Universidade do Minho
Escola de Medicina

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Immune profile characterization of an Alzheimer's disease mice model

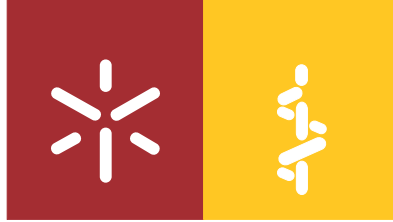**Caracterização do perfil imunológico de um murganho modelo da doença de Alzheimer**

junho de 2017

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Dissertação de Mestrado
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Trabalho efetuado sob a orientação da
Professora Doutora Fernanda Marques
e da
Professora Doutora Joana Palha

junho de 2017

À minha Família

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Immune profile characterization of an Alzheimer's disease mice model

Abstract

Alzheimer's disease (AD) is known as the most common neurodegenerative disorder, corresponding of 60 to 80% of dementia cases worldwide. Memory impairments are the principal complain of AD patients, with a huge impact in several dimensions of their daily life. The cognitive impairments developed are believed to be due to two hallmarks of the disease: the production and accumulation of amyloid-β (Aβ) in neuritic or amyloid plaques, and the accumulation of an abnormal hyperphosphorylated form of the microtubule associated tau protein in neurofibrillary plaques or tangles, outside and inside the neurons, respectively. Although the irrevocably importance of these proteins in AD appearance and progression, it is already known that AD is the result of several pathological factors, including neuroinflammatory responses. Despite the importance of the inflammatory process on AD development, as well as the prevalence of some leukocytes populations and peripheral organs on Aβ clearance, the impact of peripheral immune cells in AD is not well known. Therefore, in this work, we aimed at characterizing, at different ages, the peripheral immune profile of the J20 AD-mice model. For that, at the ages of 1, 3 and 10 months old, immune populations in the blood and in peripheral organs such as thymus and spleen, were analyzed by flow cytometry. We observed differences in thymocytes' differentiation and in blood leukocytes populations between J20 AD mice model and their littermate controls. Specifically, in the thymus of J20 animals, we observed an increase in the *single positive* for CD4 or CD8 (CD4$^+$CD3$^+$ and CD8$^+$CD3$^+$) cells, at both 1 and 3 months of age. Additionally, at the blood, J20 animals with 1 month presented an increase in the effector memory CD4$^+$ T cells. Of interest, at 3 months of age, an increase in cytotoxic CD8$^+$ T cells is observed and, at 10 months, the myeloid population of the monocytes was increased, in comparison to their controls.

In summary, this thesis shows that a variety of peripheral immune alterations accompany the development of AD in the J20 mice model, not just in the blood leukocytes populations, but also in the thymus, starting at early ages; which provides further evidence on the interplay between the peripheral immune and central nervous systems.

Caracterização do perfil imunológico de um murganho modelo da doença de Alzheimer

Resumo

A doença de Alzheimer (AD) é a doença neurodegenerativa mais comum, correspondendo de 60 a 80% dos casos de demência em todo o mundo. Problemas de memória são a principal queixa dos pacientes de AD, o qual tem um grande impacto em várias dimensões das suas vidas. Os problemas cognitivos desenvolvidos parecem ser causados por dois processos característicos da AD: a produção e acumulação de amilóide-β (Aβ) em placas neuríticas, assim como a acumulação da forma hiperfosforilada da proteína tau em placas neurofibrilares, dentro e fora dos neurónios, respetivamente. Ainda que seja irrevogável a importância destas proteínas na patologia da AD, esta é influenciada por outros fatores patológicos, como a neuroinflamação. Apesar da importância dos processos inflamatórios na AD, assim como das populações de leucócitos e órgãos periféricos na eliminação da Aβ, o impacto das células imunológicas periféricas na doença não é muito compreendido. Assim, neste trabalho, o objetivo foi a caracterização do perfil imunológico periférico do modelo da AD, o murganho J20. Para isso, nos animais com idades de 1, 3 e 10 meses, as populações imunológicas no sangue, no timo e baço foram analisados por citometria de fluxo. Os resultados mostram alterações na diferenciação dos timócitos e nas populações sanguíneas de leucócitos entre J20 e os seus controlos. Especificamente, no timo observamos um aumento das células unicamente positivas para CD4 ou CD8 ($CD4^+CD3^+$ e $CD8^+CD3^+$), a 1 e 3 meses de idade. Adicionalmente, no sangue observamos um aumento das células T $CD4^+$ efetoras de memória. Aos 3 meses, os J20 apresentam um aumento das células T citótoxicas $CD8^+$. Enquanto aos 10 meses, a população mielóide dos monócitos é encontrada aumentada, em comparação com os controlos.

Desta forma, esta tese de mestrado mostra que uma variedade de alterações imunológicas periféricas acompanham o desenvolvimento da AD no murganho modelo da doença J20, não só nas populações de leucócitos do sangue mas também no timo, desde idades muito precoces, evidenciando uma potencial interação entre o sistema imunológico periférico e o sistema nervoso central.

x

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ABBREVIATIONS

A

ACK	Ammonium-Chloride-Potassium
AD	Alzheimer's disease
apoE	Apolipoprotein E
APP	Amyloid precursor protein
Aβ	Amyloid-β

B

BBB	Blood brain barrier
BCSFB	Blood cerebrospinal fluid barrier
BHI	Brain heart infusion
bp	Pair bases
BSA	Bovine serum albumin

C

CM	Central memory
CNS	Central nervous system
Con-A	Concanavalin-A
CSF	Cerebrospinal fluid

D

DAMP	Damage-associated molecular pattern
DAPI	4,6-diamidino-2-phenylindole
dHPC	Dorsal hippocampus

E

ELISA	Enzyme-Linked immunosorbent assay
EM	Effector memory
EPM	Elevated plus maze

I

IFN	Interferon
IL	Interleukin

L

LD	Late differentiated

M

MCI	Mild cognitive impairment
MIP	Macrophage inflammatory proteins
MMSE	Mini mental state examination
MWM	Morris water maze

P

PAMP	Pathogen-associated molecular pattern
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
PSEN	Presenilin

T

TGF	Transforming growth factor
TMB	Tetramenthylbenzidine
TNF	Tumor necrosis factor α

W

WT	Wild type

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1. Introduction

1.1. Alzheimer's disease: insights

In the beginning of the XX century, a doctor from the Frankfurt Psychiatric Hospital, Alois Alzheimer studied the case of Auguste D - a patient admitted for untreatable paranoid symptomatology with fast progression and increasing intensity. The 5 years of behavioral analysis where correlated, after her death, with amyloid plaques and neurofibrillary tangles found in her brain. It was the first time that Alzheimer's disease (AD) was described (Hippius & Neundorfer, 2003).

NEARLY **44 MILLION** PEOPLE HAVE ALZHEIMER'S OR A RELATED DEMENTIA

1 IN 3 SENIORS DIES WITH ALZHEIMER'S DISEASE OR OTHER DEMENTIA

BUT ONLY **1-IN-4 PEOPLE WITH ALZHEIMER'S DISEASE** HAVE BEEN DIAGNOSED
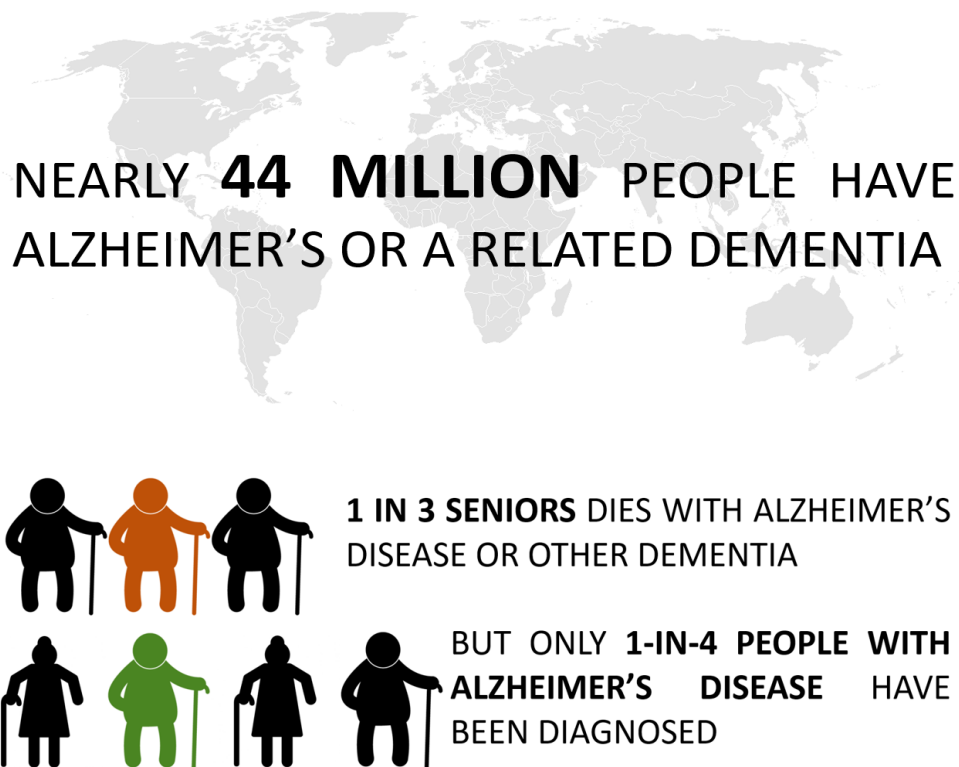
Figure 1. Facts on the prevalence of AD in a worldwide perspective. AD is one of the leading causes of death in the world, with nearly 44 million people suffering from it or a related dementia. Moreover, AD is estimated to kill one in every three elder. Despite that fact, only one in four people that have AD is correctly diagnosed for it. Although the current efforts, AD is one cause of death that cannot be prevented or cured.

Nowadays, AD is known as the most common neurodegenerative disorder of the elder, corresponding of 60 to 80% of dementia cases around the world (Figure 1) (Alzheimer's Association, 2015). Memory impairments are the principal complain of AD patients. However, several other problems are associated with the disease, namely confusion with the time and place, speaking and writing problems, inability of build new memories and personality

disturbances. The patients become totally dependent on others because of the continuous loss of mental and physical functions as the disease progresses and, at last, AD is fatal. This has a huge impact on the society, economy, familiar relations and several other personal and professional dimensions (Association, 2015).

There are 2 forms of AD: the early-onset or familial and the late-onset or sporadic. Most people with AD develop the late-onset form of the disease, in which symptoms become apparent in the mid-60s and later, and so age is the principal risk factor (Hebert et al., 2001; Hebert et al., 2013). Additionally, sex seems to play a role, being the disease more prevalent in women (Plassman et al., 2007). The causes of late-onset Alzheimer's are not completely understood, but most probably include a combination of genetics, environmental and lifestyle factors that can affect a person's risk for developing the disease. Diagnoses of mild cognitive impairment (MCI) or cases of brain traumatic injuries are also suggested to increase the probability of disease (Albert et al., 2011). Regarding the genetic risk factor it is recognized that the presence of apolipoprotein E (apoE) *e4* allele augment the possibility for AD development (Spinney, 2014); since *e3* is innocuous and *e2* even has a protective function (Holtzman et al., 2012). This is related with the efficiency of these individuals in terms of Aβ clearance, being *e2* more effective than *e3*, and this one more effective than *e4* (Holtzman et al., 2000; Kim et al., 2009).

The early-onset or familial AD is associated with mutations in the genes encoding for amyloid precursor protein (APP, the haloprotein from which Aβ is excised) or for presenilins (PSEN)-1 or 2 (the homologous proteins on the catalytic site of γ-secretase). APP and PSEN-1 mutations have a penetrance of 100%, while mutations on PSEN-2 give 95 percent chance of developing AD (Goldman et al., 2011). However, this cases account for only 1% or less of the AD diagnosis and happens in an early onset, with the AD's symptoms appearing before the 65 years old, sometimes as early as at the age of 30 (Bekris et al., 2010).

The precise influence of the various identified risk factors is still debatable. Of interest, there are also described potential protective factors (Sando et al., 2008); namely, social engagement and higher educational level. These are considered to build what is called *cognitive reserves,* that turn the individuals more capable to compensate the neurologic alterations related with AD or other dementias (Stern, 2012). Other habits, like the regular consume of coffee (Santos et al., 2010) and the practice of physical exercise (Reitz et al., 2011) and a healthy diet (Barberger-Gateau et al., 2007; Gu et al., 2010) also seem beneficial.

The cognitive impairments developed are believed to be due to two hallmarks of the disease. The production and accumulation of amyloid-β (Aβ), in neuritic or amyloid plaques, as well as, the accumulation of an abnormal hyperphosphorylated form of microtubule associated tau protein in neurofibrillary plaques or tangles, outside and inside the neurons, respectively. In addition to the irrevocable important role of these proteins in the disease appearance and progression, it is already known that AD is the result of several pathological factors. Processes such as neuroinflammation, metabolic alterations and deficits on the information transmission are now recognized to influence disease appearance and progression (Querfurth & LaFerla, 2010). Therefore, AD is a multifactorial disease that leads to cognitive and emotional impairment.

1.1.1. Aβ peptides and the amyloid hypothesis for the pathophysiology of Alzheimer's Disease

Aβ peptides are natural products of the metabolism originated from the proteolysis of the APP (Haass et al., 1992). However, there are two proteolytic processes by which APP can be catabolized and in only one of them Aβ peptides can be obtained (Figure 2). In the non-amyloidogenic pathway, APP is cleaved by α-secretase, within the Aβ peptide, and large amyloid precursor protein (sAPPα) is release, leaving the carboxyl terminal on the plasmatic membrane. γ-secretase (a protein complex with presenilin-1 at its catalytic core) is responsible for the cleavage of this membrane domain, concluding with the release of the peptide p3 on the extracellular milieu (Haass et al., 1993), while the APP intracellular domain stays inside the cell (Haass et al., 2012; Weidemann et al., 2002). However, a different sequential enzymatic action over APP can lead to the release of Aβ peptides in the extracellular medium. In the amyloidogenic pathway firstly, APP is processed by a β-secretase, specifically the beta-site amyloid precursor protein–cleaving enzyme 1, producing a shorter sAPPα. The C99 peptide - that stays at the membrane - is substrate for γ-secretase, which acts in *regulated intramembrane proteolysis* and generates Aβ peptide and APP intracellular domain (Lichtenthaler et al., 2011; Weidemann et al., 2002). Despite the apparent non functionality of this small peptide, APP intracellular domain is released in the cytoplasm where it will be further processed and directed to the nucleus where, as transcriptional factor, exert functions of activation of gene expression (Haass et al., 2012; von Rotz et al., 2004).

Unbalance between the production and elimination causes Aβ to accumulate and, this excess, can be an initiator factor to AD (Querfurth & LaFerla, 2010). This is called the "amyloid

hypothesis" and it states that the deposition of Aβ in the brain's parenchyma is the trigger event that initiates the sequence that leads to AD. This hypothesis is corroborated by the identification of autosomal dominant mutations on the genes that encodes for APP (J. A. Hardy & Higgins, 1992; J. Hardy & Selkoe, 2002), PSEN1 (Sherrington et al., 1995) and PSEN2 (Levy-Lahad et al., 1995; Rogaev et al., 1995); and/or by the association with the e4 allele of APOE (Nickerson et al., 2000).

After the release of the Aβ peptides to the extracellular milieu, they spontaneously aggregate between themselves, what results in intermediate soluble forms with several molecular weights. Aβ peptides also aggregate in fibrils, able to organize themselves in neuritic/amyloid plaques (Figure 2) (Chang et al., 2013; Kayed et al., 2003). Interestingly, while initially it was thought that amyloid in itself caused the disease, it is currently accepted that the Aβ oligomers are the toxic molecules to the cells, being the $A\beta_{42}$ peptide the most toxic form (Karran et al., 2011; Selkoe, 2001; Tanzi & Bertram, 2005). This is supported by the observation that the severity of the cognitive damages in AD is related with the level of oligomers in the brain but not with the extension of amyloid plaques (Lue et al., 1999). However, the more prevalent form has 40 aminoacidic residues - $A\beta_{40}$ (Karran et al., 2011).

As for the physiological roles of the Aβ peptides normally generated, they have been described to be participate in the release of vesicles containing neurotransmitters and their production is correlated directly with the increase of neuronal activation at the synapses. It is known that induced neuronal activity in brain slices of the hippocampus results in an increase production of Aβ due to the enhancing trafficking of APP towards β-secretase sites at the cell membrane (Kamenetz et al., 2003). However, physiologic levels of Aβ may dampen excitatory transmission and prevent neuronal hyperactivity. Without such negative feedback, synaptic activity may increase to the point of excitotoxicity (Kamenetz et al., 2003). Moreover, some reports indicate that specific stimulation of N-methyl-D-aspartate or NMDA receptors up-regulate APP, resulting in Aβ formation due to inhibition of α-secretase activity (Lesne et al., 2005). Altogether, the evidence points that APP processing and the Aβ presence provide a physiological regulation of the synaptic activity, protecting against the excessive glutamate release (Pearson & Peers, 2006). Naturally, these amyloid peptides are metabolized by specific enzymes: the PSEN (a zinc endopeptidase anchored to the membrane that degrades Aβ monomers and oligomers) (Kanemitsu et al., 2003) and the insulin-degrading enzyme (a thiol metalloendopeptidase degrading small peptides like insulin and monomeric Aβ) (Qiu et al., 1998). Of interest, the

reduction in the synthesis of these enzymes leads to cerebral accumulation of Aβ, while an overexpression of these enzymes can prevent plaque formation (Farris et al., 2003; Iwata et al., 2001; Leissring et al., 2003). As such, PSEN and insulin-degrading enzyme regulate the basal levels of Aβ peptides.

