

PROGRAMA DE DOCTORADO EN NEUROCIENCIAS

TESIS DOCTORAL:

**STUDY OF MICROGLIAL APOLIPOPROTEIN D
FUNCTION IN RESPONSE TO OXIDATIVE
STRESS**

**Estudio de la función de la Apolipoproteína D
en microglía en respuesta al estrés oxidativo**

Presentada por Beatriz Bendito Guilarte para optar al
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Dirigida por:

Dr. Diego Sánchez Romero

Dra. María Dolores Ganfornina Álvarez

Tutorizado por:

Dr. José Ramón Alonso Peña

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Universidad de Salamanca

Summary

Apolipoprotein D (ApoD) lipocalin has been studied in recent years, discovering its multifaceted functions in the central nervous system. But beyond this multifunctionality, its influence has been demonstrated in situations that lead to the production of reactive oxygen species (ROS).

At the same time, in recent years microglial cells have been redefined as a spectrum of cells of the central nervous system capable of reacting differently in each situation to coordinate a wide number of responses alone or under the influence of other cells of the nervous system through intercellular cross-talk.

This thesis **aims** to bring together the action of ApoD and microglia in specific stress situations, establishing the bases of this relationship. As well as trying to know better the influence of ApoD in microglia cytokine profile secretion under specific situations or under exposure to products of neurodegeneration.

ApoD is not produced by microglial cells, but these cells can take it up from the environment, being mainly secreted by astrocytes. Microglia can internalize ApoD in a specific manner, dependent on stress exposure, but with a shorter lifetime, compared to neurons or astrocytes. sApoD-deprived mice show a different pattern of cytokine secretion than the mouse not deprived of an ApoD-enriched microenvironment and this pattern has been found to be sex-dependent.

The interaction between astrocytes and microglia can be therefore the key to understand the role of ApoD in lipid metabolism and neurological disorders and might also help to know better the neuroprotective effect of ApoD in microglia.

Resumen

La lipocalina apolipoproteína D (ApoD) se ha estudiado en los últimos años, descubriéndose sus múltiples funciones en el sistema nervioso central. Pero más allá de esta multifuncionalidad, se ha demostrado su influencia en situaciones que conducen a la producción de especies reactivas de oxígeno (ROS).

Al mismo tiempo, en los últimos años las células microgliales se han redefinido como un espectro de células del sistema nervioso central capaces de reaccionar de manera diferente en cada situación para coordinar un amplio número de respuestas: solas o bajo la influencia de otras células del sistema nervioso a través de contactos intercelulares.

Esta tesis pretende acercar la acción de ApoD y microglía en situaciones de estrés específicas, estableciendo las bases de esta relación. Así como intentar conocer mejor la influencia de ApoD en el perfil de citoquinas secretadas por la microglía bajo situaciones específicas o bajo exposición a productos de neurodegeneración.

La ApoD no es producida por las células microgliales, pero estas células pueden captarla del medio ambiente, siendo secretada principalmente por los astrocitos. La microglía puede internalizar ApoD de una manera específica, dependiente de su exposición a situaciones de stress, pero con una vida más corta, en comparación con las neuronas o los astrocitos. Los ratones privados de ApoD muestran un patrón diferente de secreción de citocinas que el ratón no privado de un medio con ApoD y se ha descubierto que este patrón depende del sexo. Por tanto, la interacción entre astrocitos y microglía puede ser la clave para comprender el papel de ApoD en el metabolismo de los lípidos y los trastornos neurológicos y también podría ayudar a conocer mejor el efecto neuroprotector de ApoD en la microglía.

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Abbreviations

%	Percentage
AD	Alzheimer's disease
ANOVA	Analysis of variance
ApoD	Apolipoprotein D
ApoE	Apolipoprotein E
ApoE- ϵ ₂	Apolipoprotein E epsilon 2 allele
ApoE- ϵ ₄	Apolipoprotein E epsilon 4 allele
APP	Amyloid precursor protein
Arg-1	Arginase 1
ATP	Adenosine triphosphate
A β	Amyloid-beta
BBB	Blood-brain barrier
Bp	Base pairs
BSA	Bovine serum albumin
CCL	Chemokine ligand
CCR	Chemokine receptor
cDNA	Complementary deoxyribonucleic acid
cm	Centimeter
CNS	Central nervous system
COX-2	Cyclooxygenase-2
CSF	Cerebrospinal fluid
CSF1R	Colony-stimulating factor 1 receptor
Ct	Threshold cycle
CTF	C-terminal fragment
DAM	Disease associated microglia
DAPI	4',6-diamino-2-phenylindol
Dil	1,1'-Diocadecyl-3,3,3',3'-Tetramethylcarbocyanine perchlorate
DIV	Days <i>in vitro</i>

Abbreviations

DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNase I	Deoxyribonuclease I
DTT	Dithiothreitol
EBSS	Earle's Balanced Salt Solution
ELISA	Enzyme-linked immunosorbent assay
EM	Electron microscopy
EOAD	Early-onset Alzheimer's disease
EVs	Extracellular vesicles
FAM-A β	Fluorescein-labeled β -amyloid peptide
FBS	Fetal bovine serum
FC	Fold change
Fwd	Forward
g	Universal gravitational force
GFAP	Glial fibrillary acidic protein
GLP-1	Glucagon-like peptide-1
h	hour
Iba-1	Ionized calcium-binding adapter molecule 1
ICC	Immunocytochemistry
IgG	Immunoglobulin G
IL-1 β	Interleukin 1 beta
IL-10	Interleukin 10
IL-13	Interleukin 13
IL-4	Interleukin 4
IL-6	Interleukin 6
kDa	Kilodalton
KO	knockout
LDL	Low-density lipoprotein
LOAD	Late-onset Alzheimer's disease
LPS	Lipopolysaccharide
M-CSF	Macrophage colony-stimulating factor

Abbreviations

MAPK	Mitogen-activated protein kinase
MetOH	Methanol
miR(miRNA)	Micro-Ribonucleic acid
mRNA	Messenger ribonucleic acid
mTOR	Mammalian target of rapamycin
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MW	Molecular weight
NCBI	National Center for Biotechnology Information
NF- κ B	Nuclear factor kappa light chain enhancer of activated B cells
NLRP3	Nod-like receptor family, pyrin domain containing 3
OS	Oxidative stress
P/S	Penicillin-Streptomycin
P/S/A	Penicillin-Streptomycin-Amphotericin B
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PDB	Protein Data Bank
PMSF	Phenylmethanesulphonyl fluoride
PQ	N,N'-dimethyl-4,4'-bipyridinium dichloride (paraquat)
PVDF	Polyvinylidene fluoride
Rev	Reverse
RNA	Ribonucleic acid
ROS	Reactive oxygen species
rpm	Revolutions per minute
RPMI	Roswell Park Memorial Institute Medium
RT	Room temperature
RT-PCR	Reverse transcription Polymerase chain reaction
s	Second
SD	Standard diet
SDS	Sodium dodecyl sulfate
SEM	Standard error of the mean
TBS	Tris-buffered saline

Abbreviations

TGF- β	Transforming growth factor beta
TLR	Toll-like receptor
T _m	Melting temperature
TNF- α	Tumor necrosis factor alpha
TREM2	Triggering receptor expressed on myeloid cells 2
vs	<i>versus</i>
WT	Wild-type
°C	Degree Celsius
λ	Wavelength

Introduction

Apolipoprotein D

Discovery and protein family adscription of Apolipoprotein D

Apolipoprotein D (ApoD) receives its name from the fact that it is the fourth apolipoprotein discovered (1). It was detected in 1963, as a distinct component of the human plasma lipoprotein system by Ayrault-Jarrier et al. (2). Although this protein was identified in the human plasma in association to high density lipoprotein (HDL), its structure and sequence is more similar to the lipocalins family structure (3).

Furthermore, the amino acid sequence of ApoD shows no similarity to any of the apolipoproteins in lipoprotein particles, except ApoM which is also considered a lipocalin (4). Within mammals, it shares high similarity (23%) with the retinol binding protein (RBP4). ApoD has been therefore termed the atypical apolipoprotein (3).

ApoD gene and protein

The human ApoD gene (hApoD) is located within chromosome 3 (3q26.2). Its orthologous gene in mice is located within chromosome 16. hApoD is composed of 5 exons. The human gene sequence contains 20 kilobase pairs (kpb) from which only 855 base pairs (bp) codify for the protein. The protein is composed of 169 residues, including a secretion signal peptide of 20 amino acids for its secretion in the lumen of the endoplasmic reticle (3). This protein organization supports a common origin between ApoD and the lipocalin family (5).

The lipocalin family are ancient proteins conserved in chordates, particularly in mammals, but present in all biological kingdoms. The lipocalins are proteins of 150 to 250 amino acids whose main function is transporting and storing vitamins and other hydrophobic molecules. In humans alone, 21 lipocalin genes have

been described. Phylogenetic analyses have shown that ApoD is at the base of the lipocalin tree, being the earliest lipocalin within the chordate lineage (Figure 1).

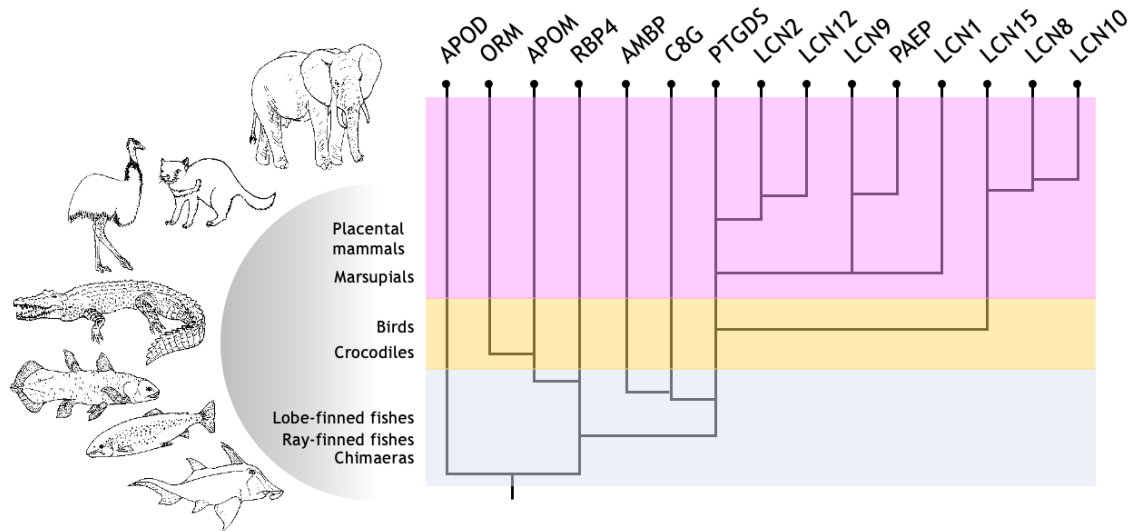


Figure 1: Phylogenetic tree of the Lipocalin family based on the maximum likelihood method.

(Illustration taken from Diez-Hernando et al., 2021 (6)).

The lipocalin secondary structure is highly specific and strongly conserved, especially the three conserved short sequences motifs known as structurally conserved regions (SCR). Lipocalins are subdivided based on the number of these SCRs present in the core. The tertiary structure of ApoD comprises a right hand β -barrel (composed of eight antiparallel β -strands) (7). These β -strands are bound by loops, giving the molecule a calix disposition with a hydrophobic region that can bind small hydrophobic ligands with more efficiency (Figure 2). At the C-terminus there is a canonical α -helix. Human ApoD has a unique fifth cysteine (Cys) residue in the 116th position that can form intermolecular disulfide bridges with another ApoD protein (dimers) or with apolipoproteins (Apo A-I and Apo A-II) (8). This free Cys provides hApoD as well as other proteins such as α -1 microglobulin, dehydrogenase/reductase properties (Figure 2). Also, thanks to the interaction of the methionine in the 93th position of ApoD with lipid hydroperoxides there is a decrease in free radicals overproduction (9).

ApoD dimers have been seen during oxidative stress situations and in later-stages of Alzheimer's disease patients hippocampi. ApoD tetramers have been observed in human breast cyst fluids (10). This oligomerization is a common feature of lipocalins and is known to impact lipocalin ligand binding.

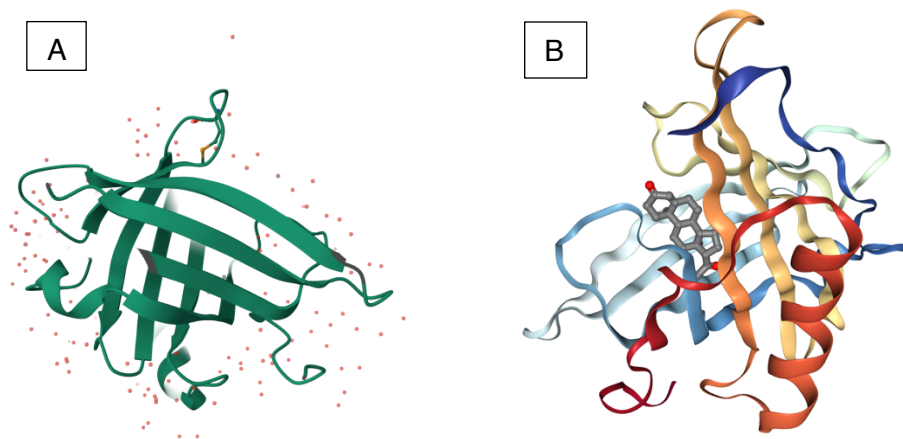


Figure 2: Tertiary structure of ApoD.

(A) Orthogonal vision of ApoD. H₂O molecules are represented in pink and the β -barrels in green. (B) Another view representing ApoD structure bound to progesterone ligand.

Images taken from www.rcsb.org.

ApoD glycosylation pattern is dependent on the tissue where ApoD is synthesized. Due to the glycosylation of the Asparagine (Asn) residues in the 45th and 78th position, its weight can vary between 19 and 32 kDa.

ApoD has been well conserved over long evolutionary distances. In invertebrates, the proteins homologous to ApoD with the greatest similarity described are in the grasshopper *Schistocerca americana* called Lazarillo, and in *Drosophila melanogaster* called Lazarillo Glial (GLaz) and Lazarillo Neural (NLaz) respectively. The last two receive their names from the type of cells where they are most expressed in the nervous system. Like ApoD, they are also widely expressed outside the nervous system (11).

ApoD ligands

The most studied *in vitro* ligands of hApoD, due to its hydrophobic pocket are lipophilic molecules such as steroids, eicosanoids, arachidonic acid (AA) and retinol (12).

Arachidonic acid is the ligand with the highest affinity and the interaction between these two molecules occurs in a flexible manner at the entrance of the ligand pocket of ApoD and relies on some exposed hydrophobic residues (13). This binding has a direct relevance to healthy brain function since this interaction is involved in the prevention of AA to turn into highly oxidative molecules (12). This is an example of ApoD 'capturing' property against potentially harmful molecules and therefore preventing lipid peroxidation chain reactions and decreasing oxidative stress in certain situations, as we will explain further in this section.

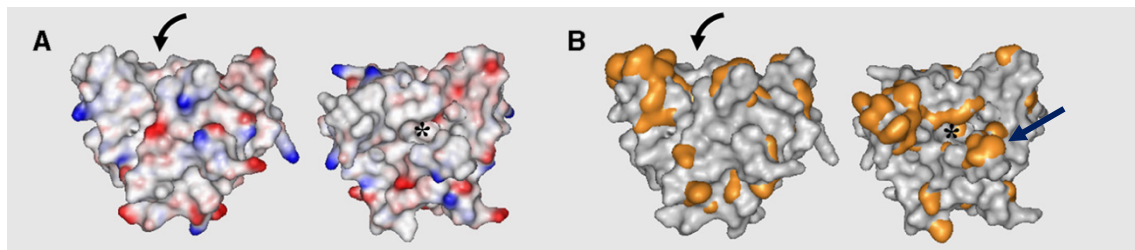


Figure 3: Molecular features of ApoD.

Gray colored space-filled of the human ApoD tertiary structure (modelled from PDB ID:2HZQ) showing charged residues in A (positive, red; negative, blue) and hydrophobic residues in B (orange). Side view of the β -barrel (left image; curved arrows point to the pocket entrance) and top view (right image) looking into the hydrophobic pocket (asterisk).

(Illustration taken from Sánchez and Ganfornina, 2021 (14)).

Steroids are another ligand of ApoD. ApoD interacts especially with progesterone and pregnenolone but with weaker affinity to estrogens. ApoD is also the major protein constituent of human breast cyst fluid (15). Cholesterol is a major membrane and lipoprotein constituent and since ApoD is a component of HDL, it could be involved in the transport of cholesterol to and from the liver (8). However several studies have reported a low affinity of ApoD to cholesterol (12,15).

Other ligands have been described, especially components derived from heme, such as bilirubin (16).

ApoD expression and subcellular traffic

The complex regulation of ApoD expression is due to the large number of regulatory elements on the ApoD promoter (17). This difference could influence ApoD function in certain species and mean different roles whether in brain or in peripheral tissues (18). ApoD is expressed in a wide range of tissues and body fluids across many species. In contrast, the expression of ApoD in mice is largely restricted to central nervous system (CNS) tissue (19).

As it is a secreted protein, its levels vary significantly from mRNA expression, since the protein accumulate in the extracellular media (20). It is most commonly found in human plasma, where concentrations are 100 times higher than in cerebrospinal fluid (CSF) (4).

Outside the nervous system, during both development and the adult stage, ApoD is expressed primarily by fibroblasts. In humans, in the peripheral nervous system ApoD is expressed in Schwann cells (21). In the CNS, it is expressed in white matter glial cells. In the grey matter, ApoD is mainly found in astrocytes (22). It is also expressed in cells that surround the choroidal plexus and the capillaries (23).

Adult human neurons do not express ApoD but are able to endocytose the protein from the extracellular space, particularly in oxidative stress conditions (24). It is not yet clear whether ApoD extracellular transport only happens via extracellular vesicles (EVs) or this protein also appears in the extracellular medium in a canonical way forming protein tetramers secreted from the cells that are able to produce this protein (14). Figure 4 summarizes the current hypotheses on intra and extracellular traffic of ApoD.

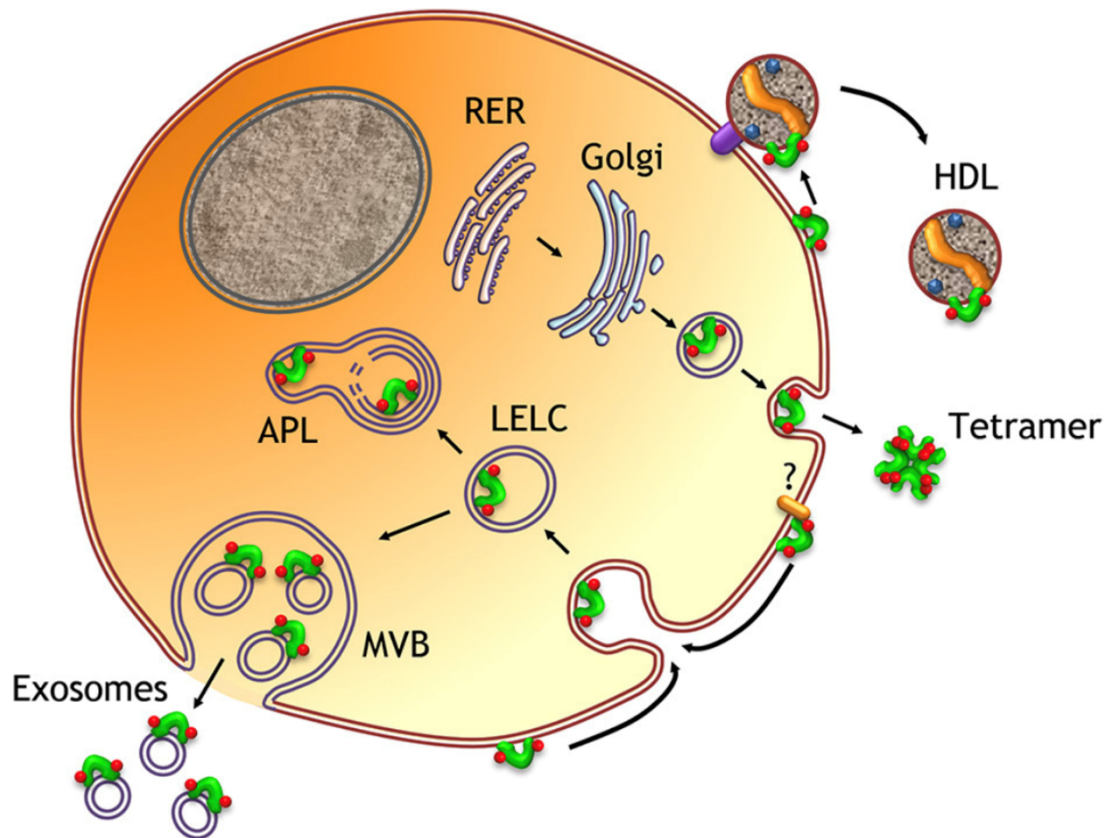


Figure 4: Schematic figure representing ApoD subcellular traffic.

A model of an ApoD-expressing cell is represented. Canonical exocytosis through the RER-Golgi pathway generates the mature, glycosylated (red dots) protein. The tetrameric form identified in the breast cyst fluid is represented as the form detected in extracellular fluids. Once at the plasma membrane, ApoD can be endocytosed (by non-expressing cells as well) and targeted to lysosomes and autophagolysosomes. When endolysosomes develop into multivesicular bodies, ApoD would be carried on the outer surface of exosomes. Finally, ApoD can be transferred to HDL during biogenesis, or during their lipid-efflux activity (upon HDL-receptor interaction).

(Illustration taken from Sánchez and Ganfornina, 2021 (14)).

Nuclear factors that are induced in pathologic situations such as PARP-1 (Poly ADP-ribose Polymerase-1) and APEX (Apurinic-Apyrimidinic Endonuclease-I) can bind the ApoD promoter and induce ApoD expression (25).

As explained in more detail in the following chapters, ApoD expression is increased during oxidative stress, inflammation, growth arrest and aging. At the same time, its expression is reduced during cell growth. The influence of ApoD in angiogenesis is variable and its expression is only suppressed at specific steps during the process of angiogenesis (26). ApoD can also selectively modulate

proliferative response to growth factors playing a role in cell growth and differentiation (27).

In regular cell culture conditions ApoD is secreted and reinternalized. However, some authors still support the idea of ApoD in a perinuclear location also under stress conditions (28). This may be caused by the fact that under stress, there is an increased in ApoD expression. And this protein is being stored in the RER which is located perinuclear.

ApoD function

ApoD function in metabolism and inflammation

ApoD main function was always explained in co-relation with other lipocalins as transporter of hydrophobic molecules. However, a recent systematic review by Sánchez and Ganfornina (14) describes ApoD function as buffering or quenching function. Indeed, ApoD act as very local shuttling of the ligand, rather than to a generalized long-range ligand transport between cells. For this reason, ApoD influences a big variety of tissues in the body.

ApoD is involved in different aspects of lipid metabolism. Polymorphisms in ApoD genes induce different levels of ApoD in plasma. Desai et al.(29) found a unique ApoD allele in African population from Nigeria that lead to higher levels of all types of lipoproteins (HDL, LDL and VLDL) and triglycerides in plasma.

In addition, ApoD binds a reaction product of the lecithin-cholesterol acyl transferase (LCAT) and that may exert negative feedback on LCAT activity. This influences the lipid transport towards and from the liver (30).

ApoD was also described as a Liver X Receptor (LXR) ligand in adipose tissue. Liver X receptors α and β (LXR α and LXR β) play a key role in lipogenesis/lipolysis modulation in adipocytes transporting lipids from the adipose tissue to the liver (the so called “inverse lipid transport”) (31).

When it comes to inflammation, ApoD plays an important role in the regulation of AA signaling and metabolism. Phospholipase 2 (PLA2) is one of the most important enzymes for AA degradation, and this enzyme was found upregulated in mice with hApoD overexpression (32).

