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A role for T cells in the induction of memory deficit in mice with Alzheimer's-like disease

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ABSTRACT

Alzheimer disease (AD) is a progressive neurodegenerative disorder described for the first time by dr. Alois Alzheimer in early 1900 and it is the most common form of dementia worldwide. Nowadays, more than 35 millions of people are affected by AD and this number is expected to rise due to the increase of life expectancy and the incidence of other risk factors for AD, including obesity and diabetes. The leading causes of AD are the accumulation of beta amyloid (Aβ) fragments, which are able to form Aβ plaques outside neurons and the accumulation of abnormal forms of tau protein, which induces the formation of neurofibrillary tangles (NTFs) inside neurons. In addition, studies conducted in AD patients and mouse models of AD have demonstrated that vascular inflammation and a dysfunctional blood-brain-barrier (BBB) are implicated in the pathogenesis of AD. In addition, circulating leukocyte subpopulations were identified in the brains of patients with AD and in the corresponding animal models of this disease. In this regard, the adaptative immune system, and, in particular T-lymphocytes, emerged as pivotal players in the inflammatory events exacerbating neuronal damage in AD brain. Since the role played by CD4+ and CD8+ T cell in AD is still unclear, in this PhD thesis our aim was to investigate the contribution of these cells into the disease pathogenesis in 3xTg-AD mice a transgenic mouse model of AD developing both Aβ and tau-pathologies. We first evaluated by flow cytometry the accumulation profile of T cells at different disease stages in the brain of 3xTg-AD mice in comparison to wild-type (WT) mice. Interestingly, our data showed that T lymphocytes differently infiltrate the brain during AD progression. At early disease stage, the frequency of CD8+ T cells compared to CD4+ T cells was higher in 3xTg-AD brain, showing a progressive reduction with disease progression. Conversely, CD4+ T cells gradually increased with time, showing a peak of accumulation at 9 months of age. We also analyzed the effects of the pharmacologic depletion of CD4+ and CD8+ T lymphocytes from the peripheral circulation by treating 3xTg-AD mice at the onset of cognitive impairments (6 months of age). Our results showed a significant restoration of the spatial memory in 3xTg-AD mice depleted of CD4+ or CD8+ cells. Moreover, immunohistochemical studies showed that CD4 and CD8 depletion reduces microglia activation and Aβ deposition in cortical and

hippocampal regions, the most vulnerable areas during AD progression. Of note, only 3xTg-AD mice devoid of CD8+ T cells displayed a significant reduction of tau phosphorylation, suggesting an association between NFT formation and CD8+ T lymphocytes. Next, we investigated the intravascular mechanisms used by T cells to migrate into the brain during AD. We have previously reported the up-regulation of the integrin-ligands ICAM-1 and VCAM-1 in cortical and hippocampal regions of 3xTg-AD mice compared to WT mice. Thus, we examined the expression of LFA-1(αLβ2) and VLA-4 (α4β1) integrins on T cell subsets in the circulation of 3xTh-AD mice. We observed a differential expression of these molecules on the surface of peripheral T-cells, with VLA-4 being more abundant on CD4+ compared to CD8+ T cells in 3xTg-AD mice, suggesting that its blockade may interfere with CD4+ T cell recruitment to the brain. Furthermore, the therapeutic targeting of VLA-4 using antibodies that specifically block α4-integrins improved the memory of 3xTg-AD mice compared to the isotype control mAb. Additionally, α4-integrin blockade in 3xTg-AD mice reduced neuropathological hallmarks of AD such as microgliosis, Aβ load and tau hyperphosphorylation. We next examined LFA-1 expression on peripheral T cells from 3xTg-AD mice and found the presence of a distinct T cell population highly expressing LFA-1 (LFA-1^{high}) which correlated with disease progression. Accordingly, we also observed a significant reduction of brain infiltrating lymphocytes, mainly CD8+, in 3xTg-AD mice genetically depleted for LFA-1 (3xTg-ADx*Itgal*$^{-/-}$). Moreover, taking advantage of 3xTg-ADx*Itgal*$^{-/-}$ mice, we observed that LFA-1 depletion led to an amelioration of cognitive functions and a reduction of neuropathological hallmarks of the disease. Together, our data show a key role for integrins VLA-4 and LFA-1 in disease pathogenesis in 3xTg-AD mice, and suggest that targeting leukocyte extravasation mechanisms may be considered a new therapeutic strategy for AD.

To further understand the mechanisms responsible for leukocyte-dependent damage in AD, we also set up an *in vitro* culture system to further investigate the interaction between lymphocytes and brain cells. Indeed, nowadays innovative fields on modeling AD using *in vitro* models came out making possible new avenues for the discovery of new AD therapeutic approaches. During this PhD thesis program, we also developed a new 3D model using neuronal cells isolated from the brain of

3xTg-AD mice and WT controls. To characterize our new in vitro system, we first analysed through qPCR and Western Blot which cells recapitulate AD hallmarks in culture. Importantly, after 15 days of *in vitro* culture, neurons and astrocytes isolated from 3xTg-AD mice showed higher expression of Aβ compared to cells obtained from WT mice, whereas, as expected, the expression of tau protein was detected only in neurons, but not in astrocytes. Moreover, after 21 days *in vitro,* the supernatant of neuronal cells accumulated soluble tau confirming the validity of our new *in vitro* AD model. We next used our 3D model to evaluate the functional impact of Th1 and Th17 cells on astrocytes and microglia. Previous studies showed that CD4+ T cells, particularly Th1 and Th17 cells, can infiltrate the brain and may have a role in the pathogenesis of AD, but how T cells interact with neural cells is still unclear. We isolated CD4+ T lymphocytes from lymphoid organs and differentiated them into Th1 and Th17 subsets producing IFN-γ and IL-17, respectively and then were co-cultured for 24 hours with neuronal cells obtained from 3xTg-AD mice. We performed preliminary studies and observed that the co-cultures with Th1 cells enhanced microglial expression CD68 and MHC II expression, which represent classical markers of microglia activation. Additionally, Th1, but not Th17, cells induced astrocyte activation by upregulating CD44 on the surface of these cells. Together, these preliminary in vitro results suggest that Th1 cells may play a harmful role on neuronal cells in AD pathology by triggering inflammatory changes in glial cells.

In conclusion, our data obtained in 3xTg-AD mice as well as our preliminary studies in an *in vitro* 3D system suggest that CD4+ and CD8+ T cells contribute to disease pathogenesis in transgenic mice with AD-like disease and that interfering with the pro-inflammatory functions of these cells may represent a new therapeutic strategy in AD.

ABBREVIATIONS

2D	*Two-dimensional*
3D	*Three-dimensional*
3xTg-AD	*Triple transgenic Alzheimer's disease mouse*
AD	*Alzheimer's disease*
ADAM	*A disintegrin metalloproteinase*
ADDLs	*Amyloid derived diffusible ligands*
AICD	*Amyloid precursor protein intracellular domain*
APC	*Antigen-presenting cell*
APOE	*Alipoprotein*
APP	*Amyloid precursor protein*
Aβ	*Beta amyloid*
BACE1	*β-Site APP cleaving enzyme 1*
BACE2	*Beta-site amyloid precursor protein-cleaving enzyme 2*
BBB	*Blood brain barrier*
C99 or βCTF	*99-residue carboxy-terminal fragment*
CAA	*Cerebral amyloid angiopathy*
CBF	*Cerebral blood flow*
CDK-5	*Cyclin-dependent kinase-5*
CFC	*Contextual fear conditioning*
CNS	*Central Nervous System*
CO	*Cerebral organoids*
CSF	*Cerebro- spinal fluid*
CTL	*Cytotoxic T lymphocytes*
CTF	*C-terminal fragment*
CTFα	*83-residue carboxy-terminal fragment*
CVD	*Cardiovascular disease*
DAG	*Diacylglycerol*
EAE	*Experimental autoimmune encephalomyelitis*
ECM	*Extracellular matrix*
EOFAD	*Early onset familial Alzheimer's disease*
ER	*Endoplasmatic reticulum*
FAK	*Focal adhesion kinase*
FDG	*18F-fluorodeoxyglucose*
fMLP	*N-formylmethionyl-leucyl-phenylalanine*
GEFs	*Guanine-nucleotide-exchange factors*
GFAP	*Glial fibrillary acid protein*
GPCRs	*G-protein-coupled receptors*
BBB	*Blood brain barrier*
HA	*Hyaluronic acid*
HEVs	*Venular endothelial cells*
HPK1	*Hematopoietic progenitor kinase 1*
IBA1	*Ionized calcium-binding adapter molecule 1*
I.P.	*Intraperitoneal*
IPSCs	*Induced pluripotent stem cells*
ICAM-1	*Intercellular adhesion molecule-1*
IL	*Interleukin*

IFN	*Interferon*
InsP3	*Inositol 1-4-5 triphosphate*
LDL	*Low-density lipoprotein*
LFA-1	*Lymphocyte function-associated antigen-1*
LOAD	*Late onset Alzheimer's disease*
LRP-1-1	*LDL receptor related protein-1*
LTP	*Long-term potentiation*
MAPK	*Mitogen-activated protein kinase*
MAPs	*Microtubule-associated proteins family*
MCI	*Mild cognitive impairment*
MHC	*Major histocompatibility complex*
MIDAS	*Metal- ion-dependent adhesion site*
MRI	*Magnetic resonance scans*
MS	*Multiple sclerosis*
MTL	*Medial temporal lobe*
MVB	*Mutivescicules body*
NETs.	*Neutrphils extracellular traps*
NFTs	*Neurofibrillary tangles*
PAMPs	*Patterns associated molecules pathogens*
PBS	*Phosphate buffered saline*
PDL1	*Phospholipase D1*
PET	*Positron emission tomography*
PFA	*Paraformaldehyde*
PHF	*Paired helical filament*
PiB	*Pittsburgh compound-B*
PIP5KC	*Phosphatidylinositol-4-phosfate 5-kinase isoform 1γ*
PLC	*Phospholipase C*
PRRs	*Patterns recognition receptors*
PSEN-1/2	*Presenilin-1/2*
PSGL-1	*P-selectin glycoprotein ligand-1*
PtdIns (4,5)P_2	*Phosphatidylinositol 4-5-bisphosphate*
PTx	*Pertussin toxin*
Pyk2	*Proline-rich tyrosine kinase-2*
RAGE	*Receptor for advanced glycation end products*
ROS	*Reactive oxigen species*
sAPPα	*Soluble N-terminal fragment*
SORL1	*Sortilin-related receptor 1*
SP	*Senile plaques*
TCR	*T cell receptor*
TGN	*Trans golgi network*
TH	*T helper*
TMD	*Transmembrane domain*
TNF	*Tumor necrotic factor*
TPM	*Two photon microscopy*
VCAM-1	*Vascular cell adhesion molecule-1*
VLA-4	*Very-late antigen-4*
VLDLs	*Very-low-density lipoproteins*
vWFA	*Von Willebrand Factor*

α2-M	*α2-macroglobulin*
βAPPs	*Smaller ectodomain derivative*

FOCUS OF THE WORK

Increasing evidence suggests that the underlying mechanisms of AD may begin years or even decades before symptoms start to manifest. Clinicians and researchers believe that treating those affected at the earliest possible stage will be the most effective way of slowing down the disease or stopping its progression. The brain immune system is considered a key factor in the development of AD, particularly a specialized type of resident immune cell called microglia. However, it appears that these cells are dysfunctional in AD and contribute to disease pathogenesis. In addition to glial cells, several recent reports also highlighted the presence of migrated peripheral T lymphocytes and suggested a role for these cells in the adaptive immune responses and chronic neuroinflammation in AD. Based on these evidences, the focus of this Ph.D thesis was to understand the role of T cells in the pathogenesis of disease using mice with Alzheimer's-like disease. Using a transgenic mouse model of AD (3xTg-AD mouse) we aimed to characterize the presence of the two main subpopulations of T cells: CD4+ and CD8+ T cells and study their role in AD pathogenesis. In order to get access to the brain, T lymphocytes have to pass through two main barriers: the blood-cerebrospinal fluid barrier (BCSFB) and the blood-brain barrier (BBB). Thus, we evaluated the role of two main integrins in the process of T cells extravasation in AD mice: $\alpha_4\beta_1$ integrin (CD49d/CD29), also known as very late antigen-4 (VLA-4), and $\alpha_L\beta_2$ integrin (CD11a/CD18), also known as Lymphocyte Function Associated Antigen 1 (LFA-1), using pharmacological and genetic approaches. Finally, to gain new insight into disease mechanisms we developed an in *in vitro* model recapitulating key features of AD pathology potentially useful to study the molecular mechanism controlling the interactions between T cells with neural cells. Together, our studies will increase our knowledge on the cellular and molecular mechanisms contributing to AD and will point out new therapeutic strategies and biomarkers to better address AD.