While there is substantial evidence supporting the role of Aβ in AD pathology, the involvement of Tau and the interplay of both proteins deserves consideration (Heppner et al., 2015), as will be next discussed.
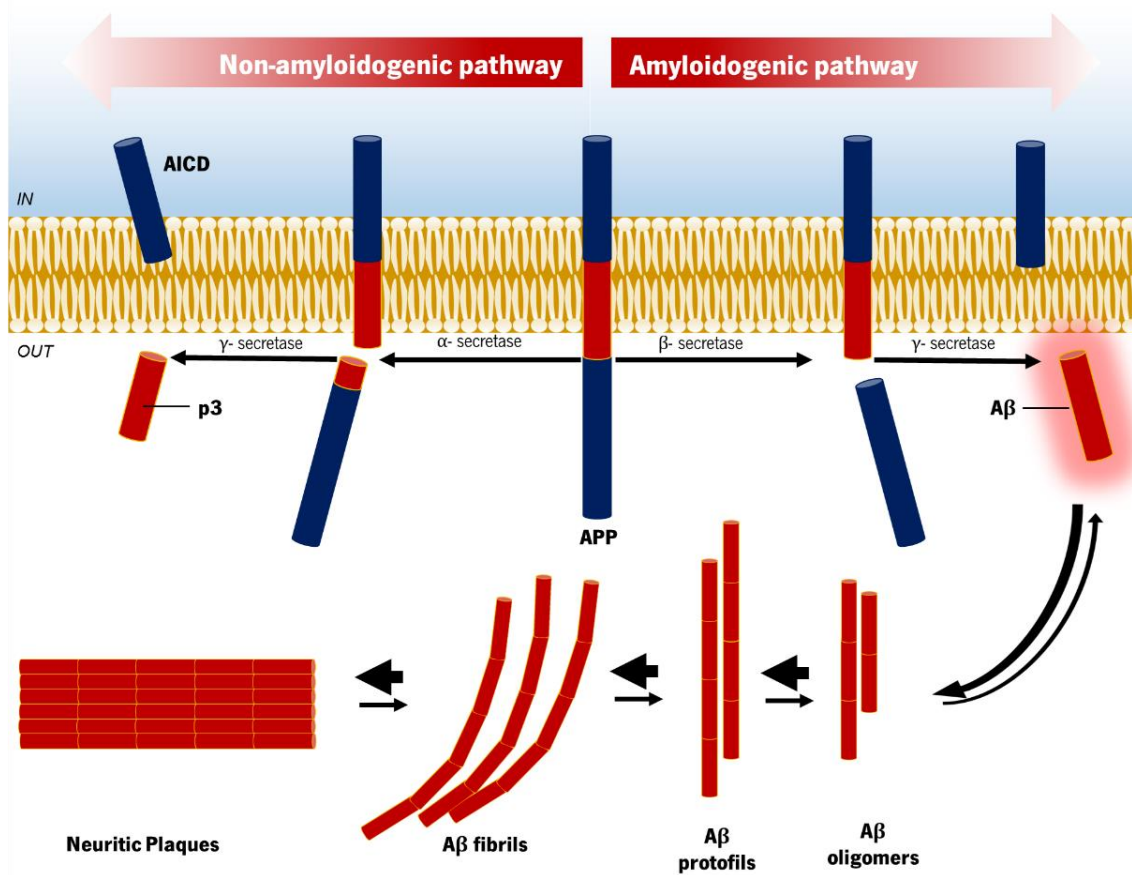
Figure 2. Amyloid pathological events in AD. Aβ peptides are originated from natural metabolism of the amyloid precursor protein (APP) (Haass et al., 1992). There are two possible pathways for APP processing, different from each other either on the enzymatic sequence that follow and the final products that are produced. One is known as non-amyloidogenic pathway, where APP is cleaved by α-secretase within the Aβ peptide, releasing sAPPα (a large APP) and leaving on the plasmatic membrane the carboxyl terminal. γ-secretase cleaves this domain, releasing the APP intracellular domain (AICD) in the intracellular milieu. The other pathway is known as, the amyloidogenic parcel of the APP catabolism and a different sequential enzymatic action over APP could lead to the extracellular release of Aβ peptides. Firstly, the APP is enzymatically cleaved by the β-secretase producing a shorter sAPPα. γ-secretase uses the C99 peptide at the membrane as substrate generating Aβ and AICD (Lichtenthaler et al., 2011; Weidemann et al., 2002). AICD is directed to the nucleus where it plays role of a transcriptional factors. Aβ peptides on the extracellular milieu, when in excess can self-aggregate, resulting in several existent forms: monomers, oligomers, protofibrils, fibrils and insoluble neuritic or amyloid plaques.

1.1.2. Tau Protein in Alzheimer's Disease

Neurofibrillary aggregations of hyperphosphorylated Tau protein are observed in AD and in other neurodegenerative disorders called *tauopathies* (Lee et al., 2001).

The Tau soluble form is normally located in high concentrations in the axons of the neurons, given its function as a microtubule-associated protein that promotes the stability and assembling of the microtubules and transport vesicles. The interaction between Tau and microtubules is a dynamic process that plays an important role in the structural remodeling of the cytoskeleton during neuronal plasticity, with functions in neurite elongation, synaptic and spine formation and memory formation (Rosenmann et al., 2012; Wang & Mandelkow, 2016).

Three main different mechanisms are responsible for the regulation of the binding between Tau and the microtubules. Firstly, the alternative splicing of the pre-mRNA that gives rise to 6 Tau isoforms. These isoforms are different due to the presence of 3 (3R) or 4 (4R) binding domains to the microtubules in the C-terminal, that associated with the presence of none, one or two amino terminal inserts (Buee et al., 2000; D'Souza & Schellenberg, 2005), give rise to the 6 possible isoforms. The 3R isoform, due to the less number of binding domains, has a lower affinity for microtubules binding turning the cytoskeletal more permissive, favoring plasticity and cellular transport (Goedert & Jakes, 1990; Goode et al., 2000). The 3R is the only isoform present in the fetal brain, while in the adulthood both 3R and 4R are present in approximately equal abundance (Goedert & Jakes, 1990; Kosik et al., 1989). However, alterations of the ratio between 3R and 4R is suggested for the appearance of tauopathies (Connell et al., 2005), even though, no differences on this ratio has been observed between AD and controls (Boutajangout et al., 2004). Tau is normally degraded through the ubiquitin-mediated pathway in the proteasome (Wang & Mandelkow, 2012). Despite the importance of ubiquitin in Tau's elimination, and the fact that it is found in the inclusions within the cells, an apparent impairment on its function is described in neurodegenerative pathologies, such as AD, that may be related with the accumulation of defective protein (Almeida et al., 2006; Johnston et al., 1998; Upadhya & Hegde, 2007).

Post-translational modifications of Tau can occur at various sites and by several molecules. One of the possible alterations is hyperphosphorylation/desphosphorylation phenomena that alter the affinity of these peptides to microtubules (Figure 3). Hyperphosphorylated tau is not soluble and has no affinity to the microtubules, self-aggregating in helical insoluble filaments. In this way and due to the fact that are described 79 phosphorylation site on these peptides, several kinases (such as, GSK-3B, cdk5, MAPK/ERK, CAMK II, JNK, c-jun and AKT/PKB) (Baumann et al., 1993;

Drewes et al., 1992; Goedert et al., 1997; Hanger et al., 1992; Liu et al., 1995) and phosphatases (PP2A, PP2B and PP1) (Garver et al., 1994; Matsuo et al., 1994) have an important role on the regulation of Tau functions within the cells, in a site-specific and context-dependent manner (Martin et al., 2013). However, these enzymes alter the functionality of these proteins without altering the total Tau protein population.
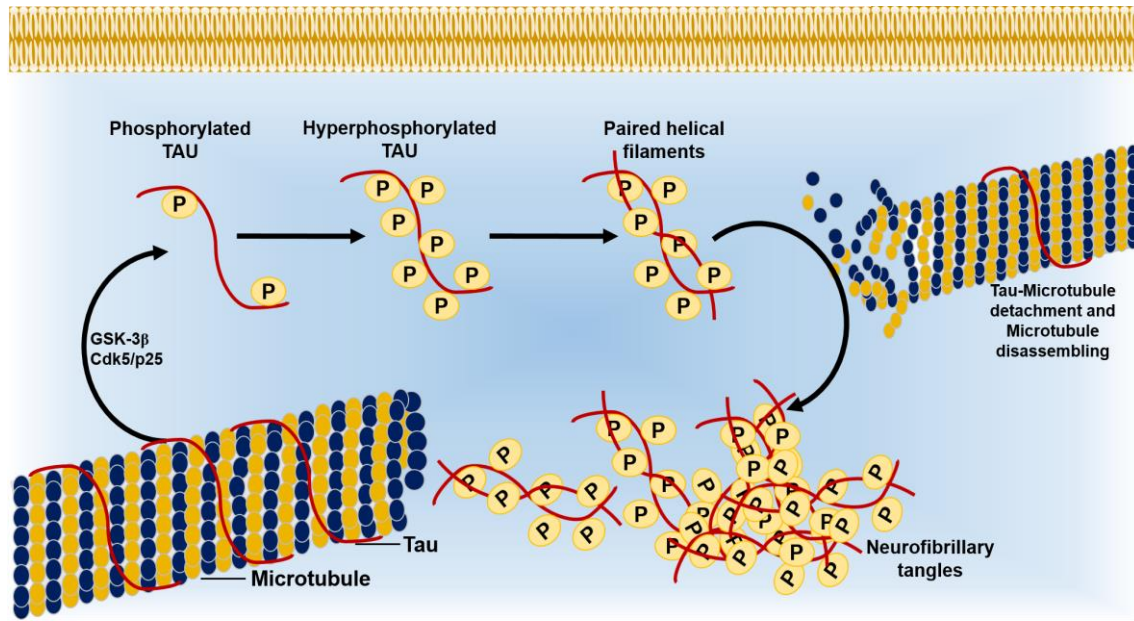
Figure 3. Tau hyperphosphorylation and aggregation in AD. Tau is a microtubule associated protein playing an important role in the maintenance of cytoskeleton of the cell by the stability of the microtubules assembling. However, in AD Tau is hyperphosphorylated by several kinases, losing its affinity for microtubules and gaining affinity for self-aggregation in neurofibrillary plaques or intracellular neurofibrillary tangles. As it happens with Aβ, the soluble intermediates are suggested to be cytotoxic and cause cognitive damages. On this perspective, the aggregates (responsible for the sequestration of toxic species of tau) could have a primary neuroprotective function.

In AD, Tau is hyperphosphorylated and aggregates as intracellular neurofibrillary tangles (Figure 3). The Tau intermediate aggregates are cytotoxic to the cell and have been associated with cognitive damages. Interestingly, neurofibrillary tangles are able to sequester toxic species of tau, having, on this matter, a neuroprotective effect. In AD, there is no direct connection between the extension of neuronal loss and the number of Tau tangles (Gomez-Isla et al., 1997). However, the increase concentration of hyperphosphorylated Tau in the cerebrospinal fluid (CSF) can be used as a biological marker of AD (Wallin et al., 2006).

Regardless of the synergetic crosstalk between the AD pathological features, namely Aβ and Tau, the truth is that the dysfunction of Tau is independent of the Aβ presence, and sufficient to cause neuronal dysfunction leading to dementia and neuronal death. As an example: Tau mutations can cause autosomal dominant Frontotemporal dementia in an AD-like manner but without the Aβ

plaques (Hutton et al., 1998). Interestingly, studies in APP transgenic mice (a model of AD) showed that some behavioral and other deficits mediated by Aβ were ameliorated by reduction of endogenous levels of tau (Roberson et al., 2011; Roberson et al., 2007).

1.1.3.   Neuroinflammation in Alzheimer's disease

Neuroinflammation is usually present whenever an insult is imposed to neural cells and is a common feature of different neurodegenerative disorders (Chen et al., 2016). In the last two decades, the role of the inflammatory response in aging and in AD has been largely scrutinized. In AD, brain inflammation occurs in response to the presence of damaged neurons and highly insoluble neuritic and neurofibrillary aggregates (Akiyama et al., 2000). As for the precise involvement of neuroinflammation in AD and in other aging-related neurodegenerative disorders, it is still not clear whether inflammation is a consequence or a cause of neurodegeneration.

There are two main types of resident cells with immune functions in the central nervous system (CNS): microglia and astrocytes. Microglia are known as the resident macrophages of the CNS with ubiquitous distribution within the brain. Microglia recognizes not only pathogen- associated molecular pattern (PAMPs) and danger-associated molecular patterns (DAMPs) but also, the presence of cellular debris; secreting factors that support the maintenance of the tissues. This type of cells has highly motile processes, allowing a survey of the brain region where present. Their ability to easily adapt has an important role also in the plasticity of neuronal circuits, protection and remodeling of synapses. The motile processes are extended when some injury is detected within the CNS, migrating to the site, and initiating an immune response (Wake & Fields, 2011). Particularly in AD, microglia cells bind to Aβ oligomers and fibrils via cell-surface recognition proteins, beginning an inflammatory response (Solito & Sastre, 2012).

Astrocytes also play a role in the neuroinflammation process. Reactive astrogliosis leads to remodeling of the astrocytes, normally aiming neuroprotection or/and recovering of damage tissue. With microglia, these cells accumulate around amyloid plaques. And despite de relation between these peptides and the astrocytic activation, it seems that even before Aβ deposition, an increase in the expression of glial fibrillary acidic protein (GFAP) and functional impairment is described. An early response in AD mice model is the presence of astrocytic atrophy, impacting negatively on synaptic connectivity and thereby contributing to the cognitive deficits (Markiewicz & Lukomska, 2006; Verkhratsky et al., 2010).

Initially, both cell types are responsible for the promotion of Aβ phagocytosis and degradation. However, when chronic activated, microglia and astrocytes, lead to the release of chemokines and a cascade of damaging cytokines, harmful for the neurons (Russo & McGavern, 2016). This is associated with glutamate and nitric oxide production, resulting in a consequently exacerbation of the inflammatory responses (Querfurth & LaFerla, 2010) (Figure 4). Presumably, cytokines and chemokines are under similar intercellular and intracellular signaling pathways in microglia and astrocytes as they do in the periphery. In AD, it is described that molecules like interleukin-1β (IL-1β) (Griffin et al., 1995; Griffin et al., 1998; Prehn et al., 1996), IL-8 (Xia & Hyman, 1999), IL-6 (Papassotiropoulos et al., 1999; Vallieres & Rivest, 1997), transforming growth factor-β (TGF-β) (Chao, Ala, et al., 1994; Chao, Hu, et al., 1994; van der Wal et al., 1993), macrophage inflammatory protein-1α (MIP-1α) (Meda et al., 1999), and tumor necrosis factor-α (TNF-α) (Tarkowski et al., 1999), are upregulated when compared to healthy controls. The secretion of inflammatory molecules such as cytokines and chemokines can also promote extravasation of monocytes from peripheral blood into plaque-bearing brain (Simard et al., 2006).
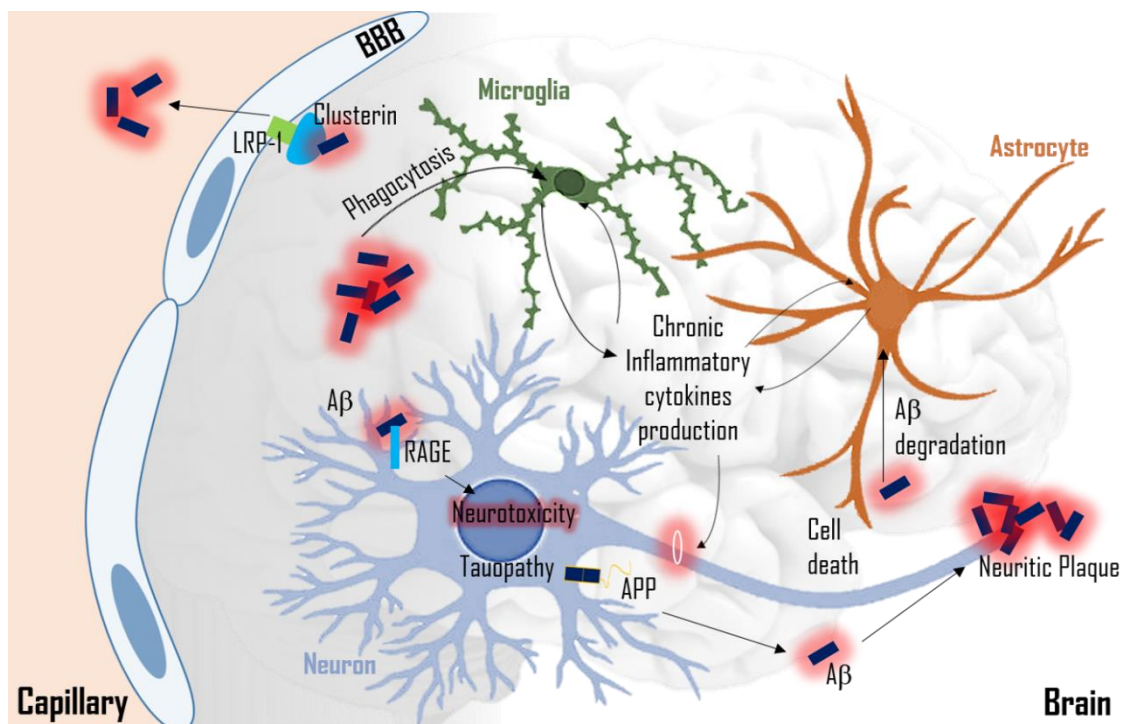
Figure 4. The role of neuroinflammatory processes in AD. The cells with immune functions within the brain: microglia and astrocytes, are activated AD, and can play an important role on the progression of the neurodegeneration. In the beginning, these cells phagocyte Aβ, eliminating it. However, with the progression of the disease, these cells stay in an activated state leading to a chronic neuroinflammatory profile accompanied by the release of chemokines and a cascade of damaging cytokines, deleterious for the neurons. Also, other molecules like glutamate and nitric oxide, are produced, exacerbating the neuroinflammatory response already in course. Altogether the activation of all these processes compromise treatments; increases neurodegeneration and accelerates progression of the disease. LRP-1 and RAGE are just examples of proteins that interact with Aβ peptides.

The activation of glial cells associated with the presence of fibrillary Aβ can also stimulate the classic complement pathway. This is corroborated by the presence of C1q and C5b-9, complement cleavage products in neuritic and neurofibrillary plaques, suggesting the occurrence of opsonization and autolytic attack (McGeer et al., 2001). Acute-phase reactants are also released by stimulated astroglia which can both ameliorate or aggravate AD; this is the case of molecules like C-reactive protein and α1- and α2-antichymotrypsin. These inflammatory events were suggested to be helping the disruption of the brain barriers namely the blood brain barrier (BBB) and the blood CSF barrier (BCSFB) in AD. However, in humans, there is no evidence that this could lead to monocyte or amyloid peptides influx from the circulation (Clifford et al., 2007; Matsumoto et al., 2007) (Kempuraj et al., 2016).

These paradoxical actions of microglia, eliminating Aβ but secreting proinflammatory molecules, compromise treatments (Fiala et al., 2005). Due to these findings, in the last years, the importance of neuroinflammation has been intensely studied in several neurodegenerative disorders. The available evidence suggests that these processes within the brain are crucial in the onset and progression of disease.

1.2. Peripheral immune alterations in Alzheimer's disease

Inflammation is a protective mechanism that is responsible for the restitution and regeneration by the removal of damage cells/tissues or parasites, toxins or infective agents from the body (Kulkarni et al., 2016). However, a chronic inflammation could be damaging and deleterious to the organism by inhibiting processes like cell regeneration (Russo & McGavern, 2016). Immune cells are responsible for inflammatory responses and includes T and B-cells, neutrophils, macrophages, microglia and mast cells (Shabab et al., 2016). Even though neuroinflammation is influenced by all of these peripheral cells – and by their secreted factors - the underlying mechanisms and how they are impacting on the development of neurodegenerative disorders is not well explored or understood. In fact, until very recently, the relevance of peripheral immune cell/mechanisms in the CNS structures in health and diseases was highly contested due to the previous dogma that the brain was "an immunologic privileged site" (Akiyama et al., 2000). However, now it is already known that peripheral inflammation episodes can influence inflammatory pathways in the brain, thought the brain barriers both at the BCSFB at the choroid plexus level (Marques et al., 2009) and at the BBB (Huber et al., 2001; Takeda et al., 2014) influencing the state of neuroinflammation within the brain (Kempuraj et al., 2016).

1.2.1.   Innate immunity

The role of peripheral innate immune cells in neurodegenerative diseases, namely in AD, is very obvious when considering monocytes/macrophages, due to their primary phagocytic function that may limit Aβ load (Rezai-Zadeh et al., 2009). Indeed, several observations suggest that the pathogenic mechanism of AD results from deficits in Aβ peptides phagocytosis by microglia and monocytes/macrophages (Town, 2009; Zhang et al., 2006). Of relevance, it was already described a decreased in the phagocytosis of monocytes/macrophages isolated from AD patients (Avagyan et al., 2009). When populations of monocytes/macrophages were characterized in AD patients, an higher percentage of CD14$^+$CD69$^+$ monocytes was present when compared to age-matched controls (Kusdra et al., 2000). Of interest, *in vitro* neural cells experienced increased cell death when stimulated with media from AD patients' monocytes/macrophages (with higher expression of CD14$^+$CD69$^+$) cultures (Figure 5). Interestingly, and despite the fact that this elevation may indicate a general activation profile of the innate immune system, this immune phenotype is not specific of AD (Kusdra et al., 2000).