A study by Thomas et al. (33) in the context of psychiatric disorders, show that ApoD acts by stabilizing AAs in the phospholipid bilayer of membranes. It also prevents its release and sequesters free AAs in cells. Another study shows that free AAs are major precursors of inflammation and that after major cell damage there is a release of membrane lipids, specially AA (34).

Upregulation of ApoD was demonstrated in humans with meningoencephalitis. Terrise et al. (35) and subsequently Do Carmo et al. (32) described an upregulation of ApoD mRNA and ApoD itself after acute viral encephalitis caused by HCoV-OC43. This upregulation seems to induce in neurons a neuroprotective effect. Do Carmo et al. work also show that this ApoD upregulation in neuroinflammation is co-related with microglial activation, limited leukocyte infiltration and proinflammatory cytokine downregulation in the CNS (especially TNF- α , IL-1 β and IL-6). This cytokine downregulation reduces PLA2 activity and therefore prevents the accumulation of AAs in the cytosol. This cytokine shift is also responsible for the decrease of the innate immune response by reducing the recruitment of immune cells to the site of infection, especially the attraction of T cells (32).

ApoD in the nervous system is also expressed in pericytes and perivascular cells in the capillaries that form the blood-brain barrier (BBB) in the choroidal plexus. In addition to that, ApoD controls the proliferation and migration of vascular smooth cells (27,36). The findings of Do Carmo et al. (37) experiments show that ApoD upregulation reduces leukocyte infiltration and T cell attraction. Therefore, ApoD expressed by cells in the BBB may act as a protector of the BBB integrity in neuropathologies.

Do Carmo et al, (38), years later, described that transgenic mice that overexpressed hApoD had more insulin resistance and glucose intolerance than their wild-type counterparts. This increased resistance affected only adult animals, which led to linking the ApoD effect with aging. Later in the introduction we would talk more about the process of aging. These transgenic mice also showed lipid accumulation in the liver, where it causes hepatomegaly and abnormal liver function.

Finally, ApoD was proposed to interact with the cytoplasmic portion of the long form of the leptin receptor Ob-Rb in the hypothalamus. Leptin is a key hormone secreted by adipose tissue that acts as an important regulator of both neuroendocrine function and energy homeostasis (39). However, in a recent systematic review it was found that this interaction between ApoD and the intracellular domain of OB-Rb is quite weak and, unless further experiments would be carried out, we may not consider this interaction as biologically relevant (14).

ApoD mRNA levels in the paraventricular and arcuate nuclei is also stimulated by dietary fats and are correlated with body fat mass in rats and mice. Therefore, ApoD might play a role in the control of food intake and body weight and can be used as a genetic marker for obesity, hyperinsulinemia, and insulin resistance (40).

ApoD function in the nervous system

ApoD function in the nervous system is crucial in maintaining adequate and dynamic neuro-glial crosstalk, lipid transport, cell membrane integrity and myelin extracellular leaflet compaction. Meanwhile, its loss generates dysfunction in growth promoting and lipogenic signaling cascades (41).

ApoD expression increases under nerve crush injuries (42). This phenomenon can be explained by the fact that after a peripheral nerve lesion there is a big

release of lipids due to myelin degradation. These lipids are somehow recycled and then stored and reused. This process of well-orchestrated morphological and biochemical changes that occurs in axons after nerve injury is called “Wallerian degeneration” (Figure 5). ApoD acts in this situation as a lipid transporter (21).

Another important process in which ApoD function is required is the compaction of myelin. There are two phases of myelin compaction and ApoD interacts in the second phase via adequate lysosomal-dependent myelin membrane recycling and remodeling (43).

ApoD-dependent lysosomal functional integrity is essential for myelin glycosyl management. ApoD deficiency in mice is associated with improper glycosyl clearance. Negatively charged glycosyl proteins can prevent the extensive bilayer-bilayer membrane interactions essential for myelination process (43).

ApoD transport within the glial cells, whether via extracellular vesicles (EVs) from astrocytes to neurons, where it gets internalized, or via canonical extracellular ApoD oligomers, has several functional implications. This is a paracrine neuroprotection system, called neuron-glia crosstalk and it is reduced in ApoD-KO individuals under stressful conditions (44). Since ApoD is also expressed in the cytosol of perivascular cells and in the pericytes lysosomes of neocortex blood vessel walls, it may act as a transporter of small hydrophobic molecules to or from glial cells to blood vessels (36). In addition to this, Apo D is a very specific marker of the exosome-containing fraction of the CNS (44).

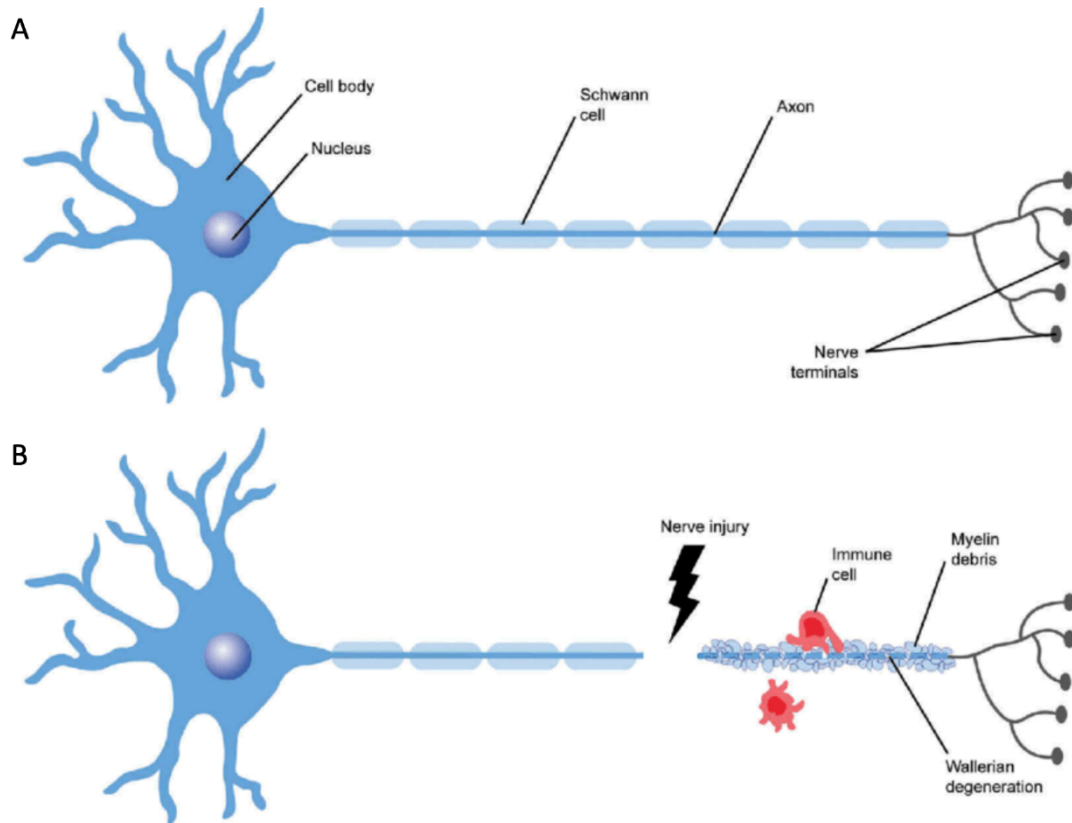


Figure 5: Schematic representation of nerve injury followed by Wallerian degeneration that precedes remyelination and nerve repair.

(A) Non-injured neuron with its intact components; soma and axon wrapped by myelin.

(B) Post-injured neuron undergoing Wallerian degeneration orchestrated by immune cells recruitment to the injury site. (Illustration taken from Saffari et al. (45)).

ApoD in aging and oxidative stress

ApoD in vertebrates is not only secreted by glial cells during development, but also during aging (22). Aging is defined as a sustained decline in Darwinian fitness over an organism's "typical lifetime" due to internal physical deterioration (46). It also comes along with an increase in mortality. It is a universal, progressive, endogenous, and irreversible process affecting all types of cells in an organism. A number of mechanisms at molecular level have been described to prevent or reduce damage-induced aging processes such as telomerase restoration of DNA endings or protein stabilization by chaperones (47). In the case of brain cells, most of them are post-mitotic and they are not influenced by telomerase action (48).

The ApoD gene is one of the few genes with a consistent and evolutionarily conserved up-regulation in the aged brain. Lack of ApoD causes premature aging but it does not alter mouse lifespan due to different cells contribution and molecular age-dependent patterns. This physiological ApoD neuroaging up-regulation may represent an homeostatic anti-aging mechanism (49).

As briefly explained before, when it comes to the antioxidant activity of ApoD, we should count on the action of a methionine sulfoxide reductase (MRS). Oxidized ApoD tend to self-associate and then ApoD redox cycling might be blocked if Met93 does not return to its native form. Therefore ApoD antioxidant capacity may be limited (14).

In the cortex of the aging human brain several studies demonstrate an increase in the number of ApoD cells by immunohistochemical analysis. An upregulation by 6- to 8-fold has been demonstrated (50). Transcriptional analyses and meta-analyses in datasets from humans, rats and mice show ApoD as the most up-regulated gene in brain aging (51). This raises the question of cause and consequence: Is the known ApoD upregulation the trigger for the aging process or is it a consequence of this process? To answer this question, a study in *Drosophila* and mouse shows that there is a clear conserved protective function of ApoD in CNS (11). Furthermore, hApoD also has a protective role when expressed in *Drosophila*, it extends lifespan and increases stress resistance (52).

Two models have been described to explain the role of ApoD and its homologs in aging: one for loss of function (GLaz-KO) and one for overexpression. It has been shown that axonal regeneration and remyelination are delayed and there is an increased sensitivity to oxidative stress with Paraquat (PQ) and H₂O₂ in GLaz-KO animals. On the other hand, in overexpressing transgenic flies, there is an increase in longevity and more resistance against powerful oxidative stress situations such as hyperoxia (53). Therefore, ApoD mechanism of action in aging is based on a lipid peroxidation protection as shown by Sánchez et al. in 2006. They found in GLaz-KO flies, higher concentration of lipid peroxidation products.

Interestingly these flies have also reduced fat body tissue abdominal and pericerebral due to a relative decreased content of triglycerides.

Remyelination means a sort of lipid re-use and recycle that obviously need a correct lipid transport and ApoD has therefore a function in maintaining the myelin that surrounds neuronal axons in peripheral nervous system and central white matter. Oxidative stress and chronic inflammation appear to be the main triggers of age- and disease-related neurological decline. Due to the inability to repair, reactive oxygen species (ROS) appear.

In mouse and *Drosophila*, ApoD has a protective effect on organisms under stress by decreasing lipid peroxidation (54). ApoD-KO mice and over-expressing ApoD mice show different survival curves upon stress induced by intraperitoneal injection of PQ. There is a reduction in both the median and the maximum lifespan showing a decline in global functionality in ApoD-KO mice. An acute upregulation of ApoD transcription in the mouse brain after a single exposure to PQ was also observed. This up-regulation was only seen in the brain and not in other organs such as lung or kidney (24).

The control of lipid peroxidation can have two different explanations. ApoD can directly '*a priori*' prevent the oxidation of bound lipids or, '*a posteriori*' removing peroxidated lipids from membranes avoiding positive feedback with more oxidative damage produced. This protein can also be involved in membrane lipids composition leading to membranes susceptibility under stress conditions (24). In this second scenario, Thomas et al.(33) in studies with neuropsychiatric drugs showed that the absence of ApoD alters the regulation of AA. It points to ApoD function linked to membrane physiology. This effect is common to all ApoD homologs. As already described above, ApoD is a very conserved protein whose protective function is similar in all animals. This could support the hypothesis that ApoD is overexpressed under stressful conditions to promote endogenous and protective antioxidant pathways (33).

ApoD and neurodegenerative diseases

Within neurodegeneration, the most well-known disease is Alzheimer's disease (AD). Although there are still many questions about AD physiology and development, an increase of ApoD in Alzheimer's disease is described (55). High levels of ApoD in CSF and hippocampus have been described, as well as in temporal and prefrontal cortex of patients with AD (35,56,57). CD68-positive microglial cells colocalize with A β aggregates immunostained with ApoD in AD samples (29). Over-expressing GLaz in a *Drosophila* cell line shows an increased viability when exposed to A β peptides in comparison with normal-expressing GLaz cells (52). ApoD levels correlate with ApoE genotype and the association between the E4 ApoE isoform and the risk of developing AD is described. A hypotheses of compensatory function between ApoD/ApoE in the organism was discarded because under ApoE deficit there is an increase of ApoD but the contrary effect was not described (53). ApoE2/3/4 isoforms can affect the ApoD promoter leading to a decrease in ApoD expression.

ApoD is also increased in multiple sclerosis (MS) (58). Both diseases involve neuron degeneration, but this damage is caused by different mechanisms with a common feature: oxidative stress.

In conclusion, a neuroprotective and anti-inflammatory effect of ApoD could result from its capacity to stabilize AA at the cellular membrane level or to sequester it by preventing its transformation into pro-inflammatory molecules in certain situations as growth arrest, cell senescence and stress. Also, ApoD has a main role in transporting lipids, especially in the CNS where myelination means lipid transport and reuse.

ApoD is paradoxically associated with good prognosis in cancer (59) but also tumor progression. A correlation was found between increased levels of ApoD and decreased tumor growth in human colorectal cancer cells (60).

In addition, ApoD shows multiple other functions such as regulation of immune response (7).

Therefore, ApoD can be considered an endogenous controller of the redox state in the CNS upon neurodegeneration, aging, and different types of damage. Due to his apparent salt-and-pepper spatiotemporal pattern expression, a pleiotropic role as a buffer or quenching protein with diverse ligands has been proposed for ApoD (14,53).

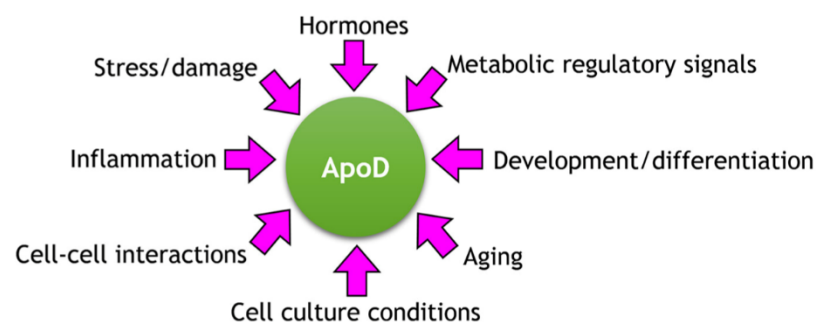


Figure 6: Schematic figure representing all the factors involved in ApoD polyvalent function and spatiotemporal distribution.

(Illustration taken from Sánchez and Ganfornina (14)).

Microglia

During the past decades knowledge on microglia has undergone a remarkable development regarding the comprehension of its fate and function during development, homeostasis, and neurodegeneration. Since their discovery in 1919 by the Spanish neuroscientist Pio del Río Hortega as a separated glial cell type in the CNS, microglia have been considered tissue-resident macrophage-like innate immune cells (61).

With the development of new image techniques, it has been recognized that microglia are multi-faceted, multi-functional and multi-context cells that interact with numerous other cells. Moreover, thanks to massive microarray sequencing it was possible to assess the expression of several defined microglial genes that act as risk factors for several neuropathologies (62).

Microglia origins

Microglia have a neuroectodermal origin. In humans, they derive from the yolk sac and arise in the CNS on the 9th day post-conception. They are believed to have potential for primitive erythropoiesis (63). Most macrophages outside the CNS are shortly thereafter replaced by a restricted hematopoietic wave originated by definitive erythroid myeloid progenitors (eEMPs). These cells then move to the fetal liver where they give rise to intermediate monocytes (64). The macrophages in peripheral barrier tissues such as the gut or the skin are progressively replaced by circulating blood monocytes. Fate mapping studies have shown that microglial cells are the only adult cells found in the brain that maintain the eEMP-cells origin (65). Also as an exception, the macrophages of the choroid plexus are cells that are able to replace their pool via monocyte-derived cells of hematopoietic stem cells (HSC) over time (66).

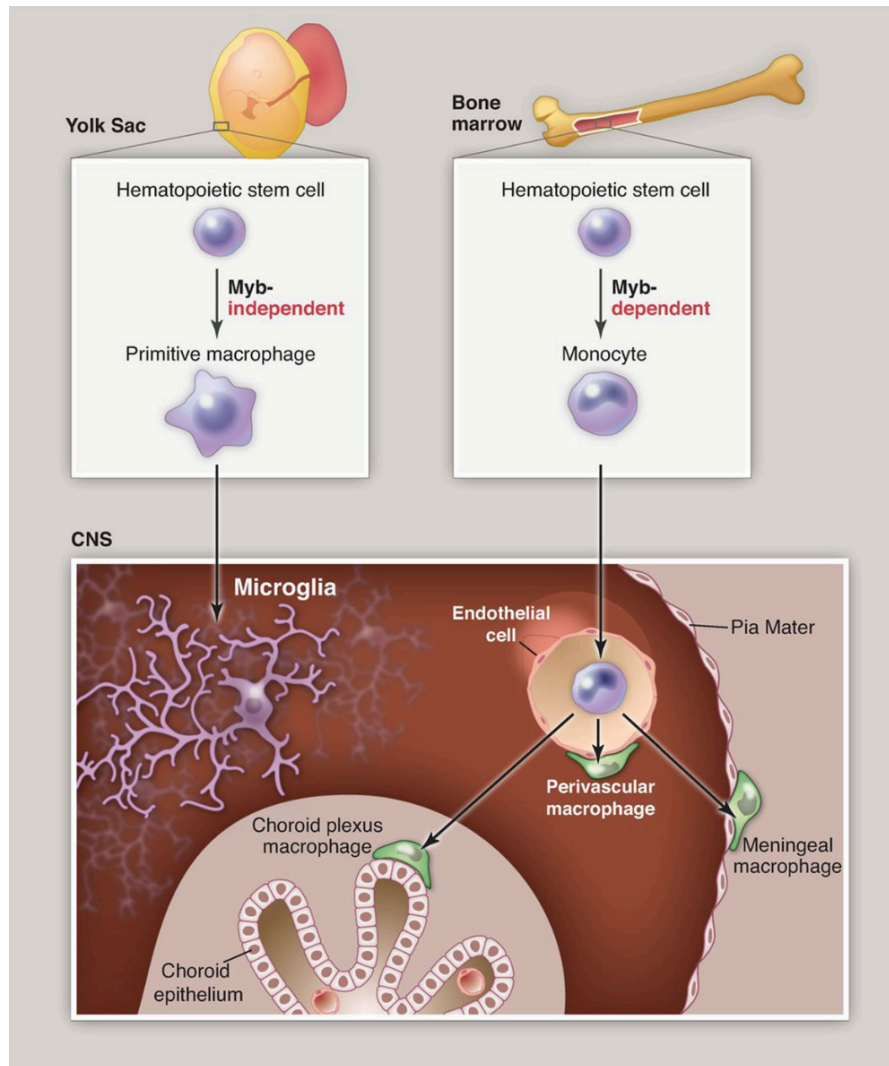


Figure 7: Schematic figure representing microglia origins.

Microglia derive from yolk-sac macrophages formed during primitive hematopoiesis beginning at embryonic day 7.5 (E7.5). Primitive macrophages migrate to the developing nervous system where they become microglia and reside throughout life. Local progenitors sustain the microglial population. Contrary, other CNS macrophages (found in the perivascular space, meninges, and choroid plexus) derive from precursor blood monocytes. Monocytes are formed in the bone marrow (BM) from hematopoietic stem cells and are dependent on Myb transcription factor.

(Illustration taken from Aguzzi et al. (67)).

This knowledge leads to assume a certain cellular compartment and niche. Loss of microglia is compensated by the high self-renewal potential, in particular thanks to the strong and constant stimulation of their colony stimulating factor 1 (Csf-1) receptor and their alternative ligand interleukin 34 (IL-34) (68). IL-34 is secreted by neurons throughout the brain, but Csf-1 is secreted not only from neurons but also from glial cells, promoting microglia proliferation and survival. It is also strongly influenced by environmental stimuli. In fact, microglia can display

distinct morphologies during development but especially throughout adulthood. It is thought that microglia on average renew their population three times during the two-year lifespan of a mouse (69). Humans microglia turnover is similarly low, with cells living several decades (70). This is called 'microglial repopulation' which is different from 'microglia replacement'. It means that endogenous microglia is also at a lower level replaced by exogenous cells such as peripheral blood cells, bone-marrow-derived myeloid cells or pluripotent-stem-cells (PSC) (71).

Differences between microglia and macrophages

Due to their distinct origins, microglia and other macrophages display different transcriptomes and epigenomes (72). Also because microglial identity results much more from the influence of local tissue factors and environmental influences (73). Macrophages engrafted in the CNS can acquire a microglia-like morphology without reaching a transcriptomic signature like the host microglia, even after prolonged time in brain parenchyma and similar colonization niche (74). However, it should be kept in mind that microglia, as well as macrophages, are very dynamic cells and that for instance P2RY12, one of the most commonly used markers to distinguish microglia from macrophages, is in some cases downregulated in AD and other neuropathologies (75).

Microglia-neuron crosstalk

The so-called microglia-neuron crosstalk implies that microglia controls abundance of neuronal and oligodendrocytes progenitor cells (NPCs and OPCs) as well as their processes. Such a control involves active apoptosis and removal of excess cells or unused synapses. It also involves a selected pruning of specific cells and synapses (76). This shaping of neuronal circuits by clearing apoptotic neurons are seen mostly in the hippocampus region (77). Other control process, such as the elimination of tagged synapses, is made via complement receptor 3 (CR3) signaling cascade (78).

This process is maintained throughout all the phases of the organism life due to the fact that the microglia are one the first colonizing cells in the CNS (63). This crosstalk can also be seen as a check-point mechanism with direct inhibitory interactions of microglia with neurons through the receptor ligand pair CX3CL1-CX3CR1 to restrain microglia immune activity in homeostasis situations (79).

Microglial function

Since microglia persist in the CNS from embryonic period until adulthood, they are exposed to very different environments and have therefore different roles, morphologies, and molecular signatures.

Embryonic microglia show higher abundance of ApoE as well as exhibition of transcripts for lysosomal proteins such as cathepsins (80). This may be explained by the fact of the importance of phagocytic activity in this period (81).

The classical theory of two distinct and polarized microglia patterns of “activated” or “homeostatic” adult microglia has developed into a new understanding of these multi-faceted cells (80). Within the so called “activated” microglia, two different patterns were found depending on the stimulus and secreted cytokines. The so called M1 pattern or pro-inflammatory microglia type is activated under stress conditions such as lipopolysaccharides (LPS) and PQ. The specific cytokines secreted by this type of microglia are mainly TNF- α , IL-1 and IL-6. M2 microglia, also defined as an anti-inflammatory microglia type, are characterized by upregulation of ApoE and MAFB and secretion of TGF- β , IL-4, IL-10 and IL-13 (82).

A new consensus of experts prompts them to be better called “monitoring” microglia because microglia are the most dynamic cells of the healthy, mature brain and, in fact, they never rest. Nowadays, these cells are considered as a continuum of an spectrum of different patterns and expressions since they are always active and respond differently depending on the context (83).

On one side of this spectrum, are the classic “activated” or “reacting” macrophage-like amoeboid microglia with enlarged cell bodies, as seen during development and under stress conditions, suggesting phagocytic functions (37). This is in most cases a reversible state once the stimulus is removed (38). On the other side, we find “resting” branched microglia or M0 in the normal adult brain. The latter are more involved in synapse pruning and other homeostatic functions. Microscopically, we can distinguish these cells with long projections that constantly advance and retract to scan the environment and cover large areas of the brain, constantly monitoring the functional state of synapses.

Microglia phenotypes can also be seen as a kaleidoscope and not a straight line with two edges. Often, in certain situations and in different brain regions, different microglial phenotypes co-exist at the same time. Although there is a difficulty in obtaining fresh microglia from human CNS tissue, RNA sequencing of nuclei (ncRNA-seq) has shed some light on this issue (84). Some studies have shown distinct transcriptional states of human microglia during homeostasis and across different anatomical locations (85). Interestingly, microglia from the white matter-rich subventricular zone expressed higher levels of CD68 and HLA-DR than microglia in other CNS regions (86). This may be because it is the BBB region where we already have pointed that strict control of molecules entering the brain fluid is paramount.