OVERVIEW OF THESIS SECTIONS

Sections 1 to 3 cover Alzheimer's disease definitions and features. Particularly, in sections 4 and 5, we describe neuroinflammation events that may represent a potential driving force in AD with a special emphasis for T cells and neutrophils.

Section 6 provides important information for the thesis, focusing the attention on classic mechanisms of leukocytes recruitment mainly related to LFA-1 and VLA-4 integrins.

In section 7 we describe new avenue for AD researcher based on the use of *in vitro* model mimicking AD features.

In sections 8 to 14 of *Material and Methods*, we describe the methods and technologies used throughout this thesis, such as mice typization, isolation of brain leukocytes, flow cytometry analysis, mice treatments, behavioural assessments and histological assay.

In sections 15 to 18 we explain the methods for neuronal cell isolation, culture and characterization using molecular approaches and qualitative assay.

Sections 19 and 21 cover the methods used for CD4+ T cells isolation, differentiation and co-culture with glial 3xTg-AD derived cells.

From sections 22 to 25 we describe the results obtained in all our experiments in animal models of Alzheimer's disease and in in vitro settings.

Finally, the *Discussion* contains the main conclusions drawn from this project and possible directions for future research. The emerging role for lymphocytes recruitments in CNS diseases provides insight into the mechanisms of brain damage during AD and may contribute to the development of novel therapeutic strategies.

CONTENTS

INTRODUCTION

1. ALZHEIMER'S DISEASE

Alzheimer's disease (AD) is one of the most common form of neurodegenerative disorder affecting more than 35 million people worldwide and represent an important public health problem due to the increase of life expectancy. It was defined for the first time by Alois Alzheimer in 1906 when he published his now famous case study of a 51-years old woman named Auguste Deter. The description made of her symptoms represent the first neuropsychological characterization of the disease. When the woman died, Alzheimer used the then-new silver staining histological technique to examine her brain microscopically, observing the neuritic plaques, neurofibrillary tangles, and amyloid angiopathy which became the hallmarks of the disease that now bears his name. However, our modern understanding of the disease and its neurophysiological consequences were understood only starting from 1980s (Schachter AS, *Dialogues Clin Neuro* 2000; Bondi MW, *J Int Neuropsychol Soc* 2017). Indeed, it is largely accepted that from the neuropathological point of view, AD is characterized by: neuronal loss in specific brain regions, especially in the medial temporal lobe structures and the temporo-parietal association cortices, intraneuronal neurofibrillary tangles (NTFs) composed by hyperphosphorylated form of tau protein and extracellular neuritic plaques, which consist in deposits of β-amyloid peptides (Aβ) mainly its 42-amino-acid isoform (Blennow K, *Lancet* 2006). Besides, it is possible to identify three main stages of Alzheimer's disease: mild, moderate and severe (**Fig. 1**). The mild stage is the initial stage of the disease, in which people experience memory loss and other cognitive complications such as repeating questions and personality and behavior changes. Diagnosis is often made in this stage, but it can take many years before any clinically or detectable signs or symptoms appear. The moderate stage is typically the longest stage and it involves damages in areas of the brain that control conscious thought, language, sensory processing and reasoning. Memory loss and confusion become worse and people may experience hallucination, paranoia and behave impulsively. The severe stage is characterized by a strong presence of Aβ plaques and NTFs in the brain. People are completely dependent on others for their care, because the pathology has a growing impact on physical and

movement capabilities (Galvin JE, *Neurodegener Dis Manag* 2012). Once diagnosticated, the progression of AD is widely variable and patients with AD could have a life expectancy between three to 11 years after diagnosis, but some survive 20 years or more. Furthmore, several studies report that a high proportion of AD deaths are attributable to pneumonia due to a silent dysphagia that cause aspiration of oral contents from the pharyngeal space into the respiratory tract and result in the development of aspiration pneumonia. Other common causes of death include dehydration, malnutrition, falls and other infections (Beard CM, *Ann Epidemiol* 1996).

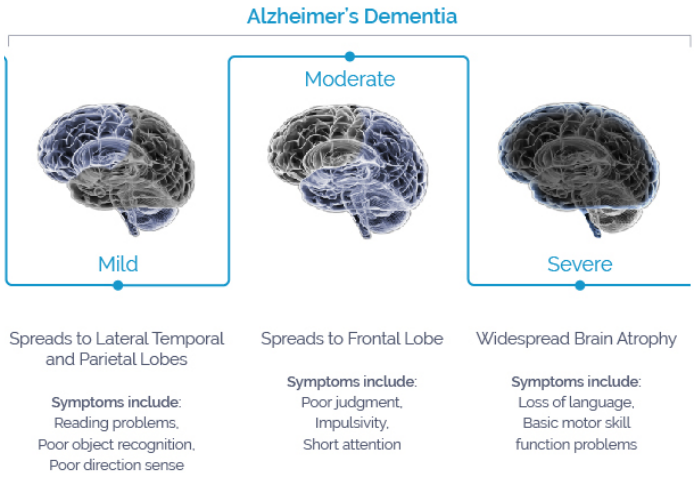


Figure 1. Different stages of AD. Picture adapted from AgeneBio.

1.1 NEUROPSYCHOLOGICAL SIGNS AND SIMPTOMS OF AD

The disruption of brain cells by Aβ and NFTs in regions involved in forming new memories during AD pathogenesis represent the key causes of neuropsychological symptoms which clearly affected AD patients. In particular, several studies showed that episodic memory impairments like amnesia could denote an earliest feature of AD dementia syndrome. Accordingly, neuropathological studies showed that extensive AD pathology occurs earliest in medial temporal lobe (MTL) structures (e.g., hippocampus, entorhinal cortex) important for episodic memory (Hyman BT, *Science* 1984). Additionally, studies demonstrated that due to the spreading of AD beyond MTL structures to adjacent temporal, parietal, and frontal association cortices, several others cognitive abilities became affected. Indeed, deficit in

language abilities were also observed quite early in AD pathogenesis with deficits in: verbal fluency, semantic categorization, and a reduced ability to recall over-learned facts (Hodges JR, *Neuropsychologia* 1995). Furthermore, visuospatial deficits occurred in patients with AD, but these deficits were usually less frequent than other cognitive deficits in the early stages of the disease (Cronin-Golomb A, Elsevier 2001). Once the identification of AD is made, the progression of the disease depends on both the age of the diagnosis and other health concurrent issues, although the course of the disease is not the same in every AD patient, symptoms seem to develop over the same general stages. In that scenario, the advances made in characterizing the neuropsychological deficits associated with AD had a major impact on the ability to accurately diagnose the disease in its early stages (Salmon DP, *Neurology* 2002).

1.2 BIOMARKERS OF AD

The criteria of the National Institute of Neurological and Communicative Diseases and Stroke and the Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA), which are commonly used for the clinical diagnosis of Alzheimer's disease, were published more than 20 years ago, and represent a useful tool for the exclusion of other form of dementias (McKhann G, *Neurology* 1984). Besides behavioral symptoms, a new avenue was made with the identification of in vivo biological markers for AD. In particular, several proteins concentrations can be measured in the cerebrospinal fluid (CSF) by antibody-based techniques (e.g ELISA assay) or by antibody-independent techniques (e.g mass spectrometry). It is well verified that reduced concentrations of Aβ42 can be detected in the CSF of patients with mild cognitive impairment (MCI) or in the pre-clinical stages of AD (Bateman RJ, *N Engl J Med* 2012; Olsson B *Lancet Neurol* 2016). The reduction is due to the sequestration of Aβ42 in senile plaques in the brain. Additionally, phosphorylated tau release by tangle-containing neurons can be measured in the CSF by ELISA. Studies reveal an increased concentration of P-tau in the CSF of AD patients (Olsson B, *Lancet Neurol* 2016); even if this higher P-tau concentration correlates weakly with neurofibrillary tangle pathology in the brain of AD patients (Buerger K, *Brain* 2006; Seppala TT, *Neurology* 2012). Together with CSF

analysis, Magnetic Resonance Scans (MRI) and Positron Emission tomography (PET) **(Fig. 2)** represent sophisticated imaging systems that may help measure earliest changes in brain function or structure to identify patients in the very first stages of AD (Scheltens P, *Dialogues Clin Neurosci* 2009). Structural MRI in AD is a powerful tool to measure gray matter atrophy related to the loss of neurons, synapses, and dendritic de-arborization and white matter atrophy related to the loss of structural integrity of white matter tracts as a result of demyelination of axonal processes and *ex vacuo* expansion of CSF spaces (Vemuri P, *Alzheimer's Research & Therapy* 2010). The earliest site of atrophy is the entorhinal cortex, followed by hippocampus, amygdala and para-hippocampus. The distribution and the amount of cerebral atrophy are strictly correlated with cognitive deficits (Johnson KA, *Cold Spring Harb Perspect Med* 2012). Instead, to assess hypometabolism in the temporal, parietal and posterior cingulate cortex 18F-fluorodeoxyglucose (FDG) Positron Emission Tomography (PET) could be used in AD patients (Jagust W, *Alzheimers Dementia* 2006). FDG-PET has a high ability to differentiate Alzheimer's disease from other dementias, especially from frontotemporal dementia, moreover, is also able to predict the progression of the pathology from MCI to AD with high accuracy. (Arnaiz E, *Neuroreport* 2001, Chetelat G, *Neurology* 2003). Furthermore, PET is also used to detect Aβ plaques in the brain using the radiolabelled Pittsburgh Compound-B (PiB) that marks Aβ (Johnson KA, *Alzheimer's Dement* 2013). AD patients typically show marked PIB retention in cortical areas known to contain large amounts of Aβ plaques. Controversial studies on this technique made on healthy and AD patients, support the principle that amyloid imaging alone has not been approved to diagnose AD or to measure the extent of cognitive impairment but rather as a diagnostic method to exclude AD in subjects who are cognitively impaired and amyloid PET-negative (Sperling RA, *Alzheimers Dement* 2011; Villemagne VL, *Future Neurol* 2012). In contrast, PET imaging of NFTs correlate with progressive neuronal degeneration and cognitive impairment allowing early detection of AD cases and when performed together with amyloid diagnosis, may also provide a tool to distinguish between AD and non-AD dementias (Villemagne VL, *Cold Spring Harb Perspect Biol* 2017).