Other cells of the innate immune system, namely granulocytes or polymorphonuclear cells, are normally underestimated in the context of AD (Figure 5). Although some studies suggest that Aβ can lead to the activation of these cells, there is no conclusive indication of a relation between granulocytes and AD neuropathology. In terms of percentages of these cells in the bloodstream, there are studies that observed an increase in the percentage of CD14$^+$ granulocytes in patients with mild cognitive impairment. While others claim a decrease of basophils in AD patients (Song et al., 1999). Furthermore, alterations in the metabolic activity of circulating neutrophils were suggested, with increment in AD patients of both oxidative stress and induction of CD11b expression (Licastro et al., 1994; Scali et al., 2002).

Finally, natural killers (NK) are cells usually taken as a bridge between innate and adaptive immune systems (Sun & Lanier, 2009) and normally are involved in the eradication of both virally infected and tumorigenic cells, due to their role as cytotoxic lymphocytes. Despite the lack of evidence that NK populations differ between AD patients and healthy age-matched controls, alterations in the responsiveness of NK cells to stimuli – either positive or negative ones - have been reported in AD patients (Araga et al., 1991). Of interest, it was described a reduction in the activity of NK cells isolated from AD patients when induced by IL-2 or IFN-α, when compared with normal control cells. In contrast, there are also evidence of increase cytotoxicity of NK cells (Solerte et al., 1996) and sensitivity to physiological modifiers in AD (Masera et al., 2002).

Moreover, alterations on the response of NK cell can be related with AD progression (Prolo et al., 2007).

1.2.2.   Adaptive immunity

While the implication of innate immune system in the pathogenesis of AD is established, the role of adaptive immune system in AD remains poorly understood. Some clinical trials are ongoing with the basis that T and B cells play important roles in the development of the disease, relaying on the evidence that these cells play a pivotal role in the control of AD. This was described in the AD mice model RAG-5Fad, which is an immune-deficient AD mouse model that lacks T, B, and NK cells, where the ability to modulate cytokine production and Aβ phagocytosis by microglia was attributed to the adaptive immune cells (Marsh et al., 2016).

With respect to the adaptive immunity-related cell types, a switch from naive to memory T lymphocytes, visible by the expression levels of CD45 on the membrane, the CD45RA (typical of naïve T cells) and the CD45RO$^+$ (typical of memory T cells), with a decrease in the CD45RA levels and an increase of the ratio CD45RO/CD45RA was observed in AD patients when compared to elderly healthy controls. In addition to this switch, there are some studies suggesting that peripheral T-cell subsets may be altered in AD patients and they were also found in the brain's parenchyma (Itagaki et al., 1988; Rogers et al., 1988). In elder AD patients, it was showed an increase in the CD4$^+$ cells but not in the CD8$^+$ when compared to healthy age-matched controls (Larbi et al., 2009; Town et al., 2005), accompanied with an increase also in CD45$^+$ T-regulatory cells (Town et al., 2005). When cultures of human CD4$^+$CD45RO$^+$ T-cells were analyzed, it was observed that the expression of a negative costimulatory factor was augmented, suggesting that these memory T cells may be anergic or somehow experiencing tolerance, perhaps due to persisting antigenic challenge (Jago et al., 2004). Of interest, the lymphocytes from the majority of AD patients seemed not reactive to the amyloid peptides, suggesting the possibility of the T cells hyporesponsiveness (Monsonego et al., 2001). This conclusion is in accordance with some studies that implied T-cells anergy to Aβ (Ferretti et al., 2016), claiming a reduction of INF-γ and IL-2 in transgenic models of AD; while there were no differences in the response to other nonrelated proteins - such as, growth hormone releasing factor - confirming Aβ-specific hyporesponsiveness (Monsonego et al., 2001). In mice Aβ-immunized, a peptide consisting of an Aβ B-cell epitope coupled to bovine serum albumin (BSA) can revert the diminished T-cell proliferation. By the aforementioned, it seems that, also in mice, the life-long exposure to Aβ

results not in a B-cell tolerance, but in T-cell hyporesponsiveness (Town et al., 2005). In contrast, depletion of B cells and consequently the decreased activation of T cells leads to a loss of adaptive-innate immunity cross talk and accelerate the disease progression (Marsh et al., 2016; Town et al., 2005) (Figure 5).

However, these alterations in adaptive immune cells populations seem to be time dependent, because studies in younger subsets of AD patients showed a selective decrease of CD8+ lymphocytes (Pirttila et al., 1992; Shalit et al., 1995). Other reports conflict with the aforementioned, falling to demonstrate differences between CD8+ and CD4+ in the peripheral blood of mild-to-moderate AD patients when compared to healthy subjects. However, this last study found a positive correlation between the CD4+ T-cell percentages and patients' cognitive score (Bonotis et al., 2008).
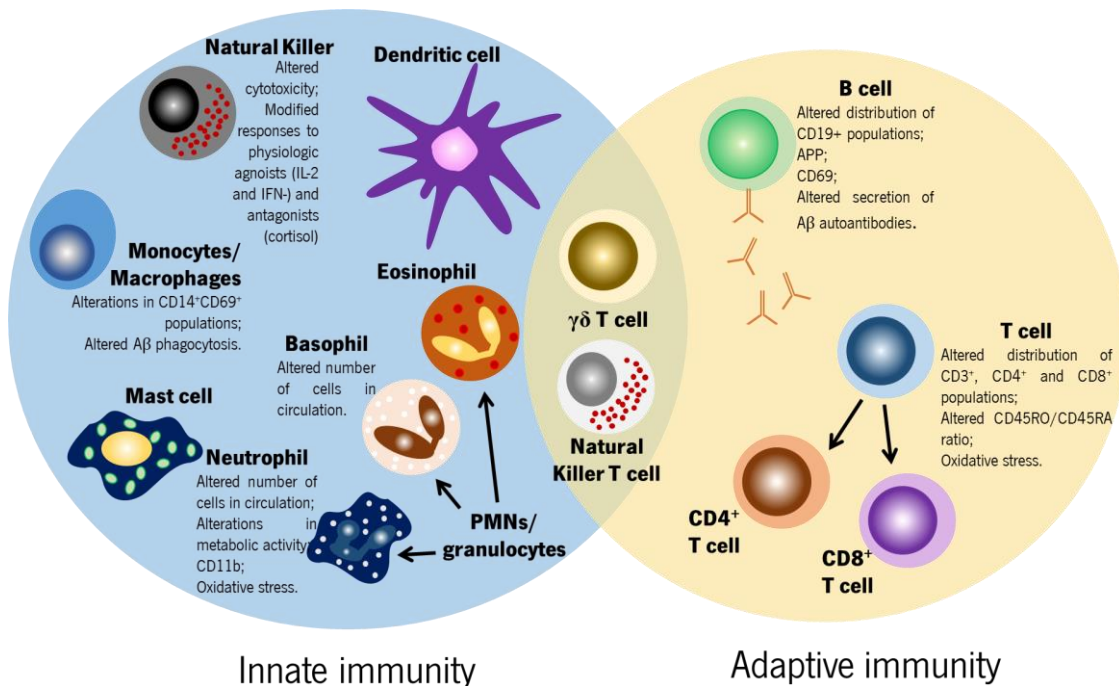
Figure 5. Leukocytic alterations described in AD. Despite the irrevocably role of the known hallmarks of AD, such as, protein abnormalities and neurodegeneration neuroinflammatory processes are also relevant. For the neuroinflammatory responses also the peripheral immune cells seem to participate in the development and progression of the disease. These cells can, for instance, increase microglia and neuronal activation and BBB permeability; or even be recruited and infiltrate the brain's parenchyma through a more permeable BBB.

Despite these alterations in the T-cells subsets distribution, it is relevant to analyze not just the quantity of these cells but also their activation state and to analyze if these cells are able to recognize, produce and secrete immunomodulators. One example of that is the observation that T-cells of AD patients have a reduced ectopic expression of interferon-γ (IFN-γ) receptors,

meaning a reduced binding of T-cells to these cytokines (Bongioanni et al., 1997). In fact, both cells and secreted factors can modulate brain's function. Secreted cytokines, such as INF-γ, released when T-cells are activated, and other proinflammatory cytokines may enter the CNS, activating microglia and astrocytes (Town et al., 2005). T-cells can also lead to the activation of myeloid cells, like monocytes/macrophages and dendritic cells, culminating with the production and secretion of proinflammatory cytokines like TNF-α, IL-1β and IL-6. Of relevance, some of these cytokines were suggested to be overexpressed and secreted by monocytes of AD patients (Huberman et al., 1995; Shalit et al., 1994). Overall, these cells promote neuroinflammation indirectly by the production of immune molecules and/or by crossing the defective BBB, directly exacerbating the inflammatory responses to the amyloid plaques (Town et al., 2005).

The involvement of B cells is also controversial. Some studies suggest a general lymphopenia accompanying AD-like pathologies and that immunesenescence may be an indicator of the disease, with a diminishing of both T cells (CD3+) and B cells (CD19+) in AD (Richartz-Salzburger et al., 2007). However, other reports did not find differences on B lymphocytes (Dysken et al., 1992).

Interestingly, APP processing disturbances are also seen in the periphery. Specifically in immune cells, an increase in surface expression of APP is suggested to occur primarily on B cells (Bullido et al., 1996; Jung et al., 1999; Magaki et al., 2008; Pallister et al., 1997). And these peripheral disturbances on APP metabolism in lymphocytes might be associated with dysregulations of APP processing within the brain (Jung et al., 1999; Pallister et al., 1997). In AD patients also a reduced expression of activation cell surface antigens in B cells, namely CD69, is described (Stieler et al., 2001). Connecting all these findings, it seems that B cells are important cells in AD.

Altogether, from the available evidence the precise participation of the peripheral immune system in AD is a relevant area of research for which this thesis intends to contribute.

1.3. Objectives

This thesis intends to contribute to the characterization of leukocytic populations, and the involvement of peripheral organs, such as the thymus and spleen, in the J20 mouse model of AD. Specifically, we will:

I. **Characterize the blood leukocytes populations in a longitudinal way in the J20 model, at the ages of 1, 3 and 10 months.** Alterations in the leukocytes populations in early time points of the disease might suggest possible biomarkers for specific stages of AD development. Also, a longitudinal study will give us the understanding of how the flexibility of the immune cells populations may be altered in the aging processing of J20 mice.

II. **Describe immune cells populations at the thymus and spleen of J20 mice.** Cell characterization by flow cytometry, accompanied by determination of spleenocytes IFN-γ production upon stimulation with different Aβ concentrations. Alterations of these organs content might indicate that the immune system can have a crucial role on the disease progression and that other organs besides the brain can suffer the impact of AD.

III. **Study the presence of leukocytic populations in the brain of J20 mice,** since the presence of these cells in the brain parenchyma can have an impact in the exacerbation of mechanisms that could increase the rate progression of AD.

IV. **Test the efficacy of the J20 mice immune cells to fight infection by *Listeria monocytogenes*,** to understand if possible alterations in the leukocytic populations found in aim I and II can have an impact in the efficiency of these animals to cope with an infection.

2. Materials and methods

2. MATERIALS AND METHODS

2.1. Ethics statement

Animal handling and experimental procedures were conducted in accordance with *Direção Geral de Veterinária*, the Portuguese national authority for animal procedures (ID: DGV9457). Animals were kept in accordance with the guidelines for the care and handling of laboratory animals in the Directive 2010/63/EU of the European Parliament and the Council. The housed animals were maintained in a controlled environment at 22-24°C and 55% humidity on 12 hours light/dark cycles and fed with regular rodent's chow and tap water *ad libitum*. Animals were housed 5 per box with regular paper as environmental enrichment.

2.2. Animals

In this work, we used hemizygous males from an AD transgenic mouse model, specifically Cg-Tg(PDGFB-APPSwInd)20Lms/2J/Mmjax (J20) mice, in a C57BL/6J background. This model was firstly developed by Professor Lennart Mucke of the J. David Gladstone Institutes (San Francisco, California, USA), and we purchased them from The Jackson Laboratory (Bar Harbor, Maine, USA).

The J20 mice express a mutant form of the human APP with two mutations (APPSwInd), the Swedish (J670N/M671L) and the Indiana (V717F) ones; therefore, being a model for the familial form of AD and not the sporadic one. The human platelet-derived growth factor beta polypeptide promoter directs the expression of the transgenic insert on the mice specifically for the neurons (Mucke et al., 2000). Hemizygous mice have the highest expression of the transgene product in neurons of the hippocampus and the neocortex. Diffuse Aβ peptides deposition is detected in these regions by the age of 5-7 months. At 8-10 months, amyloid deposition is progressive and all transgenic mice exhibit plaques (Mucke et al., 2000). Nonetheless, this model does not present tauopathy associated with the pathology, and as such does not entirely mimic the human disease. However, it presents increased neuroinflammation associated to the amyloid pathology, with increase number of reactive GFAP+ astrocytes and CD68+ microglia in the hippocampi of J20 mice when compared to wild type (WT) controls (Wright et al., 2013). Accompanying these alterations, it is also observed synaptic loss, with decrease of synaptophysin, synaptotagmin, PSD-95 levels, confirmed by electron microscopy at 3 months (Hong et al., 2016); and basal

transmission, long-term potentiation and long-term depression impairments in the hippocampus at 3-6 months, associated with smaller amplitude of extracellular recorded field EPSPs at the Schaffer collateral to CA1 synapse (Saganich et al., 2006). These physiological alterations are related with cognitive impairments in this AD model, corroborated by results on behavioral analysis with poor performances on Morris water maze (MWM) when compared with WT controls, indicating deficits in learning and memory abilities as soon as 3 months old (Mesquita et al., 2015; Palop et al., 2003; Wright et al., 2013). All these characteristics described are summed up on the Figure 6.
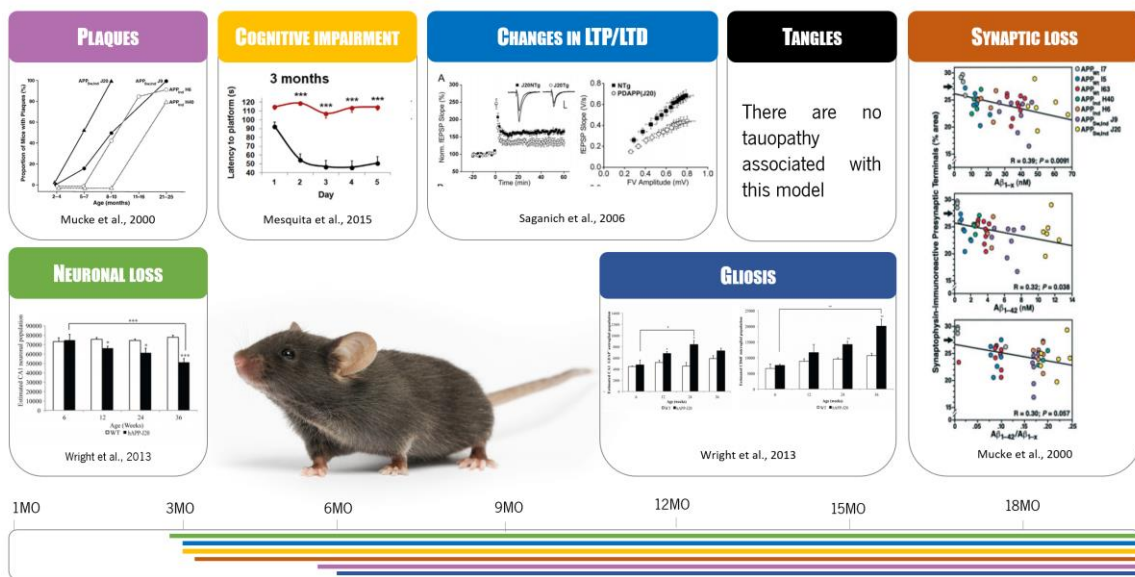
Figure 6. Schematic review of what is described about J20 AD mice model. In hemizygous mice, the transgene is highly expressed in neurons of the hippocampus and neocortex. At the age of 5-7 months old only diffuse Aβ peptides are detected in these structures and at 8-10 months, amyloid deposition is progressive and all transgenic mice exhibiting plaques. However, tauopathy is not part of the pathophysiology of the disease. Nonetheless, it is described an increased number of reactive GFAP+ astrocytes and CD68+ microglia in the hippocampi of J20 mice when compared to WT controls, suggesting augmented neuroinflammation. Simultaneously, it is also observed synaptic loss and electrophysiological deficits in the hippocampus at 3-6 months old. These alterations are implicated with functional alterations, with cognitive impairments in J20 mice, assessed by Morris water maze (MWM), where these animals present a poor performance when compared to age-matched controls, indicating deficits in learning and memory abilities as soon as 3 months (Mesquita et al., 2015; Mucke et al., 2000; Saganich et al., 2006; Wright et al., 2013).

On the experiments described on this report, both hemizygous transgenic J20 mice and age-matched non-carrier WT littermates, at 1, 3 and 10 months old, were used. To obtain the mice used for these experiments, breeding pairs were made by joining hemizygous J20 male mice with WT female mice. The progeny is exclusively hemizygous transgenic J20 mice or WT littermate. These two genotypes were used on the experiments as group of interest and age-

match controls, respectively. Newborn animals were maintained in the parental cage until postnatal day 21. At this point, a gender-dependent screening was made and the animals were separated to new cages, in groups of no more than 5 animals per cage.

2.3. Genotyping

Animals were genotyped by polymerase chain reaction (PCR) of biological sample of the ear in a distinct way between animals of the same box, allowing the identification of the animals.

The genomic DNA was extracted using NaOH method. In this method, we emerge the biological sample in NaOH 50Mm and let the mixture at 98°C for 50 minutes. After being homogenized, it was neutralized with 1/10 of the volume of NaOH of 1M Tris (pH of 8). The remaining tissue was discarded and the solution containing the genomic DNA was transferred for a new eppendorf and was frozen and kept at -20°C until further use.

To identify the animals in relation to their specific genotype, J20 or WT, we take advantage of PCR technique to amplify small sequences of the genome that are different between the two groups. The J20 animals are transgenic, meaning that they have an insertion on their genome, in this case a mutated version of the human APP gene of 360 pair bases (bp), and this insertion allowed the identification of this group. For the WT littermates' identification, we use an internal control of 500bp. To allow the PCR reaction to occur in the specific program conditions, the samples were mixed in a solution with H2O, Taq buffer, MgCl2 (25mM), dNTPs (10mM), Taq polymerase (5U/L) (from Thermo Fisher Scientific, Waltham, Massachusetts, USA) and the primers specified at the Table 1. After the DNA amplification, an electrophoresis in a 2% agarose gel was proceed to sort out the two different DNA sequences in a size-dependent manner, stained with GreenSafe Premium (NZYTech, Lisbon, Portugal).