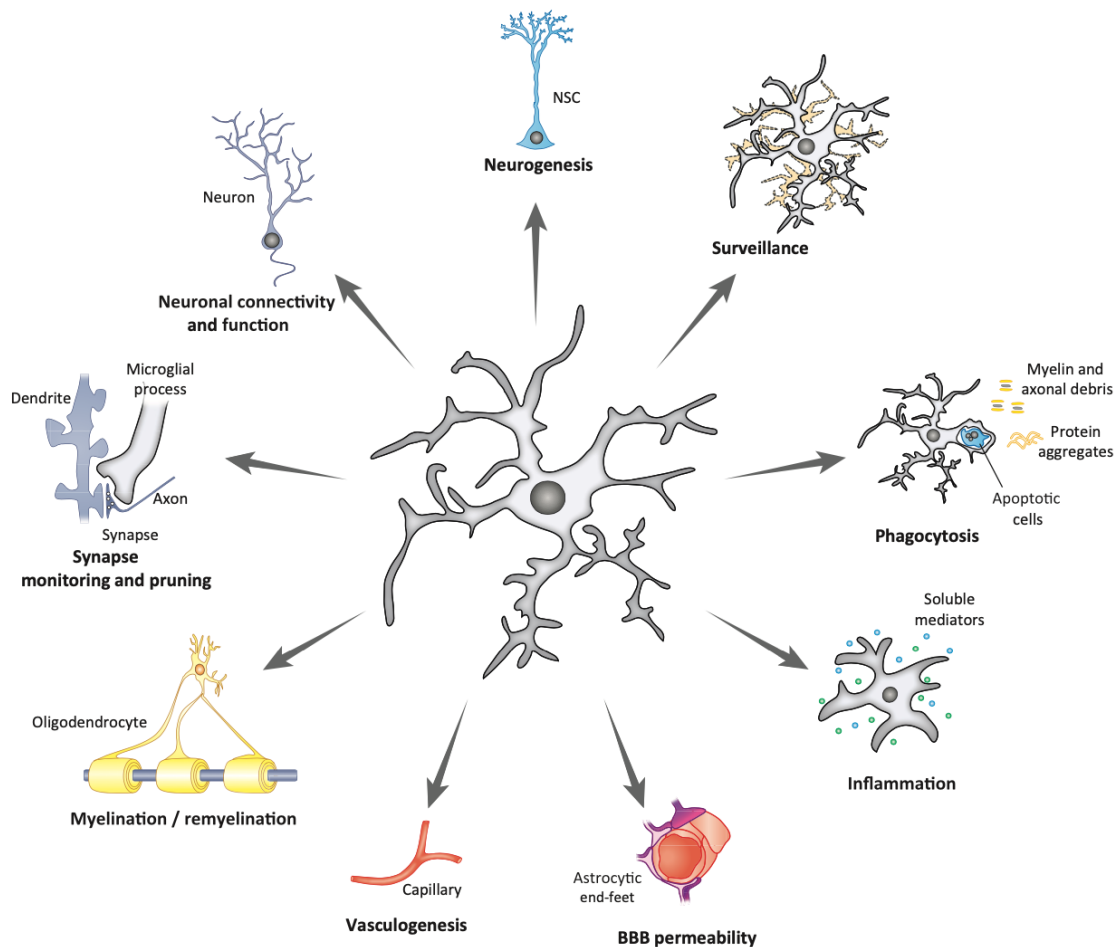


Figure 8: Schematic figure of microglia key functions.

One salient feature of microglia is their processes' motility, which mainly leads to parenchymal surveillance tasks. Under pathological conditions, microglia perform classical immune functions, such as release of pro-inflammatory cytokines and cellular debris clearance via phagocytosis process. Synapses monitoring and pruning are also microglia key functions that seem to be altered in pathological situations. Microglia is also able to establish cross-talks with other brain cells such as neurons, neural stem cells, astrocytes, and blood-brain barrier cells. It has therefore an impact on these cell's functions.

(Illustration taken from Sierra et al. (87)).

Representations of microglial state and function approaches in recent years have led to two very interesting concepts: the concept of "fifty shades of microglia" means a continuum of a spectrum of phenotypes in the same lineage, but with slightly different functions and different factors affecting one temporal-spatial difference are involved (81).

The kaleidoscopic concept also leads to a completely new approach to microglia that not only considers the spatiotemporal influence but also adds layers of

complexity such as phenomics, metabolomics, proteomics, transcriptomics and epigenomics.

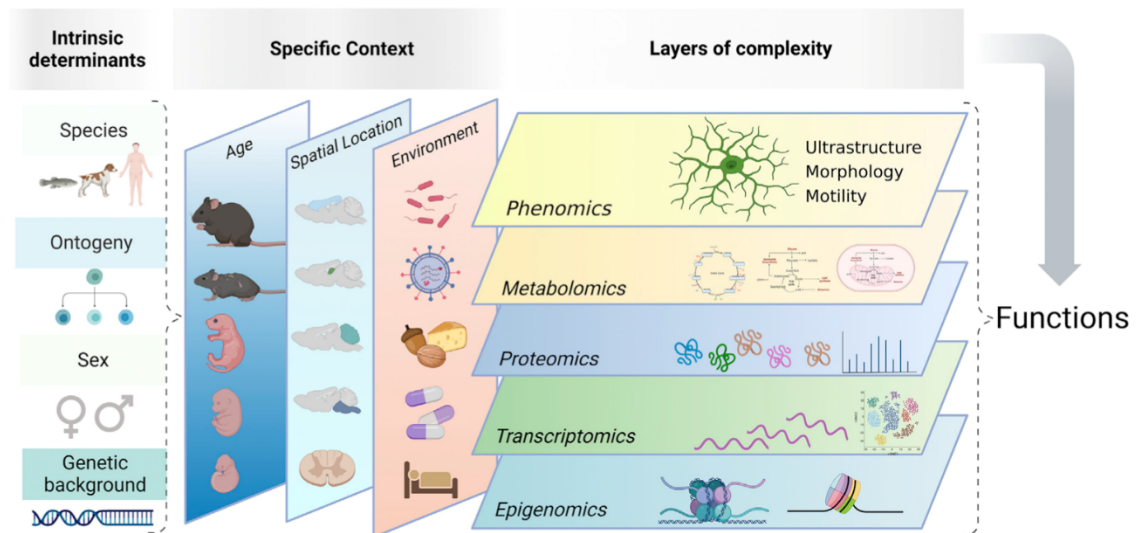


Figure 9: Figure representing microglia states defined by their intrinsic and extrinsic determinants, spatiotemporal context, and layers of complexity.

Microglia states depend on intrinsic determinants (such as species, ontogeny, sex, or genetic background) as well as the specific inhabitant context, including spatiotemporal location and environmental factors (such as nutrition, microbiota, pathogens, drugs, etc.) All these factors, impinge on microglia at multiple levels.

(Illustration taken from Paolicelli et al., 2022(88)).

Neurodegenerative diseases and aging

Oxidative stress in neurodegeneration and aging

Oxidative stress is one of the major causes of aging and neurodegenerative diseases and is likely to play a major role since the brain is the human organ with the highest energy demand (89). The brain produces most of the adenosine triphosphate (ATP), which leads to the formation of a large number of reactive oxygen species (ROS) and nitrogen-containing reactive species (RNS) due to oxidative phosphorylation.

The first association of ROS and several pathological conditions were made by Gerschmann et al. (90) that found an excessive production of ROS after ionized radiation. This process later led to the development of certain cancers (90). Oxidative stress is a natural consequence in all biological systems that depend on oxygen to survive. Therefore, ROS are not only damaging agents but also serve as signaling agents in normal conditions (91). ROS species include superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical ($\bullet OH$), singlet oxygen ($^1O_2$) and nitric oxide (NO). NO is the main neurotransmitter in many organisms and can diffuse in a paracrine manner.

The theory of ROS being the main source of aging lies in the fact that these compounds are products of the aerobic metabolism and therefore react with complex macromolecules such as proteins, lipids or even nucleic acids and aggressively damage them. This action leads eventually to age-dependent physiological decline due to inadequate antioxidant mechanisms (92). Other hypotheses shift to the idea of ROS as modulators of catalytic or regulatory reactions, for instance via Ras or MEKK1 leading to pro-oxidative situations and over-oxidation of redox-sensitive proteins (93). These species are under normal conditions controlled by a variety of antioxidant mechanisms (32).

These increased ROS levels directly lead to an increase in cellular senescence. Cellular senescence is a cell fate similar to apoptosis, but these cells remain

locked in a non-proliferating state, having less possibilities to evolve in tumorigenesis (94). Since it is an action less harmful than apoptosis, senescent cells are still metabolically active. The continuous secretion of pro-inflammatory cytokines, chemokines and proteases by senescent cells lead to the term senescence-associated secretory phenotype (SASP) with the result of brain immune cell infiltration. This process unintentionally becomes chronic in aged tissue. This CNS age-dependent chronic low level inflammatory situation is called “sterile inflammation” or “inflammaging” since there are no pathogens implicated (95). This situation has interesting positive feedback since oxidative phosphorylation is the main source of ROS in a cell and these species induce damage to mitochondria which, in turn, increases ROS production. Mitochondrial damage induces NLRP3-dependent inflammasome activation and many authors suggest that this activation also precedes the implementation of the SASP (96). PQ is a good model when it comes to mimicking ROS generating situations due to aging. This product is able to oxidate all types of macromolecules (proteins, DNA and lipids) (Figure 10).

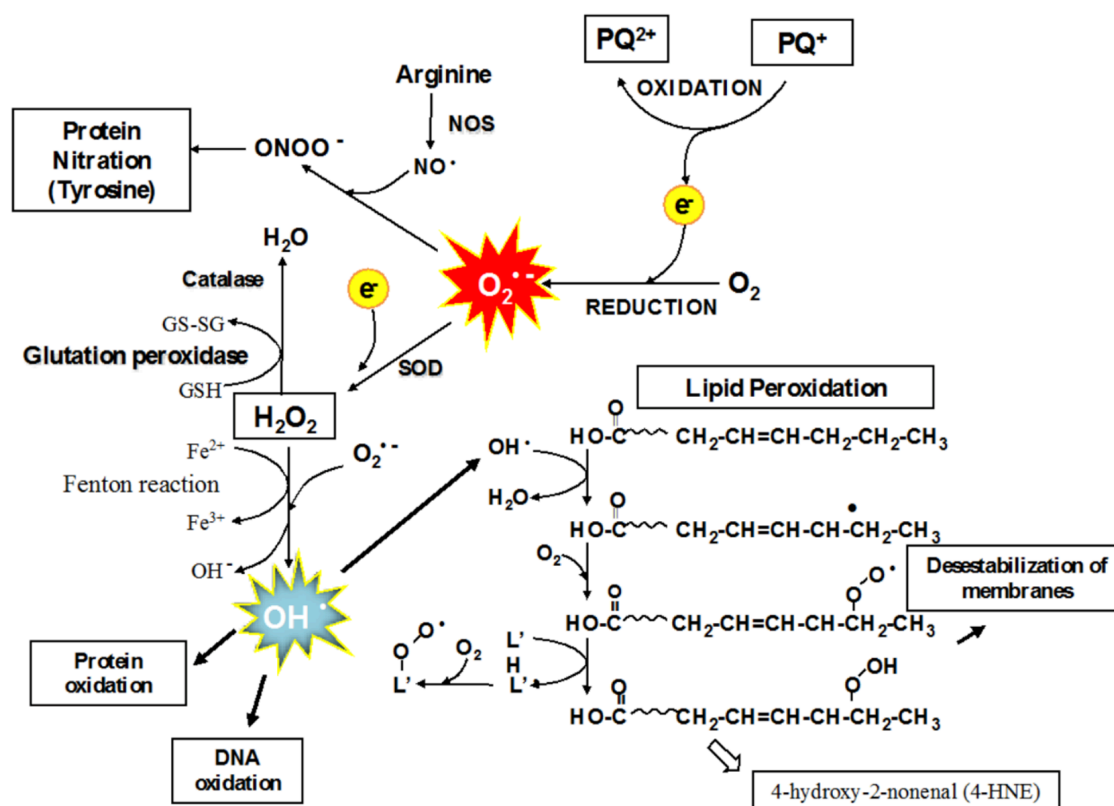


Figure 10: Schematic representation of reactive oxygen species (ROS) pathways and the main enzymes involved in these reactions.

Neurological disorders

Despite the vast number of neurological diseases, I will focus on the most prevalent ones in our society and thereby the most studied because of its clinical implications in the developing of new treatment therapies. Alzheimer's Disease (AD) is a neurodegenerative disease with aging as the main risk factor. Since its discovery more than a century ago by the German neuropsychiatrist Alois Alzheimer, it has become the leading cause of dementia worldwide. Annually, the number of deaths attributed to this disease increases compared to previous years, resulting in this disease being ranked as the sixth leading cause of death. Prevalence was in 2019 of 50 million people living with Alzheimer worldwide (as reported by the world health organization) and it is expected to increase to 125 million people in 2050.

Most *in vitro* validated AD models focus on the neurodegenerative process and the irreversible accumulation of A β aggregates and neurofibrillary tangles (NFTs) of tau protein within CNS cells and in the extracellular space.

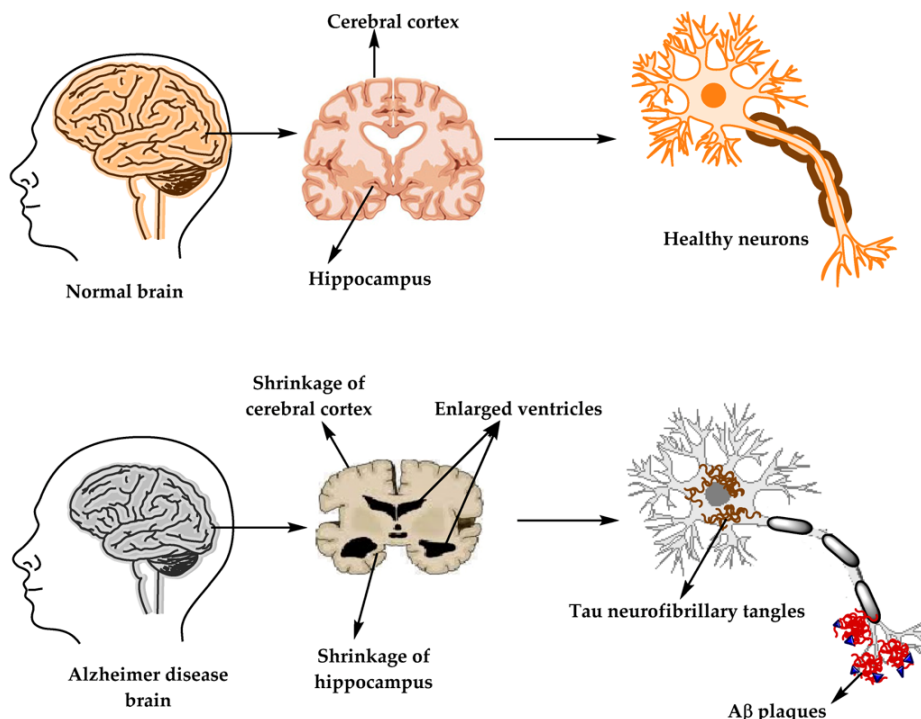


Figure 11: Illustration of the physiological structure of the brain and neurons in a healthy brain and in an Alzheimer's disease brain.

(Illustration taken from Breijyeh et al., 2020(97)).

Mutations in amyloid precursor protein (APP) lead to the enhanced generation of A β aggregates. However, these mutations only explain hereditary AD and not the more common sporadic AD. Most of the cases are late onset (LOAD) after the age of 65 with no clear cause, with aging as the largest risk factor. The cause here appears to be multifactorial, as not only age but also environmental and genetic factors increase the risk and progression of the disease (98). The clinical symptoms are characterized by progressive memory decline and cognitive dysfunction. AD typically progresses in three different stages: early, mild-moderate and severe (98). The most common genetic risk factors are alleles of the apolipoprotein E (ApoE). ApoE allele ϵ 4 increase 4-fold LOAD risk while the ApoE allele ϵ 2 is associated with a reduction and later onset of this disease (99). Unfortunately, there is still no effective treatment that promises long-term remission, despite numerous studies and lines of research addressing this disease.

Multiple sclerosis (MS), along with AD, is one of the most common neurodegenerative diseases worldwide, with a prevalence of more than 6.5 million adults (55). The risk is higher in women, especially in their early forties and has a diverse and yet unknown etiology. MS is an immune-mediated neurological disorder of the CNS characterized by inflammation, loss of oligodendrocytes, demyelination, and axonal degeneration. 85% of the patients present with the relapsing-remitting MS (RRMS) form which consist of partially reversible symptomatic periods of neurological deficits followed by phases of recovery where the patients have few or no symptoms. These patients often convert to a secondary-progressive (SPMS) form with shorter and less frequent asymptomatic periods and significant irreversible neurological impairment. In the rest of the patients, mainly women, appears the primarily progressive form (PPMS) which result in rapidly progressive neurological decline (100). As the disease progresses, the symptom free periods get shorter. The neurological symptoms reflect the progressive focal degeneration of oligodendrocytes (OLGs)

and myelin membranes around the axons in areas of the CNS and the PNS (101,102). Although the primary pathogenic mechanism is unknown, this OLGs dysfunction has been suggested to be caused by an enhanced adaptive immune response with an overly permeable BBB, autoreactive T cell recruitment and B cell activation. All these inflammatory processes directly and indirectly lead to the formation of ROS, which promote demyelination as in other neurological diseases such as AD or in neuroaging. Nevertheless, some studies in the past years have suggested that the first event that triggers the secondary autoimmune response is an initial biochemical change of myelin. Regardless, both the classical “outside-in” theory and the new “inside-out” theory led to ROS production and the activation of microglia. In order to understand the main differences between the two hypotheses and the models developed from them we might consider the primary causes of the disease. In the “inside-out” hypothesis the main primary cause is OLG injury and myelin destabilization (Figure 12). On the contrary, in the “outside-in” hypothesis, the main primary pathogenesis is the autoimmune inflammation (103) (Figure 13).

The different theories led to the development of different MS mouse models that mimic one or the other primary pathological cause. Since current therapies for MS only modify the disease or treat symptoms, the development of drugs based on both hypotheses of pathogenesis is of enormous importance.

In this context, the most used “inside-out” model is the cuprizone autoimmune encephalitis (CAE) model. A diet with cuprizone, a copper chelator, destabilizes myelin and this myelin breakdown leads to secondary immune response (104). The cuprizone model (CPZ) is the most used model worldwide to study MS *in vitro* since it allows to reverse the impact on mitochondrial function once that this copper chelator is removed. The mechanism is a demyelination in the corpus callosum (CC) visible via immunohistochemistry. Once the chelator stimulus is removed there is a visible increase in mature OLGs in the proximity. There is an impact in the subventricular zone (SVZ) due to the proximity of the CC to this area (105).

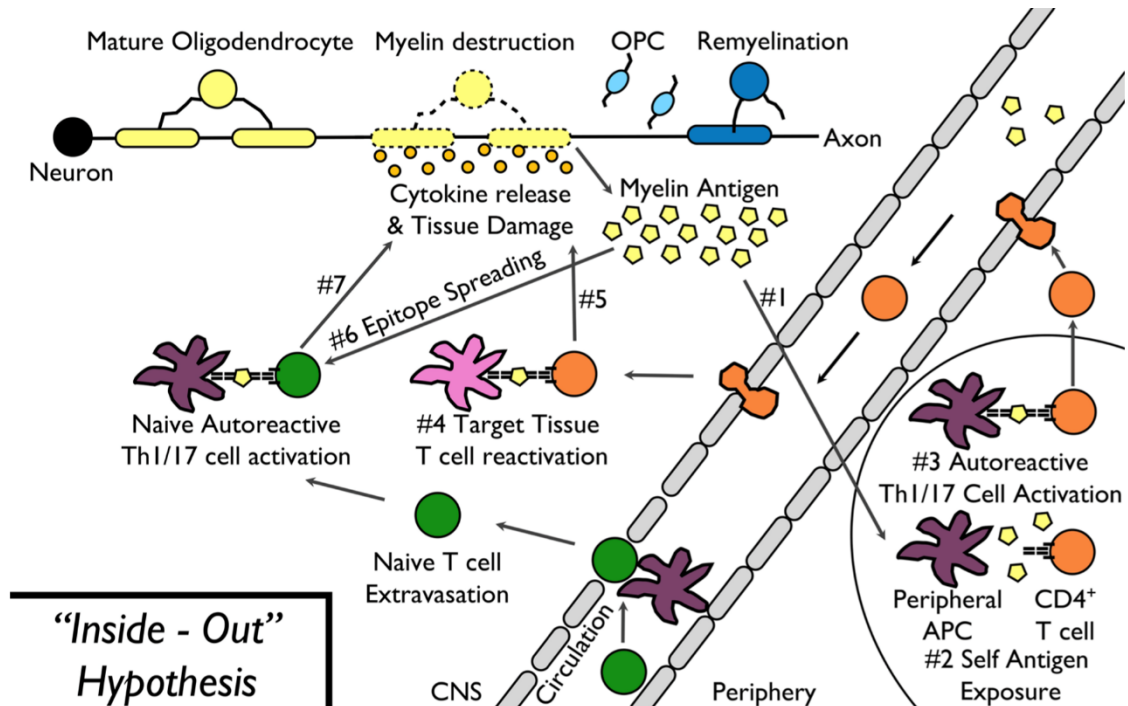


Figure 12: Figure showing "inside-out" model of MS pathophysiology.

The release of myelin antigens from injured neurons (#1) is followed by the presentation of myelin epitopes (#2) and subsequently activation of autoreactive T cells (#3). These cells migrate into the CNS and release cytokines leading to direct and indirect damage to myelin (#5). (Illustration taken from Titus et al., 2022(103)).

Within the "outside-in" models exist a chronic experimental autoimmune encephalomyelitis (C-EAE) and a relapsing remitting experimental autoimmune encephalomyelitis (RR-EAE) model. In both of them, an immune CNS infiltration is followed by secondary CNS degeneration (106).

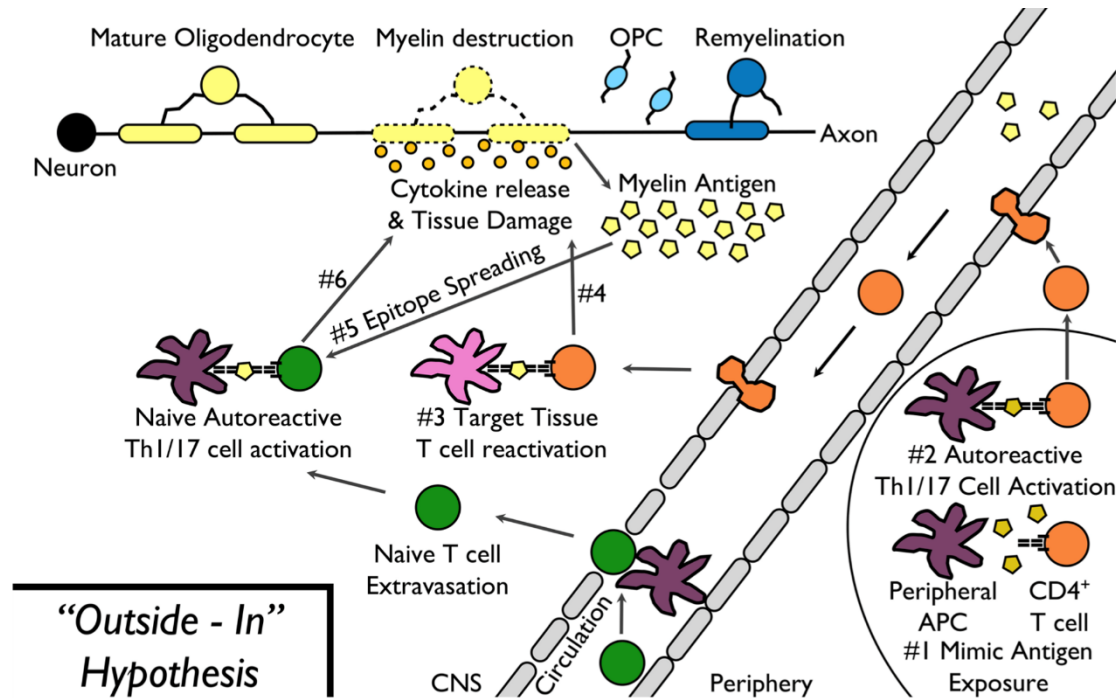


Figure 13: Figure representing "outside-in" model of MS pathophysiology.