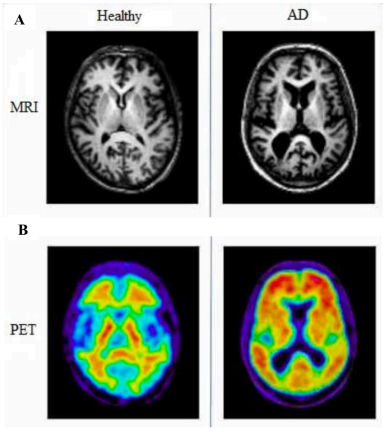


Figure 2. Illustration of MRI and PET imaging. (A) MRI scans of a healthy individual who has no evidence of brain atrophy and an AD one whit marked brain atrophy and **(B)** PET scans of a healthy individual with no evidence of Aβ and an AD brain with a significant Aβ deposit. Picture adapted from Zhang XY, *Front Mol Neurosci* 2017.

1.3 LIFESTYLE RISK FACTORS

Alzheimer's disease is a very common form of neurodegenerative disorder in elderly people, thus represent an important public-health problem. Besides ageing, which is the most obvious risk factor for the disease, epidemiological studies have suggested other aspects with a strongly association with the disease development. Recent studies highlighted the importance of brain reserve capacity which is determined by the number of neurons together with lifestyle-related cognitive strategies. A decrease in the reserve capacity of the brain which include reduced brain size, low educational and occupational attainment, low mental ability in early life, and reduced mental and physical activity during late life it has been shown to strict correlated with AD (Mayeux R, *Annu Rev Neurosci* 2003, Mortimer JA, *J Clin Exp Neuropsychol* 2003). In addition, another interesting risk is related to cardiovascular disease (CVD) including hypercholesterolemia, hypertension, atherosclerosis, coronary heart disease, smoking, obesity, and diabetes. Indeed, these risk factors facilitate the Aβ plaque and NFTs formation, which can lead to cognitive decline or induce inflammatory responses impairing cognitive function (Mayeux R, *Annu Rev Neurosci* 2003). Additionally, several studies focused on a series of life-style "correction" with which it is possible to reduce the risk of getting AD, such as diet and mental and physical exercise (Grant WB, *J Alzheimer's Dis*

2002). Evidences suggests that dietary intake of homocysteine-related vitamins (vitamin B12 and folate); antioxidants, such as vitamin C and E; unsaturated fatty acids; and also, moderate alcohol intake, especially wine, could reduce the risk of Alzheimer's disease (Luchsinger JA, *Lancet Neurol* 2004).

1.4 GENETIC RISK FACTORS

Genetic rick factors increasing AD development are widely accepted. Indeed, individuals who have a family member with AD have more susceptibility to develop the disease (Donix M, *Am J Geriatr Psychiatry* 2012). Apolipoprotein E gene (APOE4) is the major gene recognized as risk factor for late-onset sporadic AD. In fact, there are evidences that between 40% and 65% of people diagnosed with AD have one or two copies of the *APOE4* gene. *APOE4* encodes for *APOE4* protein which facilitates the pathophysiology of AD through promotion of NFTs formation, Aβ plaques deposition, neurotoxicity and oxidative stress, as well as increasing the permeability of the Blood-Brain-Barrier (BBB). BBB as a highly specialized endothelial cell membrane surrounding cerebrals micro vessel mediates solute exchange between blood plasma and brain fluid leading to an alteration in peripheral to central relationship (Altman R, *Clin Sci* 2010). Additionally, three genes have been identified to be correlate with early onset familial AD inherited in an autosomal dominant fashion: amyloid precursor protein (APP), presenilin 1 (PS-1), and presenilin 2 (PS-2) genes. Mutations in the *APP* gene might result in altered metabolism of *APP*, leading to increased production of the Aβ protein, or an increased production of the 42 amino acid form of Aβ (Aβ 1–42). Whereas, the *PS-1* gene is generally thought to be involved only in early onset familial Alzheimer's disease, although allele sharing between affected family members with late onset disease has been observed suggesting a further role for PS-1 in late onset Alzheimer's disease (Goate AM, *Geriatrics* 1997). In some cases, there are affected families in which both *APP* and PS-1 mutations have been excluded. A third mutated gene was identified as *PS-2*. Only two mutations have been recognized in the *PS-2* gene leading to AD with an onset between 40 and 88 years of age, typically later than that seen in *PS-1* linked cases. However, it is not clear how this

accumulation of fragments contributes to neurodegeneration (Goate AM, *Geriatrics* 1997).

1.5 CURRENT AD THERAPY

AD represents a major public health problem due to its tremendous personal and social impact. The long duration of illness implies that the costs of long-term care services become very high because they should be continuous, holistic, and integrated. Therefore, with the expected increase in the prevalence of AD cases among elderly individuals, also the financial toll on society will increase. Global leaders have set a deadline of 2025 for finding an effective way to treat or prevent AD. France, Australia, Japan, US and Great Britain are among the countries that have adopted national policies to address the growing numbers. Multilateral organizations have also prioritized the disease, and possibly most significantly, the G8, under British Prime Minister David Cameron's leadership, set its focus on dementia starting December 2013 (Vradenburg G, *Expert Rev Neurother* 2015).

To date, there is currently neither a cure nor adequate clinical treatment for AD. Medications fall into two broad categories–drugs: I) to treat cognitive symptoms, such as memory problems and other mental deficits of Alzheimer's, and II) to treat behavioral symptoms that do not respond to non-pharmacological behavioral-management approaches. Symptomatic treatments provide only modest benefits and however do not ameliorate cognitive worsening (Yiannopoulou K, *Ther Adv Neurol Disord* 2013). However, without affecting the underlying causes of the disease, they are only a bandaid effect.

The Pharmaceutical Research and Manufacturers of America (PhRMA) reported that between 1998 and 2017 there were some 146 failed attempts to develop medicines to treat and potentially prevent AD. And only four new medicines were approved to treat the symptoms of the disease. Still, there are currently 92 medicines for the treatment of Alzheimer's and other dementias in clinical development today.

The first targets of this research were based on the "amyloidocentric concept of AD", and the developed drugs aimed at interfering with the aggregation of Aβ and its deposition in Aβ plaques. These approaches led to the production of anti-Aβ aggregation agents, such as for example the synthetic glycosaminoglycan 3-amino-

1-propaneosulfonic acid (3APS, tramiprosate) (Gauthier S, *J Nutr Heal Aging*. 2009). It was designed to block the binding between glycosaminoglycans and Aβ, but recent data have demonstrated that this drug promotes an abnormal tau aggregation inside neuronal cells (Santa-Maria I, *Mol Neurodegener* 2007). Another Aβ aggregation inhibitor developed was Colistrin, a proline-rich polypeptide complex derived from sheep colostrum. Several studies demonstrate that Colistrin can efficiently inhibit neurotoxicity and cognitive impairments in animal models (Jin L, *Antimicrob Agents Chemother* 2013), but is also associated with adverse effects promoting antibiotic resistance. Furthmore, also the enzymes that lead to Aβ production were considered as therapeutic targets, in particular BACE1, in fact many of these compounds are at the moment in clinical trial, such as CTS-21166 actually at Phase I. This molecule has shown preliminary positive response, such as the reduction of Aβ when injected in APP transgenic mice (Luo X, *Int J Clin Exp Pathol* 2010). However, BACE1 has also other important physiological roles, therefore its inhibition could have toxic consequences. For example the γ-secretase inhibitor Semagacestat (LY-450139) has been shown to decrease the generation of Aβ in the CSF of AD subjects (Siemers E, *Clin Neuropharmacol* 2005) but it also showed severe collateral effects such as the accumulation of the neurotoxic precursor of Aβ (the C-terminal fragment of APP or CTFβ) resulting from the inhibition of the γ-secretase cleavage activity on APP (Imbimbo BP, *Curr Top Med Chem*. 2011). The multiple failures in Aβ-targeted trials have led researchers to question the feasibility of this strategy and opened the door for alternative approaches. Interest has grown on tau recently, however, far fewer drug trials have focussed on tau, although there is a very robust correlation between tau pathology and clinical measures of dementia (Congdon EE, *Nat Rev Neurol* 2018). Some problems have emerged, mouse and primate models of AD show Aβ plaques that respond to anti-Aβ therapy, but these animal models do not replicate the tau pathology seen in human AD. A novel anti-tau agent, TRx0237 was studied in a Phase 3 trial and failed to show a difference between different doses. Studies in mouse models suggested that the agent functioned as an aggregation inhibitor and reduced the number of tau positive neurons; no target engagement biomarker was included in trial to determine if this was achieved in

humans (Congdon EE, *Nat Rev Neurol* 2018). Other anti-tau drugs are also in development for AD including epigallocatechin-3 gallate (EGCG), a polyphenolic flavanoid extracted from green tea 25, and E2814, an anti-tau monoclonal antibody that is designed to slow the progression of AD actually in Phase I. Preclinical studies suggest that these molecules targeting pathological tau, improve cognition and function, reduce microglia activation, and are also capable of crossing the BBB (Congdon EE, *Nat Rev Neurol* 2018). Despite numerous studies and significant investment by biopharmaceutical companies and others, setbacks continue to outnumber successes in Alzheimer's drug development. Scientists still do not have a full understanding of the underlying causes and mechanisms of Alzheimer's disease. Only the strategy of reducing the chronic inflammatory process in AD has shown promising results, indeed preliminary clinical trials strongly suggest that medications such as nonsteroidal-anti-inflammatory drugs (NSAIDs) and immunomodulatory agents may have an important role in altering the course of AD (Townsend KP, *FASEB J* 2005; Wang J, *J Alzheimers Dis* 2015). Moreover, new biomarkers capable to identify the neuropathologic determinants underlying cognitive changes within a given individual, and to detect neuropathology in its earliest stages before the onset of significant cognitive change will represent a useful tool for AD therapy.

2. PATHOGENESIS

AD can be classified in two different forms: early-onset familial (EOFAD) and late-onset AD (LOAD). EOFAD is an uncommon form of AD with an autosomal dominant pattern of 19 inheritance, which means that one copy of the altered gene is sufficient to cause the disorder. EOFAD accounts for less than 5% of total cases and refers to families in which onset is consistently before age 60 to 65 years and often before age 55 years (Bird TD. *Genet Med* 2008). The three clinically indistinguishable subtypes of EOFAD based on the underlying genetic mechanism are: Alzheimer disease type 1 (AD1), caused by mutation of *APP* (10%-15% of EOFAD); Alzheimer disease type 3 (AD3), caused by mutation of *PSEN1*, (30%-70% of EOFAD); and Alzheimer disease type 4 (AD4), caused by mutation of *PSEN2* (<5% of EOFAD) (Bird TD, *Genet Med* 2008). LOAD is also known as

"sporadic AD" because appears to be no genetic factor or family link involved. LOAD represents the most common form of AD affecting about 90% of all AD cases and occurs in people of age 65 or older (Tanzi RE, *Cold Spring Harb Perspect Med* 2012). LOAD form is probably related to variations in one or more genes in combination with improper lifestyle and environmental factors. In fact, the only genetic risk associated with LOAD is the presence of the mutated allele *APOE E4* is linked with a higher risk of LOAD (Strittmatter WJ, *Proc Natl Acad Sci* 1993). The ε4-allele of *APOE* increases AD risk by approximately fourfold when inherited in one copy and by greater than 10-fold for two doses of the allele (Corder EH, *Nat Genet* 1994). However, this allele is neither necessary nor sufficient for developing AD.