Table 1. *Primers* use in the PCR for mice genotyping.		
	Designation	**Sequence 5'-3'**
P1	Transgene Forward	GGT GAG TTT GTA AGT GAT GCC
P2	Transgene Reverse	TCT TCT TCT ACC TCA GC
P3	Internal Positive Control - Forward	GTC CTT CTC ACT TTG ACA GAA GTC AGG
P4	Internal Positive Control - Reverse	CAC ATC TCA TGC TGC TCA GAT AGC CAC

2.4. Behavioural assessment

The analysis of the behavioral performance of J20 mice is a routinely test done to verify whether the animals present the expected cognitive deficits at the time of study. The cognitive impairments are usually assessed by the MWM test. This paradigm gives us information on the spatial learning memory deficits and on memory flexibility (Morris, 1984). However, less works study the emotional processing alterations that are also part of the human pathology, namely anxious behavior. This dimension is translated to the mice like an anxious-like phenotype and can be analyzed by Elevated Plus Maze (EPM) (Walf & Frye, 2007).

In the Jackson lab description 16 weeks is when the cognitive deficits appear (Palop et al., 2003; Wright et al., 2013) but some other studies observe them as early as 12 weeks of age (Mesquita et al., 2015). In the present study, we tested 3 months old animals in these two behavioral dimensions, cognitive and emotional processing, by MWM and EPM, respectively. These paradigms were tested on a group of 7 J20 animals and 6 WT littermate age-matched controls.

2.4.1. Elevated plus maze

EPM test is used to analyze anxious-like behavior. The apparatus has cross sign shape with two closed arms (50.8 x 12 x 40.6 cm) and two open arms (50.8 x 12 cm) that are extensions of a common central area (10 x 10 cm), elevated 72.4 cm from the floor. During the light phase of the diurnal cycle, the animals were placed individually in the central area of the cross and were allowed to freely explore the maze for 5 minutes. 10% ethanol was used to clean the apparatus between the sessions. The behavioral parameters were recorded using a video-camera and an infra-red photobeam system connected to a computer with a specific software for the EPM paradigm (MedPCIV, Medassociates, Vermont, USA). The behavior analysis was conduct in EthoVision® XT software (Noldus Information Technology, Wageningen, Netherlands) and by the absolute time spend in each area of the maze – open or close arms and central area – the percentage of time was calculated [(absolute time/300)*100] for each area. The time spend on the close arms can be used as a measure of anxiety-like behavior, since more time in the closed arms is associated with an increased anxious profile (Pego et al., 2008; Wall & Messier, 2001).

2.4.2. Morris water maze

MWM paradigm was performed during the light phase of the diurnal cycle with the objective of evaluate memory and learning performance. A white circular pool (170 cm in diameter and

50 cm in height) filled with tap water (23 ± 1 °C; 30 cm of depth) and placed in a poorly lit room with extrinsic clues, was used on this test. The water tank was divided in four imaginary quadrants (north, east, south and west), each corresponding to a fixed extrinsic clue visible to the mouse. An escape transparent platform (14 cm in diameter and 30 cm high), not visible to the mouse, was placed in the center of one of the quadrants and was maintained in that position during the four days of the acquisition. In a hippocampus-dependent task, the spatial reference-memory acquisition, the mice were placed randomly in one of the quadrants in each trial, and allowed to search for the hidden platform. Each mouse performed four trials per day, during the four days of the acquisition phase. Within the time-limit of 120 seconds (s), each trial was concluded when the platform was reached by the mice. When failing to reach the platform within this time-period, the mouse was guided to the platform and allowed to stay on it for 30s. On the fifth day, we evaluated the reversal learning task with the objective to analyze the cognitive flexibility. In this task, the first trial was conducted without the platform, which was removed from the tank, testing memory, and the distance swum in the quadrant where, on the phase of acquisition, the platform was placed was analyzed. On the others three trials, the hidden platform was changed to the opposite quadrant ("new quadrant") of where it was on the four days of acquisition, the behavioral flexibility was studied by the analysis of the total distance swum in the "new" and the "old" quadrants. Using a video-tracking system (Viewpoint, Champagne au Mont d'or, France), the mean values of the time (latency time to the platform) and travelled distance until reach the platform were calculated for the four random consecutive trials of each day. To analyze the MWM performance, mice were divided into groups according to their genotype.

2.5. Tissue samples collection and storage

After the blood collection for flow cytometry and during the light phase of the diurnal cycle, specifically in the morning, we proceed with the euthanasia of the animals. Mice were anesthetized with intraperitoneal injection of a mixture of ketamine hydrochloride (150 mg/kg, Imalgene® 1000) and medetomidine hydrochloride (0.3 mg/kg, Dorben®). When deep anesthetized, CSF samples were collected from the cisterna magna. Samples were stored at -80°C until further use. After this, blood was collected directly from the inferior *vena cava* and after a centrifugation serum was collected and store at -80°C. The mice were transcardially perfused with 0.9% saline. In experiments performed at 1 and 3 months of age, the thymus and spleen were used for flow cytometry to analyzed their leukocyte populations. To this purpose,

these organs were collected and stored in cold RPMI 1640 liquid medium (10% heat inactivated FCS, 10 mM HEPES, 1 mM sodium pyruvate, 2 mM L-glutamine, 50 mg/ml streptomycin and 50 U/ml penicillin, from Biochrom, Cambridge, UK) until further processing.

The brains were removed from the skull. The choroid plexus samples from each brain ventricle of the same mouse were rapidly removed under conventional light stereomicroscope (SZX7, Olympus, Hamburg, Germany), pooled, snap frozen and stored at -80°C. Specific brain areas namely dorsal hippocampus, ventral hippocampus, prefrontal cortex, cortex, cerebellum and hypothalamus from the right hemisphere were obtained by macrodissection of the right hemisphere and snap-frozen and stored at -80°C. The left hemisphere from the mice brains were fixed in 4% paraformaldehyde (PFA, Panreac Química S.L.U., Barcelona, Spain) for 48 hours and kept in paraffin blocks until further sectioning.

2.6. Blood flow cytometry

Flow cytometry is a very powerful technique to analyze characteristics of cells or particles in suspension, by a laser-based technology. We can use antibodies for specific surface or intracellular molecules that are characteristic and can identify subpopulations of cells (Givan, 2011).

To develop immune profile characterization of J20 AD mice model with 1, 3 and 10 months old, animals were put under a red light to warm them and increase vasodilation, increasing the blood flow by relaxation of the smooth muscle cells within the vase walls. A small cut was made on the extremity of the mice tail, and the blood was collected with heparinized glass micro-hematocrit capillary tubes (VWR®, Radnor, Pennsylvania, USA), preventing clot formation. To analyze the leukocyte cell populations in the blood, cells were stained with CD49b FITC (clone DX5), CD45.2 BV785 (clone 104), CD3 PEcy7 (clone 17A2), CD4 BV421 (clone RM4-5), CD8 BV510(clone 53-6.7), CD19 APC (clone 6D5), CD44 APCFire (clone IM7), CD62L Percpcy5.5 (clone MEL-14) and GR1 BV711 (clone RB6-8C5, all from BioLegend, San Diego, California, USA). After a treatment with hemolytic solution Ammonium-Chloride-Potassium (ACK) (155 mM NH4Cl, 10 mM KHCO3, pH 7.2) and FACS buffer (PBS, 0.5-1% BSA or 5-10% FBS, 0.1% NaN3 sodium azide) the red blood cells were lysed and depleted from the samples. At this point, cell surface staining was detected (100,000 events) in a twelve-color LSRII flow cytometer (BD, Pharmingen, San Jose, California, USA) using FACS Diva Software (Becton Dickinson, New Jersey, USA) and analyzed with FlowJo software version 10 (FlowJo LCC., Ashland, Oregan, USA).

A.

B.
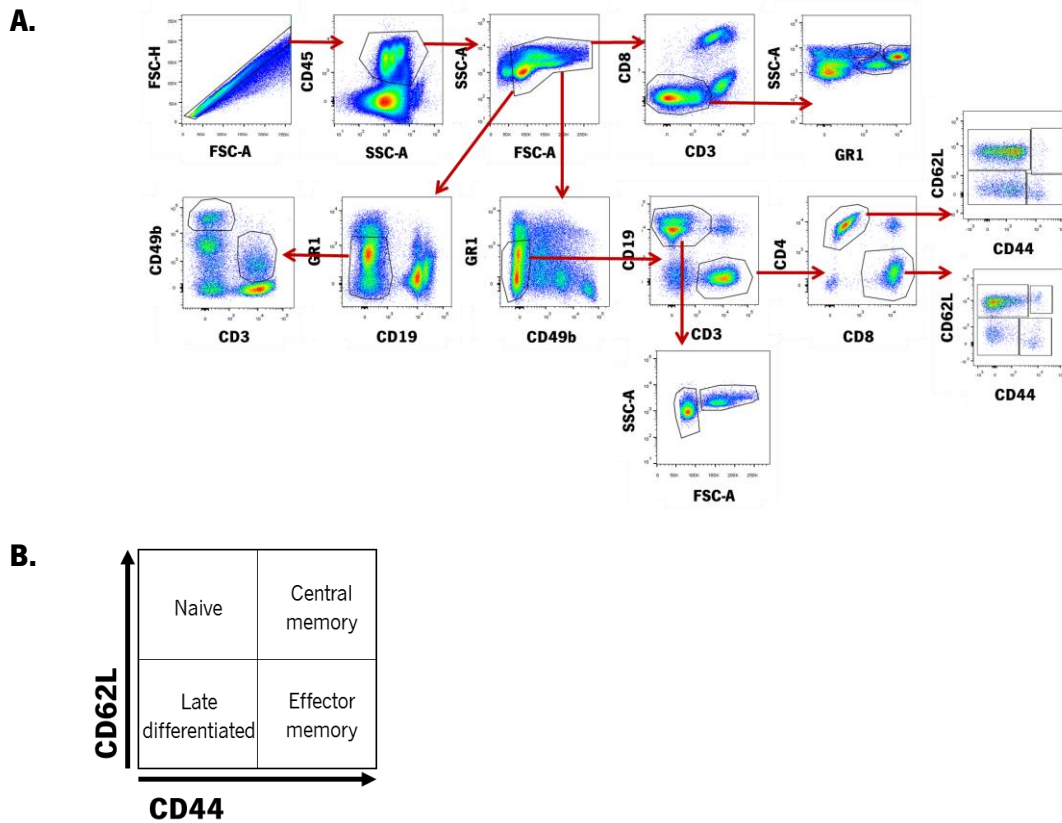
Figure 7. Gating strategy defined for the blood flow cytometry experiments. A. Firstly, in FSC-A versus FSC-H the singlets were gated; we defined CD45+ cells and then in FSC-A versus SSC-A we gated the viable leukocytes subset. Inside of this population we had three different strategies to reach the major leukocyte subpopulations: we gated CD3-CD8- cells to defined granulocytes, monocytes and eosinophils related to their GR1 expression; inside cells CD19-GR1- we defined NK and NKT cells, in the plot CD49 versus CD3; and lastly, CD49-GR1- were divided in terms of CD3 or CD19 surface expression, for T and B cells, respectively. Inside the CD3+ cells, we analyzed the expression of CD4 and CD8, and inside these populations we analyzed the expression of CD62L and CD44 surface markers. **B.** Inside the CD3+ cells, the T cells, we analyzed the expression of CD4 and CD8, and inside these populations we studied the subgroups by the expression of CD62L and CD44 surface markers, dividing the T cells in Naive, Central memory, Effector memory and Late differentiated.

Briefly, the gating strategy (defined in Figure 7.A) applied in all blood flow cytometry experiments was firstly gate the singlets from the FSC-A versus FSC-H. In this population, we defined CD45+ cells and then in FSC-A versus SSC-A we removed mostly every cell debris and other non-interest components of the suspension, getting the viable leukocytes subset. At this point we defined three different strategies inside of this population to reach the major leukocyte subpopulations: we gated CD3-CD8- cells and analyzed them in terms of GR1 expression to defined granulocytes, monocytes and eosinophils; the cells from CD19-GR1- gate were gated in CD49 versus CD3 defining the NK and NKT cells; and lastly, the viable leukocytes were gated in CD49-GR1- for CD3 versus CD19 expression for T and B cells, respectively. Inside the CD3+ cells, the T cells, we

analyzed the expression of CD4 and CD8, and these populations were studied in terms of their subgroups by the expression of CD62L and CD44 surface markers, diving the T cells in Naive, Central memory (CM), Effector memory (EM) and Late differentiated (LD), like exemplified in Figure 7.B.

Blood flow cytometry was performed in J20 and WT animals at 1, 3 and 10 months of age. At the time-point of 1 month, we had 2 independent experiments with 4 J20 and 6 WT; and 6 J20 vs 6 WT animals. For the experiment at the age of 3 months old we had 4 independent experiments with the following set of animals, respectively, 7 J20 and 6 WT; 7 J20 and 6 WT; 4 J20 and 8 WT; and 13 J20 and 13 WT mice. For the time-point of 10 months we did one experiment with 4 animals of each genotype. This will give us an overview on the leukocyte subpopulations longitudinally, as AD progresses on J20 mice.

2.7. Thymus and spleen flow cytometry

The thymus and spleen stored were turned into cell suspensions by gentle disruption of the organs between slide glasses. After centrifugation, the cells were ressuspended in FACS buffer. The quantification of the number of cells were made on a Countess Automatic Cell Counter (Life Technologies, Carlsbad, California, USA) using a staining with 4% of trypan blue. This allows us to only stain a specific number of cells of the organs, on this case $5x10^6$ cells. A surface staining was performed with mice antibodies for, on the thymus: CD3 PEcy7 (clone 17A2), CD4 BV421 (clone RM4-5) and CD8 BV510 (clone 53-6.7, all from BioLegend); on the spleen: CD49b FITC (clone DX5), CD45.2 APC (clone 104), CD3 PEcy7 (clone 17A2), CD4 BV421 (clone RM4-5), CD8 BV510 (clone 53-6.7), CD19 APCcy7 (clone 6D5), CD11b PE (clone M1/70), CD11c PercpCy5.5 (clone N418), GR1 BV711 (clone RB6-8C5, all from BioLegend). Cell analysis was performed (100,000 events) in a twelve-color LSRII flow cytometer using FACS Diva Software and posteriorly analyzed with FlowJo software version 10.

For the thymus (Figure 8.A), we defined the singlet population in the plot FSC-H versus FSC-A. Inside it, we got the viable cells in SSC-A versus FSC-A. The populations were then screened for their surface expression of CD4 and CD8. Due to the sequence of the T cell differentiation on the thymus, next we analyzed the CD3 expression in each one of the previous populations. In the spleen (Figure 8.B), the gating strategy was very similar to that used for the blood analysis. We defined the singlets in FSC-A versus FSC-H plot. Next, in the population of singlets we defined CD45+ cells and after the viable leukocytes in FSC-A versus SSC-A. Inside the viable leukocytes

we defined three divergent strategies. Firstly, we gated CD3⁻CD8⁻ cells to defined granulocytes, monocytes and eosinophils related to their GR1 expression. Inside cells CD19⁻GR1⁻ we plot CD49 versus CD3 to defined NK and NKT cells; and moreover, CD49⁻GR1⁻ were divided into T and B cells, in terms of CD3 or CD19 surface expression respectively. Inside the CD3⁺ cells, the expression of CD4 and CD8 was also analyzed.

A.

B.
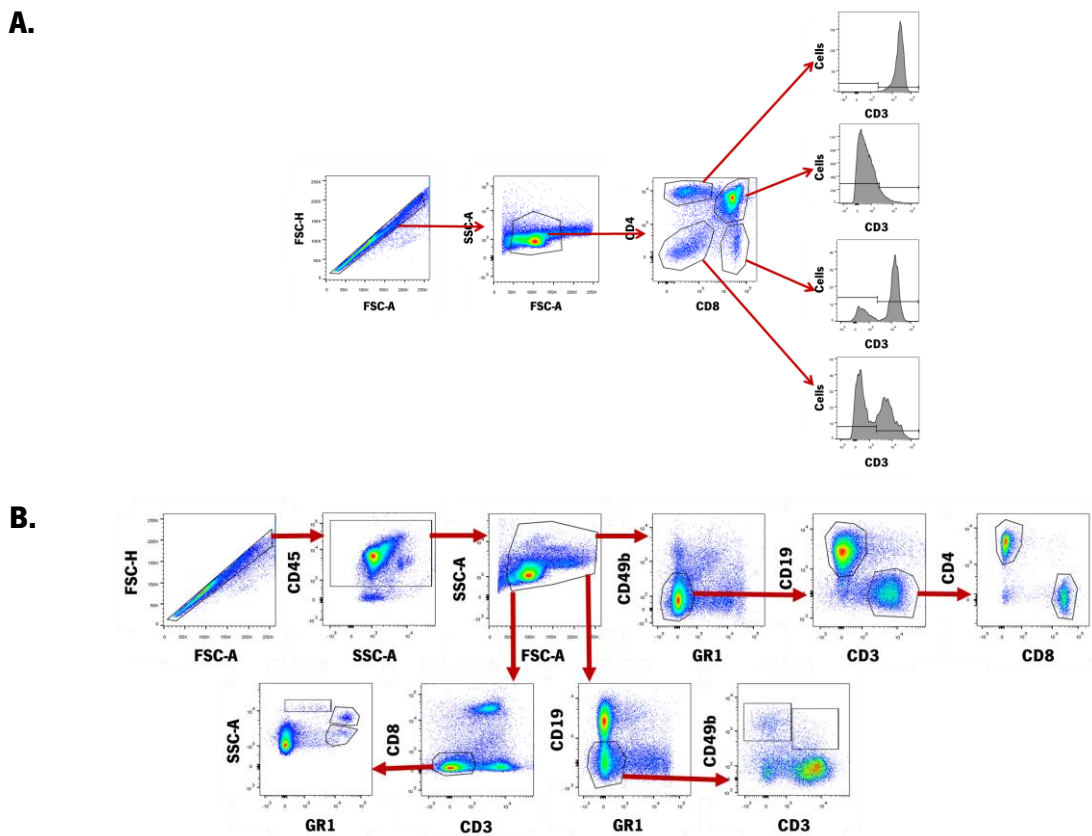
Figure 8. Gating strategy defined for the thymus and spleen flow cytometry experiments. A. Gating strategy used for the thymus data, firstly was defined the singlet population in FSC-H and FSC-A. The singlet population lead to the viable cells pool in SSC-A versus FSC-A. A screening was made in terms of the surface expression of CD4 and CD8, and then we analyzed the CD3 expression in each one of the previous populations. **B.** Related to the spleen's gating strategy, we defined the singlets in FSC-A versus FSC-H plot. Next, CD45+ cells in FSC-A versus SSC-A results in the gating of the viable leukocytes subset. In this population, we defined three different strategies: we gated CD3-CD8- cells to defined granulocytes, monocytes and eosinophils related to their GR1 expression. Inside cells CD19-GR1- we plot CD49 versus CD3 to defined NK and NKT cells; and moreover, CD49-GR1- were divided T and B cells, in terms of CD3 or CD19 surface expression respectively. Inside the CD3+ cells, the expression of CD4 and CD8 was also analyzed.

Flow cytometry of thymus and spleen was performed in J20 animals of 1 and 3 months and only at 3 months, respectively. At the time-point of 1 month we had 1 experiment with the thymus of 4 J20 and 6 WT, without spleen analysis. For the experiment at the age of 3 months we had 2 independent experiments with the following set of animals, 7 J20 and 6 WT; and 4 J20 and 8 WT

mice, for the thymus; and only one experiment for the spleen flow cytometric analysis with 7 J20 and 6 WT.

2.8. Splenocytes stimulation and cytokine analysis

In an attempt to understand if J20 animals presented any impairment in response to immune cell stimulation we next used splenocytes in suspension (developed as described in the point 2.7.) from 4 J20 and 8 WT animals with 3 months of age, to proceed to a stimulation protocol and we assessed their production of IFN-γ.