This model begins with activation of myelin-specific T cells (#1) in response to a myelin peptide mimic epitope expressed on a pathogenic virus or other microbe exposure. Activation of autoreactive T cells (#2) follows and migration of these cells to CNS and are reactivated by CNS-resident APCs (#5). There is a subsequent release of cytokines producing direct and indirect damage to myelin. (Illustration taken from Titus et al., 2022(103)).

ApoD in neurological disorders

Most of the neurological disorders are associated with cellular stress and inflammation. Since ApoD is known to play an important role in protecting against lipid peroxidation in the brain, it is not surprising that ApoD is upregulated in various pathologies, and it is likely that this protein has similar functions in neurodegeneration as in aging (24).

Regarding Alzheimer's disease, ApoD has been found in reactive microglial cells surrounding the A β plaques (29), however the accumulation of A β plaques itself can induce a direct upregulation of ApoD, especially in the hippocampus via ROS triggered production (107). As previously explained ApoD highly conserved methionine residue in humans (Met93) reduces lipid hydroperoxides that result in oxidation of the methionine sulfhydryl to form methionine sulfoxide (MetSO), and as a result dimerization of ApoD (13). This compound is reduced back to

methionine sulfhydryl via MetSO reductase enzyme. A substantial decline of this enzyme has been found in AD (108). Since ApoD acts as an antioxidant, such as in aging processes, ApoD is found in dimeric aggregates in the brains of AD patients (9). However, this protective mechanism at the beginning of the disease can have a detrimental effect over time, as oxidized ApoD accumulates as dimeric aggregates and is ultimately deposited together with peroxidized lipids as amyloid plaques (29).

Interestingly, there was a significant reduction in hippocampal and cortical amyloid plaque pathology in transgenic mice with neuronal expression of human ApoD (109). The mechanism of action of ApoD in AD is through alteration of the amyloid precursor protein (APP) pathway. APP is cleaved by secretase to generate A β peptides. During this process, proteolytic residues such as membrane associated APP-CTFs are produced. The main APP-CTF produced is the β -(BACE1)-cleavage (110). Significantly increased BACE1 levels were found in murine ApoD-KO models. These data suggest an inverse relationship between ApoD levels and BACE1 activity/levels and thus possible BACE1 modulation (111).

Interestingly, an immunohistochemical colocalization with BACE1 and Iba1 (a well-known microglial marker) was found in the vicinity of amyloid plaques. Therefore, the altered lipid peroxidation does not appear to be one of the main and initial contributors of the AD pathology and that raises the question if ApoD represents a protective function in AD or whether it is simply a redox protector.

When it comes to MS, it has also been shown that ApoD is upregulated in CSF of patients with MS and that this protein is increased in astrocytes with a characteristic expression pattern in MS lesions (112,113). As has been found in other neurological diseases, ApoD is thought to play a protective role in MS by preventing or reducing the production of ROS and protecting mitochondrial function. As well as in AD, it is not clear if this is the main function that ApoD plays in MS, or this protein is involved in more mechanisms/pathways of the disease.

To shed some light in this question, a recent study using the cuprizone model showed that ApoD can prevent the toxic effect of CPZ on brain cells after 24 hours of treatment. However, this neuroprotective effect is independent of ROS levels (114).

Disease associated microglia.

The identification of microglia states in humans during pathology is important to understand microglia's role in CNS diseases (85). Thanks to sub-tissue-focused single-cell RNA sequencing, it was possible to identify eight microglia-specific clusters and determine two clusters representing microglia states. A highly phagocytic subtype of microglia was identified in animal models of neurodegeneration that develops in a two steps Trem2/ApoE-dependent manner (115). These microglia are called disease associated microglia (DAM). They were discovered in a mouse model of AD that contains five human familial genes mutations for AD (5XFAD), but also in a mouse model for amyotrophic lateral sclerosis (ALS) called mSOD1 (G93A) and in the brain of aged mice. These results were further validated in other AD's models such as PS2APP and APP/PS1 (116) or in multiple sclerosis models (117).

The DAM phenotype is activated in a two-steps process in which the triggering receptor expressed on myeloid cells 2 (TREM2) is involved (118). TREM2 genetic variants are also associated with increased risk of certain neuropathologies (119). TREM2 is a cell surface transmembrane glycoprotein receptor of the Immunoglobulin (Ig) superfamily present in the CNS only in microglial cells populations. It is located in human chromosome 6p21 and encodes a 230-amino acid glycoprotein. It binds phospholipids, sulfatides and other polyanionic ligands. Lipoproteins such as HDL and LDL which may contain A β as a cargo, can also act as TREM2 ligand (120). Although TREM2 binds this protein directly in *in vitro* studies, this effect could not be demonstrated in *in vivo* studies, or at least not in all areas of the brain and without the presence of ApoE (121). Recent studies propose that TREM2 also facilitates brain monocytic infiltration in AD (122). This suggests a multifactor-dependent or A β burden-dependent effect of TREM2 on

A β deposits. TREM2 is also cleaved from the microglial cell surface by proteases such as ADAM10 and ADAM17 generating a soluble form of TREM2 (123).

This model also made it possible to determine the spatial location of DAM in the proximity of the A β plaques within the cortex but not in uninvolved regions such as cerebellum. Furthermore, a high number of microglia positively stained for intracellular A β particles and a reduction in these two cell clusters of classical microglia homeostatic genes were shown (124). Of utmost importance is the fact that ApoE, one of the most well documented ligands of TREM2, is also one of the main AD risk factors in LOAD. ApoE is upregulated with a 25-fold increase in DAM microglia (115).

Specific analyses at different disease stages also show that microglia in this AD model undergo a transition from homeostatic microglia to a DAM population in parallel with the development of the disease. This DAM program proceeds in two steps, as two different clusters were found, and one appears to be the intermediate microglial phenotype. The first activation implies a reduction in the expression of homeostatic microglial check point genes due to danger signals called neurodegeneration association molecular patterns (NAMPs). Similar to the general damage associated stress signals (DAMPs), they are recognized by constitutively expressed receptors and trigger the transition into stage I DAM or intermediate DAM (125). NAMPs may contain myelin debris, apoptotic cell bodies, extracellular protein aggregates such as A β plaques or lipid degradation products. Intermediate DAM has a decreased expression of purinergic receptors; therefore, these cells are less influenced by purine micro gradients. These gradients serve as “find me” signals to induce microglial chemotaxis and phagocytosis of debris (115). Further activation is dependent on TREM2 and involves upregulation of phagocytic and lipid metabolism genes.

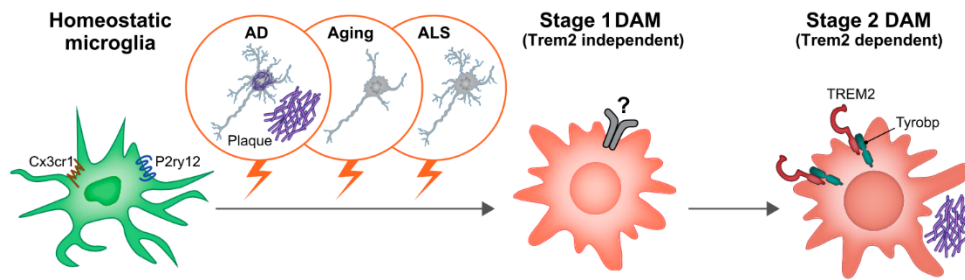


Figure 14: Schematic illustration representing two-step DAM activation mechanism.

First step, microglia switching from homeostatic to stage 1 DAM (Trem2-independent) and stage 2 DAM (Trem2-dependent) following signals such as those associated with AD pathology, aging, and ALS pathology. (Illustration taken from Kheren-Saul et al., 2013(115)).

Other authors, notably Krasemann et al. (117) also describe a specific type of microglia through cluster RNA analysis and see a new different type of microglia in a mouse model of AD with a loss of expression of 68 genes related to homeostatic functions and an upregulation of 28 inflammatory genes. This specific pro-inflammatory microglia with loss of homeostatic functions was termed microglia with neurodegenerative phenotype (MGnD). ApoE and TGF β are major upstream regulators of MGnD. Krasemann et al. also describe that phagocytosis of apoptotic neurons, the hallmark of neurodegeneration, suppresses homeostatic microglia and induce MGnD microglia via TREM2/ApoE pathway. While LPS or IFN γ stimulate and suppress many homeostatic genes, these two molecules are mainly leading to the switching in microglia to the classical M1 state. However, in this state of microglia ApoE is suppressed in contrast with this new MGnD where ApoE is upregulated. In conjunction with other studies on the impact of ApoE in axotomy of nerves, this may lead to the assessment that global and conditional deletion of ApoE in microglia reduces neuronal apoptosis and thus ameliorates neuronal loss (69). Interestingly, ApoE pathway suppresses the tolerogenic function of microglia by suppressing T cell proliferation especially during the disease peak where the highest expression of ApoE is found (117).

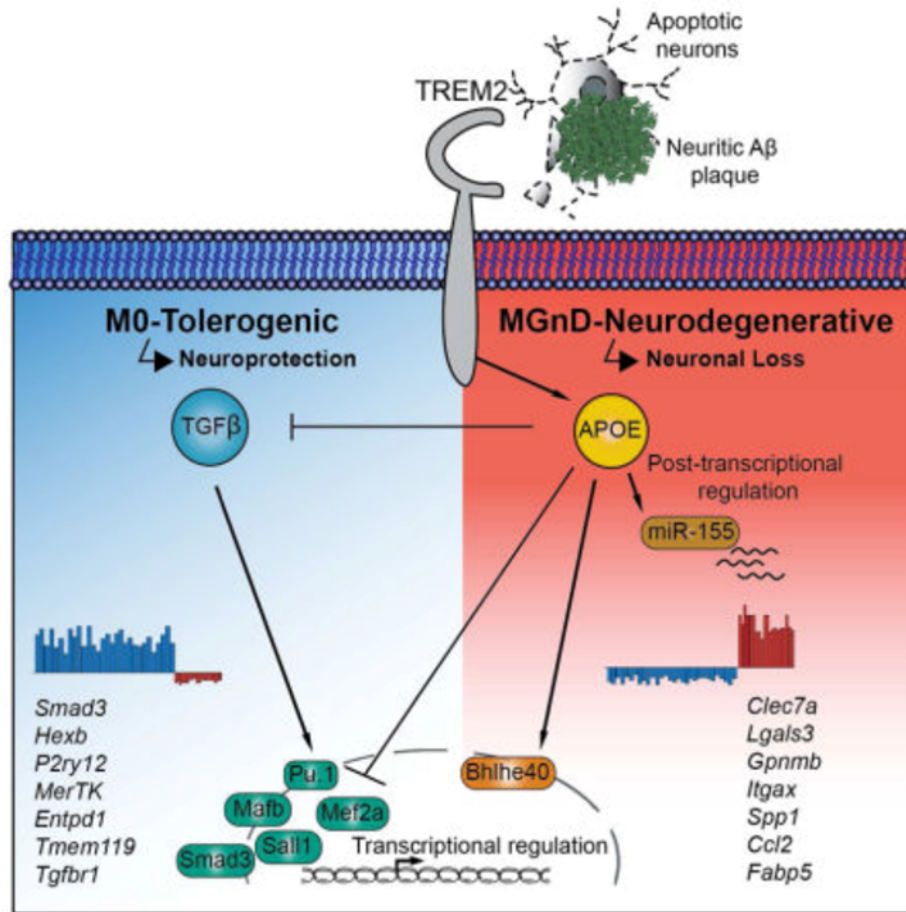


Figure 15: Schematic figure of the new termed microglia with neurodegenerative phenotype (MGnD).

It shows the TREM2 influence and the most up-regulated transcriptional factors within these cells. (Illustration taken from Krasemann et al., 2017(117)).

Microglia have similar regulatory mechanisms at moments when neuronal processes are occurring such as early development and neurodegeneration. These moments share a common feature, focal apoptosis, and therefore an increase in ApoE has been described in both processes. This ApoE overexpression correlates with the suppression of homeostatic genes (48). MGnD microglia, dystrophic neurites, Aβ plaques form a microenvironment rich in ApoE which subsequently leads to more amyloid plaques accumulation and Aβ aggregation. ApoE exerts then positive feedback. TREM2/ApoE signaling occurs via mirR-155, a key microglia-specific signaling pathway (126).

To understand this switch to phagocytic microglia, TREM2 role in human organism and in other cells has to be clarified. It has a pro-proliferative and pro-

survival function through certain molecules such as the mammalian target of rapamycin (m-TOR) (127). Cell autophagy is partially regulated by m-TOR dependent pathways (128). Autophagy reflects and responds adaptively to stress that may occur when cells cannot meet increased demands for energy and protein synthesis, while preventing apoptotic mechanisms (129). Impaired m-TOR signaling is associated with phosphorylation of AMP-activated protein kinase (AMPK), a sensor of low energy states (130). Microglial mice deficient or lacking Trem2 and AD patients carrying a TREM2 mutation have more multivesicular/multilamellar structures indicative of an autophagosome. An impairing in energetic pathways and ATP levels was also found in these microglia. It was also shown that alternative energetic and signaling pathways can compensate for the lack of TREM2 signaling, for instance addition of cyclocreatine *in vitro* can bypass TREM2 deficiency, rescue energetic metabolism and increase m-TOR signaling and cell viability. This fact suggests that TREM2 sustains microglial activation more than it promotes the action of specific transcriptional programs via microglia-specific immune receptors. TREM2 deficiency has shown a reduction in codification of ribosomal genes and glucose transporters (127).

Since TREM2 is required to recruit microglia to plaques and to compact these plaques “in situ” (121), a study of plaque morphology was performed. It was found that plaques from TREM2 deficient mouse models were less compact with smaller cores and many fiber-like structures. Therefore, it is suggested that the plaque density and biochemical profile of the subspecies of A β also depend on TREM2 (131).

Interestingly, activation of microglia through NLRP3 inflammasome promotes A β accumulation due to activation of an inflammatory pathway with the release of IL-1 β (132). This proinflammatory signaling is inhibited by TREM2 activation. This also explains the broad and long-term impact of TREM2 on different microglial functions and in global microglial dysfunction. Increasing autophagy may be beneficial in the short term to reduce A β load and the inflammatory cascade, but

in the long term, a defect in m-TOR signaling is detrimental and compromises microglial fitness and ability to control A β accumulation (120).

Toll-like receptor (TLR) and NOD-like receptors have also been previously implicated in the microglial response to A β accumulation and seem to mediate the immune adaptative response that contributes to this pathology. These receptors induce a big inflammatory response with a striking metabolic reprogramming (132).

Microglia are known to degrade tau species released from AD brains and eliminate neurofibrillary tangles (NFTs). Tau is primarily an intracellular protein, critical for promoting microtubule assembly and stability (133). However, its extracellular presence in brain interstitial fluids forming neurofibrillary tangles (NFT) is another pathophysiological marker for AD. However, the effect of this mechanism in contrast to A β plaques is still unknown. Primary mouse microglia internalize and degrade phosphorylated tau rapidly and efficiently, in a time-dependent manner. It is also evident that large tau aggregates accumulate on the surface of microglia. It reaches a plateau after one hour, but the values are maintained for up to 48 hours (126). Controversial studies suggest that microglial activation by pro-inflammatory cytokines/chemokines in AD brains appears to induce tau phosphorylation, thereby promoting tau aggregation and consequently NFT formation (134).

Although neurofibrillary tangles process and assembly is still unknown, it is important to assess that TREM2 is also associated with phosphorylated tau levels in CSF and these levels are also associated with neuronal loss and cognitive decline in AD patients (135). In contrast, A β levels do not correlate with cognitive decline and usually this deposition into plaques occurs before the onset of symptoms (136). However, when investigating the association of TREM2 and NFT, the results in several studies showed unclear differences in tau phosphorylation and NFT aggregation in mice with partial or complete loss of TREM2 (137). Moreover, activated microglia have been observed to be more

significantly correlated with the development of NFT than with amyloid-containing senile plaques (138).

However, it is not yet clear whether microglial DAM in AD is a useful and protective immune response program against pathologies or whether these microglia are efficient in the early stages and become harmful with the chronic development of the process and unable to contain and to eliminate the neuronal damage. Although this phenotype of cells has been found in another neurological disease, it is not clear whether all DAM models are identical or whether there are different disease-specific subtypes of DAM (139).

To sum up, defining the role of immune cell subsets in neurodegenerative diseases as well as their onset and progression is a real challenge due to cellular heterogeneity (55). However, as described, it was possible to define two specific disease-associated microglia types using transcriptional cell sorting; DAM and MGnD (125). Both are mainly regulated by the interaction of TREM2/ApoE.

Role of ApoD in microglia

Since both microglia and ApoD have been found to be increased in the CNS under the same situations, it is of interest to investigate whether these two components act in concert within the two most prevalent neurodegenerative diseases of the CNS, and whether they are involved in the same response to these diseases: as collaborators or simply as participants in the same defined pathways during the organism's response. Not only classical roles of microglia as immune functions, phagocytosis, and cytokine secretion are examined. Other functions such as cell triggers are examined as well. Novel functions within this kaleidoscopic or 'fifty shades of microglia' approaches must also be studied.

Although the role of ApoD in neurons, oligodendrocytes and astrocytes has been extensively studied, there were no studies in the literature describing the influence of ApoD on microglial cell physiology. Since we know that ApoD helps

to downregulate the synthesis of proinflammatory leukotrienes and pro-inflammatory cytokines such as IL-1 β and TNF- α and it is also involved in oxidative stress reduction via ROS metabolism (34), we hypothesize that this protein is also involved in the activation of microglia towards one of the different levels of this pro-inflammatory spectrum.

A study by Yu et al. (140) showed that ApoD induced NLRC4 inflammasome in highly “responding” microglia from the brains of AD patients. After a bioinformatic gene analysis, using genes in mice whose transcriptional expression depends on ApoD compared with a list of genes expressed by microglia in the adult mouse brain, 42 ApoD-dependent microglial genes were discovered (141). Furthermore, early signs of aging and degeneration appear in the brain of ApoD-KO mice (49).

Considering that DAM microglia with increasing ApoE expression is actively involved in the dismantling and digestion of the amyloid plaques, at least in the early stages of the disease, and are conserved from mouse to human neuropathology, we hypothesize that at least one subtype of microglia in neurodegenerative disease and aging is beneficial and is a common signature of microglial response to CNS pathology, regardless of disease etiology.

The involvement of ApoD in both lipid metabolism and neurological disorders suggests a central role for ApoD in neurodegeneration and/or repair, but the exact role is still unclear.

The link factor among these three pillars (ApoD, microglia and neurodegenerative diseases) may be the binomial TREM2/ApoE. These two molecules act as receptor and ligand and are involved in synergy in a specific alteration of microglia in different phases of a certain number of neurodegenerative diseases. The question now is whether these changes are a common factor in all neurodegenerative diseases, such as: microglial imprinting, or whether they are part of the normal changes that microglia undergo after certain basic primary damage. This also led us to question whether these changes and effects induced

by TREM2/ApoE action play a protective or detrimental role in the development and prognosis of neurodegenerative diseases.

Objectives

The protein ApoD has already been extensively studied in CNS cells such as neurons, Schwann cells and astrocytes, and for this reason we wanted to shed light on the role of ApoD in microglial cells.

As a foundation of the work, a systematized literature search was carried out to comprehensively record the published research results of the last few years, and the results of the experimental work were placed in the context of the literature findings.

Systematized Review

The following questions guided the systematized search of the literature included in both the introduction and discussion section of this thesis:

1. Is there a link in the current literature between ApoD, microglia and neurodegenerative diseases?
2. ApoD and microglia are increased in aging and neurological disease with ROS augmentation. Have ApoD and microglia a specific neuroprotective effect in AD and MS or are mainly involved in ROS clearance and stress reduction?
3. Can both ApoD and TREM2/ApoE be markers of AD and, in this case, are there sources in the literature that prove ApoD as a possible therapeutic target?

Experimental aims

1. Assess microglia ability to take up ApoD from the extracellular medium. To this end, we sought to know the role of ApoD in primary microglial cell cultures and immortalized cell lines.

2. Determine ApoD influence in microglia cytokine secretion profile under different situations under basal conditions, under oxidative stress-producing factors and under exposure to products related to neuropathologies (A β aggregates).

Materials and Methods

Systematized review of the literature

To assess the current knowledge on microglia in neurodegenerative diseases, a literature review of primary publications was performed in a systematized and comprehensive manner, searching the National Library of Medicine database with the PubMed engine (published until June 2021. “Microglia (AND) neurodegeneration” led to 3117 articles. Using the following filters led to the stated number of articles:

(((microglia) AND neurodegenerative disease[MeSH Terms]) AND ("2010/01/01"[Date - Publication] : "3000"[Date - Publication]))) AND central nervous system[MeSH Terms] resulted in 1742 articles.

((((microglia) AND neurodegenerative disease[MeSH Terms]) AND ("2010/01/01"[Date - Publication] : "3000"[Date - Publication]))) AND central nervous system[MeSH Terms])) AND brain inflammation[MeSH Terms] resulted in 85 articles.

((((((microglia) AND neurodegenerative disease[MeSH Terms]) AND ("2010/01/01"[Date - Publication] : "3000"[Date - Publication]))) AND central nervous system[MeSH Terms])) AND phagocytoses[MeSH Terms] resulted in 86 articles.

((((((microglia) AND neurodegenerative disease[MeSH Terms]) AND ("2010/01/01"[Date - Publication] : "3000"[Date - Publication]))) AND central nervous system[MeSH Terms])) AND Alzheimer [MeSH Terms] resulted in 408 articles.

((((((microglia) AND neurodegenerative disease[MeSH Terms]) AND ("2010/01/01"[Date - Publication] : "3000"[Date - Publication]))) AND central nervous system[MeSH Terms])) AND Alzheimer disease[MeSH Terms] resulted in 868 articles.

((((((microglia) AND neurodegenerative disease[MeSH Terms]) AND ("2010/01/01"[Date - Publication] : "3000"[Date - Publication]))) AND central

nervous system[MeSH Terms])) AND *demyelination*[MeSH Terms] resulted in 36 articles.

(((*microglia*) AND *brain inflammation*[MeSH Terms]) AND ("2010/01/01"[Date - Publication] : "3000"[Date - Publication]) resulted in 856 articles.

((((*microglia*) AND *brain inflammation*[MeSH Terms]) AND ("2010/01/01"[Date - Publication] : "3000"[Date - Publication]))) AND *neurodegenerative disease*[MeSH Terms] resulted in 154 articles.

To specify the results, the words were restricted, and the Boolean operators were adjusted.

(((*microglia*[Title/Abstract]) AND (*demyelination* [MeSH Terms]) AND (*inflammation*[MeSH Terms] OR (*phagocytosis*[MeSH Terms]))) resulted in 314 articles.

(((*microglia*[Title/Abstract]) AND (*demyelination*[MeSH Terms]) AND (*inflammation*[MeSH Terms] OR (*phagocytosis*[MeSH Terms])))) AND ("2010/01/01"[Date - Publication] : "3000"[Date - Publication]) resulted in 207 articles.

In addition, the observation period was limited.

(((*microglia*[Title/Abstract]) AND (*demyelination*[MeSH Terms]) AND (*inflammation*[MeSH Terms] OR (*phagocytosis*[MeSH Terms])))) AND ("2015/01/01"[Date - Publication] : "3000"[Date - Publication]) resulted in 124 articles.