2.1 APP

The principal constituent of the plaques is Aβ, a 39±43 amino acids long peptide, which derived by proteolytic cleavage of the ubiquitous transmembrane APP. (Kang J, *Nature* 1987).

2.1.1 APP AND ITS FUNCTIONS

The *APP* gene is located in human on the long arm of chromosome 21 indeed subjects with Down syndrome have an increased risk of developing AD (Farfara D, *J Cell Mol Med* 2008). Although the connection between Down syndrome and AD is unclear, the production of excess Aβ peptide due to the presence of three copies of genes on chromosome 21 genes (including the *APP*) may account for the increased risk. The alternative splicing of *APP* gene arising the formation of three major isoforms: *APP695, APP751* and *APP770* (Goate A, *Nature* 1991). *APP695* is predominantly expressed in neurons and lacks the Kunitz Protease Inhibitor (KPI) domain which by the contrary is present in *APP751* and *APP770* within their extracellular regions (Kang J, *Biochem Biophys Res Commun* 1990; Rohan de Silva HA, *Brain Res Mol Brain Res* 1997). APP belongs to a protein family that includes APP-like protein 1 (APLP1) and 2 (APLP2) in mammals (Wasco W, *Proc Natl Acad Sci USA* (1992; Coulson EJ, *Neurochem Int* 2000); all are single-pass transmembrane proteins with large extracellular domains which are processed in a similar manner. Moreover, their shared several conserved domains such as E1 and E2 domains in the extracellular sequence, differently the Aβ domain is unique to the APP protein. Since its identification, *APP* has been the focus of several studies, but its physiological function remains largely unknown. The current hypothesis suggested a role for *APP* in neurite outgrowth, synaptogenesis, neuronal protein trafficking along the axon, transmembrane signal transduction, cell adhesion and calcium metabolism (Zheng H, *Mol Neurodegener* 2006). *APP* is proteolyzed into smaller peptides (Zhang YW, *Mol Brain* 2011) during its intracellular trafficking and these *APP* metabolites mediate various functions. More than 25 mutations in *APP* gene can cause EOFAD, between them the most common is the replacement of the amino acid valine with the amino acid isoleucine at protein position 717 in the *APP* increasing the amount of Aβ peptide.

2.1.2 APP PROCESSING

APP is a type I transmembrane protein synthesized in the endoplasmatic reticulum and then transported through the Golgi apparatus to the trans-Golgi-network (TGN)

where the highest concentration of APP is found in neurons at steady state (Xu H, *Proc Natl Acad Sci USA* 1997; Greenfield JP, *Proc Natl Acad Sci USA* 1999). Secretory vesicle then permitted the transport of APP from the TGN to the cell surface where it is either cleaved by α-secretase to produce a soluble molecule: sAPPα (Sisodia SS, *Proc Natl Acad Sci USA* 1992) or re-internalized via an endosomal/lysosomal degradation pathway (Caporaso GL, *J Neurosci* 1994)

2.1.3 THE NON-AMYLOIDOGENIC PATHWAY

Cleavage of APP by α-secretase within the Aβ domain (at the Lys16-Leu17 bond) prevents Aβ generation leading to the formation of a soluble N-terminal fragment (sAPPα) (Esch FS, *Science* 1990). Once synthetized, sAPPα is released into the lumen/extracellular space. A carboxyl terminal fragments (CTFs) of APP (αCTF) remain associated to the membrane, together with βCTF formed by β-secretase during amyloidogenic pathway. αCTF and βCTF will be further cleaved by γ-secretase to generate p83 and a membrane-bound C-terminal fragment (AICD) or and Aβ, respectively. The p83 fragment is rapidly degraded and widely believed to possess no important functions. Differently, AICD is released into the cytoplasm where it activates transcription in the nuclei. The generation of sAPPα by α-secretase is a constitutive event but can also be regulated by various reagents. It was demonstrated that this enzyme is part of a zinc metalloproteinase family (Roberts SB, *J Biol Chem* 1994) composed by several members named ADAM (A Disintegrin And Metalloproteinase). Three of them have been suggested as the α-secretase: ADAM9, ADAM10, and ADAM17. A confirmation of their involvement in α–secretase activity came up from several studies. For example, manipulation of ADAM17 can alter α-cleavage of APP and Aβ generation, with regulated α-cleavage abolished in ADAM17-deficient cells, suggesting that ADAM17 is the α-secretase regulating APP cleavage **(**Buxbaum JD, *J Biol Chem* 1998). Additionally, a strongly reduction of ADAM10 in the platelets of sporadic AD patients correlates with a significant decrease of sAPPα levels (Colciaghi F, *Mol Med* 2002) and a reduced α-secretase activity in the temporal cortex homogenates of AD patients (Tyler SJ, *Biochem Biophys Res Commun* 2002). These studies suggest that ADAM10 represents the constitutive α-secretase at the cell surface. Once released, the sAPPα has an important role in neuronal plasticity/survival and is protective against excitotoxicity (Furukawa K, *J Neurochem* 1996), furthmore sAPPα regulates neural stem cell proliferation and is important for early CNS development (Caille I *Development* 2004). Indeed, so far sAPPα has been showed to have neurotrophic and neuroprotective properties (Habib A, *J Neurosci Res* 2017).

2.1.4 THE AMYLOIDOGENIC PATHWAY

The APP which is not cleaved by α-secretase is processed in the amyloidogenic pathway by the β-secretase also called beta-site amyloid precursor protein-cleaving enzyme 1 (BACE1). BACE1 is a membrane-bound aspartyl protease with a characteristic type I transmembrane domain near the C-terminus (Vassar R, *Science* 1999). BACE1 cleaves APP at the N-terminal position of Aβ, leading to secretion of a smaller ectodomain derivative (APPs). This generates a 99-residue carboxy-terminal fragment (C99 or βCTF), which is inserted into the membrane. The membrane-associated stub C99, can then undergo an intra-membrane division that is mediated by the γ-secretase complex, a special type of aspartyl-protease with a unique active site and cleavage mechanism (Wolfe MS, *Nature* 1999). This complex is composed of PSEN1 or PSEN2, nicastrin, APH1 and PEN2 **(Fig. 3A, B)** (Kimberly WT, P*roc Natl Acad Sci USA* 2003). It has been proved that all four proteins are necessary and sufficient to reconstitute γ-secretase activity in yeast, which lacks these enzymes (Edbauer D, *Nat Cell Biol,* 2003). γ-secretase can carry out multiple intra-membrane cleavages. Current evidence indicates that the "presenilin–γ-secretase complex" can cleave at different sites (referred to as γ, ε and ζ) in the transmembrane domain (TMD). The ε-cleavage close to the cytoplasmic border of the TMD releases the free intracellular domain into the cytosol (Sastre M, *J neuroinflammation* 2008). It seems that the remaining membrane-anchored fragment undergoes an intermediate scission of about 3 N-terminal residues to the ε-cut at the so-called ζ-site (Zhao G, *Biol Chem* 2005). Thereafter, Aβ is released into biological fluids by the final cuts at the γ-site. The γ-cut is variable and occurs after Aβ amino acids 38, 40 or 42. Most secreted Aβ peptides are 40 amino acids in length (Aβ1-40), although the longer fraction of 42 amino acid species (Aβ1-42) have received greater attention due to the propensity of these peptides and other derivatives of the APP to nucleate and drive production of Aβ fibrils. These γ-cleavages have an important influence on the self-aggregating potential and resulting pathogenicity of Aβ, where only the Aβ1-42 peptide has a strong propensity to oligomerize *in vivo*.

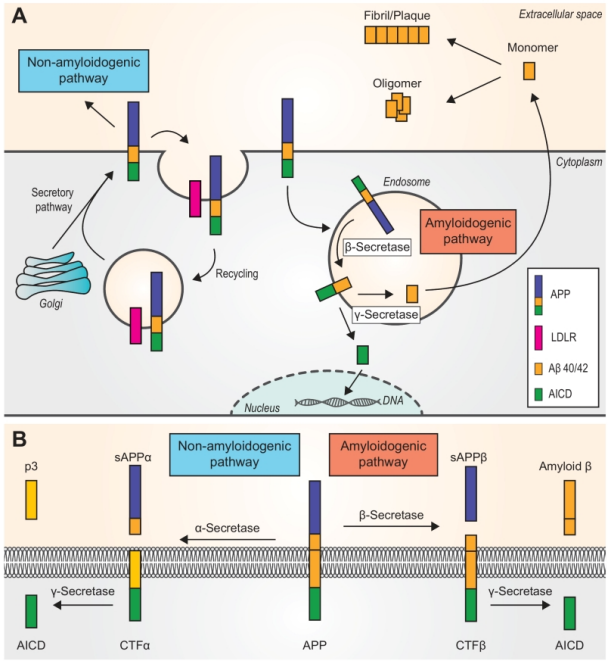


Figure 3. Two pathways of amyloid β (Aβ) peptides generation**.** Picture adapted from Zhou Y, *Cell stress* 2018.

2.2 PRESENILIN GENES

There are 2 presenilin genes that encode two different forms of proteins: *PSEN1* and *PSEN2*.The *PSEN1* gene is located on the long arm of chromosome 14 at position 24.3, from base pair 73,603,142 to base pair 73,690,398 (bp 87,256). *PSEN1* is a protein involved in the activity of γ-secretase. γ-secretase is associated in protein complex at high molecular weight, where *PSEN1* exerts its action. More than 150 *PSEN1* mutations have been identified. These mutations are the most common cause of EOFAD, accounting for up to 70% of cases. Disease shows between 28 and 50 years. Almost all *PSEN1* mutations change single nucleotide of DNA in a specific segment of the gene. These mutations result in the production of an abnormal *PSEN1* protein. Defective *PSEN1* disrupts the processing of APP, leading to the overproduction of Aβ peptide. The *PSEN2* gene is located on the long arm of chromosome 1 between positions 31 and 42, from base pair 227,058,272 to base pair 227,083,803 (bp 25,531). P*SEN2* is a protein homologous to *PSEN1*. Several studies suppose that *PSEN2* may act in synergy with P*SEN1*, in the function of □-secretase. Mutations in *PSEN2* gene account for less than 5% of all EOFAD cases of AD and the onset of the disease is later in age, between 40 and 55 years.

Two of the most common *PSEN2* mutations that cause EOFAD are due by a change of single amino acids. One mutation replaces the amino acid asparagine with isoleucine at position 141. The other mutation changes the amino acid methionine to valine at position 239. These mutations appear to disrupt the processing of *APP*, leading to the overproduction of Aβ peptide. The causes of LOAD are less clear, indeed is probably related to variations in one or more genes in combination with lifestyle and environmental factors (Zhang C, *Nature* 2009; Bekris LM, *J Geriatr Psychiatry Neurol* 2010).