The cells suspensions of the animals were divided in non-stimulated and stimulated with Concanavalin A (Con-A; 4 μg/mL; Sigma Aldrich, St. Louis, Missouri, USA), human Aβ$_{1-42}$ (Abcam Plc, Cambridge, UK) in three different concentrations – 0.1, 1 and 2 μM for 24, 48 and 72 hours of stimulation. In each time-point the supernatants were stored at -80°C until further use.

The response to the different stimuli in terms of the production of IFN-γ was measured by Enzyme-Linked Immunosorbent Assay (ELISA) on the supernatants. To do this essay we coat a 96 well plate with a coating antibody against mouse IFN-γ (1:1000; clone AN18, eBioscience) diluted in coating buffer (0,1M sodium carbonate buffer at pH=9,5). To do the standard curve we use a commercial recombined murine IFN-γ vial (PreproTech, London, UK) and diluted from 3600 μg/μL to 3.52 μg/μL in a ½ ratio. A biotin conjugated anti-mouse IFN-γ antibody (1:500; clone R4-6A2, eBioscience) was used at this point to detect the antibody-coated molecules. Detection was done with the streptavidin-peroxidase polymer (1:5000; Sigma Aldrich) using, 3′,5,5′-Tetramethylbenzidine (TMB; liquid substrate, Sigma Aldrich) as substrate. Enzymatic reaction was stopped by adding to the well a stop solution (2M H$_2$SO$_4$). The absorbance was read on a Model 680 Microplate Reader (Bio-rad, Hercules, California, USA) connected to a computer with, the Microplate Manager® 6, Version 6 (Bio-rad) software.

2.9. CD3$^+$ cells in the brain

The presence of T cells in the brain was analyzed by staining for CD3$^+$ cells in the brain's parenchyma. For that, animals were transcardially perfused with saline, under anesthesia as previously described. After perfusion, brains were removed from the skull. The left hemisphere from the mice brains were fixed in 4% PFA for 48h and kept in paraffin blocks until further sectioning. The brains' left hemispheres were sectioned in serial 4μm sections and collected to SuperFrost® Plus slides (ThermoFisher Scientific). After 20 min of antigen retrieval by heat with

10Mm, pH 6.0, acetic acid (Sigma Aldrich) and a blocking step of 1 hour at room temperature in 0.5% bovine serum albumin (Sigma Aldrich) in PBS 0.3% Triton X-100 (PBS-T), sections were probed with the biotin conjugated antibody, anti-rat/mouse CD3 diluted (1:50; clone G4.18, eBiosciences) in blocking solution, overnight. Afterwards sections were incubated with streptavidin-594 diluted in PBS-T (1:500; Sigma Aldrich) for 1 hour. The cell nucleus was stained for 5 minutes using 4,6-diamidino-2-phenylindole (DAPI) in PBS (1:1000; Invitrogen, Carlsbad, California, EUA), and mounted in Shandon Immu-Mount (ThermoFisher Scientific). Samples were analyzed using optical microscopy (BX61; Olympus).

2.10. Experimental infection with *listeria monocytogenes*

To test if the immune system of J20 mice responds differently to a peripheral infection, we infected the animals with *L. monocytogenes*.

In this experimental infection with *L. monocytogenes*, 7 J20 and 10 WT animals with 3 months were used (Figure 9). All the experiments involving the bacteria, including infected mice, were performed and maintained in the biosafety level 2 facilities, with characteristic pathogen-free conditions.
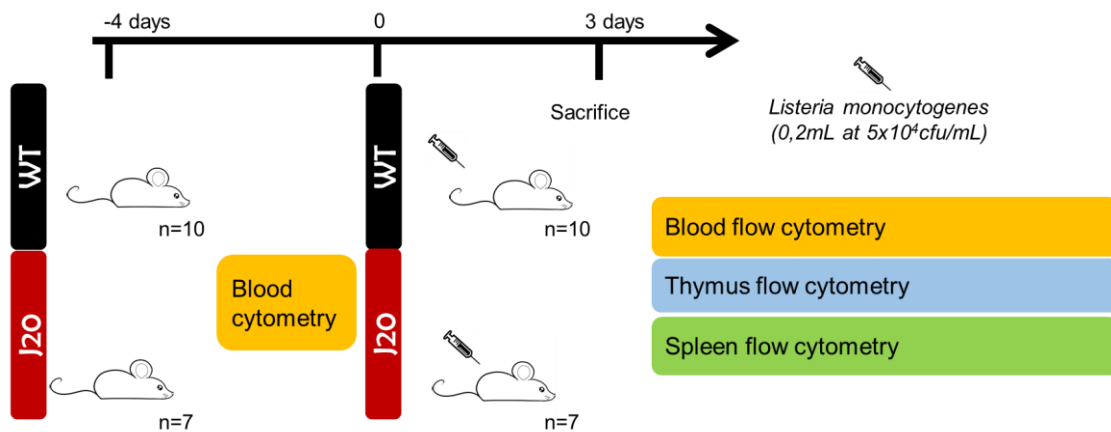
Figure 9. Scheme of the experimental design of infection with *L. monocytogenes*. A group of 7 J20 and 10 age-matched littermate controls was used. Four days previously to the infection with *L. monocytogenes*, the blood leukocyte populations were study by flow cytometry. After the occurrence of the infection, the animals were biometric monitored for the progression of the infection by weight measurement for the three days that the experiment was ongoing. On the third day the animals were sacrificed and the blood, thymus and spleen collected for the study of the leukocytic profile by flow cytometry.

2.10.1. Experimental infection: inoculum development and biometric parameters control

L. monocytogenes (provided by Dr. S. Sousa and Dr. D. Cabanes, Instituto de Biologia Molecular e Celular, Porto, Portugal) was maintained in vials at -80°C. Since the last use of this bacterial pool was on 2010, we decided to develop a new inoculum to use on the experiment described on this report.

To do that, we diluted one of the vials in saline solution and injected 200 µL in the mouse lateral tail vein. 24 hours after the infection the mouse was sacrificed by carbon dioxide and the liver and spleen were collected in aseptic conditions for evaluation of the bacterial load. Briefly, the selected organs were mechanically homogenized, serial diluted in ice cold H_2O and plated onto Brain Heart Infusion medium (BHI; Laboratorios Conda, Spain) with Middlebrook 7H10 agar medium. After 1 day of incubation at 37°C, the CFU number was assessed. We used one CFU diluted in liquid BHI medium to grow over night. The medium was then transferred to falcons and centrifuged, being ressuspended in saline solution, and this was the new inoculum of *L. monocytogenes*.

With the new inoculum developed, we plated on BHI medium and let to grow for 24 hours to count the CFU and determine its concentration, since we aimed to inject $5x10^4$ CFU/mL in 200 µL/mice.

After that assessment, animals were injected with $5x10^4$ CFU/mL of *L. monocytogenes* in the tail lateral vein. The animals were maintained in biosafety level 2 for a period of 3 days being weighted every day. Also, other 3 animals were infected to test the bacteria presence in other organs, namely the thymus and the brain.

2.10.2. Tissue samples collection and colony forming unit counts

On the third day after the infection, blood was collected for flow cytometry in the light phase of the diurnal cycle. Mice were anesthetized with intraperitoneal injection of a mixture of ketamine hydrochloride and medetomidine hydrochloride. When deep anesthetized, mice were transcardially perfused with saline solution. The spleens and thymus were aseptically collected and automatically put in ice-cold RPMI medium until further processing. The brains were removed from the skull and were fixed in 4% PFA for 48 hours, for paraffin processing in blocks.

The selected organs, namely liver and half of the spleens were mechanically homogenized, serial diluted in ice cold H_2O and plated onto BHI plates. After 24 hours of incubation at 37°C, the CFU

numbers was counted and taken as a measure of bacterial load in the animals' organism. Of interest, we also did this on the brains and thymus of 3 control animals to assess the bacterial presence in these organs.

2.10.3. Blood, thymus and spleen flow cytometry

The blood flow cytometry was performed as previously described in the section *2.5. Blood flow cytometry*. However, on this experiment we had two time-points of analysis for leukocyte subpopulations, namely 4 days before and 3 days after the infection with the *L. monocytogenes* (Figure 10).
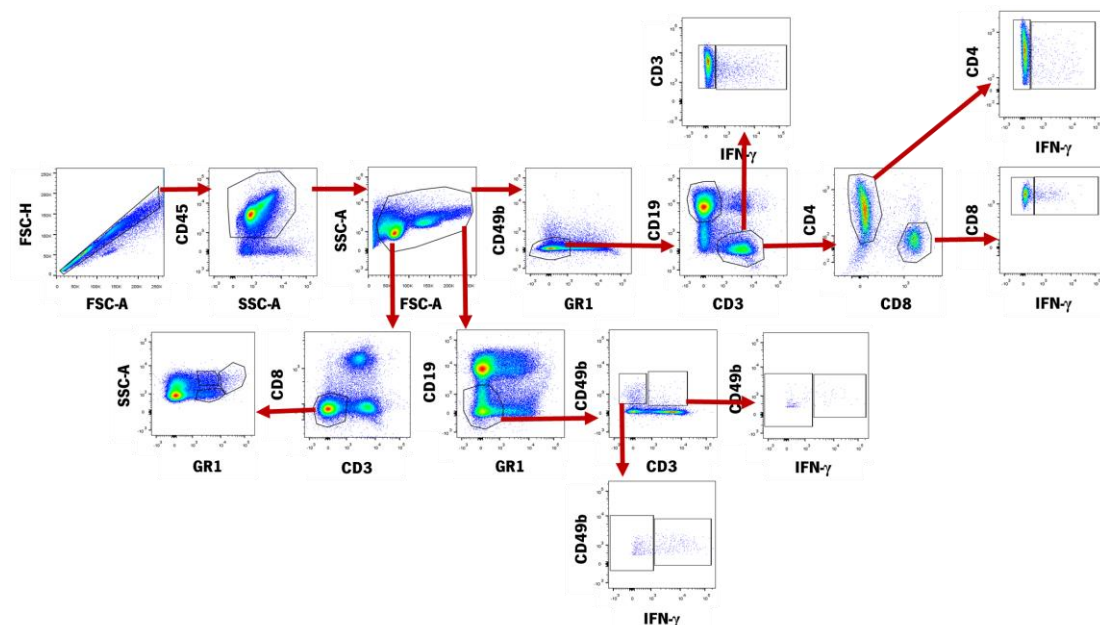
Figure 10. Gating strategy defined for spleen flow cytometry experiment including the intracellular IFN-γ production. Related to the spleen's gating strategy, we defined the singlets in FSC-A versus FSC-H plot. Next, CD45+ cells in FSC-A versus SSC-A results in the gating of the viable leukocytes subset. In this population, we defined three different strategies: we gated CD3-CD8- cells to defined granulocytes, monocytes and eosinophils related to their GR1 expression. Inside cells CD19-GR1- we plot CD49 versus CD3 to defined NK and NKT cells; and moreover, CD49-GR1- were divided T and B cells, in terms of CD3 or CD19 surface expression respectively. Inside the CD3+ cells, the expression of CD4 and CD8 was also analysed. Inside of the cells with the ability to produce INF-γ, this was also assessed, namely on CD3+, CD4+, CD8+, CD49+ and CD49+CD3+ cells.

The thymus and spleen flow cytometry protocol and antibodies panel were the same used previously, described in section *2.7. Thymus and Spleen flow cytometry*. However, and because we induced an inflammatory response we also analyzed the production of IFN-γ by immune cells with an intracellular staining. To do that we needed to stimulated the $5x10^6$ cells in suspension from the spleen with 500 ng/mL of ionomycin (Sigma Aldrich) and 50 ng/mL of phorbol 12-

myristate 13-acetate (or PMA, Sigma Aldrich) for 4 hours, that were associated with 10 µL of Brefeldin (BD Biosciences, San Jose, California, EUA) in the last 2 hours. After the surface staining, the cells were fixed with PFA and permeabilized with 0.5% saponin. At this point, cells were ready for intracellular staining, on this case, only for IFN-γ, using the IFN-γ PE antibody (clone XMG1.2, from BioLegend).

2.11. Statistical analysis

Statistical analysis was performed with GraphPad Prism version-5.0 (GraphPad, Inc., La Jolla, California, USA). The Morris water maze (MWM) latency and total distance *per* day were analyzed by repeated measures Mixed-ANOVA ($\alpha = 0.05$). The probe and reverse time in the new and old quadrant were analyzed by Student's t-test. The EPM time percentage on the different areas of the maze; the blood, thymus and spleen flow cytometry data; the results on CFU counts; and production of IFN-γ by stimulated spleen cells were also statistically compared by Student's t-test. On the experimental infection, the study of the genotype/infection influence in the response of the animals and the impact on the production of IFN-γ by the spleen cells, was analyzed using Mixed-ANOVA ($\alpha = 0.05$).

CHAPTER 3

3. Results

3.1. J20 behavioral characterization at 3 months of age

The MWM (Figure 11.A) is a behavioral paradigm used for evaluation of the memory and learning performance in mice and rats. On Figure 11.A, in the latency time to the platform (MIXED-ANOVA; $F_{(1, 44)}$=19.82; p<0.0001) and the total distance swam by the animals tested until they reach the platform (MIXED-ANOVA; $F_{(1, 44)}$=52.2; p<0.0001) is observed that the time and distance of the WT animals were inferior when compared to the J20 animals through the days of the test. This means that the J20 mice always present a worse performance when compared with WT littermate controls. Regarding the percentage of correct trials (MIXED-ANOVA; $F_{(1, 44)}$=56.08; p<0.0001) and the average for all days of the correct trials [*t test*; WT, 79.17 ± 11.91 % (n=6) vs. J20, 22.32±8.296 % (n=7); p=0.021] the same result is observed, in the sense that WT animals achieved the platform more times in all days of the test when compared to J20 mice. As aforementioned, in the probe test [*t test*; WT, 32.46 ± 2.010% (n=6) vs. J20, 25.42 ± 2.290% (n=7); p=0.0442] the platform was taken out of the tank and the mice were able to freely explore the pool. The J20 animals swam less on the quadrant where supposedly the platform was hidden on the 4 days of the acquisition test, when compared to the WT controls. These outcomes suggest that at 3 months old, the J20 animal model of AD present already cognitive impairments with learning and memory deficits.

On the fifth day of the test, it was also executed the reversal learning, where the hidden platform was placed in the opposite position (of where it been for the previous 4 days) in the tank. The mice were let to explore the pool for 120s to find the platform. The analysis of the latency to the platform [*t test*; WT, 45.39 ± 6.330 s (n=6) vs. J20, 96.90 ± 11.56 s (n=7); p=0.0034] showed that the J20 mice took significantly more time to reach the hidden platform that their WT littermates. In the same way, when the total distance in the tank was analyzed [*t test*; WT, 3104 ± 448.1 cm (n=6) vs. J20, 5803 ± 572.7 cm (n=7); p=0.0442] the J20 animals swam more until reach the platform and some of the animals do not reach it at all. These results in the reversal learning suggest that the J20 animals present an impaired behavioral flexibility when compared to the age-matched WT controls.

This advocate that the WT mice could more easily understand and learn the paradigm and the J20 mice had more difficulty on it, exhibiting an impaired spatial learning ability and behavioral flexibility.

Emotional behaviour was evaluated by the analysis of the anxious-like behavior on the EPM (Figure 11.B). No significant differences were found in the percentage of time that the animals spend on each one of the regions of the maze, namely the open arms, the closed arms and the center of the maze, suggesting that the animals, J20 and WT, presented an equal anxious-like profile.
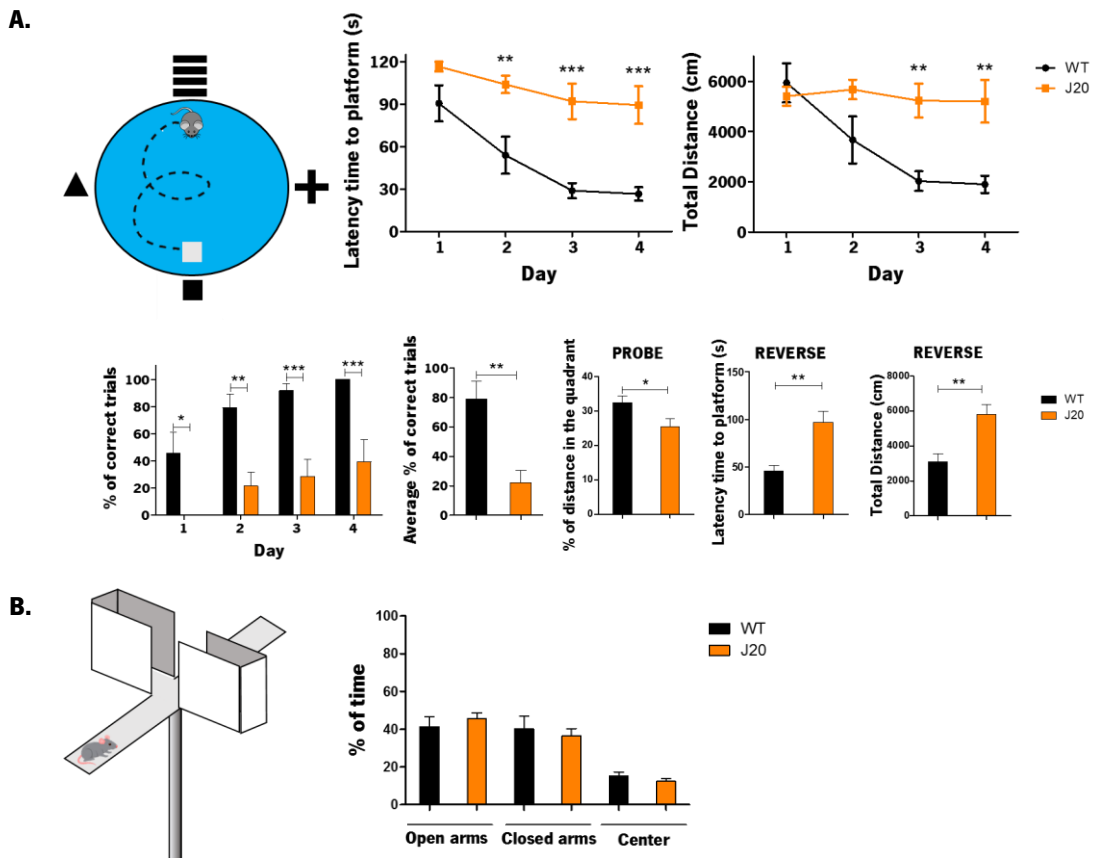
Figure 11. Assessing the cognitive and emotional processing impairments in J20 mice at 3 months. Behavioral performances of WT (N=6) and J20 (N=7) at 3 months old were evaluated by **A.** MWM to assess spatial learning memory and behavioral flexibility deficits associated with AD. In this test, we analyzed the latency time to the platform (s); that corresponds to the amount of time the mice spend until reach the platform; total distance to the platform (cm); percentage of correct trials and average of the same, to evaluate the percentage of times that the animals reach the platform. At the probe test the platform was remove from the tank and the total distance (cm) swam at the quadrant where it should be, was analyzed. On the reversal learning the platform was disposed on the opposite quadrant of where it was the previous days of the test and the latency time to the platform (s) and the total distance (cm) swam to it where evaluated. **B.** The EPM paradigm was also performed at 3 months old J20 animals and littermate WT controls to evaluate anxious-like behavior and associated emotional processing alterations. The total percentage of time spend in each in of the regions of the maze were analyzed. Each bar represents the mean ± SEM from the animals of the groups. $^*p < 0.05$; $^{**}p<0.01$; $^{***}p<0.001$.