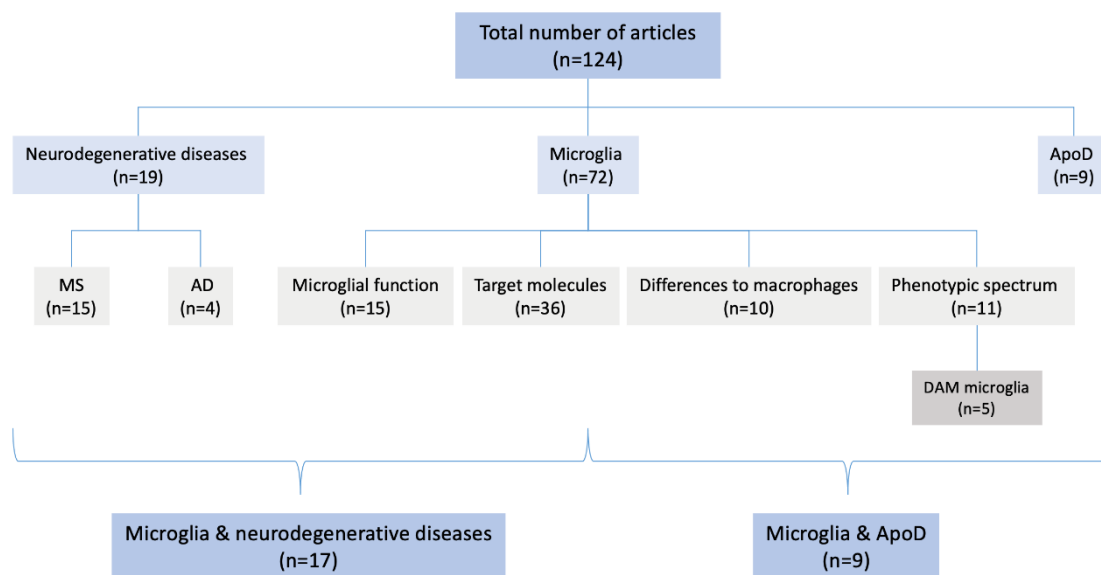


Figure 16: Results of the systematized review of the literature.

Publications (number) on neurodegenerative diseases, microglia and ApoD between 2015 and 2021.

Experimental animals

Animal facilities

The mice were maintained in positive pressure-ventilated racks at 25 ± 1 °C with 12 h light/dark cycle (light from 9:00 to 21:00) at the animal facility of the University of Valladolid (Uva, Spain). They were fed *ad libitum* with a standard diet (Global Diet 2014; Harlan Inc., Indianapolis, IN, USA), and allowed free access to filtered and UV-irradiated water. Cage enrichment included cotton bedding and opaque tubes.

ApoD knock-out mice were generated by standard homologous recombination techniques. We replaced the wild-type ApoD gene with a copy interrupted by the insertion of the neomycin resistance gen (Neo) in the opposite direction to ApoD transcription, which rendered a transcriptional null mutant without deleting any genomic fragment potentially containing regulatory regions. These mice are viable and breed normally.

In order to discard potential maternal effects of ApoD, and to generate WT and ApoD-KO mice of homogenous genetic background, the experimental cohort used in this study are F1 generation of homozygous crosses of ApoD $-/-$ and

ApoD $+/+$ littermates born from heterozygous crosses of an ApoD-KO line backcrossed for over 20 generations into the C57BL/6J background. Experimental procedures were approved by the University of Valladolid Animal Care Use Committee and Junta de Castilla y León (JCyL), following the regulations of the Care and Use of Mammals in Research (European Commission Directive 86/609/CEE, Spanish Royal Decree 1201/2005).

Brain dissection and tissue harvesting

Adult mice brains were quickly removed following decapitation after CO₂ anesthesia and subsequent cervical dislocation and cut into several coronal blocks. These blocks were then divided into two, following a mid-sagittal section. The pieces obtained correspond to olfactory bulbs, cortex regions, cerebellum, and hippocampus. The cerebellum was collected after brain dissection under stereoscope. Tissues were immediately frozen by immersion in isopentane and stored at -80 °C.

Microglial cell cultures

Cell lines

BV-2

BV-2 murine microglial cell line was generated by Blasi et al. by infecting primary female microglial cultures with a v-*raf*l_v-myc oncogene carrying retrovirus J2 (142). They were obtained after defrosting from liquid nitrogen at -80°C. BV-2 cells were grown in 25 cm² cell-culture flasks covered with 5 ml of RPMI 1640 (GIBCO) supplemented with 5% heat-inactivated FBS, 1% L-glutamine (final concentration 2 mM) and 1% P/S (final concentration 100 U/ml penicillin, 100 U/ml streptomycin). To detach cells, GHCKS solution (2g/l D-glucose, 4.77 g/l HEPES, 6.37 g/l NaCl, 0.3 g/l KCl, 3 g/l Na₃Citrate•2H₂O, pH 7.5) was used. For maintenance, cells were harvested and reseeded at 1:10 three times a week. In this complete medium with FBS, BV-2 cells display a rounded morphology, not

seeing in microglia in real homeostatic conditions. To bring BV-2 to “real or resting microglial” morphology, these cells must be grown for at least 24 hours in low-serum medium, where its replication rate is reduced.

To characterize the proliferation kinetics of the BV-2 cell line, the multiplication time of these cells during growth in complete medium was calculated. Three similar flasks of 25 cm² were seeded at a density of 500.000 cells/flask (20.000 cells/cm²) and every 24 hours one of the flasks was harvested with GHCKS solution as described above. Cells from theses flask were counted with a Neubauer chamber, and with these data the number of cell divisions (n) and the duplication time for each temporal point, using the equation $N_f = N_0 \cdot 2^n$ where N_f and N_0 are the final and initial number of cells was calculated. Using the data on the number of duplications within time, a linear regression to create an equation to estimate the number of cell divisions was performed. We got $y = 0.0418x + 0.094$, where “x” is the time in hours and “y” is the number of cell divisions, with an R² of 0.96. With these data, it was estimated that the average duplication time for the BV-2 cell line growing in complete medium is ~22 hours (Figure 17).

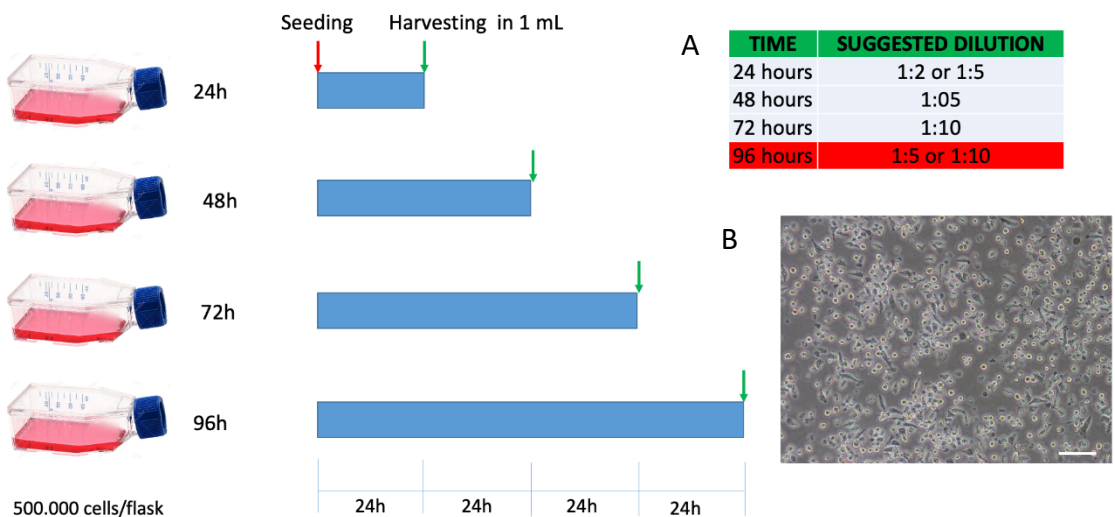


Figure 17: Illustration of the determination of the duplication time in BV2 cells.

A) Time of duplication and B) Image of BV2 48% confluency after 96 hours (scale bar: 20µm).

IMA 2.1.

The mouse astrocytes cell line IMA 2.1 was grown with Dulbecco Modified Eagle's Medium (DMEM) supplemented with heat-inactivated 5% fetal bovine serum, 1% of L-Glutamine and 1% of Penicillin/Streptomycin/Amphotericin. For maintenance, cells were harvested and reseeded three times per week at a ratio of 1:10.

Cell treatments

Under normal conditions, microglial cells are not exposed to serum components. Only when the blood-brain barrier malfunctions are CNS cells exposed to the circulating components of the blood. The presence or absence of serum is not a variable component in other cell cultures. In the case of microglia, this change in serum concentrations leads to numerous data on different microglial responses. Therefore, our studies were performed under conditions of low or no serum levels. Treatments were carried out in RPMI 1640 (GIBCO) 11.1 mM D-glucose. The following treatments were used:

- ApoD: 1-4 μ l at 3 μ M, 2-24 hours.
- LPS: 100-1000 ng/ml in RPMI 1640 without FBS, 8-24 h.
- Paraquat (PQ): 10-500 μ M in RPMI 1640 without FBS, 8-24 h.
- IL-4+ IL-13: IL-4 (20 ng/ml) + IL-13 (50 ng/ml) in RPMI 1640 without FBS, 18-48 h.
- A β oligomers: 0.1-10 μ M in RPMI 1640 without FBS, 8-24 h.
- FAM-A β oligomers: 1 μ M in RPMI 1640 without FBS, 0.5-24 h.
- Myelin-Dil: 20 μ g/ml in RPMI 1640 without FBS, 3-24 h.

Extracellular media processing

To detect secreted proteins in conditioned microglial media, culture media were collected after cell treatment and centrifuged at 300 g for 5 min at RT to pellet and discard detached cells. The supernatants were collected and centrifuged again for 30 min at 4 °C and 16,000 g to pellet the debris. Cell- and particle-free

conditioned media were then concentrated using Amicon Ultra centrifugal filter units according to the manufacturer's instructions. The concentration factor achieved was 20–40 x, depending on the molecular weight limit of the filters. Protein concentrations were quantified by BCA and 80 µg of the sample was analyzed by immunoblot.

Cell treatments with LPS (Sigma-Aldrich), the ROS generator Paraquat (PQ; Sigma-Aldrich) or oligomeric A β ₁₋₄₂ (Bachem), with or without pre-exposure to ApoD, were performed in serum-free media after a 5h adaptation period. Human ApoD (hApoD) was purified from breast cystic fluid as described before (4). A β oligomers were prepared following the protocol by Corraliza-Gomez (5). Briefly, after solubilization in 1,1,1,3,3,3-Hexafluoropropanol (HFIP; Sigma-Aldrich), aliquots were left 2h to evaporate and the peptide film was dissolved in DMSO as a 5 mM stock, which was sonicated and stored at -20°C. Before use, the stock was diluted (1.6 µl/100 µl of DMEM-F12 without additives), sonicated again, and A β peptides were allowed to polymerize for 24h at 4°C. For imaging experiments, fluorescently labeled A β oligomers (FAM-A β ₁₋₄₂, Bachem) were prepared as described below in this chapter.

Primary cell cultures

To obtain primary microglial cell cultures we used neonatal (0-1 days old) mice of two genotypes: ApoD-KO and their WT littermates. We collected newborns from breeding cages. To avoid gender possible bias, we perform a gender assessment according to the Woltering-Donselaar et al. method (143). Male newborns have pigmented area between the anus and the scrotum. In comparison to female newborn mice, male newborns have a bigger anus-genital distance of 1.9 +/- 0.1 mm versus 0.8 +/- 0.1 mm in females. To obtain primary microglial cultures we followed the method described by Saura et al. (144).

To obtain the brain, we performed decapitation of mice after CO₂ anesthesia, detached the head from the snout with a curved needle, and cut the scalp along the midline from the posterior end to the snout to expose the skull. To get rid of

the skull, three incisions with microdissection scissors were required: a transverse incision in the middle of the cerebellum, a second longitudinal incision from the sagittal suture to the midpoint between the eyes, and the last transverse on the olfactory bulb. Finally, we removed the skull by passing one end of the microsurgical forceps under the skull but over the brain tissue and pulling along the midline toward the snout. Once the cerebral cortex was obtained without the cerebellum, midbrain, and leptomeninges, the cerebral cortex was rolled onto sterile filter paper using a microspatula. Then, the tissue was immersed in 2 ml dissection media (3.2 $\mu\text{g/ml}$ BSA, 27.5 $\mu\text{g/ml}$ DNase I, 100 U/ml penicillin, 100 U/ml streptomycin and 0.25 $\mu\text{g/ml}$ amphotericin B) and transferred to a Petri dish. The tissue was cut in smaller pieces with a surgical blade and left for sedimentation on a tube for 1 minute. It was centrifuged at 200g for 2 minutes in brake low at room temperature (RT) to remove medium and blood cells. Pelleted tissue was resuspended in 1 ml digestion media (3.2 $\mu\text{g/ml}$ BSA, 27.5 $\mu\text{g/ml}$ DNase I, 100 U/ml penicillin, 100 U/ml streptomycin and 0.25 $\mu\text{g/ml}$ amphotericin B and 0.25 mg/ml Trypsin) mechanically with a Pasteur pipette. The suspension was then incubated for 15 min at RT with stirring (750 rpm) to gravity pellet cell clumps to achieve tissue disruption. Trypsinization reaction was stopped by addition of dissection media/FBS (110 μl FBS per-1 ml of dissection media). Samples were then centrifuged at 200 g for 5 minutes to stop the trypsinization by the serum proteins, to pellet cells and then discard trypsin solution. Then DNase dissociated the leftovers of nucleic acids after mechanical and chemical disaggregation. Cells were resuspended in 1 ml of dissection media and mechanically dissociated with a Pasteur pipette. The samples were incubated for 5 minutes at RT to pellet by gravity cell clumps, while individual cells remained in suspension to seed both fractions separately. Cells were plated onto different surfaces (from 2.24 to 78.5 cm^2) after cell counting with Neubauer camera (NC) at a density of 62,500 cells/ cm^2 and incubated at 37 °C in 5 % CO₂ with 95 % humidity.

To subsequently maintain the culture, samples were replaced one day after cell seeding (D1) and then every 5–7 days. After 14 days *in vitro* (DIV), cell

confluence of 90% or more was determined by NC. Samples were kept for an additional week to promote microglial proliferation.

To remove astrocytes, the culture medium was refreshed at 20 DIV and after 24 hours, at 21 DIV, the astrocytes were detached by “mild” trypsinization (500 mg/ml or 0.25% in DMEM solution). It took about 60 minutes to detach completely the astrocytic layer and left the microglia attached to the culture surface. Culture media containing astrocytic factors were collected to be used as a “conditioned media” essential for microglial proliferation in the next 24 hours. Meanwhile, cell cultures were washed twice with PBS and incubated in a solution of mild trypsin (0.8 mg/ml Trypsin-EDTA in DMEM-F12 without FBS) for 1 hour at 37 °C in 5 % CO₂. During the incubation time, conditioned media was centrifuged at 300g for 5 minutes to pellet non-desirable floating cells. The supernatants were transferred to new tubes and supplemented with macrophage colony stimulator factor (M-CSF 25 ng/ml). Trypsin was inactivated by adding 10 % FBS and removing the media afterwards. Cells were washed twice with PBS following addition of conditioned media and M-CSF. Isolated microglia remained 48-120 hours in this media to attain a basal or homeostatic state (the so called “resting microglia”) before their use in experiments.

Techniques

MTT assays

MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) is the most popular technique to assess cell viability and cytotoxicity. It is based in measuring the number of alive cells after exposition to the MTT. There is a reaction carried out by the mitochondrial dehydrogenases with the MTT that relies on tetrazolium salt as an indicator of the intracellular reducing potential. This component's reduction results in the formation of violet-blue, a water insoluble formazan product, whose absorption in the visible region in theory correlates with the number of metabolic intact (alive) cells in the sample. A different MTT-based protocol depending on the biological question was developed (145).

Standard MTT colorimetric assays

Cells were seeded in different plate formats, ranging from 6 to 96-well plates, with a cell density of 50.000 cells/cm². After being exposed to different treatments, cells were washed with PBS and MTT (62.5 µg/ml) was added for 3 hours in phenol red-free DMEM without FBS. After MTT exposure, cells were incubated in an isopropanol -10% Triton-X100 solution to solubilize formazan. Formazan production was measured by spectrophotometry using the SOFTmax Pro microplate reader (Molecular Devices). Absorbance was collected at $\lambda = 570$ nm, subtracting the background measured at $\lambda = 690$ nm.

Normalized multi-well plate formazan detection method

The MTT method cannot estimate viability after long-term treatments because after long-term exposure to MTT, all cells can be affected by MTT and the survival rate decreases. To overcome this issue, a method to estimate the number of cells present in each well was developed to be able to normalize formazan signal to the total number of cells. By using the nuclei Hoechst staining, a suitable method to easily count cells in a sample, quantitative formazan detection with nuclear labeling in each in the sample was combined. Cells were then seeded to a final density of 30000 cells/cm² in a 96 well-plate with black walls. After receiving their correspondent treatment, culture medium was removed, and Hoechst staining was performed to estimate the number of cells per sample. This was followed by a standard colorimetric assay.

RT-PCR method

We reverse-transcribed (PrimeScript Takara Bio Inc.) 0.5 µg of total RNA with Oligo-dT primers and random hexamers.

To examine mRNA expression, cells from mouse heads were frozen at -80 °C. RNA was then extracted after cells were washed twice with 1 mL of PBS and lysed with QIAzol Lysis Reagent. RNA concentration was measured with

NanoDrop spectrophotometer. To assess the quality of the RNA extracted and to check the absence of genomic DNA, samples were loaded into a 1% agarose gel for electrophoresis and run at 80 V for 30 minutes. To visualize the nucleic acids, the gel was stained with RedSafe and illuminated with ultraviolet ray (GelDoc XR, BioRad).

After quantification, 500 ng of RNA were treated with DNase I (0.1 U/ μ l) to eliminate any remained genomic DNA, in a reaction volume of 5 μ l. Reverse transcription (RT-PCR) was done with the PrimeScript™ RT Reagent Kit (Takara Bio Inc., Otsu, Japan) following manufacturer's instructions by using Oligo-dT primers and random hexamers:

Reagent	Volume	Final concentration
5X Prime Script RT Buffer	2.0 μ l	1X
Oligo dT Primer 50 μ M	0.5 μ l	5 μ M
Random 6-mers 100 μ M	0.5 μ l	10 μ M
Prime Script RT Enzyme Mix I	0.5 μ l	
RNAse-free dH ₂ O	1.5 μ l	
Sample	5.0 μ l	50 ng/ μ l

The resulting cDNA was used as a template for quantitative real-time polymerase chain reaction (RT-PCR) using BioRad Mg mini thermal cycler (BioRad) and the DreamTaq Mix (In Vitro Gen). Standard cycling conditions were 95°C, 5 minutes; 40 cycles (95°C, 30 s; 60°C, 1 minute). Rpl18 was used as the reference because neither genotype nor treatment gives a significant fold change for this gene. The genes analyzed by RT-PCR with their respective forward (Fwd) and reverse (Rev) primers are the following:

- ApoD gene: Fwd 5'-GAAGCCAAACAGAGCAACG-3' and Rev 5'-TGTTTCTGGAGGGGATAGGA-3'.
- IL-1 β gene: Fwd 5'-TGTAATGAAAGACGGCACACCCAC-3' and Rev 5'-GGCTTGTGCTCTGCTTGTGAGG-3'.

- TGF α gene: Fwd 5'-CGCAACAACGCCATCTATGA-3' and Rev 5'-ACCAAGGTAACGCCAGGAAT-3'.
- TNF α gene: Fwd 5'-TCTTCTCATTCTGCTTGTGGCAG-3' and Rev 5'-TGGTTTGCTACGACGTGGGCT-3'.
- Rpl18 gene: Fwd 5'-TTCCGTCTTTCCGGACCT-3' and Rev 5'-TCGGCTCATGAACAACCTCT-3'.

To validate the specificity of the amplified products, melting curves were analyzed and PCR was loaded into a 2% agarose gel and run at 100 V for 30 minutes. Changes in transcriptional expression were estimated with the $2(-\Delta\Delta C(t))$ method by Schmittgen and Livak (146). The following criteria were applied to our amplifications: (i) Replicates with variation coefficient > 2.5% were excluded; (ii) Undetermined Ct values (gene expression below detection levels) were assigned Ct = 35; (iii) Pairwise comparisons in which the average Ct value of the gene was >35 cycles under both conditions were excluded from the analysis; (iv) Only transcriptional changes greater than or equal to twofold were included in the analysis.

Statistical differences of the gene transcription changes found were evaluated with the SigmaPlot v11.0 program using the non-parametric U-Mann-Whitney test using the ΔCT of each replicate (calculated by subtracting the average CT of the reference gene for each sample) with a $p < 0.05$ statistical significance level.

Luminex assays

As previously described, primary microglial cultures were isolated and incubated for 5 days in astrocyte-produced conditioned media supplemented with 20 ng/ml M-CSF. Cells were then starved for 3 h before exposure to various stimuli prepared in RPMI 1640 medium (11.1 mM D-glucose, 1% L-glutamine, 1% P/S) without FBS for 18 h.

The stimuli were: 100 ng/ml LPS; 20 ng/ml IL-4 + 50 ng/ml IL-13; 25 μ M PQ and 1 μ M A β oligomers. Each condition was performed in six replicates (male and female microglial cell samples, each as triplicates). After incubation, the culture medium was collected and centrifuged at 4 °C and 1000 g for 10 minutes. It was then stored at -20 °C.

The Luminex Multiplex Assay developed by R&D Systems are bead-based multianalyte profiling kits for detection protein analytes in biological samples. The kit uses color-coded superparamagnetic microparticles (beads) coated with analyte-specific antibodies. Beads recognizing different target analytes are mixed and incubated with the sample. Captured analytes are subsequently detected using a cocktail of biotinylated detection antibodies and a streptavidin-phycoerythrin conjugate. Firstly, samples were added to a mixture of color-coded beads, pre-coated with analyte-specific capture antibodies. The antibodies bound to the analytes of interest. Secondly, biotinylated detection antibodies specific to the analytes of interest were added and formed an antibody-antigen sandwich. Phycoerythrin-conjugated streptavidin was added and bound to the biotinylated detection antibodies. Finally, beads were read using a Luminex 100® Analyzer. The excitation system in this analyzer involves two solid-state lasers: a classification laser (red) excites fluorochromes embedded in the beads as they flow single-file through the cuvette and identifies each type of bead, while a reporter laser (green) excites the phycoerythrin-derived signal on the bead surface and quantifies the analyte present in the sample. Photodiodes and photomultiplier tube receive both fluorescent signals, which are further digitalized and sent to a digital signal processor.

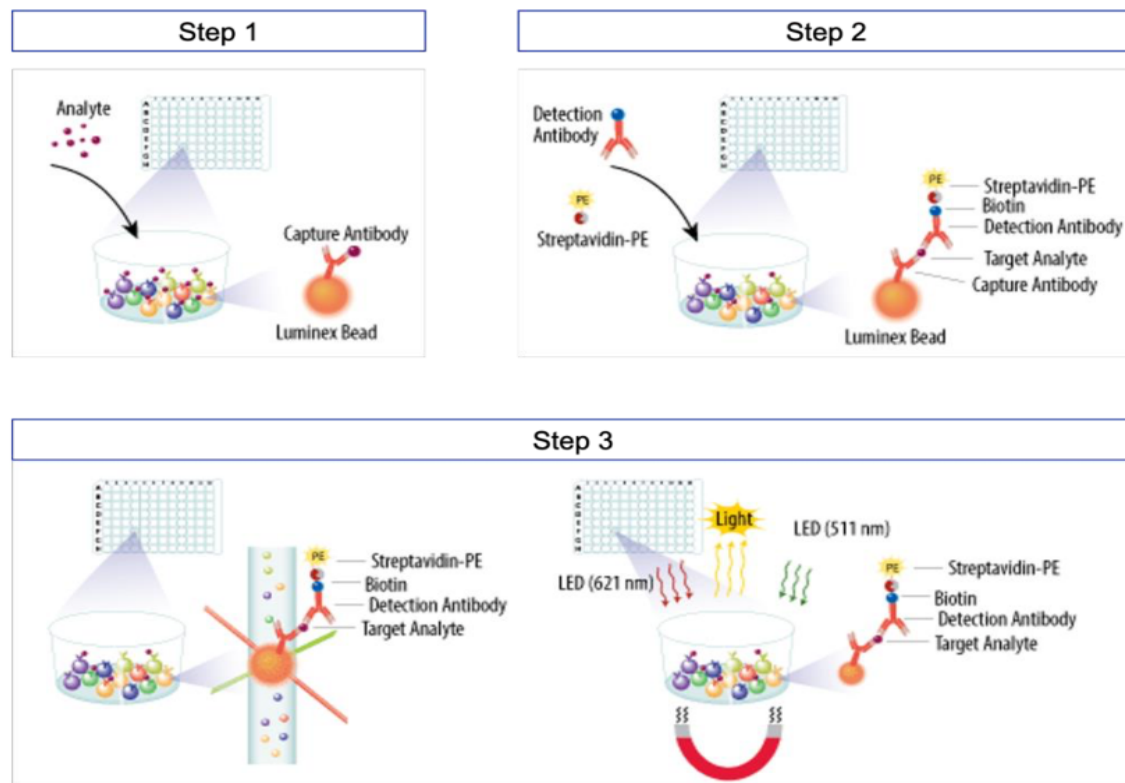


Figure 18: Luminex assay principle.