2.3 *APOE4* GENE

The most influential genetic risk factor for LOAD is the allelic variation in the *APOE* gene (Querfurth HW, *N Engl J Med* 2010). The *APOE* gene is located on chromosome 19 at position 13.2, from base pair 45,409,038 to base pair 45,412,649 (bp 3611). *APOE* acts normally to scaffold the formation of high-density lipoprotein (HDL) particles, which promote the proteolytic degradation of soluble forms of Aβ. It is synthesized and secreted primarily by astrocytes and is involved in brain development and repair (Fagan AM, *Microsc Res Tech* 2000). In CNS, *APOE* is mainly expressed by glial cells, and it transports cholesterol to neurons via APOE receptors, and normally promotes proteolytic degradation of soluble forms of Aβ plaques (Liu CC, *Nat Rev Neurol* 2013). *APOE* has three common forms or alleles: ε2, ε3, and ε4. The ε2 form may provide some protection against AD, in fact, data suggest that AD patients with ApoE2 exhibit highly reduced Aβ deposition in the neocortex. (Nagy ZS, *Neuroscience* 1995). Differently, ε3 is thought to play a neutral role. The ε4 form is a known risk-factor gene for the common LOAD, and many studies are underway to clarify its impact. Some evidences suggest that *APOE4* promotes Aβ deposition and tau phosphorylation (Holtzman DM, *Proc Natl Acad Sci USA* 2000; Holtzman DM, *Ann Neurol* 2000a). Furthmore, the APOE protein created from the ε4 allele forms a more linear conformation after a high-fat meal, which may increase permeability of the BBB, and the lipidation state of APOE is also reported to affect degradation and clearance of Aβ plaques (ltman R, *Clin Sci* 2010). Additionally, it has been shown that the E4 allele is more prone to degradation itself and has reduced stability (Dong LM, *J*

Biol Chem. 1994). Most experts believe that, in addition to APOE ε4, at least half a dozen more genes may influence the development of LOAD in some way, but studies are still needed.

3. NEUROPATHOLOGICAL HALLMARKS

3.1 NEUROFIBRILLARY TANGLES

Neurofibrillary tangles (NFTs) are made of paired helical filaments (PHFs), which are fibrils of ≈10 nm in diameter that form pairs with a helical tridimensional conformation at a regular periodicity of ≈65 nm (Kidd M, *Nature* 1963; Kidd M, *Brain* 1964; Wisniewski HM, *J Neurol Sci* 1976). A small proportion of fibrils within the NFTs do not form pairs but give the appearance of straight filaments without the periodicity of PHFs (Crowther RA, *Proc Natl Acad Sci* 1991). The major constituent of NFTs is the microtubule-associated protein tau, which is encoded by single gene on chromosome 17 but is expressed in several molecular isoforms that are generated by alternative splicing of its mRNA (Himmler A, *Mol Cell Biol* 1989). Six isoforms of the protein are known, which differ in the presence or absence of exons 2, 3 or 10. An alternative splicing of exon 10 produces Tau with four (4R Tau) or only 3 (3R Tau) repeated motives (Goedert M, *Embo J* 1989). Normally, tau is involved in the stabilization of microtubules due to the binding with tubulin stabilizing their structure (Cleveland DW, *J Mol Biol* 1977). Aberrant phosphorylation on tau protein renders the protein insoluble, decrease its affinity for microtubules with consequent neuronal degeneration. NFTs can be examined through silver impregnation methods such as the Gallyas technique (Braak H, *Acta Neuropathol* 1991) or alternatively with fluorescent dyes such as Thioflavin-S, which recognize the β-sheet pleated structure of the paired helical filaments (Arnold SE, *Cereb Cortex* 1991) or by immunostaining with anti-tau antibodies **(Fig. 4).** Three morphological stages have been distinguished: (1) Pre-NFTs or diffuse NFTs are defined by a diffuse tau staining within the cytoplasm of normal-looking neurons; (2) Mature or fibrillar intraneuronal NFTs (iNFTs) consist of cytoplasmic filamentous aggregates of tau that alter the nucleus position moving to the periphery of the soma (3) extraneuronal "ghost" NFTs (eNFTs) result from the death of the tangle-bearing neurons and are identifiable by the absence of nucleus

and stainable cytoplasm **(Fig. 5)** (Su JH, *Brain Res* 1993; Braak E, *Acta Neuropathol* 1994; Augustinack JC, *Acta Neuropathol* 2002). Both silver and Thioflavin-S stains, as well as some phosphotau antibodies such as AT8 and PHF1, preferentially identify the iNFTs and the eNFTs (Braak E, *Acta Neuropathol* 1994; Augustinack JC, *Acta Neuropathol* 2002).

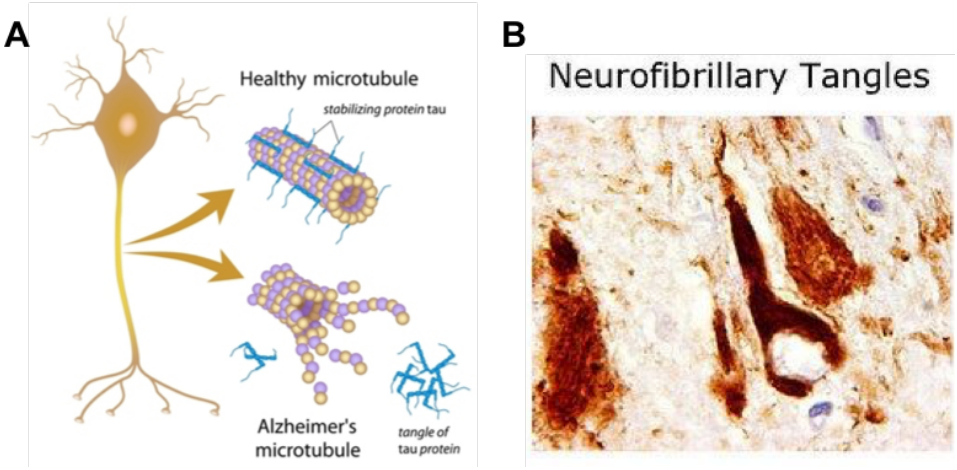


Figure 4. (A). A schematic representation of Tau function for neuronal stabilization in healthy vs AD microtubules. **(B)** Immunostaining in AD-patient derived brain showing neurofibrillary tangles accumulation (http://bigthink.com/articles/the-brain-plaques-and-tangles-that-cause-alzheimers-disease).

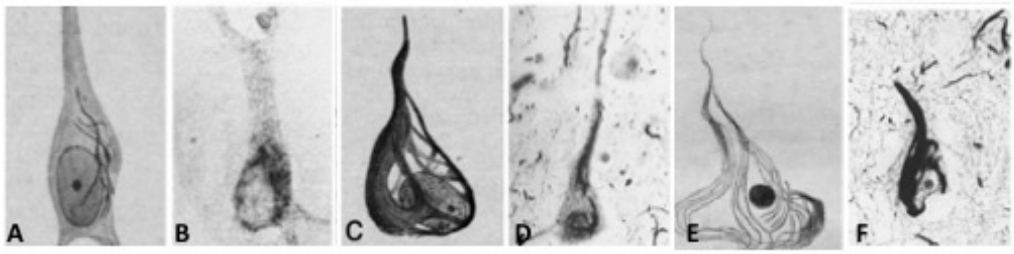


Figure 5. A schematic and histological representation of Pre-NFTs **(A-B)**; Mature-NFTs (**C-D)** and extraneuronal "ghost" NFTs **(E-F).** Picture adapted from Schwab C, *Acta Neurophatologica* 1998.

3.2 AMYLOID PLAQUES

Aβ principal forms are constituted by Aβ1-40 and Aβ1-42 peptides, two normal products of the metabolism of the amyloid precursor protein (APP). These peptides form a variety of structures, including multiple monomer conformers, different types of oligomers, Aβ-derived diffusible ligands (ADDLs), protofibrils and fibrils. Aβ42 due to its higher fraction of fibrillization and insolubility represent the most abundant form present in the plaques. Indeed Aβ1-42 tends to form stable trimeric

and/or tetrameric oligomers, whereas Aβ1-40 does not (Chen YR, *J Biol Chem* 2006). The enhanced production of the Aβ1-42 peptide results from AD-causing mutations in APP and PSEN1 or PSEN2 (Scheuner D, *Nat Med* 1996). Early senile plaques contain mainly Aβ1-42, whereas plaque maturation is associated with the progressive appearance of the shorter Aβ1-40 **(Fig. 6)** (Iwatsubo T, *Neuron* 1994). The Aβ1-40 peptide is more frequently produced than Aβ1-42 by cells and probably by further cleavage of Aβ1-42 peptide. A morphological classification distinguishes two types of Aβ plaques defines as diffuse or dense-core based on their staining with dyes specific for the β-pleated sheet conformation such as Congo Red and Thioflavin-S **(Fig. 7)**. Diffuse Aβ plaques are Thioflavin-S negative, whereas, differently, dense-core plaques are positively labeled with the same method of staining and their presence is relevant to disease prognosis. Diffuse Aβ plaques are commonly present in the brains of cognitively intact elderly people, whereas dense-core plaques, particularly those with neuritic dystrophies, are most often found in patients with AD dementia (Masliah E, *Am J Pathol* 1990; Urbanc B, *Proc Natl Acad Sci* 2002). Forms of soluble Aβ oligomers are detected in the CSF of AD patients showing a direct correlation with the severity of the cognitive impairment. (Santos AN, *J Alzheimers Dis* 2012). Indeed, studies suggested that oligomers could be the most significant biomarkers of early stage in AD pathology and may be used as future therapeutic target for the prevention of cognitive dysfunction (Lue LF, *Am J Pathol* 1999).

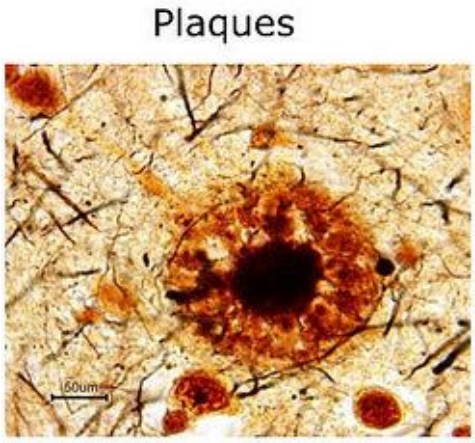


Figure 6. Immunostaining in AD-patient derived brain showing Aβ plaques accumulation. (http://bigthink.com/articles/the-brain-plaques-and-tangles-that-cause-alzheimers-disease)

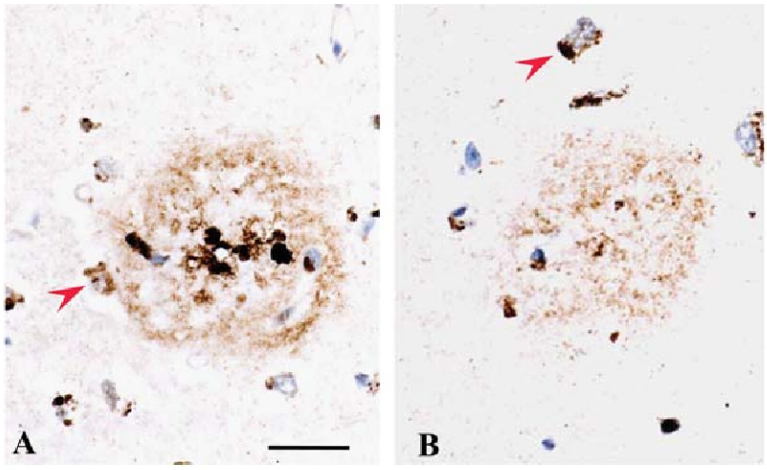


Figure 7. AD tissue sections immunolabeled for Aβ42 showing plaque morphology. **(A)** dense core Aβ and **(B)** diffuse Aβ plaque. (http://bigthink.com/articles/the-brain-plaques-and-tangles-that-cause-alzheimers-disease)