3.2. As early as 1 month, J20 animals presented differences on T cells in the thymus and in several leukocytes populations in the blood

The J20 animals presented cognitive spatial memory impairments associated with the disease at 3 months of age (Mesquita et al., 2015; Palop et al., 2003; Wright et al., 2013). Herein our aim was to understand if the animals presented alterations in the immune system. For that reason, we analyzed the blood and thymic immune populations of J20 animals at 1 month of age.

Figure 12. Evaluation of the immune cells populations in 1 month old J20 thymus and blood. Percentage of main leucocyte populations on the **A.** thymus, where the T cells maturation subsets were studied in J20 (N=7) compared with WT (N=6) age matched controls; and in the **B.** peripheral blood, assessing the percentage of both innate and adaptive immune cells populations in 1 month J20 (N=7) in comparison with littermate WT controls (N=6). Each bar represents the mean ± SEM from the animals of the groups. *$p < 0.05$; **$p<0.01$;***$p<0.001$. This is an independent experiment chosen to be example of the obtained results, having in consideration that the 2 experiments performed in the timepoint of 1 month old had the same tendencies.

When analyzing the thymic leukocyte populations (Figure 12.A) by flow cytometry, more specifically the T cells subsets, a significant increase was observed in the differentiated single

positive populations, populations that co-express CD4 and CD3 surface markers: CD4$^+$CD8$^-$CD3$^+$ [t $test$; WT, 6.133 ± 0.144% (n=6) vs. J20, 7.108 ± 0.4588% (n=4); p=0.0428]; and CD8 and CD3: CD4$^-$CD8$^+$CD3$^+$ [t $test$; WT, 1.398 ± 0.04771% (n=6) vs. J20, 1.810 ± 0.1994% (n=4); p=0.0404]. However, no differences were observed on the total number of cells, as well as on the double negative, the cells that reach the thymus (CD4$^-$CD8$^-$CD3$^-$), or on double positive cells, the population that acquire both cluster of differentiation (CD4$^+$CD8$^+$CD3$^-$).

When the blood of these animals was analyzed by flow cytometry (Figure 12.B) differences were observed on the total viable leukocytic population being increased in the J20 animals (t $test$; WT, 93.77 ± 1.047% (n=6) vs. J20, 96.85 ± 0.3274% (n=6); p=0.0185). Specifically this increase was due to alterations on different populations of both innate and adaptive immune cells, namely on NK cells [t $test$; WT, 1.390 ± 0.1768% (n=6) vs. J20, 2.508 ± 0.4688% (n=6); p=0.0497], Eosinophils [t $test$; WT, 1.932 ± 0.1146% (n=6) vs. J20, 2.680 ± 0.2265% (n=6); p=0.0146], and EM CD4$^+$ T cells [t $test$; WT, 0.4957 ± 0.1763% (n=6) vs. J20, 1.212 ± 0.2658% (n=6); p=0.0485]. Of interest, all these leukocytic populations were found to be increase in J20 animals in comparison to their littermates age-matched controls.

These results suggest as early as at 1 month, alterations in the homeostasis of these immune cells populations are occurring both in the thymus and in the bloodstream of AD mice.

3.3. At 3 months, the differences are maintained on T cells in the thymus and the blood CD8$^+$ T cells are increased in J20 animals

In the thymus (Figure 13.A), the same tendency observed at 1 month of age is still present at 3 months. This means that the J20 animals presented a significant increase of the CD8$^+$ cell population, the CD4$^-$CD8$^+$CD3$^+$ [t $test$; WT, 1.170 ± 0.1000% (n=6) vs. J20, 1.570 ± 0.0800% (n=7); p=0.0091]. In the other cellular populations, no differences are observed. These observations imply that some alterations spotted on the early age of 1 month are still present until adulthood in the J20 animals.

Regarding the populations of leukocytes in the blood (Figure 13.B), only the population of CD8$^+$ T cells [t $test$; WT, 8.008 ± 0.3617% (n=6) vs. J20, 9.177 ± 0.3783% (n=7); p=0.0492] was observed to be significantly increased in the J20 animals compared to their littermate controls at 3 months old.
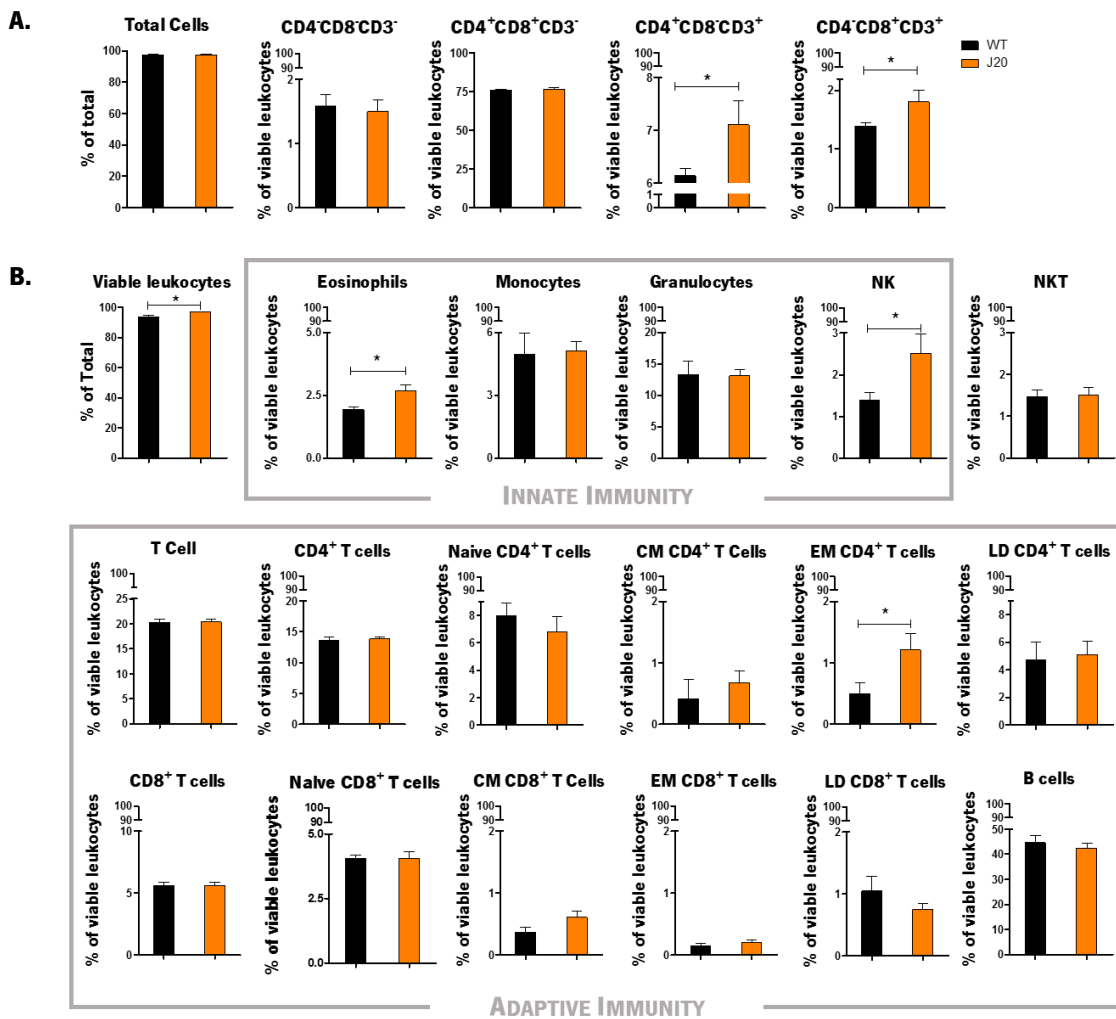
Figure 13. Evaluation of the immune cells populations in 3 months old J20 thymus and blood. Percentage of main leucocyte populations on the **A.** thymus, where the T cells maturation subsets were studied in J20 (N=7) compared with WT (N=6) age matched controls; and in the **B.** peripheral blood, assessing the percentage of both innate and adaptive immune cells populations in 3 months old J20 (N=7) in comparison to littermate WT controls (N=6). Each bar represents the mean ± SEM from the animals of the groups. *$p < 0.05$; **$p<0.01$;***$p<0.001$. This is an independent experiment chose to be example of the obtained results, having in consideration that all the 3 experiments performed at the timepoint of 3 months old had the same tendencies.

At this age, we also decided to evaluate the leukocytes populations in other important immunological organ- the spleen. This would help us to understand if the increase in the T cell differentiated populations on the thymus was related to re-circulation processes from the bloodstream to the organ. The flow cytometry analysis of this organ showed no differences in any of the populations considered, that corresponds to the same immune cells populations analyzed on the blood.

3.4. At 10 months of age, the J20 mice start to present alterations in monocytic population in the blood
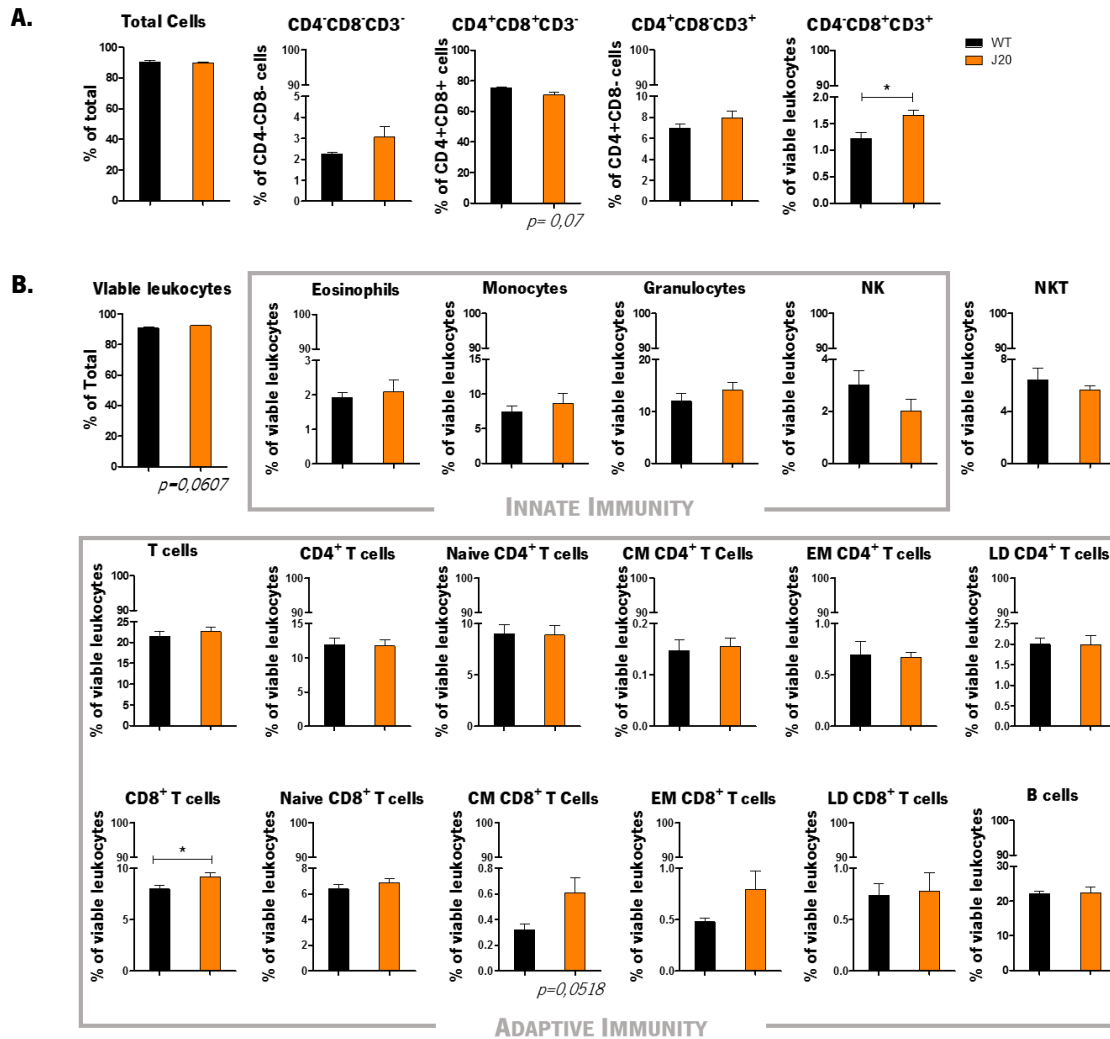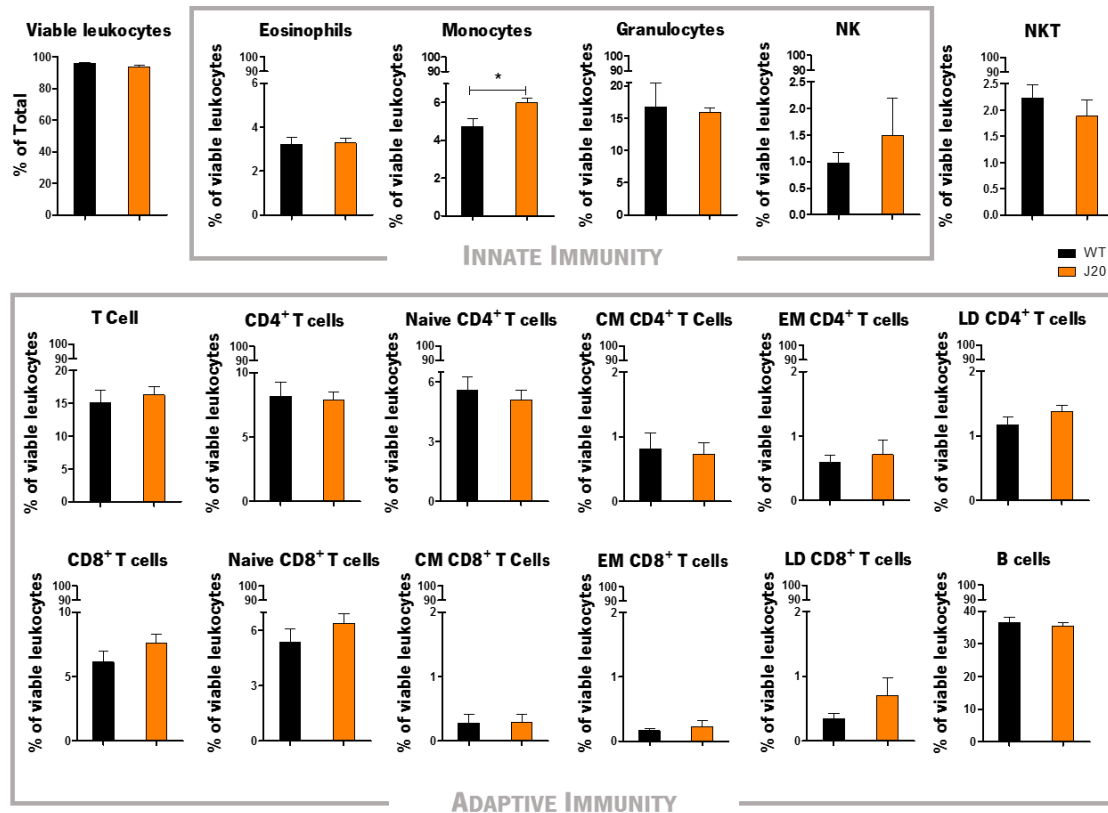

Figure 14. Evaluation of the immune cells populations in 10 months old J20 thymus and blood. Percentage of main leucocyte populations on the **A.** thymus, where the T cells maturation subsets were studied in J20 (N=7) compared with WT (N=6) age matched controls; and in the **B.** peripheral blood, assessing the percentage of both innate and adaptive immune cells populations in 10 months old J20 (N=7) in comparison to littermate WT controls (N=6). Each bar represents the mean ± SEM from the animals of the groups. $*p < 0.05$; $**p<0.01$;$***p<0.001$. This is an independent experiment chose to be example of the obtained results, having in consideration that all the experiments performed had the same tendencies.

Flow cytometry analysis of blood (Figure 14), showed that the altered percentage of CD8+ cells observed at 3 months does not persist at 10 months. However, at 10 months an increase is observed in the monocytes [*t test*; WT, 4.743 ± 0.4278% (n=4) vs. J20, 5.988 ± 0.2697% (n=4); p=0.0490] population. This shows that the homeostasis of the leukocyte populations on the periphery changes with the aging process and is different with the progression of AD J20 model when compared to their littermates WT controls.

3.5. Splenocytes of J20 mice respond as the WT to Con-A and Aβ

Also at the age of 3 months old, the production of IFN-γ by the spleen cells was measured by ELISA in a non-stimulated condition (Figure 15.B), stimulated with a positive condition – Con-A (Figure 15.C), or stimulated with three different concentrations of Aβ - 0.1, 1 and 2 µM (Figure 15.D, only 1µM is presented because none of the concentrations tested as stimuli present a response in terms of IFN-γ production)- for 24, 48 and 72h).

No production of IFN-γ was observed in the non-stimulated cells or in cells stimulated with the different concentrations of Aβ.
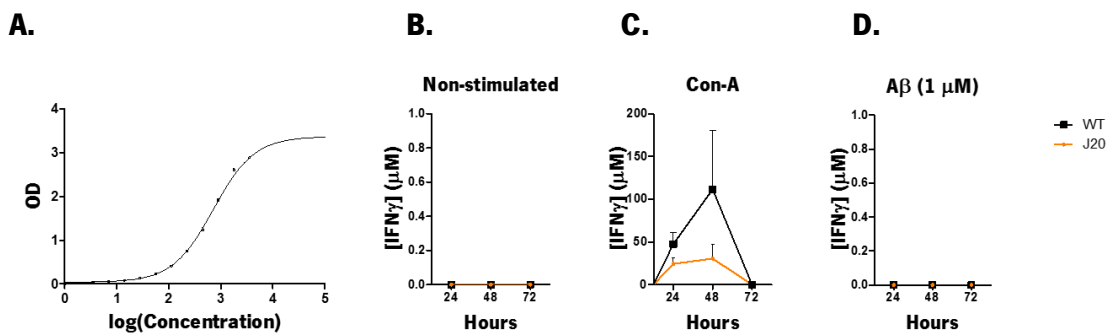
Figure 15. INF-γ production by spleenocytes non- or stimulated with Con-A and Aβ, measured by ELISA. A. Example of a standard curve that allowed the calculation of the IFN-γ levels of the different samples. The concentrations of the INF-γ molecule used was from 3600 µg/µL to 3.52 µg/µL in a ½ ratio. The obtained standard curve followed the equation log(concentration)=linear(absorvance). The values of the IFN-γ were obtained by protruded from this curve. **B. C.** and **D.** are the graphics obtained for the concentration of IFN-γ (µM) for the different conditions tested, not stimulated, stimulated with a positive stimulus Con-A and stimulated with 3 different concentrations of Aβ, but only the concentration of 1µM of Aβ is presented due to be only an example of what was obtained, since no one of the conditions had a response in terms of IFN-γ production. Each point represents the mean ± SEM from the animals of the groups

3.6. No infiltration of CD3+ cells in the J20 brain's parenchyma is observed

To understand if these alterations on the percentages of the peripheral immune adaptive cells of the J20 mice could be related with an increase recruitment of them to the brain parenchyma, immunofluorescence assays were made on brain slices of J20 and WT controls at 1, 3 and 10 months of age, for the presence of CD3+ cells (Figure 16).
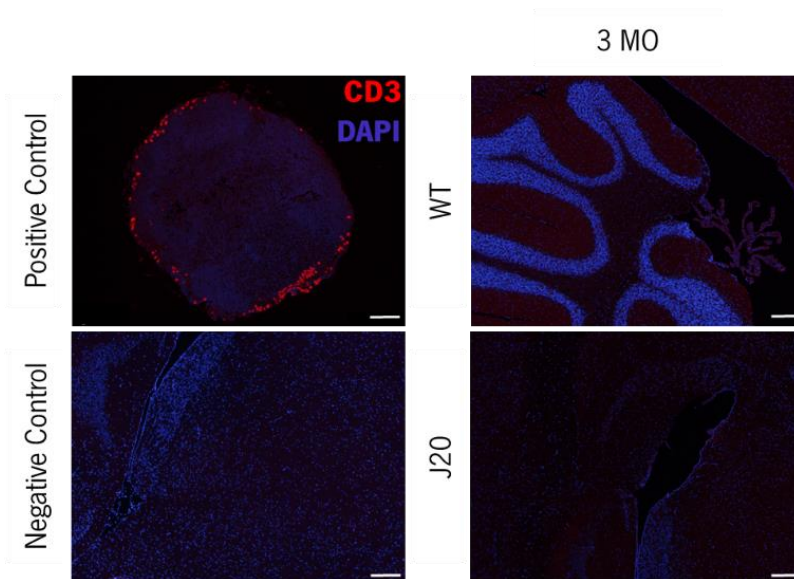
Figure 16. Immunofluorescence assay for the presence of CD3+ cells in the brain's parenchyma. Preliminary assay for staining of CD3 in the brain of WT (N=2) and J20 IgG (N=2) at 1, 3 and 10 months old, suggests that there are no increase infiltrations of these cells within brain's parenchyma. The blue labeling is the DAPI and the red is the CD3. Pictures with the objective of 4x/0,16 and the scale bar attends for 200 μm. The image corresponds to 3 months old brain and is identical of those obtained for the other ages. No CD3+ cells were seen in the brain parenchyma; only in blood vessels or in interface regions, like the stroma of the choroid plexus. The positive control corresponds to a lymphatic ganglion.