Step 1, capture antibodies, bound to a color-coded bead, bind to their specific target analytes.

Step 2, biotinylated detection antibodies are added and bind to target analytes, forming an antibody-antigen sandwich. Then, PE-conjugated streptavidin is added and binds to the biotinylated detection antibody.

Step 3, beads are read using a Luminex 100® Analyzer with two lasers: a classification laser, which identifies the beads, and a reporter laser, which quantifies the analytes. Image adapted from R&D Systems website.

A multiplex assay, in which IL-1 β , IL-6, TNF- α , IL-4 and IL-10 were measured, was performed. The Luminex assay kit provide a cocktail of standards of known concentration. A standard curve was created for each analyte by reducing the data to generate a five-parameter logistic curve-fit, and cytokine concentrations were calculated from the standard curve.

TGF- β is initially synthesized as a precursor protein consisting of a large N-terminal portion, the so-called latency-associated peptide, and a short C-terminal fragment, the mature cytokine (147). For accurate quantification, latent TGF- β must be activated to the immunoreactive form in a two-step activation procedure: first, incubation with acid (0.17N HCl) for 10 minutes at RT, followed by

neutralization with base (0.17N NaOH/0.71 M HEPES). Because of the sample pretreatment, TGF- β cannot be multiplexed with other cytokines. TGF- β active form was quantified in cell culture supernatants using the assay DuoSet ELISA Mouse TGF- β 1 (R&D Systems), following manufacturer's instructions.

Amyloid β_{1-42} (A β_{1-42}) oligomers preparation

There are several protocols to prepare A β oligomers due to its complexity and difficulty to obtain reproducible oligomers preparations. Our method is based on the method described by Núñez et al. (148) and it is published in detail in Corraliza-Gómez et al. (149).

Protein immunodetection

To characterize the intracellular localization of proteins, immunocytochemistry (ICC), which is based on the use of specific primary antibodies to recognize a specific epitope of that protein, followed by fluorescence-coupled secondary antibodies that recognize the primary antibodies, was used. Nuclear staining (DAPI) was also used to identify the nuclei and thus each individual cell. Protein detection using immunofluorescence also enables the use of specifically prepared tissue samples to visualize the respective protein, called immunohistochemistry (IHC).

To obtain the ICC samples, BV-2 and IMA.2.1 cells were counted using the Neubauer chamber. 75000 cells were plated on 12 mm diameter Poly-lysine coverslip (~ 66500 cells/cm²) in complete medium (RPMI 1640 + 5% FBS + 1% L-glutamine + 1% P/S) followed by different treatments. Cells were washed twice to remove the possible rest of the treatments. Live cells attached to glass coverslips were incubated at 4 °C for 5 minutes to stop endocytosis machinery and were exposed to an antibody cocktail in RPMI medium with 1% FBS at RT for 20 minutes. After several washes, cells were fixed with formaldehyde (4 %) in PBS. Cells were blocked and permeabilized with Tween-20 (0.1%) and 1% non-

immune (goat or bovine) serum. Cells were incubated overnight at 4 °C with the primary antibodies.

The following list includes all the antibodies used for ICC and IHC:

Antibody	Source	Fixation	Permeabilization and blocking	Concentration
Goat serum anti-mouse ApoD	Santa Cruz	4% PFA in PBS	Tween-20 (0.1%) 1% non-immune FBS	1 µg/ml (1:300)
Rabbit serum anti-human ApoD	Custom-made	4% PFA in PBS	Tween-20 (0.1%) 1% non-immune goat serum	0.5 µg/ml 1:2000
Rat monoclonal anti-mouse Lamp-2	DSHB	4% PFA in PBS	PBS-Tween-20 (0.1%) 1% BSA	1.95 µg/ml 1:500
Rabbit monoclonal anti-mouse Iba1	Santa Cruz		PBS-Tween-20 (0.1%)	1 µg/ml

Alexa Fluor® 594/488 (Jackson Labs) conjugated IgGs were used as secondary antibodies (0.75 µg/ml) for fluorescence immunocytochemistry. After washing in PBS to remove blocking solution, the slides were mounted with EverBrite™ mounting medium with DAPI and sealed with CoverGrip™ Coverslip Sealant (Biotium). We used two different fixation methods to fix the obtained samples: paraformaldehyde cross-linker reagent or methanol (an organic solvent). To fix cells and especially tissue fragments with paraformaldehyde, cells were incubated for 10 minutes at RT in freshly prepared 4% paraformaldehyde in PBS with stirring. The samples were then washed thoroughly in PBS.

For fixation in methanol, cells were incubated in ice-cold methanol for 5 minutes at –20 °C. Three washes with PBS followed. After fixation with both reagents, samples were blocked with 1% nonimmune goat serum in either PBS, 0.1%

Tween-20, or Triton X-100 for 30 min at RT. The cells were then incubated overnight at 4°C. Incubation of samples with fluorophore-bound secondary antibodies takes 1 hour without light. The rest of the process is similar to that of unfixed cells.

To prepare immunohistochemistry, mouse brains were quickly removed after sacrifice after anesthesia with CO₂ and divided into two parts after a midsagittal section. Brains were fixed by immersion in 4% paraformaldehyde overnight at 4°C and embedded in paraffin following standard procedures. Coronal slices (4 µm section) were obtained with a rotary microtome (Microm, Wayzata, MN, USA) and were mounted on Polysine Slides (Menzel-Gläser) and dried or stored at -20°C respectively. To proceed to the immunohistochemistry with paraffin samples, the sections were previously dewaxed in xylene and rehydrate through ethanol series in PBS. Endogenous peroxidase was inactivated with H₂O₂ (0.9%) for 5 min in the dark before immunohistochemistry was performed. Antigen retrieval was carried out using a sodium citrate buffer (10mM sodium citrate, 0.05% Tween 20, pH 6.0). The slices were then blocked and permeabilized with Triton X-100 (0.25% in PBS) and 1% non-immune goat serum. The same primary and secondary antibodies were used as in immunocytochemistry.

Image acquisition and analyses

Labelled cells were visualized with an Eclipse 90i fluorescence microscope (Nikon) equipped with DS-Ri1 (Nikon) digital CCD camera. Images were acquired under the same conditions of illumination, diaphragm and condenser adjustments, exposure time, background correction and color levels.

Confocal images were obtained with a 63x oil immersion objective (HCX PL Apo CS NA=1.4; Leica) attached to a confocal DMI 6000B microscope with a TCS SP5 confocal system (Leica) equipped with AOBs system and three spectral detectors. Laser power and detection gains were set by scanning control samples labeled with secondary antibody alone. To ensure that the images have similar dynamic ranges, gain, and offset were adjusted using LUTs. In this way, blood

circulation can be neglected. Negative control images showed a very faint and homogeneous background. To maintain constant conditions and to minimize fluctuations in image acquisition, confocal sections were used. Images were analyzed using FIJI software (ImageJ) and saved as 1024 × 1024 pixels and 8-bit TIFF files.

Image quantification

Image quantification was obtained with FIJI software in immunostained sections. Microgliosis was measured as Iba1 fluorescence intensity per unit area.

Statistical analyses

Statistical analyses were performed with SPSS v.19 (IBM) and SigmaPlot v.11. (Systat) software's. To check the normality of distributions, Kolmogorov-Smirnov test was used. To analyze statistically significant differences between two sets of data, Student's t-test (parametric data) or Mann-Whitney U test (non-parametric data) were used. Comparisons between more than two groups of data were performed using ANOVA as many ways as factors (for parametric data) and Kruskal-Wallis test (for non-parametric data). A p value < 0.05 was used as a threshold for significant changes.

Results

Since our research triangle is the relationship between ApoD, microglia and neurodegenerative diseases, we first lay the groundwork to establish a common/synergistic effect of microglia and ApoD. Subsequently, we introduce possible factors that mimic common features of degenerative diseases to establish a possible link between these three concepts/entities.

Effect of ApoD in microglial populations upon aging

Hippocampal microglia density is dysregulated in response to the lack of ApoD in an age-dependent manner

The aging process involves an increase in ROS due to several factors and ApoD has antioxidant potential as an MRS enzyme. Therefore, due to this potential, ApoD can then be altered in the brain of mice only due to its normal aging evolution.

Microglial cell number is known to be tightly regulated throughout adulthood, maintaining a constant value by equilibrium between cell death and cell division (150). We wanted to evaluate in a brain region from aging mice if there is a difference in basal conditions between the ApoD-KO mice and their wild type (WT) counterparts. We evaluated then microglial numbers in the hippocampus of young (100 d) and aged (655 d) WT and ApoD-KO mice using the marker Iba1 and found a statistically significant increase of microglial cell density in ApoD-KO young mice (Figure 20). This result indicates that microglia are sensing the lack of ApoD and modifying their equilibrium. This alteration early in life is consistent

with the fact that signs of aging and degeneration appear early in the brain of ApoD-KO mice (151) (Figure 19).

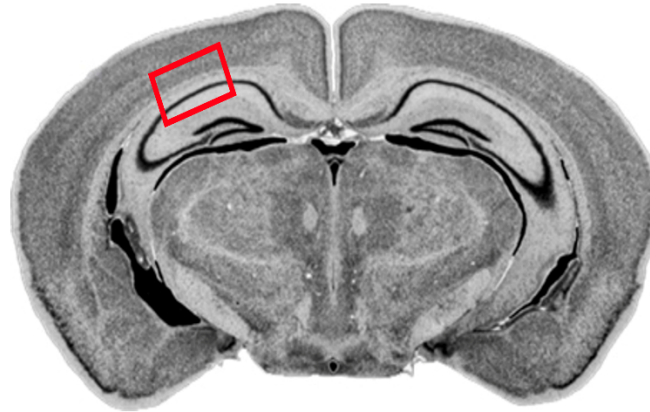


Figure 19: Coronal cerebral slice of a mouse showing the region where microglia are studied (boxed in red). The histological section comes from the mouse brain library atlas (<http://www.mbl.org/>).

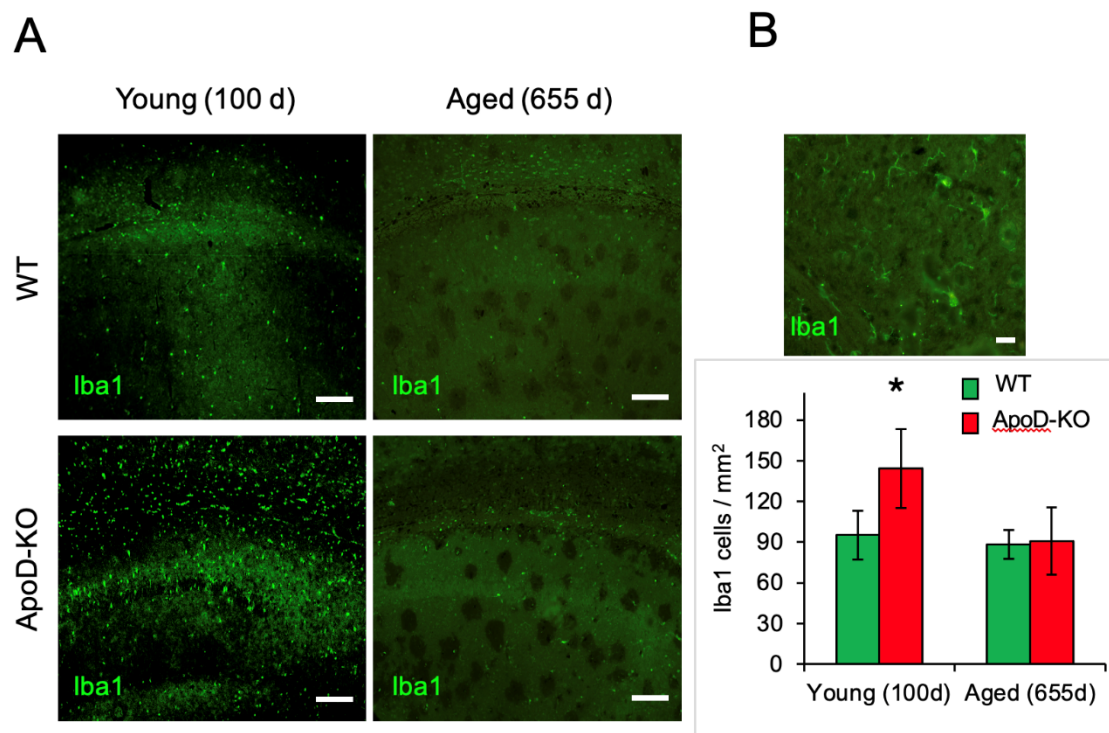


Figure 20: Immunohistochemistry of Iba1 in microglial cells.

Young adult mice lacking ApoD expression increase their number of microglial cells in the hippocampus, a genotype-dependent response that disappears upon physiological aging. Degenerated regions in brain tissue are evident in aged samples of mice lacking ApoD. Representative images are shown (A). Microglial cells labeled by Iba1 antibody and graphical quantification of Iba1-positive cells in young [100 d] (2 serial sections; n=3 / genotype) and aged [655 d] (2 serial sections; n=4 / genotype) samples. The Secondary antibody was Alexa Fluor 488. Iba1 positive cells were measured using FijiJ Image software (B). Asterisk point to significant differences between young and aged groups (Student's T-test). Calibration bars: 100 μ m (A).

Microglia do not express ApoD in contrast to astrocytes

All available evidence consistently indicates prominent ApoD expression within the nervous system, particularly in oligodendrocytes and Schwann cells. While there are reports of ApoD expression in astrocytes, it appears to be at lower levels and more widely distributed (1). Notably, macrophages have been shown not to express ApoD (2). Given the similarity in function between macrophages and microglial cells within the CNS, we questioned whether microglia, which share similar functions with macrophages in the CNS, express ApoD. To investigate this, we initially assessed whether ApoD plays a role in microglia under normal conditions before defining its role under specific circumstances. Our investigation aimed to determine whether ApoD is expressed by microglia under any condition, including basal conditions and exposure to various stressors.

BV2 do not express mApoD contrary to IMA2.1.

Fluorescence immunocytochemistry with microglia BV2 and astrocytes IMA2.1 cells was performed. Astrocytes IMA2.1 cells were used as positive control. We used Iba1 protein as a microglia membrane marker. Based on the presence of ApoD in basal conditions in IMA2.1 we did not detect direct immunofluorescence of mouse ApoD (mApoD) in microglial immortalized BV2 cell line in basal conditions (Figure 21).

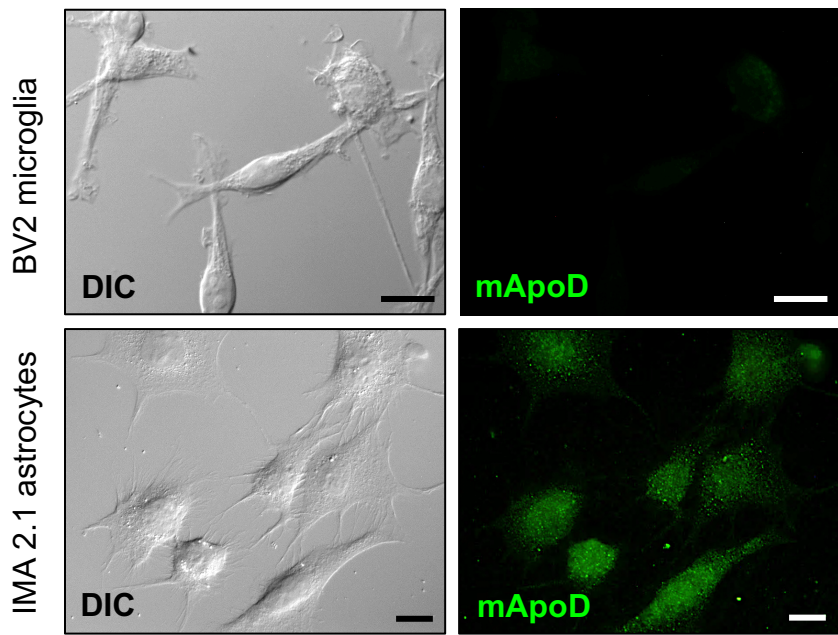


Figure 21: Immunocytochemistry mApoD.

The mouse microglial cell line BV2 is not labeled by the mouse ApoD antibody, while the astrocytic cell line IMA2.1 shows an intracellular characteristic ApoD punctate pattern. We used as primary antibodies a goat anti-mouse ApoD specific antibody and a rabbit anti-mouse Iba1 specific antibody. The secondary antibody was Alexa Fluor 488. Calibration bars: 20 μ m.

To ensure our results with immunocytochemistry we performed a RT-PCR measuring the levels of ApoD mRNA in primary microglial cells and BV2 cells. The same conclusion was reached. RT-PCR revealed the absence of ApoD mRNA transcripts in primary mouse microglia and in BV2 cells (Figure 22).

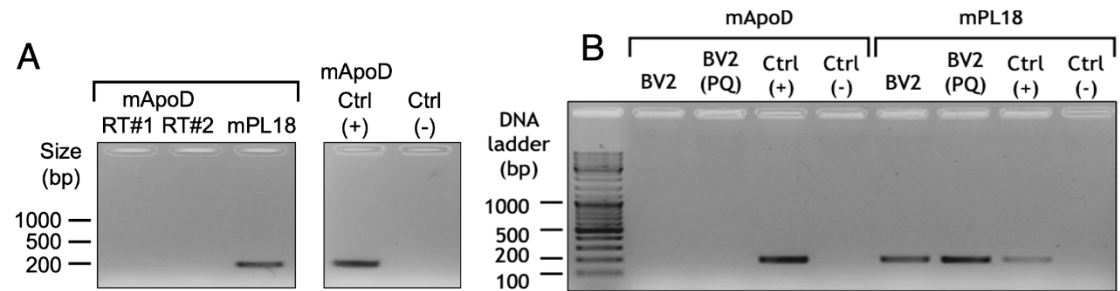


Figure 22: RT-PCR of primary mouse microglia and BV2 cells.

RT-PCR reveals the absence of ApoD mRNA transcripts in both primary mouse microglia and BV2 (A and B). The gene *rpl18* was used as a normalizer. RT#1&2 are two separate RT reactions from independent primary cultures (B). Mouse sciatic nerve expression is used as positive control. An unrelated template is used as negative control. PQ 75 μ M for 3 h does not induce ApoD expression in BV2 cells (B).

BV2 cells do not express mApoD under Paraquat exposure

After evaluating the absence of ApoD expression in microglia under basal conditions, we tried to find a condition, shared by aging and the neurodegeneration process, that would lead microglia to express ApoD. We exposed BV2 cells to experimental oxidative stress (OS) with PQ (75 μ M) for 3 hours. We found that this stressing factor does not induce ApoD mRNA expression in BV2 either (Figure 22B).

ApoD can be internalized by microglia

It is well established that ApoD is internalized by astrocytes and neurons (21,152). We, therefore, sought to demonstrate internalization of exogenous ApoD in microglial BV2 cells. We performed fluorescent immunocytochemistry with cells exposed to hApoD purified from breast cystic fluid (10 nM) for 3 hours pre-fixation. Exogenously added hApoD is internalized to intracellular compartments of BV2 cells and primary microglial cells in a characteristic punctate pattern (Figure 23).

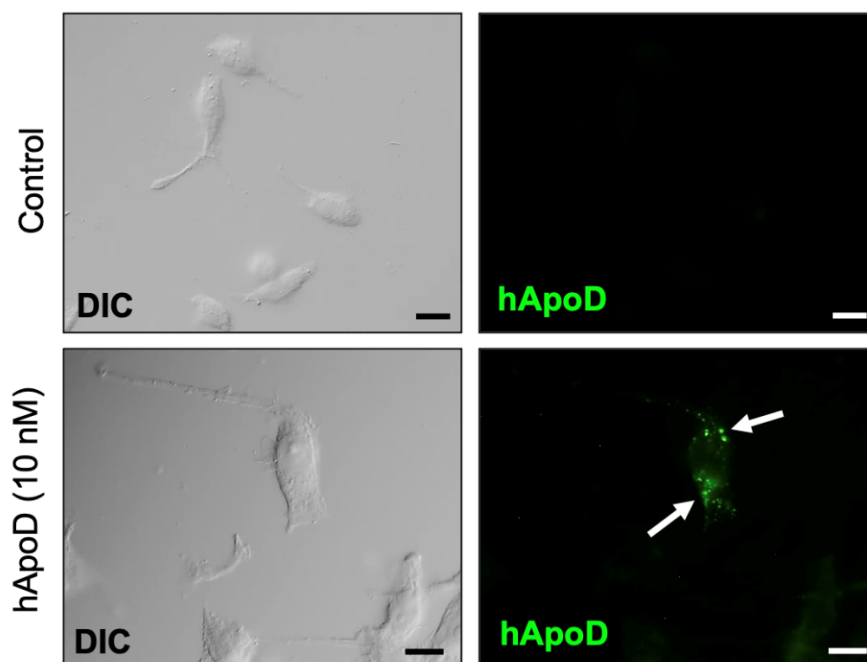


Figure 23: BV2 Immunocytochemistry of hApoD.

Results

Exogenously added human ApoD (10 nM) is internalized to intracellular compartments of BV2 cells after 3 h exposure. White arrows point to hApoD intracellular punctate. Primary specific antibody rabbit anti-human ApoD and secondary antibody Alexa 488 were used. Calibration bars: 20 μ m.

ApoD traffic in microglia differs from that of other cell types, with less prominent endolysosomal location

Exogenously-added purified or recombinant hApoD, as opposed to bacterial recombinant ApoD, was demonstrated to persist in astrocytes for over 48-72 hours. A prominent and long-lasting endolysosomal localization is found in these cells (152,153). Therefore, we investigated whether ApoD internalization in primary cells microglia cultures has a stable localization in the endosome-lysosomal compartment.

We demonstrated the co-localization of hApoD with the late endosome-lysosome marker Lamp2 and found that, as reported for astrocytes and neurons, ApoD enters this compartment in primary microglia. However, this co-localization was significantly smaller than the astrocytes hApoD quantification (Figure 24).