3.2.1 CLEARANCE OF Aβ

Aβ removal from the peripheral blood and lymphatic systems or degradation within the CNS tissues represent a key mechanism for the balance between production and clearance during its steady-state level. (Shibata M, *J Clin Invest* 2000). In pathological conditions, Aβ deposition in the vasculature leads to pro-inflammatory and cytotoxic events that contribute to the accelerated blood-brain barrier (BBB) permeability in the AD brain (Roher AE, *Mol Med* 2003; Erickson MA, *J Cereb Blood Flow Metab* 2013). Aβ reaches the peripheral circulation via chaperone-mediated transport across the BBB, perivascular drainage, or the glymphatic system. Once arrived in the parenchyma, myelomonocytic cells such as monocytes and macrophages are able to phagocytize fibrillar and soluble oligomeric Aβ. Additionally, also neurons and astrocytes contribute to Aβ degradation and are involved in its homeostasis. Several mechanisms mediating an extracellular enzymatic degradation of Aβ have been proposed. For example, angiotensin-converting enzyme (ACE) is a zinc-dependent peptidase highly present in the endothelium and cortical neurons in the brain. Even if this molecule is known for their contribution in the coagulation process, it was also shown to cleave Aβ42 into the less toxic Aβ40 alloform (Hemming ML, *J Biol Chem* 2005). Insulin-degrading enzyme (IDE) is a zinc metalloprotease that is capable of

degrading soluble Aβ40 and Aβ42 into non-toxic fragments. (Kurochkin IV, *FEBS Lett* 1994). Although it is mainly localized in the cytosol, a small portion of IDE is secreted by glial cells (Bulloj A, *JAD* 2010) or expressed on the cell surface of neurons where its acts as critical enzyme for extracellular Aβ degradation (Vekrellis K, *J Neurosci* 2000). Matrix metalloproteinase-9 (MMP-9) are enzymes well known for their role in extracellular matrix degradation, but MMP-9 has been shown to degrade compact plaques (Yan P, *J Biol Chem* 2006) and soluble forms of Aβ42 and Aβ40 (Yin KJ, *J Neurosci* 2006). In the CNS, MMP-9 is expressed by neurons (Backstrom JR, *J Neurosci* 1996), microglia (Gottschall PE, *J Neurosci Res* 1995), astrocytes (Muir EM, *Brain Res Mol Brain Res* 2002), and infiltrating monocytes. Neprilysin (NEP) is a type II integral membrane zinc metalloprotein mainly localized on pre- and post-synaptic neuronal membranes, it is also secreted by microglia and astrocytes (Fisk L, *Neurochem Res* 2007; Hickman SE, *J Neurosci* 2008). It is considered the most powerful Aβ-degrading enzyme able to cleave oligomeric Aβ42 and Aβ40 but not fibrillar forms. Another important mechanism of Aβ catabolism is its removal into the peripheral circulation which represent the majority of physiologic Aβ clearance. Upon Aβ transport cross the BBB made by chaperones. In particular, members of the LDL receptor (LDLR) family, such as the low-density lipoprotein-related protein 1 (LRP-1) and ATP-binding cassette (ABC) transporters, are primary receptors for Aβ efflux (Tarasof-Conway JM, *Nat Rev Neurol* 2015). LRP-1 is expressed in reactive astrocytes and in brain capillary endothelium where it can bind LRP-1 ApoE-Aβ complexes or Aβ alone subsequently stimulating endocytosis of either species. To completely perform its function LRP-1 needs specific adaptor protein, for example phosphatidylinositol binding clathrin assembly protein (PICALM) which is a molecule expressed mainly on endothelial cells acting as adapter protein for the transcytosis of the Aβ-LRP-1 complex across the BBB. ApoE is also a carrier protein that maintains cholesterol and phospholipid homeostasis under physiological conditions. Major ApoE receptors include LDLR, LRP-1, the very low-density lipoprotein receptor (VLDLR), and ApoE receptor 2 (ApoER2). However, the exact role of ApoE in AD pathogenesis remains unclear, but several evidences suggest their importance as carrier protein (Schmechel DE, *Proc Natl Acad Sci USA* 1993; Holtzman DM,

Proc Natl Acad Sci USA 2000). Indeed, the hypothesis is that the complex made by ApoE-Aβ bind to and are internalized by LRP-1 for delivery to the vasculature and removal from the brain. Conversely, the Aβ influx into the CNS is mediated by receptor for advanced glycation end products (RAGE) (Deane R, *Stroke* 2004). RAGE is a multiple ligand-receptor of the immunoglobulin superfamily of cell surface molecules and is expressed on endothelial and glial cells. The binding of Aβ to RAGE leads to the secretion of endothelin 1, a potent vasoconstrictor causing blood flow suppression Down-regulation of RAGE can inhibit the influx of Aβ (Deane R, *Nat Med* 2003) while an excessive amount of Aβ leads to the up regulation of RAGE through a positive-feedback mechanism. Dysfunction in the clearance of Aβ through deregulated LRP-1/RAGE with arterial alteration may initiate neurovascular uncoupling, Aβ accumulation, cerebrovascular regression, brain hypoperfusion and neurovascular inflammation (Deane R, *Curr Alzheimer Res* 2007). In fact, severe AD is associated with significant changes in the relative distribution of RAGE and LRP-1 in the hippocampus, as compared with age-matched controls (Donahue JE *Acta Neuropathol* 2006). Moreover, LRP-1 and other Aβ-binding receptors (LDLR, RAGE, and CD36) were shown to be expressed on pericytes from post-mortem AD brains associated with cerebral amyloid angiopathy (CAA), and in vitro treatment of human pericytes with Aβ induces the expression of LRP-1 and LDLR, suggesting that these receptors are involved in the Aβ-mediated death of cerebral perivascular cells (Wilhelmus MM, *Am. J. Pathol* 2007).

3.3 CEREBRAL AMYLOID ANGIOPATHY

The Aβ peptide not only is able to form deposits in the brain parenchyma as Aβ plaques but also in the vessel walls leading to cerebral amyloid angiopathy (CAA). Indeed, CAA is major constituted by Aβ40 peptide accumulating mainly in the interstitium between the smooth muscle cells of the tunica media. Even if CAA can also appear independently to AD, approximately the ≈80% of AD patients showing mild form of CAA. The true incidence and prevalence of CAA are difficult to be specified, also because the pathologic diagnosis is typically obtained post-mortem. It seems that the failure of the elimination of soluble and insoluble form of Aβ along

with the perivascular pathways that serve as lymphatic drainage channels for the brain, probably causes CAA thus compromising the BBB integrity explaining a link between CAA and AD (Weller RO, *Brain Pathol* 2008). The same methods described for the investigation of Aβ plaques are valid for the CAA: Thioflavin-S or Congo red staining or immunohistochemical studies with anti-Aβ antibodies are largely used to describe the severity of CAA within a single vessel. In that way it is possible distinguish between: "grade 0 or absence of staining; grade 1 or congophilic rim around an otherwise normal-appearance vessel; grade 2 or complete replacement of the tunica media by congophilic material; grade 3 or cracking of ≥50% of the circumference of the vessel, and grade 4 or fibrinoid necrosis of the vessel wall" (Greenberg SM, *Stroke* 1997). CAA usually affects cortical capillaries, small arterioles and middle-size arteries causing a vascular weakening which tend to rupture and lead to intracerebral hemorrhage (Zhang-Nunes SX, *Brain Pathol* 2006). Differently, venules, veins, and white-matter arteries are rarely involved.

3.4 SYNAPTIC LOSS

Synapse loss is another contributor to the cortical atrophy of the AD brain, which can match with neuronal loss although in not the only cause. Indeed, synaptic loss can precede neuronal loss in a specific cortical area, as a result, the remaining neurons become less well connected to their synaptic partners leading to a progressive cognitive decline with is typical in AD (DeKosky ST, *Ann Neurol* 1990; Ingelsson M, *Neurology* 2004). The initial triggers may be Aβ plaques toxicity or disrupted intracellular transport of aggregated tau (Pereira C, *Drug Targets* 2005). Indeed, in the intrasynaptic space the presence of Aβ plaques impairs synaptic plasticity and important postsynaptic receptors, such as acetyl choline receptor. In addition, excitotoxicity, oxidative stress, and apoptosis have all been claimed to contribute to synaptic dysfunction. Studies have shown that in AD patients there is the 55% of synaptic loss in the CA1; instead patients with MCI exhibited 18% synaptic loss in comparison to control cases (Scheff SW, *Neurology* 2007). These evaluations were made using immunohistochemical approaches

(antibodies against pre- or postsynaptic proteins), typically against the presynaptic protein synaptophysin or with electron microscopy studies.

3.5 NEURON LOSS AND BRAIN ATROPHY

During the progression of AD pathology neurons can be damaged altering their physiological function and leading to their death. The cause of this process can be related to the accumulation of fibrillar Aβ forming plaques that damage neurons through a direct or indirect mechanism. In the first case, Aβ interacts with membrane components and damages directly neurons, causing neuronal injury and synaptic dysfunction (Koh JY, *Brain Res* 1990). Whereas, during the indirect processes, Aβ activates microglia and astrocytes to produce inflammatory mediators, as nitric oxide, cytokines and free radicals, causing neurons death for apoptosis or necrosis (Meda L, *Nature* 1995). Together, these events lead to neuronal death in a process called brain atrophy. The severity of the neuronal loss is usually evident in sections stained with hematoxylin and eosin or it can be more readily shown with a Nissl staining or a NeuN (neuronal-specific nuclear antigen) labeling in immunohistochemistry.

4. NEUROINFLAMMATION IN AD

Prior to the early 1990s, one of the main scientific dogma in the neurology field was to consider the brain as an immunologically privileged organ due to the presence of the BBB. It was widely accepted that this barrier was able to prevent the passage of immune cells and humoral factors, whereas resident brain cells were unable to support an efficient immunological response. In the past two decades, the concept of neuroinflammation has evolved and represents nowadays a generally accepted idea whose mechanisms and consequences are still actively under investigation, particularly with regard to AD. Brain inflammation appears to have a dual function playing a neuroprotective role during an acute-phase response, but becomes detrimental when a chronic response is mounted (Kinney JW, *Alzheimer's Dement* 2018). However, it is now clear that during AD the disruption of the BBB mediated by soluble factors and plasma proteins, leads to a close communication with peripheral immune system cells, thus establishing neuro-immune interactions,

supporting the view that neuroinflammation contributes to AD pathology (Quan N, *Brain Behav Immun* 2007; Heneka MT, *Lancet Neurol* 2015). Accordingly, enhanced levels of TNF-α and IFN-γ were typically found up-regulated in the serum of AD patients. Therefore, soluble Aβ oligomers induce expression of vascular adhesion molecules, which may promote leukocyte adhesion and transmigration during AD. Additionally, their soluble forms are released into the circulation, thus providing useful biomarkers of endothelial dysfunction and vascular inflammation (Rentzos M, *J Neurol Sci* 2005; Nielsen E, *Neurobiol Dis* 2007). Indeed, higher levels of soluble VCAM-1, ICAM-1, E-selectin and P-selectin are found in plasma samples from AD patients compared to control subjects, suggesting that vascular inflammation occurs during AD (Zuliani G, *J Neurol Sci* 2008; Huang CW, *Thromb Haemost* 2015). All together, these events may induce the recruitment of cells belonging to both the innate and acquired arm, as demonstrated by several histological and biochemical analyses that showed the presence of immunity associated cells to the brain parenchyma in AD, especially in and around classical AD-defining histological lesions like the senile plaques and NFTs (Rogers J *Neurobiol Aging* 1988; Griffin WS, *Proc Natl Acad Sci USA* 1989). In this scenario, the characterization of molecular mechanisms controlling vascular inflammation and leukocyte trafficking could therefore help to determine the alterations developing during AD and lead to the discovery of new therapeutic approaches.