We selected the hippocampal region since it is a brain regions affected in the disease and where we know that there is Aβ accumulation. However, no CD3+ cells were detected in none of the tested ages. These results are preliminary, given the number of animals analyzed, but suggest that there is no infiltration of CD3+ cells in the brain parenchyma. However, some CD3+ cells can be observed inside blood vessels or in regions outside the brain barriers – namely on stroma of the choroid plexus, but never on our experiment, inside the brain parenchyma.

3.7. The efficiency to respond to *L. monocytogenes* infection is not compromised in 3 months old J20 mice

Since, at 3 months old, we found differences in the CD8+ T cells population, we next performed an infection with *L. monocytogenes* - in J20 and WT controls at the age of 3months old - to understand if these alterations in the peripheral immune cells homeostasis could have an impact on the response of these animals to an infection that is directly dependent on the CD8+ T cells population.

The results next presented correspond to a single experiment and are therefore preliminary. As shown in Figure 17, no differences were observed in the weight loss in the first 3 days after

infection (Figure 17.A) nor on the bacterial load in the spleen (Figure 17.B) and liver (Figure 17.C).
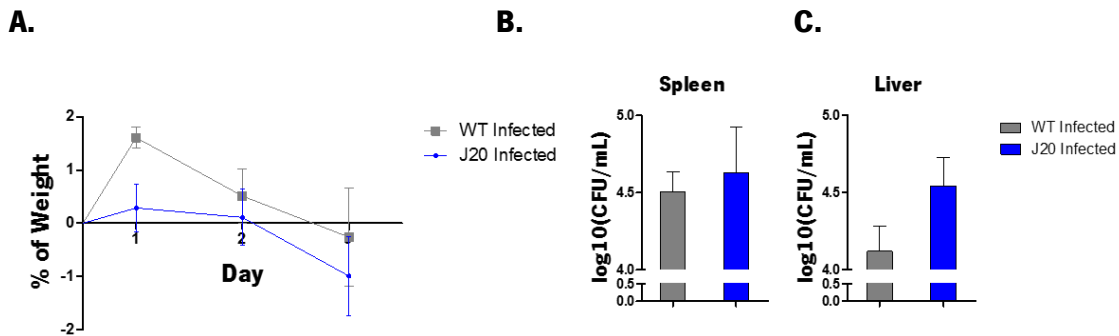
Figure 17. Weight monitoring and bacterial load in the spleen and liver of J20 and WT animals infected with *L. monocytogenes*. A. The infected J20 (N=7) and WT (N=10) were controlled for the 3 days that the infection was on going by the monitoring of their weight, and the percentage of weight is shown on the graph. After sacrifice the animals at the 3rd day of infection, the bacterial load in the **B.** spleen and in the **C.** liver were calculated by the count of the CFU in plaque, being presented on the graphs as log10(CFU/mL). Each point or bar represents the mean ± SEM from the animals of the groups. *p<0.05; **p<0.01;***p<0.001.

3.8. The differences in the blood immune cells populations are related with the infection.

In this analysis, due to the low number of animals that we had available we decide to analyze, by flow cytometry, the blood using the same animals before and after the infection. For the analysis of the thymus we only had the opportunity to assess immune cell populations after the infection when the animals were sacrificed. When the leukocyte population in the blood of the J20 and WT animals (previous and after the infection with *L. monocytogenes,* Figure 18.A) were analyzed, a significant increase NK cells (MIXED-ANOVA; $F_{(1, 30)}$=145.89; p<0.0001) and NKT cells (MIXED-ANOVA; $F_{(1, 30)}$=80.19; p<0.0001), associated with a significant decrease on granulocytes (MIXED-ANOVA; $F_{(1, 30)}$=24.43; p<0.0001) and monocytes (MIXED-ANOVA; $F_{(1, 30)}$=145.89; p<0.0001) are observed, similarly in both genotypes in response to infection. Regarding the thymus characterization (Figure 18.B) the results showed that in response to infection, a significant decrease in the double positive T cells, CD4+CD8+CD3- [*t test*; WT, 77.94 ± 0.5624% (n=10) vs. J20, 75.04 ± 1.209% (n=7); p=0.0297] was observed (Figure 18.B).

Related to the analysis of the leukocyte populations in the spleen (Figure 18.C) and more specifically, the cells that are producing IFN-γ, no differences were observed when J20 animals and WT animals that were infected with *L. monocytogenes* for 72 hours are compared.

A.

INNATE IMMUNITY

Eosinophils — **Monocytes** — **Granulocytes** — **NK** — **NKT** — **B cells** — **T cells** — **CD4+ T cells** — **CD8+ T cells**

ADAPTIVE IMMUNITY

B.

Total Cells — **CD4−CD8−CD3−** — **CD4+CD8+CD3−** — **CD4+CD8−CD3+** — **CD4−CD8+CD3+**

WT Infected
J20 Infected
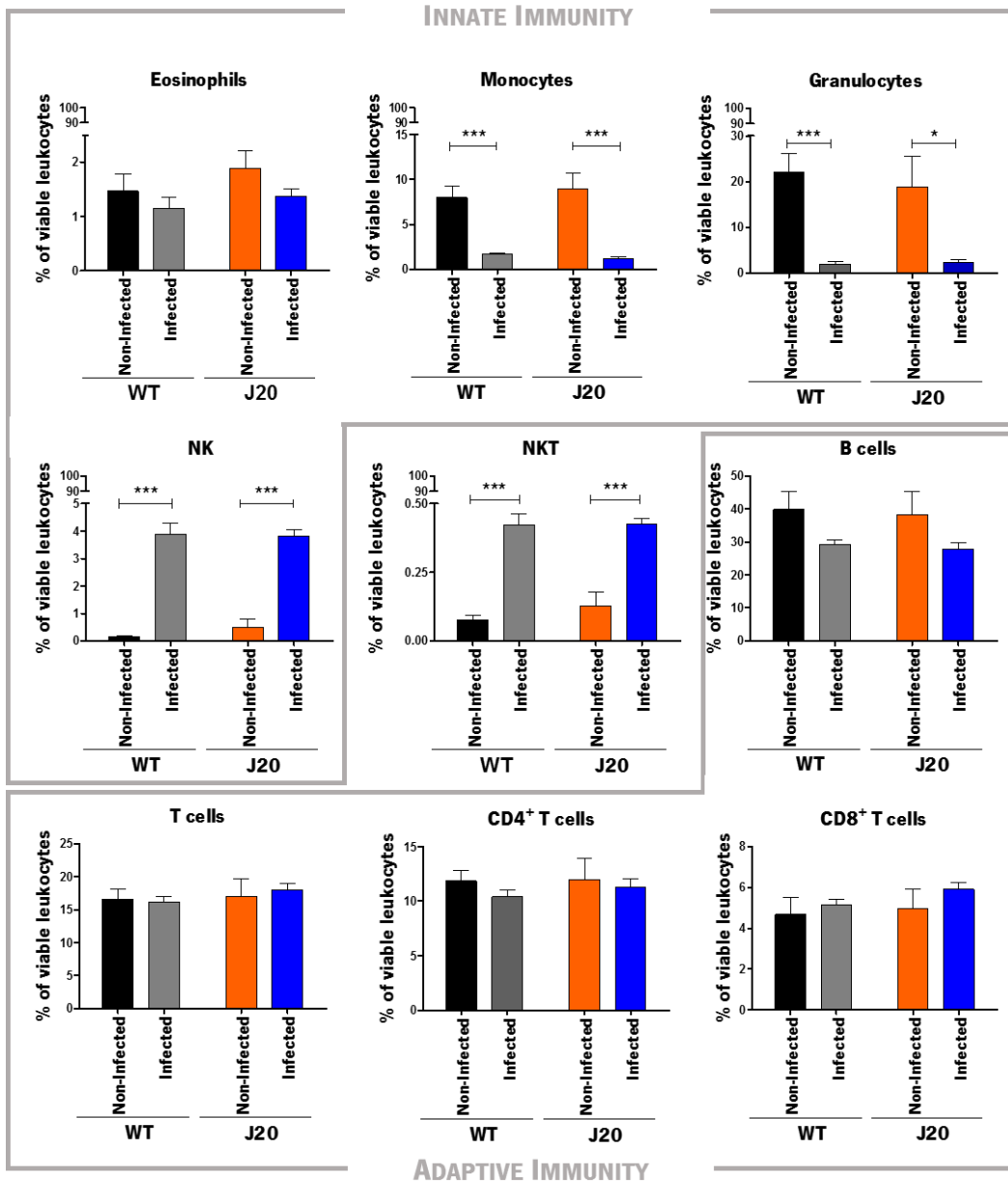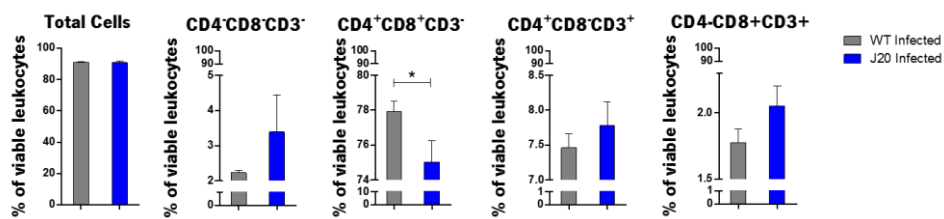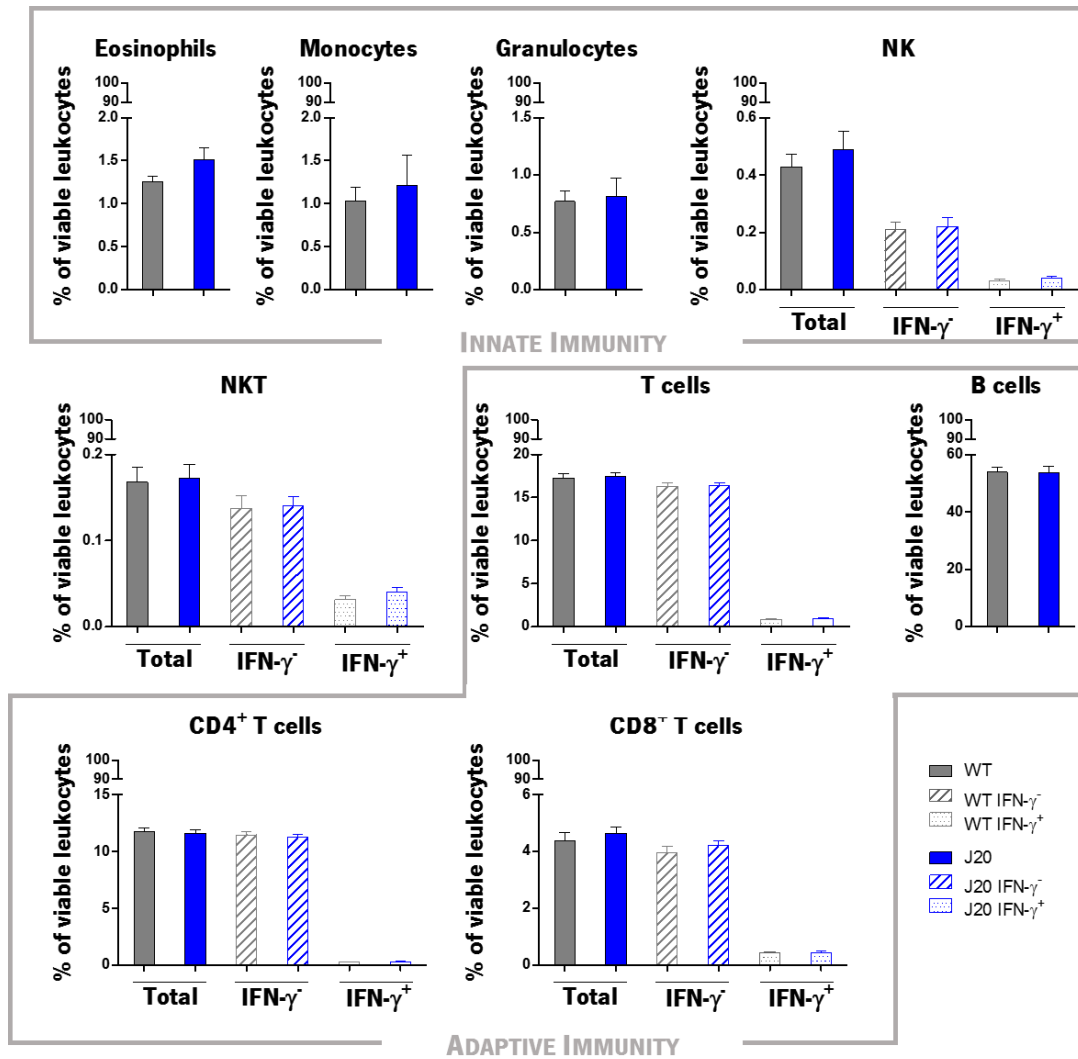
Figure 18. Evaluation of the immune cells populations in J20 blood, thymus and spleen infected with _L. monocytogenes_. Percentage of main leucocyte populations on the **A.** peripheral blood, assessing the percentage of both innate and adaptive immune cells populations J20 (N=7) in comparison to littermate WT control (N=10) animals, previous and after the infection with _L. monocytogenes_ at 3MO; and in the **B.** thymus, where the T cells maturation subsets were studied in J20 (N=7) compared with WT (N=10) age matched controls after the infection. **C.** The immune cells percentage in the spleen was also assessed and even further the cells that were producing IFN-γ in response to the infection with _L. monocytogenes_, were also studied. Each bar represents the mean ± SEM from the animals of the four group. *p<0.05; **p<0.01;***p<0.001.

CHAPTER 4

4. Discussion

4. DISCUSSION

In this work, we characterized the alterations in the peripheral immune system that go along with AD progression in the J20 model, namely on the immune cells populations of the blood and in important immunological organs, such as the thymus and the spleen. Herein, we analyzed the immune cell profile of the J20 animals at different ages: 1, 3 and 10 months of age, showing that the leukocytic profile varies between the analyzed timepoints. Also, we described the impact of the disease in peripheral organs of J20 mice, specifically in the thymus, where alterations in the percentage of differentiation stages of thymocytes were observed.

We consider this study relevant since it is currently accepted that the brain is not an immunological privileged organ (Kipnis et al., 2008; Schwartz & Shechter, 2010), and the role of the peripheral immune system, either on its cellular component or the molecules produced and secreted by them, have crucial impact on basic processes in the brain such as cognition. Of interest, immune peripheral cells have been associated with healthy cognition (Kipnis et al., 2004; Ron-Harel et al., 2008) and in the development and progression of cognitive impairments, which may be relevant for AD. Nevertheless, in AD is still missing the characterization of the immune cell populations profile at different ages. This characterization is significant since it may open new possibilities for biomarkers that can allow an earlier diagnose of the disease, or even it may unravel new therapeutic approaches.

The J20 AD mice model is a very well-studied model in terms of neurologic alterations that occur in the brain along with the progression of the disease (Hong et al., 2016; Mucke et al., 2000; Saganich et al., 2006). The J20 model has the advantage to present cognitive deficits very early, with spatial learning memory deficits observed already at 3 months of age, which we confirmed in the present work. However, no emotional processing impairments were observed, namely on the anxious-like behavior tested in the EPM, since J20 and WT animals spend equal time in the open/closed arms, as previously described (Wright et al., 2013). The results of cognitive impairments, but not at the emotional levels observed at this early age in the J20 model, may suggest that the amyloid-pathology starts in the dorsal part of the hippocampus, mostly related with this cognitive processing mediation, and not at the ventral hippocampus, which is more related with emotional processing (Fanselow & Dong, 2010).

4.1. Effector memory CD4+ T cells as predictors of a worse cognitive performance

The spatial memory cognitive impairments appeared in J20 AD mice model at the age of 3 months old, as previously reported (Mesquita et al., 2015), which validates the model to the experimental approaches taken here. Previous to that age, some cognitive alterations are described, being J20 animals' worse performers in recognition tasks when compared to the WT controls (Webster et al., 2014). We were particularly interested in the peripheral immune profile, given recent findings that cognitive performance is associated with the blood immune cell profile in healthy elder individuals. Specifically, CD4+ T cells were found to be increased and correlated with the worse performance in cognitive tasks (Serre-Miranda et al., 2015). Interestingly, this increase in EM CD4+ T cells was also observed in our youngest set of J20 animals, with 1 month of age, when compared to their littermate WT-controls. Noticeably, this increase is only seen at the earlier age where the cognitive dysfunction is not fully present, which might lead us to hypothesize that this cell profile precedes the development of cognitive impairments. Additionally, EM CD4+ T cells, in comparison to other T cells' types, are one of the majors producers of cytokines (Okada et al., 2008). This, in association with the correlation found between proinflammatory profile and cognitive deficits in older individuals (Dimopoulos et al., 2006; Trollor et al., 2010), can suggest that the EM CD4+ T cells are likely increasing the pool of proinflammatory cytokines, thus resulting in a worse performance of the J20 mice. Further studies to understand the relation between the different CD4+ T cells populations, their cytokine production profile, and the cognitive scores of animals and humans, are needed to fully understand the involvement of these cells in the cognitive decline in normal aging and in AD.