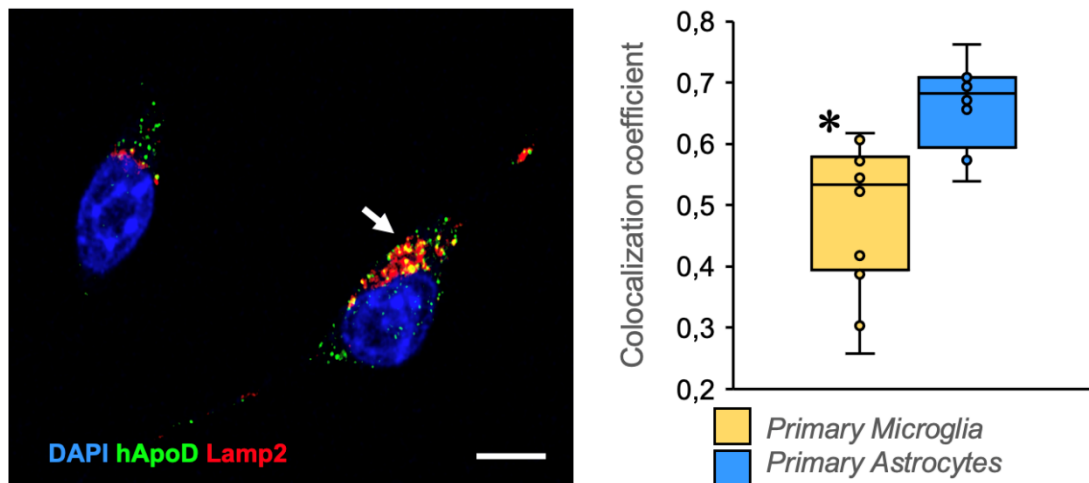


Figure 24: Immunocytochemistry of primary microglia.

We performed fluorescence immunocytochemistry after 2 hours addition of hApoD. Primary antibodies rabbit anti-human ApoD and rat anti-mouse LAMP2 were used. Secondary antibodies Alexa 488 and Alexa 594. Nuclei were stained with DAPI. Exogenously added ApoD traffics to the late-endosome-lysosomal compartment of primary mouse microglia in control conditions, but with a lower co-localization index than that of primary astrocytes (Student's T-test). Arrow points to colocalizing signals. Calibration bar: 50 μ m.

ApoD, oxidative stress and A β oligomers interaction

Oxidative stress generated by Paraquat increases hApoD internalization in BV2 cells

Both long-term and acute exposure of microglial cells to ApoD, in conjunction with relevant stimuli associated with aging, neurodegeneration, or demyelinating diseases, reveal distinct roles compared to the effects observed in astrocytes, Schwann cells, neurons, and other previously studied cell types. ApoD appears to be an integral part of the endogenous response to a diverse range of diseases, with ROS emerging as the primary candidates as common factors underlying these diseases, potentially influencing changes in ApoD expression. Therefore, we sought to investigate whether oxidative stress (OS) modulates ApoD internalization.

In astrocytes and neuronal cells, pro-oxidant stimuli promote ApoD internalization, a process evident within the initial two hours of ApoD exposure in these cell types (7). However, in microglial cells, we observed that longer periods are necessary to detect increased ApoD internalization.

We use PQ as an oxidative stress agent to determine possible changes in hApoD internalization in microglial BV2 cells after certain periods of time (PQ exposition of 3 hours or 6 hours). Addition of hApoD (10 nM) and PQ (50 μ M) was performed and a significant increase in hApoD signal when BV2 cells have longer exposition to PQ (6 hours) was found. HApoD signal was measured from fluorescence immunocytochemistry marking hApoD (Figure 25).

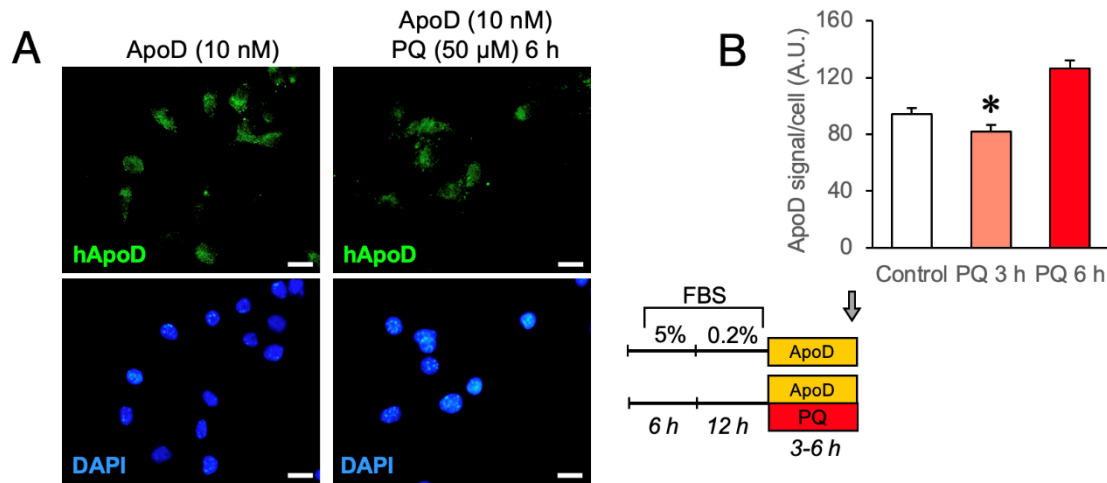


Figure 25: Immunocytochemistry of BV2 microglial cells.

Internalization of exogenous ApoD in BV2 is time and PQ-dependent. After 3 h exposure to 50 nM ApoD, the internalized ApoD steadily decreases with time in BV2 cells. Primary antibody was rabbit anti-human ApoD and secondary Alexa Fluor 488. Nuclei were stained with DAPI (A). ApoD signal/cell in control conditions and after 3 or 6 hours exposure to PQ. Only after 6h of PQ treatment there is a significant increase in internalized ApoD (ANOVA followed by Holm-Sidak pairwise comparisons.) (B). Calibration bars (A): 20 μ m.

These findings underline the importance of understanding the dynamics of ApoD intracellular trafficking to accurately interpret its influence on microglial physiology. They clearly indicate that ApoD likely influences microglial responses and functions through an alternative mechanism, which could involve modulation of signaling cascades, rather than relying solely on its stable presence and maintenance in the endolysosomal compartment.

ApoD does not exert a pro-survival effect in BV2 acutely challenged by oxidative stress or neurodegenerative producing conditions

ApoD has been reported to protect astrocytes, neurons and fibroblasts when exposed to stressing factors such as ROS (44,152,154,155). To test whether ApoD exerts a similar function on microglial cells, we pre-exposed BV2 microglial cells to purified hApoD at concentrations showing protective effects in astrocytes, before treating them with two stimuli relevant to aging and neurodegeneration (OS and A β oligomers). Neither long (24h) nor short (3 h) intervals of ApoD pre-exposure rescue microglia significantly from the cell death provoked by PQ. A

Results

small pro-survival effect was detected in control conditions or with low dose stimuli (Figure 26).

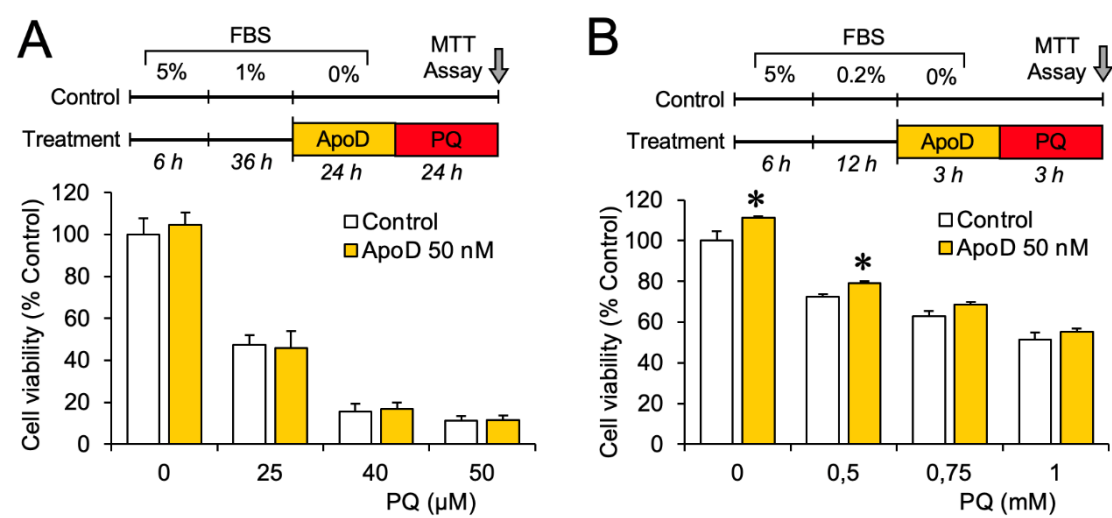


Figure 26: MTT analysis of BV2 microglial cells (1).

Cell viability analysis of BV2 cells by MTT assay upon PQ exposure with or without pre-exposure to ApoD. Treatment started after adaptation of cells to serum starvation (5% - 1% - 0% for 24 h experiments (A); 5% - 0.2% - 0% for 3 h experiments (B)). Addition of hApoD (50 nM) to the culture medium exerts a mild pro-survival effect on BV2 cells in control conditions but is not sustained with increasing doses of PQ (0-50 μM for 24 hours experiments and 0-1 mM for 3 hours experiments). Also, this mild pro-survival effect is more evident in short term expositions (3 hours). All experiments were performed in triplicates. Asterisks point to significant differences in comparison with control conditions evaluated by ANOVA followed by Holm-Sidak pairwise comparisons.

The same results were found when the cells are treated with increasing Aβ oligomers quantities (Figure 27).

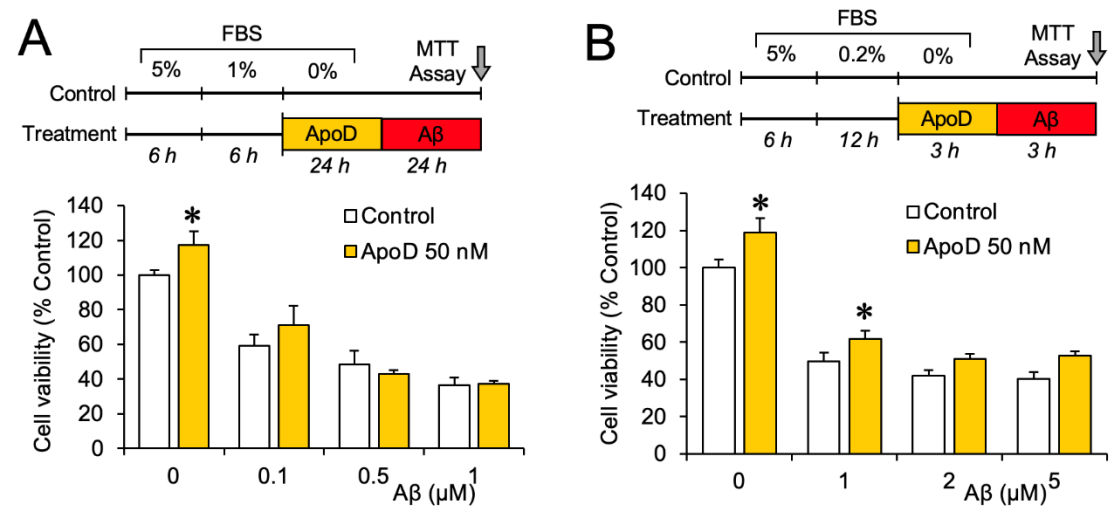


Figure 27: MTT analysis of BV2 microglial cells (2).

Cell viability analysis of BV2 cells by MTT assay upon Aβ exposure with or without pre-exposure to ApoD. Treatment started after adaptation of cells to serum starvation (5% - 1% - 0% for 24 h experiments; 5% - 0.2% - 0% for 3 h experiments). Addition of ApoD (50 nM) to the culture medium exerts a mild pro-survival effect on BV2 cells in control conditions but is not sustained with increasing doses of Aβ (0-1 μM for 24 hours experiments and 0-2 μM for 3 hours experiments). All experiments were performed in triplicates. Asterisks point to significant differences in comparison with control conditions evaluated by ANOVA followed by Holm-Sidak pairwise comparisons.

effect on BV2 cells in control conditions but is not sustained with increasing doses of A β oligomers (0-1 μ M for 24 hours experiments (A) and 0-5 μ M for 3 hours experiments (B)). Also, this mild pro-survival effect is more evident in short term expositions (3 hours). All experiments were performed in triplicates. Asterisks point to significant differences in comparison with control conditions evaluated by ANOVA followed by Holm-Sidak pairwise comparison.

ApoD alters the microglia cytokine secretion profile towards a pro-inflammatory phenotype in basal conditions, Paraquat and A β exposure in a sex-dependent way

Firstly, we aimed to evaluate whether ApoD could alter the cytokine secretion profile of microglia in response to various stressful stimuli (PQ, LPS) at concentrations that do not compromise the viability of primary microglial cells. To achieve this, we utilized primary microglial cultures obtained from either WT or ApoD-KO mice, serving as a simplified model reflecting long-term exposure or absence of astrocyte-derived ApoD. The experiments started with cells adapted to serum starvation (0.2% FBS) for 12 hours. Subsequently, an acute exposure to ApoD was introduced to simulate a damaging event in the nervous system, known to induce acute peaks of ApoD production. We assessed the concentrations of five cytokines (IL1 β , TNF α , IL6, IL4, and IL10), traditionally associated with either pro-inflammatory (IL1 β , TNF α , and IL6) or anti-inflammatory (IL4 and IL10) phenotypes of immune system-related cells. Primary cortical microglia were cultured from newborn mice of both genders and genotypes, following a protocol involving extended periods of adaptation to allow cells to recover from phenotypic changes induced by the extraction/culture process and to attain a basal or 'resting state' characteristic of a healthy brain. Multiplex analysis was performed on the culture media after 18 hours of culture under control or experimental conditions, with or without prior acute exposure to ApoD.

To validate the correct cytokine induction of our cells, we exposed microglia to a well-known potent stimulus, LPS. We treated our WT male microglia cells with LPS (100 μ g/l) and the treatment resulted in strong inductions of TNF α , IL6, IL1 β

Results

and IL10, while it did not significantly induce IL4 secretion. Pre-exposure to exogenously added purified ApoD (50 nM) did not change the cytokine profile in response to LPS, excepting a significant lower induction of IL6 (Figure 28).

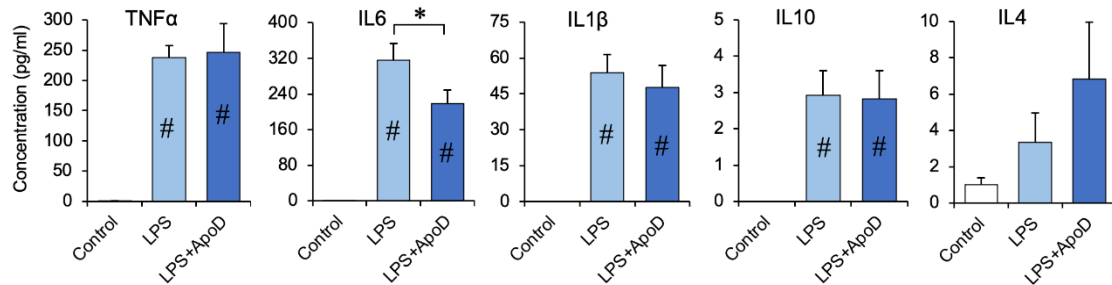


Figure 28: Luminex® assay analysis of male primary microglial cell culture (1).

Cytokines secretion evaluated in WT male primary microglia upon LPS (100 ng/ml) exposure (light blue) or LPS preceded by ApoD (50 nM) addition (dark blue). Asterisks point to significant differences among groups of each graph, and # represent differences with respect to control conditions, evaluated by three-way ANOVA followed by Holm-Sidak pairwise comparisons.

After validating the cytokine secretion capacity of our cells with LPS, we investigated the effect of a ROS-producer agent, PQ. We found that WT microglia maintained the same profile in control or with PQ addition (0-50 μ M) without an acute exposure to ApoD, for TNF α , IL6, and IL4. IL1 β and IL10 appeared though undetected.

Analysis of the male microglia responses after acute exposure to ApoD revealed a significant increase (over ten-fold) of TNF α and IL10 secretion in control conditions. ApoD pre-treatment did not change IL4 and IL1 β secretion, it was still undetected. Moreover, an exacerbated induction of IL6 upon PQ insult (with a similar trend in IL10 response) was demonstrated (Figure 29).

The sex of microglial cells did influence their response. Female microglia showed response patterns for TNF α and IL6 that were globally similar to that of WT or ApoD-KO male microglia. However, they differed from male microglia in the fact that they did not secrete detectable levels of IL10 and IL1 β in any of the conditions explored. Contrary to male-mice cells, they showed an overall higher

level of IL4 secretion (~ five-fold difference with males) and they had a stronger differential TNF α and IL6 response to PQ (Figure 30).

An interesting finding in these experiments was the absence of significant amounts of the pro-inflammatory cytokine IL1 β in response to any of the experimental conditions tested.

We performed the same experiments described above but instead of using PQ as a stressor stimulus we expose microglia to neurodegenerative mimicking situation with A β (0-1 μ M) oligomers addition.

We found with A β similar results to the above-described experiments with PQ. WT male microglia maintained the same profile in control or A β conditions for TNF α , IL6 and IL4, while IL1 β secretion still appeared undetected. However, ApoD-KO microglia turned down the secretion of TNF α in response to A β oligomers. Lower values of IL6 secreted by ApoD-KO microglia were also observed with A β addition, though the differences were not statistically significant possibly due to the low quantity of this cytokine, close to the detection threshold of the assay.

Analysis of the responses after acute exposure to ApoD revealed a significant decrease of TNF α and IL6 and IL10 in response to A β (with a similar trend in IL4 response but without enough significance) (Figure 29).

The sex of microglial cells also influenced the response upon A β exposure and ApoD addition with similar a trend to PQ addition (Figure 30).

When we analyzed globally the responses of microglia derived from ApoD-KO brains, a noticeably different functional scenario emerged. We observed a consistent decrease of cytokines release, both by male and female microglia in every condition and with every specific stimulus.

Results

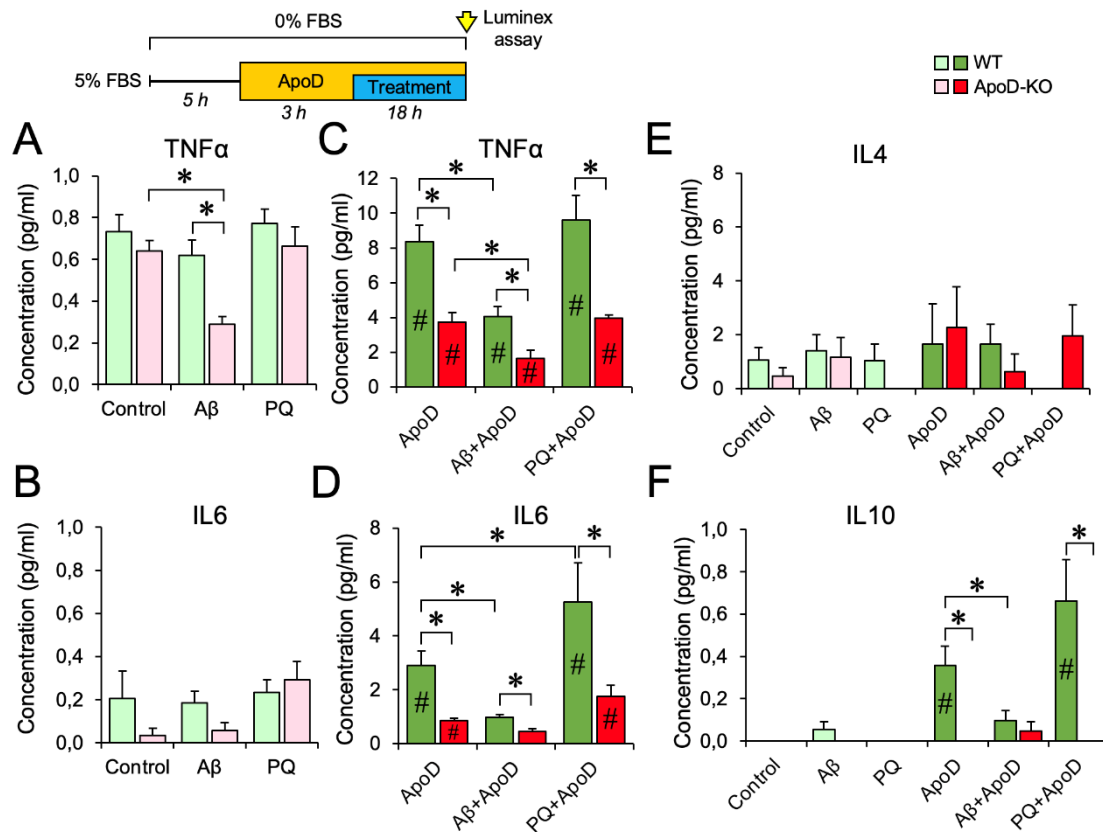


Figure 29: Luminex® assay analysis of male primary microglial cell culture.

Cytokine secretion profiles of male primary microglia in control conditions, PQ-induced OS and Aβ-oligomers exposure. Luminex multiplex assay is used on microglia culture media after 18 h incubation under different conditions (control or 25 μM PQ). Light green and pink bars represent concentration of each cytokine under control conditions for primary microglia from WT (light green) or ApoD-KO mice (pink). Dark green and red represent concentration of each cytokine secreted by WT or ApoD-KO primary microglia when stimuli were preceded by the addition of purified human ApoD (50 nM) to the culture medium. ApoD is maintained during the treatment period. Each profile represents two technical replicas of two primary cultures, each obtained from two separate newborn mice per sex. Asterisks point to significant differences among groups within each graph, and # point to differences between conditions, with or without ApoD acute exposure, evaluated by ANOVA followed by Holm-Sidak pairwise comparisons.

Results

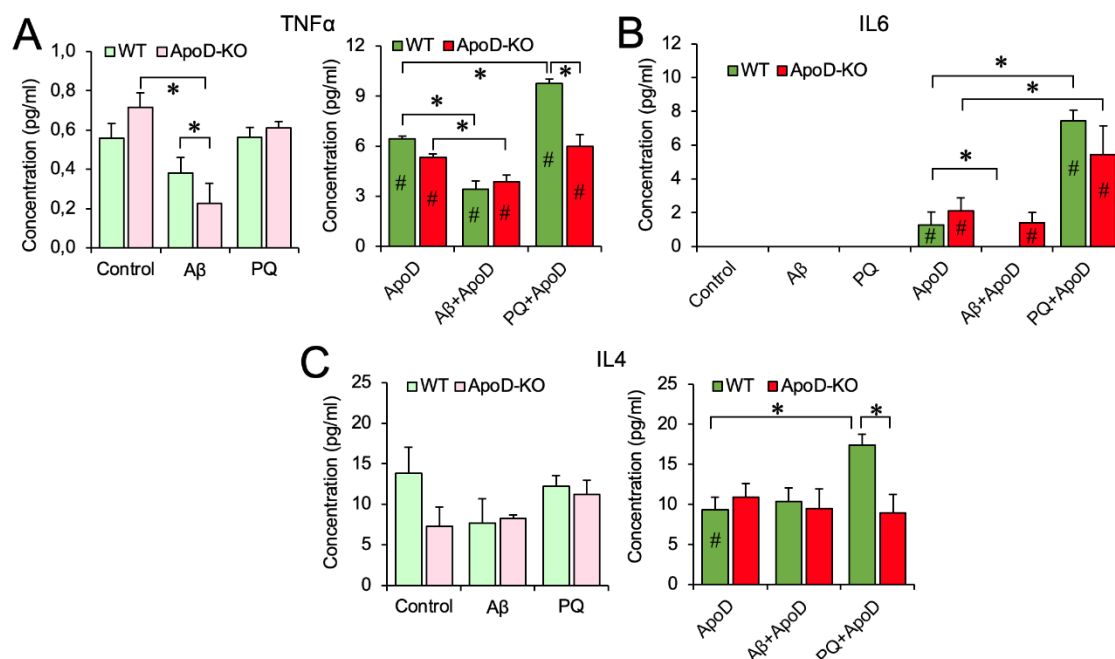


Figure 30: Luminex® assay analysis of female primary microglial cell culture.

Cytokine secretion profiles of female primary microglia in control conditions, PQ-induced OS and Aβ-oligomers exposure. Luminex multiplex assay is used on microglia culture media after 18 h incubation under different conditions (control or 1 μM Aβ oligomers). Light green and pink bars represent concentration of each cytokine under control conditions for primary microglia from WT (light green) or ApoD-KO mice (pink). Dark green and red represent concentration of each cytokine secreted by WT or ApoD-KO primary microglia when stimuli were preceded by the addition of purified human ApoD (50 nM) to the culture medium. ApoD is maintained during the treatment period. Each profile represents two technical replicas of two primary cultures, each obtained from two separate newborn mice per sex. Asterisks point to significant differences among groups within each graph, and # point to differences between conditions, with or without ApoD acute exposure, evaluated by ANOVA followed by Holm-Sidak pairwise comparisons.