4.1 BLOOD-BRAIN BARRIER LEAKAGE IN AD

The neurovascular unit (NVU) is composed by microglia, astrocytes, neurons, brain endothelium, pericytes, vascular smooth muscle cells (VSMC), and perivascular macrophages. Dynamic communication between the cells of the NVU is required for normal brain functioning. Numerous studies suggest that neurovascular dysfunction contributes to the onset and progression of AD. Indeed, during AD vascular anatomical defects are observed mainly associated to an atrophy and irregularities of arterioles and capillaries, swelling and increased number of pinocytic vesicles in endothelial cells, increase in collagen IV, heparan sulfate proteoglycans and laminin deposition in the basement membrane, disruption of the basement membrane and reduced total microvascular density

(Zlokovic BV, *Acta Neuropathol* 2009). Although age-dependent deterioration of BBB normally occurs during aging in the human hippocampus, several studies showed a more accelerated degradation in patients with mild cognitive impairment (MCI) suggesting that this phenomenon contributes to early cognitive impairment (Montagne A, *Neuron* 2015). Aβ deposition in the vasculature leads to pro-inflammatory and cytotoxic events that contribute to the greater BBB permeability in the AD brain (Roher AE, *Mol Med* 2003; Erickson MA, 2013). The accumulation of Aβ on the cerebral blood vessels induce CAA, thus altering the integrity of the blood vessel wall resulting in micro or macro intracerebral bleedings that exacerbates neurodegenerative process and inflammatory response and may lead to hemorrhagic stroke. Furthermore, evidence from *in vitro* studies and transgenic mouse tauopathy models suggests that tau may also promote BBB deterioration (Vidal R, *Acta Neuropathol* 2000; Forman MS, *J Neurosci* 2005; Kovac A, *J Alzheimers Dis* 2009). BBB dysfunction correlates with the appearance of perivascular tau around major hippocampal blood vessels (Blair LJ, *Acta Neuropathol Commun* 2015). Thus, both tau and Aβ may directly promote the loss of BBB integrity, exacerbating the neurodegenerative process and associated inflammatory responses. Besides, a decreased of cerebral blood flow (CBF) typically occurrent in AD patients negatively affects the synthesis of proteins required for memory and learning and may eventually lead to neuritic injury and neuronal death. Furthermore, impaired clearance of Aβ from the brain by the cells of the neurovascular unit may lead to its accumulation on blood vessels and in brain parenchyma. Finally, it is now clear that the BBB has the ability to respond to soluble factors and plasma proteins, and to communicate with peripheral immune system cells thus establishing neuro-immune system interactions, supporting the view that neuroinflammation contributes to AD pathology (Quan N, *Brain Behav* 2007; Heneka MT, *Lancet Neurol* 2015).

4.2 MICROGLIA ACTIVATION

The key mediators of the neuroinflammatory response in the brain are its resident immune cells, microglia. Microglia represent the 10–15% of all cells found into the CNS. Generally, microglia cells are identified by Iba1 staining that allows

unequivocal morphological differentiation of microglia status. In a healthy brain, microglia are in a "resting" state and are described morphologically as ramified cells with small soma **(Fig. 8)** (Glenn JA, *J Anat* 1992). In this conformation the cell some is stationary, and the cell processes are used to constantly survey the presence of pathogens and cellular debris, simultaneously contributing to the protection and modeling of synapses for the maintenance of neurons plasticity (Kettenmann H, *Physiol Rev* 2011; Ji K, *PLoS ONE* 2013). The surveillance action is mediated by various receptors for classical neurotransmitters, receptors for various cytokines and chemokines, and a number of receptors, such as fractalkine (CX3CR1), that bind ligands constitutively released in healthy neuronal environments (Lee YB, *J Neurosci Res* 2002). Once activated by pathological triggers, microglia enlarge their soma and extend the processes to the site of injury, and later start migrating to the lesion and initiate an innate immune response. In AD, microglia are able to bind to soluble Aβ oligomers and Aβ fibrils via receptors including class A scavenger receptor A1, CD36, CD14, α6β1 integrin, CD47 and toll like receptors (TLR2, TLR4, TLR6 and TLR9) (Bamberger ME, *J Neurosci* 2003; Liu Y, *Brain* 2005). As a result, activated microglia starts to produce proinflammatory cytokines and chemokines exacerbating neuroinflammation and neuronal damage. Additionally, the constant microglia activation reduces their efficacy to bind and phagocytose Aβ. Besides, more recent data indicate that, as microglia become less able to clear Aβ, peripheral macrophages may be recruited to Aβ plaque deposition in an effort to clear Aβ (Jay T, *J Exp Med* 2015). The recruitment of peripheral macrophages into the brain likely exacerbates the effects of sustained inflammation and thus AD pathology. Finally, recent data demonstrated that a rare missense mutation in the gene encoding for Triggering Receptor Expressed on Myeloid Cells 2 (TREM2), which was predicted to result in an R47H substitution, was found to be related to a significant higher risk to develop AD (Jonsson T, *N Engl J Med* 2013). This result was confirmed by another study in which researchers showed an upregulation of TREM2 on myeloid cells surrounding Aβ deposits in AD mouse models and human AD tissue. However, the cell types and mechanisms underlying TREM2 involvement in neurodegeneration remain to be completely established (Jay TR, *J*

Exp Med 2015). Furthermore, in response to Aβ or NFTs, microglial cells produce proinflammatory cytokines, chemokines, and complement peptides, which can recruit leukocyte subpopulations through the BBB into the brain during brain inflammation (Heneka MT, *Lancet Neurol* 2015). Several reports indicate that microglia promote the sustained migration of lymphocytes and monocytes through the BBB into the CNS during brain inflammation (Persidsky Y, *Am J Pathol*. 1999; Hudson LC, *Brain Res* 2005; Lécuyer MA, *Biochim. Biophys. Acta* 2016). Indeed, in a later disease stage, chronic activation of microglia is accompanied by diminished phagocytosis and more abundant secretion of pro-inflammatory cytokines, leading to the accumulation of Aβ and the amplification of neuroinflammation (El Khoury J, *Nat Med* 2007; Heneka MT, *Lancet Neurol* 2015). Furthermore, IL-1β, a major pro-inflammatory cytokine released from activated microglia, increases the permeability of the BBB and abolishes the ability of astrocytes to maintain BBB integrity (Wang Y, *PLoS One* 2014). Studies in animal models revealed that the stimulation of microglia with Aβ or injection of Aβ 1-42 oligomers lead to the release of proinflammatory cytokines such as IL-1β and TNFα. As a result, these cytokines promote the production of chemokine CXCL8 by endothelial cells (Lou J, *J Neuroimmunol* 1997; Zhang W, *J Cereb Blood Flow Metab.* 2000; Eisenhauer PB, *Microb Pathog* 2004; Galanakis E*, Eur Cytokine Netw*. 2006). Interestingly, T cells of AD patients are enriched in the chemokine receptor CXCR2 which, upon binding to CXCL8, promotes T cell transmigration across endothelial barriers, including the BBB (Liu Y, *J Neurobiol Aging* 2010). A more permeable BBB together with stimuli inducing lymphocytes extravasation triggered by microglia may exacerbate brain neuroinflammation.

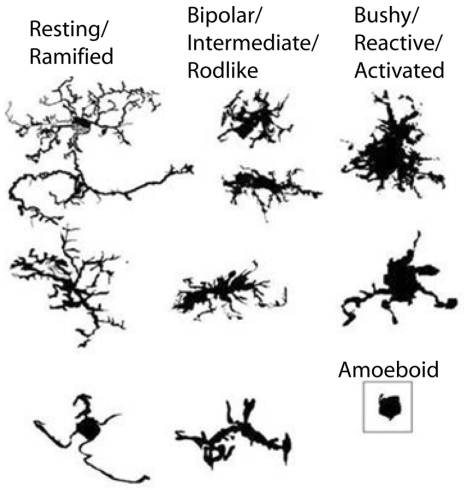


Figure 8. A schematic representation of progressive changes in microglia morphology associated with distinct activation status. Picture adapted from Fernández-Arjona M, *Front Cell Neurosci* 2017.

4.3 ASTROCYTES IN AD

Astrocytes are specialized glial cells existing over fivefold than neurons in the brain where they exert many essential complex functions in the healthy CNS. Astrocytes respond to different forms of CNS insults starting a process called astrogliosis which has become a pathological hallmark of CNS structural lesions. Expression of glial fibrillary acid protein (GFAP) has become a classical marker for immunohistochemical identification and classification of astrocytes, indeed traditionally astrocytes have been distinguished in protoplasmic and fibrous. Protoplasmic astrocytes are cortical astrocytes, with low or absent GFAP immunoreactivity in normal conditions, and with profuse ramification of their processes in fine prolongations through which they can reach the pre-and post-synaptic elements of the neurons. By contrast, fibrous astrocytes are GFAP-immunoreactive astrocytes found in the white matter along the myelinated axon. In physiological condition astrocytes have critical function in CNS development promoting the formation of excitatory synapses and to engulf and eliminate both excitatory and inhibitory synapses in adult brain and prenatal development (Allen NJ *Nature* 2012). Furthermore, astrocytes can modulate synaptic activity transmission and plasticity mainly by the re-uptake of glutamate from the synaptic cleft through their membrane glutamate transporters GLT-1 (also called excitatory amino acid transporter 2 or EAAT2) and GLAST (also called excitatory amino acid

transporter 1 or EAAT1). Finally, astrocytes represent an essential structural part of the BBB together with endothelial cells, basement membrane and pericytes (Hall CN *Nature* 2014). It was demonstrated that astrocytes in the BBB compartment are able to coordinate blood flow with neuronal activity in a process known as *neurovascular coupling* (Mishra A *Nat. Neurosci* 2016). In addition, astrocytes control water flux between the brain and the bloodstream and are involved in the perivascular clearance of toxic solutes. In neurodegenerative diseases, like AD, a relationship between reactive astrocyte and Aβ plaque is widely accepted. Indeed, senile Aβ plaques commonly are surrounded by cluster of reactive astrocytes that embrace Aβ deposits with their processes isolating them from the surrounding cells **(Fig. 9)** (Itagaki S, *J. Neuroimmunol* 1989), moreover neuropathological studies on postmortem brains demonstrated a higher occurrence of reactive astrocytes in the proximity of Aβ plaques that increases with the disease progression **(Fig. 10)** (Vehmas AK, *Neurobiol Aging* 2003). Finally, studies report that reactive astrocytes can take up and degrade extracellular deposits of Aβ42 (Wyss-Coray T, *Nat Med* 2003) and that this function is attenuated in ApoE−/− astrocytes (Koistinaho M *Nat Med* 2004) suggesting that reactive astrocytes functions or dysfunctions could play a role in the progression and severity of AD. Whereas an increase in GFAP intensity level associated with a strongly astrocytes activation and later stages of AD, the levels of astrocyte glutamate transporters have been reported to decline, which may increase the vulnerability of local neurons to excitotoxicity (Simpson JE, *Neurobiol Aging* 2008). Reactive astrocytes also exhibit increased expression of presenilin in sporadic AD, but the consequences of this expression are not known (Weggen S, *Neuroreport* 1998). Furthermore, astrocytes as part of the BBB, actively contribute to the immune responses especially by integrating signals from the periphery and the brain. Studies revealed that unstimulated human astrocytes *in vitro* express cytokines and chemokines such as granulocyte and granulocyte-macrophage colony stimulating factors (G-CSF, GM-CSF), CXCL1, IL-6, IL-8, monocyte chemoattractant protein 1 (MCP-1/CCL2) and migration inhibitory factor (MIF) (Choi SS, PLoS One. 2014). In addition, in response to stimuli classically associated with neuroinflammation (e.g IL-1β and TNFα), cultured human astrocytes produce IL-1β, IL-1ra, TNFα,

interferon gamma-induced protein 10 (IP-10/CXCL10), macrophage inflammatory protein 1 alpha (MIP-1α/CCL3), soluble ICAM-1 (sICAM-1) and complement component 5 (Choi SS, PLoS One. 2014). Besides, astrocytes could regulate T cell recruitment through chemokine production (e.g CCL5, CCL2, CCL3, CCL12, CXCL1, CXCL2, CXCL8, CXCL10), although, the exact role of astrocytes on T cell recruitment needs further investigation (Dong Y, *Glia* 2001, Choi SS, *PLoS One* 2014). Together, these data suggest that soluble factors released by astrocytes may promote the trafficking of cells related to innate and acquired immunity in the brain (Choi SS, *PLoS One*. 2014).