4.2. Longitudinal analysis of the blood leukocytes populations in J20 model can be associated with the variation of IFN-γ expression

Previous results of our group and others have shown that J20 mice present alterations in the IFN expression throughout age and in the J20 AD model. Of interest, in the J20 AD mice model, IFN-γ levels are higher at 3 months of age and decrease with the aging of the animals (from 3 up to 12 months of age) both in periphery (liver) and in the CNS (at the choroid plexus and in the dorsal hippocampus (Mesquita, 2015; Mesquita et al., 2015) (Figure 19). Given the data obtained in the present study with respect to the peripheral immune cell profile, it would be

interesting to next study whether this altered IFN-γ expression profile also occurs as early as at 1 month of age.
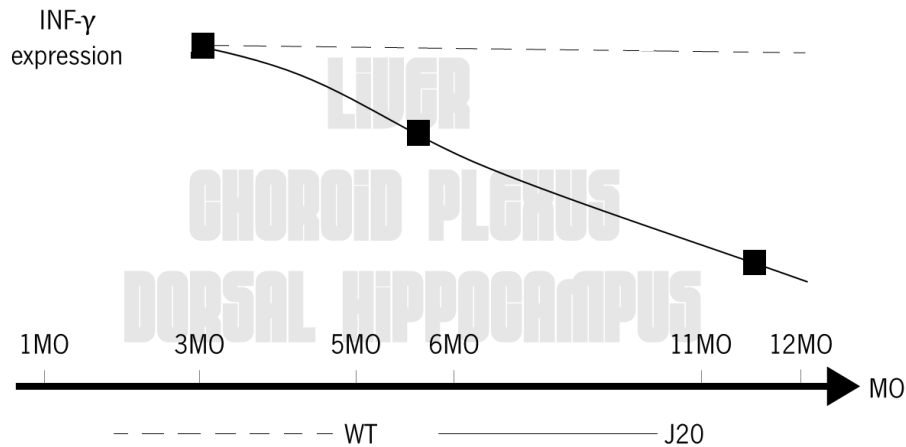
Figure 19. IFN-γ levels variation in J20 mice. Previous studies of our group (Mesquita, 2015; Mesquita et al., 2015) showed altered dynamics in IFN-γ levels in J20 animals compared to their littermates age-matched controls. This work concluded that throughout the life of J20 animals they have a continuous decrease of the IFN-γ expression in both, periphery and brain, namely on the liver, choroid plexus and dorsal hippocampus, while an increase of the expression of type I IFN was observed. The alterations on the normal levels of cytokines could have an impact on the modulation of the immune system of these animals.

In addition, understanding whether these alterations are cause or consequence of the expression pattern observed for IFN-γ still needs to be clarified.

The IFN-γ levels influence the T cell differentiation, resulting in a more proinflammatory profile of T cells (Derecki et al., 2010), which may lead to an increase of NK cells (Asadullah et al., 2003). Of interest, this seems to occur in our animal model, since with aging J20 animals presented a decrease in IFN-γ levels that is accompanied by a decrease in the number of EM CD4+ T cells and NK cells (Figure 20). Of relevance, already at 1 month of age the increase in the percentage of these cells is observed, when compared to the age-matched WT controls. Also, the same profile was observed for eosinophils (Figure 20). Interestingly, eosinophils can be activated by the NK increase *per se.* Some studies assessed this topic and reported an increase of the activation marker CD69 in eosinophils, after co-culture with NK cells (Awad et al., 2014). Of notice, it was also showed that when co-cultured with NK-cells eosinophils also showed an increase in the CD63 surface expression, suggesting their degranulation (Awad et al., 2014). The activation of eosinophils is important for several inflammatory processes (being the most well described the case of allergic diseases), culminating with the exacerbation of responses by the release of large amounts of several lipid mediators and cytokines (Hogan et al., 2008). For this activation to

occur, the cells need to contact with each other (Awad et al., 2014), but also secreted factors from NK cells seems to play an important role on the recruitment and activation of eosinophils (Walker et al., 1998).

The increase on the aforementioned populations – NK and eosinophils - is only observed in J20 mice at the age of 1 month (Figure 20). Previous studies showed that elder AD patients present diminish in the eosinophilic population when compared to age-matched healthy controls, having in account variables like sex, age, and comorbidities (such as, diabetes, hypertension, cardiac problems, among others) (Jaremo et al., 2013). Studies in older animals are needed to see whether this model mimics what is observed in the humans.

Of interest, we have a simultaneous increase, at 3 months of age, of both IFN-γ and CD8+ T cells which may be explained by the fact that CD8+ T cells when activated produce IFN-γ (Balmer et al., 2016) (Figure 20). Of notice, and simultaneously, IFN-γ levels are described to be responsible for the abundance of CD8+ T cells, which is well studied in cases of viral infection (Whitmire et al., 2005). The increase in the levels of CD8+ T cells only at 3 months, and then the progressive normalization with aging of the J20, is relevant because it seems that the animals, at this early age, are trying to respond to the Aβ peptide, but with aging they seem to be losing this capacity. One phenomenon that can be occurring and that can be responsible for this profile is the already described hyporesponsiveness of the cells when in the presence of a chronic situation. Of relevance, this concept was already described in AD (Monsonego et al., 2001) and incorporates not just a low T cell activation, which may lead to the dead of the cells, but also a down-regulation of antigen presentation. Also, the results obtained in the ELISA assay for the IFN-γ production by spleen cells of J20 and WT animals seems to corroborate such hyporesponsiveness hypothesis. No response was found to different Aβ concentrations in both genotypes, and the cells from J20 animals seemed to have less IFN-γ production (although with no statistical significant differences) in response to a positive stimulus, that is known to lead to a response.

The altered levels of CD8+ T cells led us to further study whether this could impair the immune response to infection. For that, we performed a preliminary single infection study with *L. monocytogenes*. Despite of the higher number of CD8+ T cells, J20 seem to cope as well as the controls to this infection.
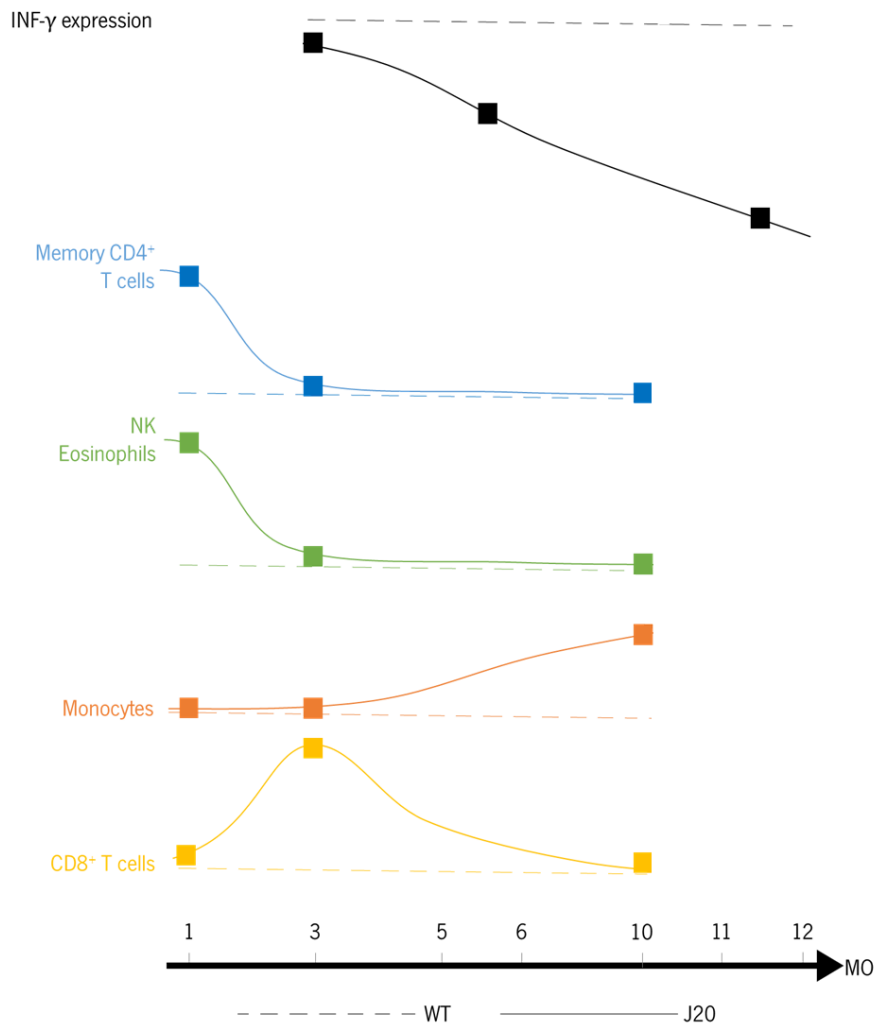
Figure 20. IFN-γ levels and leukocytic populations alterations in J20 mice – longitudinal study. In this thesis is described a longitudinal study of the immune cells populations of the J20 animals. Previous work of our group showed alterations of immune modulators, namely the cytokines IFN. Of interest, IFN-γ levels were find to be decrease throughout time in the periphery and CNS, while the type I IFN were find to have an increase with the aging of J20 animals when compared to their littermates age-matched controls. This could be having an impact in the different leukocytes population, changing their percentage like it is described for the timepoints analyzed, namely 1,3 and 10 months old. In this figure the alterations in each timepoint analyzed are normalized to the WT littermate controls levels. The squares represent the timepoints were the experiments were developed.

4.3. Increased neuroinflammation in AD can lead to augment in the recruitment of monocytes

The increase in the monocytes population observed in the blood of 10 months old J20 animals in comparison to their littermate WT-controls (Figure 20), may be the result of the increased neuroinflammation that is described in this model during the aging process and in disease development (Wright et al., 2013).

It is a past vision the dogma that postulated the brain as an immune privileged organ, and it is already described several interactions in both directions, either periphery alterations influencing

the brain (Marques et al., 2009), or from the CNS to the periphery (Koyuncu et al., 2013). More specifically, the interactions between microglia and peripheral monocytes have drawn little attention and the real mechanisms behind them are still poorly understood. However, it is known that microglia and monocytes present alike functions, such as phagocytosis and secretion of proinflammatory molecules, and even express similar surface markers (Beumer et al., 2012; Prinz & Priller, 2014; Schmitz et al., 2009; Shechter & Schwartz, 2013). The bone-marrow derived phagocytes are suggested to be recruited, playing a role in the elimination of the amyloid load in the brain's parenchyma in AD models (Mildner et al., 2011; Prinz & Priller, 2014; Simard et al., 2006). Moreover, when microglia and macrophages from AD were compared for their gene expression profiles, both innate immune cells presented over 100 genes relate to AD (Schmitz et al., 2009).

The neuroinflammatory process is characterized by the activation of microglia and astrocytes. This activation leads to the production of cytokines and chemokines that, in a chronic manner, may be deleterious to the brain (Russo & McGavern, 2016). These molecules produced by microglia may lead to the activation and recruitment of peripheral immune cells, like monocytes, normally to help in the immune responses, cooperating with microglia (Takahashi et al., 2016). This phenomenon of activation, increase and recruitment of peripheral monocytic population is described for several neurologic pathologies including schizophrenia, depression and bipolar disorder (Beumer et al., 2012; Hickman & El Khoury, 2010; Prinz & Priller, 2014; Shechter & Schwartz, 2013; Takahashi et al., 2016). It was already suggested that the same could be occurring in AD (Hickman & El Khoury, 2010). This results in J20 mice seems to highlight the same: an increase of the monocytes population at the age of 10 months old may occur due to the observed increase in the activation of microglia. More deep studies need to be conducted in order to understand how these alterations can have an impact on AD progression and are related with each other.

4.4. No evident infiltrations of CD3+ cells were found in the brain parenchyma of J20 animals or WT littermates

Even though peripheral immune cells can cross the BBB and infiltrate the brain parenchyma in several AD mouse models (Ferretti et al., 2016), our preliminary data on the infiltration of CD3+ cells on the brain parenchyma showed that T cells are not present. However, this does not mean that the brain is not sensing the alterations that are occurring in the periphery. The peripheral

immune cells can be secreting molecules that in contact with the cells from the barrier can lead to their activation and to the production of molecules that can be secreted to the brain parenchyma-side that will modulate brain's cells function, such as, increasing or inhibiting the activation of microglia (Hoogland et al., 2015). Of interest, we observed some T cells inside the vessels and in the surrounding meningeal space, which corroborate also the idea that these cells can directly activate the brain barriers by the secretion of molecules.

Apparently, the brain region that we selected for the analysis is where Aβ is aggregating and for that reason it also corresponds to the region where inflammation is taking place. We cannot exclude that other immune cells can infiltrate the brain, neither that immune T cell infiltration occur at later ages as observed by others (Ferretti et al., 2016).

4.5. Changes in T cells maturation in the thymus are observed in the J20 animals since an early age

Herein, we also characterized the T cells maturation process in the thymus by flow cytometry. In this primary lymphoid organ, the bone marrow derived progenitor cells, that will be maturated in T cells, undergo differentiation and maturation processes, culminating with a functional T cell repertoire (Pearse, 2006). The thymus is composed by thymocytes in several differentiation states and by supporting stromal thymic epithelial cells (Gruver & Sempowski, 2008). The maturation process of the thymocytes in the thymus gives rise to the CD4+ T cells (commonly known as T helper), CD8+ T cells (or T cytotoxic cells) and some T cells with regulatory function (the Treg cells) (Germain, 2002).

In terms of the differentiation of the T cells in the thymus, they acquire distinguishable cell surface markers, namely CD4 and CD8 markers as being the most important ones, as well as T cells receptor molecules (Gruver & Sempowski, 2008). This process begins with the immature T cells progenitors reaching the thymus (Allman et al., 2003). These progenitor cells have a low expression of both CD4 and CD8 surface markers (CD4-CD8- cells). Already in the thymus, they acquire them both (CD4+ and CD8+ cells, cells called *double positive* cells). With the differentiation process, one of the above-mentioned clusters of differentiation start being less expressed, and the cells start expressing CD3 (CD4+CD3+ or CD8+CD3+ cells, *single positive* cells) (Boyd & Hugo, 1991). Only after a process of positive and negative selection, where the T cells showed their ability to link and recognize endogenous and exogenous antigens (which leads to the apoptosis of defected cells), the T cells are ready to enter the bloodstream. Due to this

primary function of the thymus, it is easy to understand its role on preventing or leading to autoimmunity (Boyd & Hugo, 1991; Gruver & Sempowski, 2008).

We show here that J20 mice present, since a very young age of 1 month old, differences on the thymic homeostasis. The T cells populations were already altered with a tendency to decrease of *double positive* population of cells and a significantly increase of the *single positive* cell populations (CD4$_+$ and CD8+$_+$) that are maintained and seen also at 3 months of age for CD8$_+$ T cells. This suggests a change in the differentiation kinetics in the thymus of J20 animals, with acceleration of the T cells maturation from *double-positive* cells to *single positive* ones. It is not clear the cause of this increased differentiation in the thymus, but is described, while not mechanistically explained, in some works with thymic atrophies in tumor models and animals injected with vascular endothelial growth factor (Adkins et al., 2000; Garcia-Suarez et al., 2002; Laronne-Bar-On et al., 2008; Ohm et al., 2003). Of interest, this increase in the number of *single positive* cells in the thymus can be also due to an increase in the recirculation rate to this organ since it was shown that an increase in the recirculation of cells to the thymus could occur (Thiault et al., 2015). However, when the spleens of the animals were tested, no differences were spotted in terms of immune populations, suggesting an alteration in the thymus *per se*.

Future studies analyzing alterations on the thymus' normal architecture of the cortical and medullary areas, can give us more information about how these AD-causing mutations - that result in the amyloid pathology in the J20 mice - can alter other organs besides the brain.

5. Concluding remarks

5. CONCLUDING REMARKS

Until very recently, the role of the peripheral immune system was underrated specially the impact of it in the healthy brain and in pathogenesis, namely in neurodegenerative disorders, including AD. Nowadays, it is known that the brain is not immunologically privileged but, instead, enjoys the privilege of an immune-dependent maintenance with the immune system, playing important mediation in brain function, like in cognitive dimensions, learning and memory (Kipnis et al., 2004; Ron-Harel et al., 2008; Serre-Miranda et al., 2015).

The present work corroborated that the immune system is characterized by high dynamics and mutability of its populations. However, contradictory studies related to the alterations of the immune cells populations in AD (with the most of the studies executed in elder individuals with already stablished pathology and intense cognitive deficits) are published (Rezai-Zadeh et al., 2009). Herein, this longitudinal study characterizes these alterations in leukocytes populations in the blood, thymus and spleen, providing further evidence that the peripheral immune system should be studied in AD.

The main conclusion of the present study pertains with the observation that AD progression is associated with a continuous change in the peripheral inflammatory profile. However, why this increase is occurring and how it impacts on the disease development deserves further investigation.

Also of interest is how changes in the immune cell profile influence the ability of animals to respond to infection. In the present study, given the increased percentage of CD8$^+$ T cells, which are crucial to the immunological response developed, we asked whether J20 are more resistant to infection (because they have more CD8 T cells) or if they are less prone to fight it because these cells may be less active. Our preliminary findings indicate no differences between the immune response to *L. monocytogenes* between J20 animals and littermates controls

Of interest, apparently related with the fluctuations in the IFN-γ levels: where it was described that there is an initial increase in the J20 animals at 3 months but after this age it starts to progressively decrease with aging (Mesquita, 2015; Mesquita et al., 2015) are the alterations in the immune cells populations discussed in this thesis. This is a possible relation since IFN-γ is a very well-known modulator of the immune system, important not just for the activation, but also differentiation and proliferation of leukocytes. However, it is not clear if the IFN-γ levels are the cause or consequence of the immune cells alterations described in this work. Further studies are

required to understand the chronology of events and even the capability of these cells to produce cytokines such as IFN-γ. Other relevant question that remains to be addressed is if there are other immune modulators that can be inducing the alterations in the immune system cell populations described in this work.

Another novel finding of the present work is that at 1 month of age, J20 mice have an increased population of the EM CD4+ T cells. This increase was already described to be happening in elder individuals with no cognitive impairments but with worst cognitive performances (Serre-Miranda et al., 2015). In the present work, the worst performers are the J20 mice - that at this age already present some difficulties in recognition tasks (Webster et al., 2014). This result support the conclusion of Serre-Miranda et al., that this subpopulation of CD4+ T cells may be an indicator of bad cognitive performance. However, more studies are needed to understand if this increase of the EM CD4+ T cells can be associated with an augmented predisposition to have a more quickly cognitive decline and possibly dementia by AD, even opening the possibility of being used as a biomarker for early diagnosis. This is particularly relevant since these cells are only increased at earlier ages in the J20 model, meaning that the increase of this cells may be temporary, which deserves further investigation.

The characterization of the thymus is also new, and revealed alterations already at 1 month of age that persist until 3 months. Whether these remain at later ages still needs to be investigated. The increase in *single positive* populations observed here suggests an increase of the differentiation rate that can impact in the alterations seen in peripheral blood, even though thymus and bloodstream homeostasis are separated and differently controlled phenomena.

Despite the increase observed in several immune cells populations, this does not seem to result in immune cell infiltration in the brain parenchyma, at least during the time points studied. However, communication between the periphery and the brain does not necessarily require cell infiltration, since released molecules can interact with receptors at the brain barriers and elicit particular responses towards the brain parenchyma.

Altogether, this thesis suggests that both the immune and inflammatory responses may play a very important role in AD pathogenesis. The characterization of the J20 mouse model showed age-related changes in several cell populations that warrant further investigation.

6. References

6. REFERENCES

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