All the results presented in this study were carried out in the context of a collaboration project from the members of the C5 laboratory in the IBGM (Valladolid, UVA) between 2017 and 2021. I was part of a team involved in these experiments that led to the publication of the first paper about the role of ApoD in microglia (141).

Discussion

ApoD, a lipocalin primarily associated with lipid metabolism and stress response, has attracted attention for its intriguing involvement in various aspects of neurobiology. The role of ApoD in the mammalian nervous system has been extensively studied in neurons, astrocytes, and myelinating glia (14). Microglia play a critical role in immune surveillance, maintenance of CNS homeostasis, and response to injury. Emerging evidence suggests that dysregulation of microglial function contributes to the pathogenesis of neurodegenerative diseases (156). In this context, understanding the interplay between ApoD and microglia holds great promise for elucidating novel mechanisms underlying neurodegeneration.

Recent research has shed light on the potential role of Apolipoprotein D (ApoD) and microglia in the pathogenesis and progression of neurodegenerative diseases. In order to establish a link between ApoD and microglia in our laboratory we asked whether we could find an ApoD-specific transcriptome profile in microglia during the aging process in mice to fill this gap. The research was initiated by a gene enrichment bioinformatic analysis using lists of mouse brain genes whose transcriptional expression depends on ApoD in response to physiological aging in the cortex and hippocampus (151). A total of 322 genes were identified as being specifically expressed by microglia. Estimating that the number of genes expressed by mouse microglia to be approximately 15,661, the results indicate that there is a significant enrichment of microglial genes in our lists among the ApoD-dependent age-related genes in both the cortex (26 out of 452) and hippocampus (21 out of 647) (141). Subsequent functional enrichment analysis using the DAVID platform identified a total of 42 ApoD-dependent enriched microglial genes. These genes are associated with known ApoD functions in other cells and subcellular locations, such as glycoproteins and lysosomes. It is suggested that the transcriptional response of the aging nervous system to the constitutive absence of ApoD expression is enriched in microglial genes (141).

Furthermore, the functional enrichment of ApoD-dependent microglial genes in pathways related to immunity, antigen presentation, and lysosomal activities underscores the potential involvement of microglial ApoD in the neuroinflammatory response associated with neurodegenerative diseases. Given the critical role of microglia in neuroinflammation and synaptic remodeling, ApoD-mediated modulation of microglial function may influence disease progression and neuronal survival.

In humans, ApoD has been found to colocalize with microglial cells in neurodegenerative diseases with Alzheimer's disease being the most well-studied among them (29). Loss of ApoD increases the hippocampal A β load in experimental animals by 50% (109). Based on these previous studies, we know that A β accumulates preferentially in the hippocampus which was the reason why we performed our experiments in this area of the telencephalon. Interestingly, we were able to show that the number of microglial cells is different in the hippocampus of young and aged WT and ApoD-KO mice. An increase in microglial cell density in ApoD-KO young mice was found (Figure 20). Microglia chronically exposed to ApoD absence may alter their balance to reach an aged state with more potential redox protective mechanisms. Of course, this is in contrast to the stable microglial density observed throughout the lifespan of the mouse, as only a minimal amount of microglia is replaced by cells derived from the bloodstream (157). However, we found normal levels of microglia in aged ApoD-KO mice, which may indicate an ApoD-dependent ability of microglia to avoid homeostatic control in early adulthood.

Therefore, ApoD does not seem to be the only/main effector in microglia under aging or stress conditions.

Microglia does not express ApoD

Based on the previous results and knowing that many cells of the mammalian nervous system express ApoD and others do not express ApoD but are able to internalize it in certain situations, we confirmed ApoD non-expression by microglia. Although there is a consensus in the current literature that ApoD plays a role in glial cell reactivity (14), microglia, which are an important part of glial cells controlling CNS homeostasis, do not express ApoD. ApoD is an endogenous regulator of the redox state, and it is also involved in lipid-containing structures intra- and extracellularly. However, ApoD is not a ubiquitous protein; it is expressed in cells in a spatio-temporal pattern (14). This expression pattern is not clearly identified but may be closely related to the extracellular crosstalk between astrocytes and microglia, and not only by microglia themselves.

Our experiments show, both by immunocytochemistry and RT-PCR, that microglial BV2 cells and primary microglial cells do not express ApoD under any conditions (Figure 22). These data are similar to those found in macrophages (158).

ApoD internalization by microglia and the concept of the “upper limit”

Although microglia cannot express ApoD, internalization of this protein in microglia BV2 cell line and primary cell cultures after exogenous addition of purified hApoD was found. This internalization was dose-dependent. This may be related to the widely evoked concept in the current literature about microglial “upper limit” (14). ApoD acts in situations of utmost stress of microglia and its role is to be integrated by microglia and help these cells to be involved in several actions, such as the secretion of cytokines, which mainly help to recruit other glial cells. However, the ApoD “upper limit” concept is based on the fact that once

ApoD MRS enzyme activity can no longer work because ApoD is oxidized then ApoD is no longer able to neutralize any more hydroxiperoxidized lipids, and therefore cannot protect cells under stress situations. This situation can be deleterious due to the accumulation of ApoD in the dimeric form. This may be the case in chronic exposure to oxidative stress, such as in neurodegeneration or aging. The number of microglial cells increases with age, in contrast to other glial cells, but they also display signs of dystrophy such as reduced ramifications and swollen, tortuous processes (159). Returning to the microglial “threshold limit”, the status of microglia in late neurodegenerative diseases may represent an exaggerated state of aging or senescent microglia. This effect may affect microglia at certain stages of the neurodegenerative disease when these cells become unable to perform their main functions and become detrimental to the progression of the disease. Firstly, microglial phagocytosis of A β in AD may be beneficial, but chronic stimulation of microglia by A β may be detrimental and cause prolonged general inflammatory pathways. This microglial failure has been demonstrated in several studies of AD (160).

There comes a point in the progression of the disease when microglia reach their limit and are unable to clear A β products. Instead, microglia switch to a pro-inflammatory state, recruiting more immune cells and contributing to the damage. In these situations, ApoD may also have reached its own upper limit and been unable to exert its antioxidant functions. Both effects simultaneously can develop together into a more serious cell damage. Or maybe ApoD can be involved in the microglia to reach its “upper limit” faster only by the fact that there is a co-relation between two processes; microglia is aging at the same time that ApoD is increasing. Such functional relationships may underlie the biological limits of microglia in the advanced Alzheimer’s brain, where both, ApoD and A β oligomers, are elevated (141). Being aware of both the upper limit of ApoD antioxidant function and microglial failure should be useful to further study and determine its consequences in neurodegenerative diseases.

ApoD traffic in microglia differ from those of other cells

ApoD is internalized by microglial cells, but this process differs in certain details from ApoD internalization in other cells. In our experiments, we showed that ApoD co-localize with the late lysosomal-endosomal compartment and is more internalized in the presence of certain pro-oxidant stimuli such as PQ. But in contrast to other cells in the CNS such as astrocytes (44), we found in our studies that microglia required longer exposure to the stress factor to increase ApoD internalization. Furthermore, in contrast to astrocytes, ApoD persistence within microglial cells is shorter (43). About 40% of the peak ApoD concentration in microglial cells is reduced in the first 24 hours after our team's experiments (141). This transient presence of ApoD in microglial lysosomal compartments suggests that the role of ApoD is not mediated by lysosomal protections. Instead it might be mediated by triggering downstream processes or signaling cascades after interaction with specific membrane domains such as TREM2/ApoE (149) rather than acting as a major effector protein.

AD is one situation that induces microglial cells to uptake ApoD, as previously shown in the literature (29). However, the open question about these findings now lies in understanding the role of ApoD in AD. Is ApoD just more internalized by microglia in the early stages of AD, as in any other brain injured situation where ROS are released, or is there a specific protective mechanism in AD involving ApoD?

To assess this issue, our laboratory performed some further experiments and found that the internalization of A β oligomers by microglia, measured by cytometry, is not affected by ApoD addition. As we already described above, ApoD is located in the lysosomal compartment but A β is located extracellularly or inside endosomal vesicles, which means that to test the theory of a possible ApoD-A β interaction requires further experiments. Since we could not

demonstrate a specific effect of ApoD in the internalization of A β oligomers, ApoD may be involved in different processes in AD.

Pre-exposure to ApoD does not rescue microglia BV2 cells from stressor stimuli

The laboratory experiments measuring cell viability showed that ApoD does not protect microglia under acute stress stimuli (PQ) or stimuli associated with aging and neurodegeneration (A β oligomers and myelin release). A small pro-survival effect was found with low dose PQ addition after 6 hours exposition in control conditions or with low dose A β stimuli (141). However, ApoD-KO mice show more A β plaques and more microglia than their WT counterparts (109). This finding might seem contradictory, but it might simply indicate that ApoD effects on amyloid plaque accumulation is dependent on other mechanism, different from A β internalization by microglia.

A β internalization in microglia can occur by different mechanisms: phagocytosis, endocytosis or pinocytosis (161). Since microglia play a role in extracellular A β degradation by the secretion of proteases but also in A β lysosome-mediated degradation, further analysis will lead to study the influence of ApoD in A β clearance (162). Previous studies suggesting that female microglia have a higher phagocytic activity (163) and experiments performed in our laboratory have shown ApoD- and sex-dependent myelin phagocytosis and degradation (141). These results with myelin show a possible influence of ApoD on A β phagocytosis in microglia and this might help to clear specific products in AD.

ApoD modifies the cytokine response of microglia after oxidative stress in a time-dependent manner

To test one of the main microglial functions, which is cytokine secretion, we performed a Luminex assay to measure the release of five known pro- and anti-inflammatory cytokines in primary microglial cells.

It was found that WT microglia, without an acute exposure to ApoD, maintained the same low secretion profile under control, or PQ conditions, for TNF α , IL6, and IL4. Meanwhile, IL1 β and IL10 secretion was undetected (141). The addition of PQ at concentrations that do not compromise cell survival in primary microglia does not induce a differential secretion of the cytokines studied. This lack of cytokine response to noxious stimuli has been reported by others and confirms that our cultured primary microglia are in a homeostatic/ground state (141,164,165).

Interestingly, after acute exposure to ApoD, an intriguing result was found: a significant increase (more than ten-fold) in TNF α , IL6 and IL10 secretion was observed, while no change in IL4 or IL1 β secretion was detected (141). There was also an overall increase in the classically considered pro-inflammatory cytokines when ApoD was paired with PQ (similar to previous results reported for other ROS-generating stimuli). However, when microglia ApoD-KO were analyzed, there is a completely different result. A consistently attenuated cytokine release by male/female microglia was found. Since ApoD-KO microglia have always lived in a constitutive absence of ApoD, this difference may be due to the exposure of WT microglia to astrocyte-expressed ApoD culture media. This may suggest a long-term instructive or memory function of ApoD in microglia, and this may be the basis of the previously introduced concept of astrocyte-microglia crosstalk. In response to various environmental signals and stimuli throughout the lifespan of the brain, crosstalk between glial cells is necessary to develop organized and coordinated responses, especially in situations such as neuronal diseases (141).

We also found that the sex of the microglia has an influence on the responses that are induced. Female WT microglia show a slightly different response pattern than male microglia. They do not secrete detectable levels of IL10 and IL1 β in any of the conditions studied, they showed an overall higher level of IL4 secretion (~five-fold difference from males), and they have a stronger differential TNF α , IL6 and IL4 response to PQ (141). However, for TNF α and IL6 they are globally similar to WT or ApoD-KO male microglia (141). The difference in the number and appearance of male and female microglia in adult mouse models is well known, as is the influence of estradiol on the inflammatory potential of microglia (166). However, these two assessments should also be extrapolated to humans. This possible overexpression of IL4 in female primary microglial cells, which mean stimulation of female microglia towards a more anti-inflammatory phenotype, may shed a light on the fact that women have a lower risk of vascular diseases (due to the anti-inflammatory potential of IL4) but more risk to neurodegenerative diseases, which require a strong phagocytic initial phase to remove debris. Our results are then in line with the previous result that highlighted a neuroprotective function of IL4 in female mice subjected to experimental cerebral stroke (167).

As with previous studies on the role of other apolipoproteins or lipocalins, ApoD appears to play opposing roles depending on whether it activates or inhibits certain microglial inflammatory responses specifically to different physiological or pathological situations. This means that acute exposure to ApoD, mimicking a punctual expression of neighboring cells, induces a switch in microglia to a more secretory and less phagocytic phenotype, whereas long-term absence of ApoD (ApoD-KO) leads to attenuated cytokine secretion and increased myelin uptake (141).

Therefore, ApoD expression in astrocytes, neurons, oligodendrocytes, and endothelial cells may influence microglial function indirectly through paracrine signaling or by modulating the CNS microenvironment. Further investigation of

cross-signaling from microglia to other CNS cells may help to understand the full function of microglia in neurodegenerative diseases. Thus, we propose that ApoD may play a regulatory role in microglial phenotype and function, particularly under conditions of oxidative stress or aging, ultimately influencing neuroinflammatory responses and potentially contributing to the pathogenesis of neurodegenerative diseases.

ApoD modifies the cytokine response of microglia after amyloid stress

However, there is a consistent decrease in $\text{TNF}\alpha$, IL6 and IL10 release produced by the combination of acute exposure to ApoD and $\text{A}\beta$ oligomers. This may indicate a putative competitive effect of $\text{A}\beta$ oligomers on the cytokine released induced by ApoD. It is in this situation of advanced AD that microglia has reached their “upper limit” and therefore both ApoD and $\text{A}\beta$ oligomers are found elevated, but there is no ApoD contribution to $\text{A}\beta$ oligomers clearance.

In ApoD-KO microglia, there is a dampening of $\text{TNF}\alpha$ and IL6 secretion in control and $\text{A}\beta$ conditions, though the differences are not statistically significant, possibly due to the low levels of this cytokine, close to the detection threshold of the assay (141).

The role of TREM2/ApoE

Some authors describe an improvement in the survival of glial cells exposed to ROS-generating stimuli (14). However, our experiments in microglia showed an apparent lack of a pro-survival role for ApoD. Similarly, microglia exposed to noxious $\text{A}\beta$ are not protected by ApoD, although ApoD-KO mice showed more $\text{A}\beta$ plaques as previously described. Only limited microglial amelioration by

soluble factors, mainly involving glial-derived factors CSF-1/TGF β and TREM2/ApoE receptor, has been described previously (168). Interestingly, the increased risk of developing AD is significantly associated with rare variants of innate immune receptors expressed in microglia (48). The most important of these receptors is TREM2/ApoE receptor.

Recent studies have shown an association between certain ApoD polymorphisms and the risk of developing sporadic AD (169). This is similar to the ApoE polymorphism that are currently used as a diagnostic tool for AD (120).

TREM2/ApoE also shows a time-dependent function in AD, but this function is in contrast to the possible function of ApoD. *In vivo* studies suggest that TREM2/ApoE is detrimental in early stages, as it can promote both tau phosphorylation and tau aggregation due to tyrosine kinase dysfunction (170). Nonetheless, in later stages, when there is a complete loss of TREM2/ApoE function, it becomes beneficial and protects against neurodegeneration (170).

Since TREM2/ApoE is cleaved from the microglial surface, soluble TREM2 may regulate microglial function and/or expression. Therefore, cerebrospinal fluid levels of TREM2 can be used as a potential biomarker of microglia activity in early stages of AD (48).

Heterogeneity of microglia; kaleidoscope vs. classical microglia signature

Microglial cells are neither a homogeneous population nor a “patchwork of black and white cells” (81). The term microglia M1/M2 is abandoned because microglia cannot be simplified as a dichotomous good versus bad. The term “neuroinflammation” does not imply bad but rather an adaptive response to damage that can get out of control in some situations.

Using TREM2/ApoE as a link protein, we move into the microglia called DAM identified by Streit et al. (160) as well as the neurodegeneration-associated microglial phenotype (MGnD) identified by Krasemann et al. (117) in an AD mouse model. It comprises a broader term to refer to a microglial phenotype that suppresses its homeostatic functions, specifically influenced by the TGF β pathway, and upregulates its phagocytic functions, mainly mediated by the TREM2/ApoE pathway. In addition, the term MGnD microglia has been validated in other neurodegenerative diseases such as MS, ALS and aging. This phenotype overlaps with the classical pro-inflammatory M1 phenotype previously induced by INF γ and LPS, but without the overexpression of TREM2/ApoE. On the contrary, it seems to be closer to the M2 phenotype, also called anti-inflammatory, where there would be an up-regulation of TREM2/ApoE and TGF β .

Thus, MGnD microglia, dystrophic neurites, amyloid plaques form a microenvironment highly enriched in TREM2/ApoE which could play a major role in AD while providing tolerogenic signals to T cells and not preventing neuronal loss (117).

There are also studies suggesting the pro-inflammatory effect of microglia, promoting tau formation and aggregation, similar to the results found for TREM2/ApoE, promoting tau formation in early stages (171). However, we need to consider the temporal-spatial effect in all the neurodegenerative diseases as well as in aging. These dystrophic microglia may be a cause of the disease and precede NFT formation and neurodegeneration in all the cases. DAM encompasses MGnD and all the other different context-dependent microglia states described in the different disease models. Therefore, DAM is the core signature. However, we should avoid microglial acronyms that suggest stable microglial states. Further investigation in the role of ApoD and its association with MGnD need to be done due to its similarity with ApoE as a lipid transporter and other functions. ApoD may have a specific role in neurodegenerative diseases that may help us to understand the pathophysiology of these devastating diseases and the long term being able to find better therapeutic targets.

ApoD influences neurodegenerative diseases

Neurodegenerative diseases represent a significant challenge in modern healthcare, with conditions such as Alzheimer's disease, Parkinson's disease, and multiple sclerosis posing considerable burdens on affected individuals and healthcare systems worldwide. Understanding the intricate molecular mechanisms underlying these diseases is crucial for the development of effective therapeutic interventions.

ApoD belongs to the small selection of genes whose manipulation is life-extending (4). Oxidative stress and inflammation are also generated at some point in time either directly or indirectly in neuroaging and most neurological disorders (4). ApoD is increased in stressed neurons, so it affects in a phase previous to neuronal damage. Stress is always an initial factor in each of the neurological disorders. In AD, but also in any of the MS theories. Then, ApoD can act in the early phases of these diseases as priming or immune training factor and as an endogenous controller of the redox state before the classical clinical patterns of the diseases are established (which means in a pre-symptomatic phase of the diseases) (141). ApoD, acts as an ancient and conserved anti-stress factor in the short term. But in the long run, due to microglia and ApoD coincident “upper limit”, their beneficial functions are no longer available in the later stages of the disease.

At this point, the concept of the “prion paradigm” for all the neurodegenerative diseases should be explained. It talks about two histopathological explanations of the primary cause of the disease and can be explained for all neurodegenerative diseases, not just for MS. All of these diseases develop from a seeded aggregation of endogenous proteins (48). The protein aggregation is different in each disease, but they lead to similar pathogenic cascades which always include early ROS release. These misfolded proteins which aggregate into small oligomers that end up in fibrils form extracellular or intracellular

complexes that are very difficult to eliminate. Neurodegeneration then implies an imbalance between generation of aggregates and protein clearance.

ApoD is expected to act in concert with other lipid-binding proteins in different physiological or pathological conditions; for example, with ApoE. We have already described the TREM2/ApoE effect in microglial clearing of A β oligomers, and thus contributing to the protection of neural cells in certain stages of the disease (141). However, in this context, there are studies showing that in the absence of ApoD there is no compensatory increase in ApoE (172). Despite these contradictory results, there may be some link between these two lipoproteins and therefore a possible therapeutic target in AD.

The implications of ApoD-microglia interactions in neurodegenerative diseases are multifaceted. Microglial cells are enormously powerful and capable of a duality, beneficial homeostatic roles in physiology and detrimental roles in disease (87). Further research should seek to elucidate the mechanisms, such as ApoD and other factors, to be able to turn microglia from a hostile neighbor to a friendly one (87). This work also opens the door to further research into epigenetic imprinting in microglia and the factors involved in it.

The emerging evidence implicating ApoD and microglia in the pathophysiology of neurodegenerative diseases highlights the complexity of these disorders and the importance of exploring novel molecular targets for therapeutic intervention. ApoD and other moonlighting proteins require further investigation to elucidate the mechanisms underlying microglial interactions and their impact on neuroinflammation and neuronal survival. This holds promise for the development of innovative strategies for the treatment of neurodegenerative diseases.

ApoD, seen as a potential therapeutic target and its implications for neurodegenerative diseases, may open new avenues for therapeutic interventions. Targeting ApoD expression or function in microglia may represent

a novel strategy to modulate neuroinflammatory processes and mitigate neuronal damage in conditions such as AD, PD, and MS. Pharmacological agents or gene therapy approaches aimed at enhancing or suppressing ApoD effects in microglia could be explored for their therapeutic potential.

In conclusion, elucidating the role of ApoD and microglia in neurodegenerative diseases and understanding their interactions at the molecular and cellular levels hold great promise for the development of innovative therapeutic strategies aimed at modulating neuroinflammation and preserving neuronal function in these devastating disorders. Continued research efforts in this field are essential for advancing our understanding of disease mechanisms and identifying novel therapeutic targets for neurodegenerative diseases.

Conclusions

1. iMicroglial cells, whether in immortalized cell lines or in mouse primary cultures, do not express ApoD, neither under basal conditions nor under stress conditions.
2. Microglial cells are able to endocytose ApoD from the extracellular compartment in a dose-dependent manner and in a stress-dependent manner.
3. ApoD is internalized by microglial cells in the late endosomal-lysosomal compartment, but its time course is different than in other glial cells.
4. ApoD does not exert a pro-survival effect in BV2 cells acutely challenged by oxidative stress or neurodegenerative producing conditions.
5. ApoD changes microglial cytokine secreted profile under situations involving ROS release such as PQ or products of neurodegeneration such as A β , in a sex-dependent manner.
6. The involvement of ApoD in antioxidant situations as well as its role inside the lysosomes to prevent its permeabilization might point more to a neuroprotective role of ApoD in microglia. Nevertheless, the precise role is still undetermined, and the 'upper limit' theory might help to elucidate this question.

Conclusiones

1. La falta de ApoD altera los mecanismos de control de la densidad de microglía en el hipocampo de ratones de una manera dependiente de la edad.
2. Las células microgliales, ya sea de líneas celulares inmortalizadas o de cultivos primarios de ratón, no expresan ApoD ni en condiciones basales ni bajo condiciones de estrés.
3. Las células microgliales son capaces de endocitar ApoD procedente del espacio extracelular de forma dependiente de la dosis y la exposición a estrés.
4. ApoD es internalizada por células microgliales en el compartimento endosomal-lisosomal con una vida media diferente a otras células gliales
5. ApoD no ejerce un efecto protector en células BV2 sometidas a estrés oxidativo o situaciones de neurodegeneración.
6. ApoD cambia el perfil de citoquinas secretadas por las células microgliales bajo situaciones de estrés o tras exposición a productos de neurodegeneración como A β de forma dependiente del género.
7. La función de ApoD dentro de los lisosomas impidiendo su permeabilización es menos importante en microglía ya que en dichas células ApoD no tiene un gran efecto pro-supervivencia. Sin embargo, ApoD tiene un papel regulador en una función importante de la microglía: su perfil de secreción de citoquinas en respuesta a estrés inducido por oxidación o por acumulación de productos amiloides. No obstante, el mecanismo de esta regulación por ApoD es todavía desconocido.
8. La revisión sistematizada revela una coincidencia entre ambos 'límites superiores' en las respuestas de microglía y la actividad oxidante de ApoD. Esta teoría puede ayudar a descubrir nuevas estrategias

terapéuticas para prolongar el periodo en el cual la microglía actúa como factor protector en los procesos de neurodegeneración.

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