Stored for cytokines studies
wt	17	14 & 13	5,10E+06	3,00E+05		Stored for cytokines studies
wt	23	14 & 13	5,54E+06	2,41E+05		Stored for cytokines studies
wt	25	14 & 13	6,25E+06	2,50E+05		Stored for cytokines studies
wt	30	14, 13 & 12	9,68E+06	3,23E+05		Stored for cytokines studies

Discussion & Conclusion

The precise relationship between different pools of amyloid and the memory deficits characteristic of human AD are still under intense investigation (Kayed et al., 2003; Walsh et al., 2002). It is known that fAβ has some toxic effects on neurons directly, but the strong presence of activated microglia at sites of fAβ deposits in AD suggest that these cells may also be contributing to the progression of the disease (Li et al., 2004). It has been reported that in *in vitro* studies of primary microglia, addition of IFN-γ is required for fAβ induced microglia-mediated neurotoxicity (Goodwin et al., 1995; Murphy et al., 1998). In this paper, we report that fAβ and C5a synergize to enhance nitric oxide production. However, we saw a lot of variability in our system. Unexpectedly, in the studies with CD88 knockout mice, we found NO release in the cells treated with C5a. Since there was no receptor for C5a and NO was released anyway, an explanation could be that removing CD88 may have altered signaling and possible led to compensatory mechanisms by which NO can be produced in the absence of CD88. In addition, it has been shown that CD88 plays an important role in regulating TLR4 signaling (Zhang et al., 2007), thus its possible changes in TLR4 on cells lacking CD88, could possibly explain the production of NO by C5a.

We also studied the effect C5a receptor antagonist PMX53 had on primary neonatal microglia from wild type mice. Complement activation products, including C5a, are thought to play a role in the pathogenesis of numerous neurological diseases. An interesting point shown in our results was that PMX53 alone on some of our experiments seemed to activate our cells. When added to cells treated with fAβ and C5a, there was essentially no decrease in NO production compared to cells treated with fAβ 20μM + C5a 10nM. In this experiment shown in figure 11, we did not see synergy from cells treated with fAβ + C5a although we did see synergy in other experiments. In that particular experiment the untreated cells were producing a big amount of NO probably caused by some noise in the media, so we have to consistently repeat the experiment with those conditions to find out the explanation that C5a receptor antagonist PMX53 has on treated cells.

As highly specialized cells, microglia can either trigger neurotoxic pathways leading to progressive neurodegeneration, or exert important roles in promoting neuroprotection, downregulation of inflammation, and stimulation of repair (Czeh et al., 2011). Microglia are related to resident tissue macrophages despite disctinct embryonic origins. Monocyte-derived macrophages are classified into phenotypic subsets: M1, M2a, M2b and M2c (Figure 11) (Geissmann et al., 2010). It is plausible that microglia acquire one or other of these unique phenotypes based on their exposure to variable stimuli. M1 is a phagocytic phenotype state associated with the production and release of pro-inflammatory cytokines (interleukin (IL)-1β, IL-6, IL-23, tumor necrosis factor alpha (TNF-α)) and cytotoxic substances (oxygen-free radicals, nitric oxide (NO), quinolinic acid). M2 is also a phagocytic phenotype state, but triggers anti-inflammatory responses (IL-10, transforming growth factor beta (TGF-β)), or alternatively promotes tissue repair. It is important to note that M1 and M2 represent a spectrum of activation patterns and not separate cell subtypes. Thus, a continuum of different intermediate phenotypes may exist, (Mosser and Edwards, 2008) and microglial cells will switch from one to another according to both the nature of the stimulus provoking the initial insult, and subsequent events influencing phenotype.

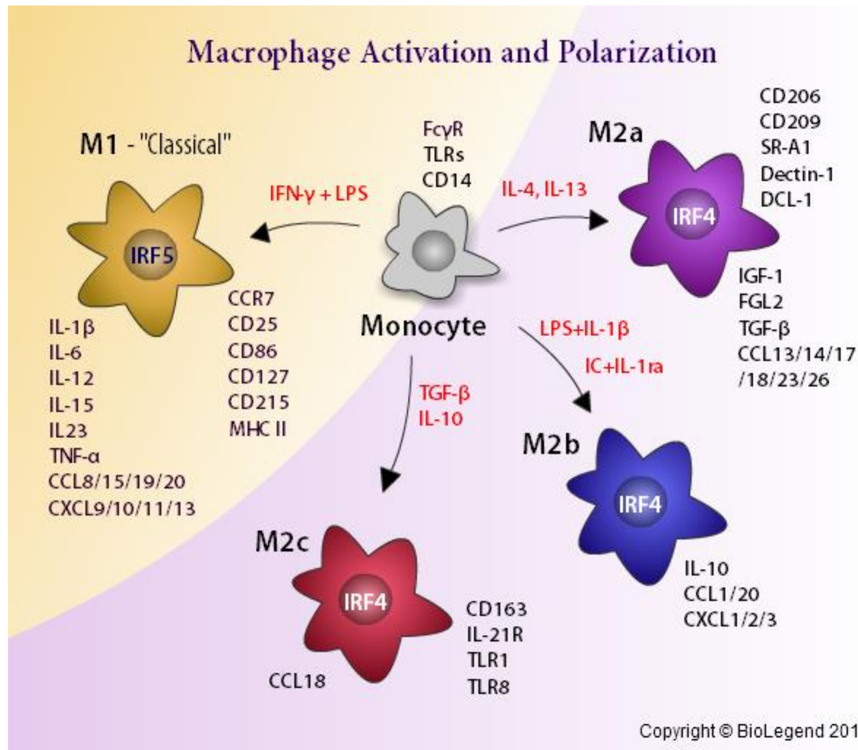

Figure 11. Phenotypic subsets of microglia. *Like T helper cells, macrophages also polarize to distinct phenotypes expressing unique cell surface molecules and secreting discrete sets of cytokines and chemokines. The classical M1 phenotype supports pro-inflammatory Th1 responses driven by cytokines such as IL-12 and IL-23, while the alternate M2 phenotype is generally supportive of anti-inflammatory processes driven by IL-10. M2 cells can be further classified into subsets, M2a, M2b, and M2c, based on the type of stimulation and the subsequent expression of surface molecules and cytokines. Source: BioLegend.*

In summary, microglia are extremely sensitive to minor alterations on the CNS microenvironment, even ionic unbalance and stress (Ransohoff and Perry, 2009; Sugama et al., 2007) thus suggesting a possible explanation for the variability of the results obtained along the experiments performed. Moreover, microglial cells are a heterogeneous population with respect to their sensitivity to neurotransmitters/neurohormones and that they are more responsive in defined activation states.

Acknowledgements

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