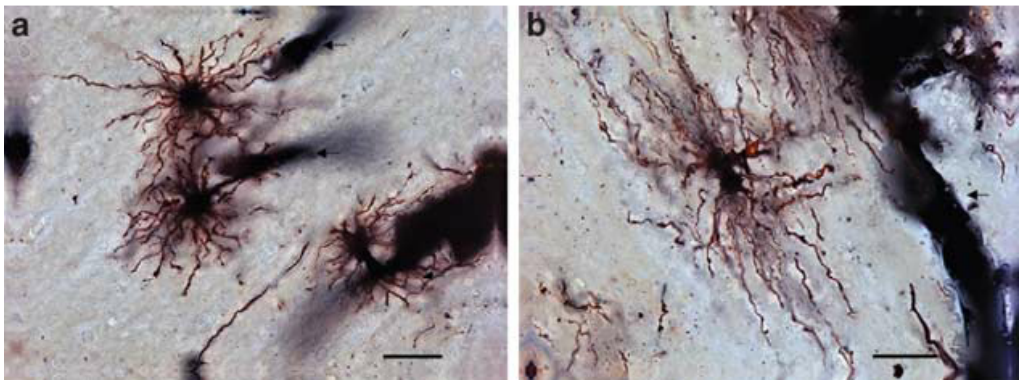


Figure 9. Representative examples of Golgi-stained protoplasmic and fibrous astrocytes in the human brain. Protoplasmic astrocytes **(A)** showed a characteristic spherical cell body, with tortuous varicose and thorny processes radiating in all directions. Fibrous astrocyte **(B)** presented a more oblong cell body, with relatively long and unramified varicose and thorny processes extended mainly in two opposite directions. Picture adapted from Torres-Platas SG, *Neuropsychopharmacol.* 2011).

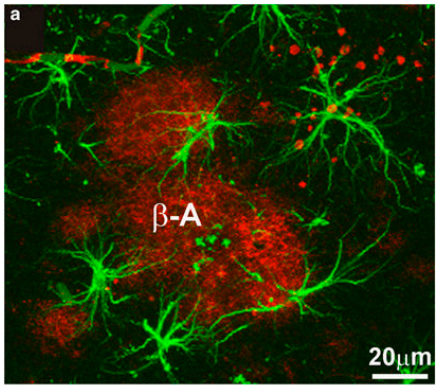


Figure 10. Confocal image showing GFAP-positive (green) reactive astrocytes surrounding Aβ plaques. Picture adapted from Rodríguez JJ, *Cell death and differentiation* 2009.

5. THE CONTRIBUTION OF IMMUNE CELLS IN AD PATHOGENESIS

The immune system is composed of an army of cells whose duty is to protect us from invasion by external pathogens. Several evidences have erased this ideology and demonstrated that dysfunction in the immune system may be a primary factor in neurodegenerative disease, such as AD. The suggestion that inflammation may participate in Alzheimer's disease pathogenesis was first articulated about 20 years ago by documented presence of phenotypically activated microglia that surrounded amyloid deposits in the brain and invested the plaques with their processes (Akiyama H, *Neurobiology of Aging* 2000). Afterwards, other publications confirmed those findings, and, accordingly, in the brain of AD subjects elevated levels of cytokines and other immune mediators were reported, reflective of a chronic inflammatory environment (Wyss-Coray T, *Cold Spring Harb Perspect Med* 2012). More than 30 Alzheimer disease risk loci have been identified via a combination of genetic linkage studies, candidate gene studies, genome-wide association studies (GWAS) and whole-genome sequencing and/or whole-exome sequencing studies (Pimenova AA, *Biol. Psychiatry* 2017). Of these risk loci, over 50% of gene variants validated by functional genomics are implicated in microglial and innate immune cell function, including the top two risk genes, *APOE* and *TREM2* **(Fig. 11) (**Karch CM, *Biol Psychiatry* 2015)**.** In addition, epigenomic analysis has shown that Alzheimer disease GWAS loci are preferentially enriched in enhancer sequences implicated in innate immune processes (Gjoneska, E, *Nature* 2015). Variants in genes involved in lipid metabolism, the inflammatory response, and endocytosis have been identified through these GWAS. These findings indicate an essential role of the innate and adapative immune response in Alzheimer disease pathogenesis.

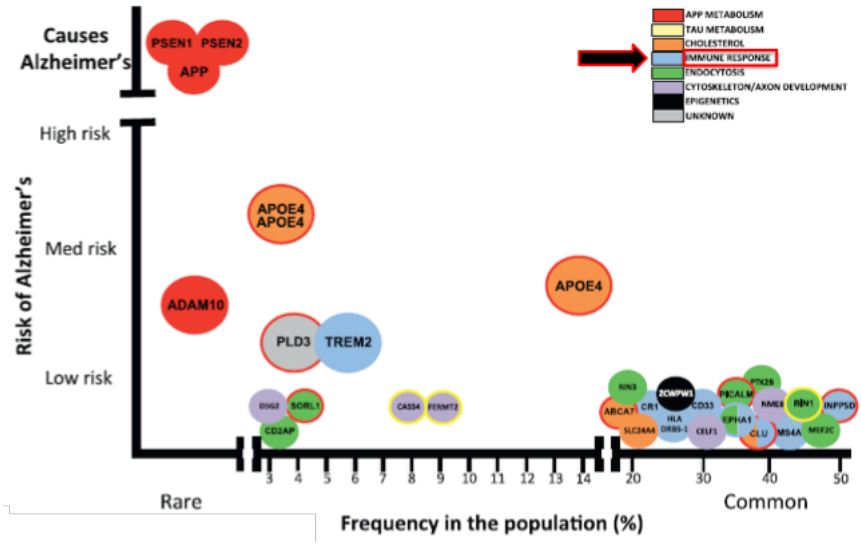


Figure 11. Rare and common variants contribute to Alzheimer's disease risk. Picture adapted from Karch CM, *Biol Psychiatry* 2015.

5.1 NEUTROPHILS IN AD

Neutrophils, also known as polymorphonuclear (PMN) leukocytes, are produced in the bone marrow in large numbers and represent the most abundant cell type in human circulatory system (Mocsai A, *J Exp Med* 2013). Neutrophils are important effector cells in the innate immunity and their most well-known function is defending the host against infectious pathogens. Neutrophils have a very short half-lives for this reason they promote very rapid responses like phagocytosis, degranulation and release of nuclear material in the form of neutrophil extracellular traps (NETs). NETs are composed by decondensed chromatin and antimicrobial/granular proteins that allow neutrophils to eliminate extracellular pathogens system (Mocsai A, *J Exp Med* 2013). Under homeostatic conditions, neutrophils enter the circulation, migrate to tissues to perform specific functions and finally are eliminated by macrophages. Additionally, they also facilitate the repair of wounds and mediate inflammation resulting from infectious and sterile injuries (Kruger P, *PLoS Pathog* 2015). In addition to their classical functions, it is known that they are able to respond to numerous signals and activate a well-organized transcription programs which lead to the production of cytokines and other inflammatory factors that influence and regulate inflammation and modulate the activities of nearby cells (Nauseef WM, *Nat Immunol* 2014; Scapini P, *Blood.*

2014) Tecchio C, *Semin. Immunol* 2016). Recently, the involvement of neutrophils in AD has emerged, however their role of is poorly understood. The first report was published in the 2002, where researchers found a higher level of CD11b in neutrophils isolated from the blood of patients with sporadic form of AD compared to controls, demonstrating a close correlation between CD11b up-regulation and the severity of mental decline (Scali C, *Neurobiol Aging* 2002). Other studies disclosed increased levels of ROS in peripheral blood neutrophils from AD patients compared with controls confirming a function for neutrophils in AD development which may exist in a more activated state during AD (Vitte J, *J Clin Immunol* 2004). Recently, two reports published in mouse model of AD confirmed a pivotal role of neutrophils in AD pathogenesis (Baik SH, *Neurobiol Aging* 2014; Zenaro E *Nat Med* 2015). In 2014, Baik et al. showed through 2-photon microscopy that neutrophils enter in the brain parenchyma of 5XFAD mice and accumulate around Aβ plaques. In agreement, Zenaro et al. demonstrated increased neutrophil accumulation in the brain parenchyma of 3xTg-AD mice, and authors hypothesized the mechanism of neutrophils recruitment into the brain during AD. They demonstrated that Aβ increases the affinity state of LFA-1, a leukocyte integrin expressed on neutrophils able to bind ICAM-1 expressed by activated brain endothelial cells. In AD mice devoid of LFA-1, neutrophils did not infiltrate the brain parenchyma, moreover these mice showed an amelioration of cognitive function, decreased microgliosis and Aβ1–42 deposition together with a reduction in hyperphosphorylated tau protein levels. Recently, a study focused on the evaluation of modification in cerebral blood flow (CBF) in AD patient and related mouse models showed that events of stalled flow was due to neutrophils that had adhered in capillary segments and blocked blood flow. As a confirmation, treatment against neutrophil marker Ly6G reduced the number of stalled capillaries leading to an increase of CBF and to an improvement in spatial and working memory (Cruz Hernández JC, *Nat Neurosci* 2019). Once established the presence of neutrophils in AD brain and their possible involvement with the pathogenesis of the disease, several studies investigated the mechanisms used by brain accumulating PMN to exacerbate neuroinflammation. In that scenario, intraparenchymal migrating neutrophils produce NETs and this mechanism could be triggered by Aβ. Indeed, it

was shown that neutrophils migrate inside the parenchyma in areas of Aβ plaques. Aβ as damage-associated molecular patterns (DAMP) is capable to promote ROS release by activating NADPH oxidase. The production of ROS is an essential step for NET formation, thus indirectly Aβ may further contribute to intravascular NETosis through its interaction with the receptor RAGE expressed on brain endothelial cells, thus promoting the generation of ROS and the secretion of pro-inflammatory cytokines (Giri R, *Am J Physiol Cell Physiol* 2000; Askarova S, *Neuroscience* 2011). Finally, pro-inflammatory cytokines, (e.g TNFα, IL-1β, and IL-8) produced by neural cells or activated endothelial cells can trigger the formation of NETs by neutrophils during AD (Keshari RS, *PLoS One* 2012). However, the mechanisms of neutrophil-dependent damage in AD are still unknown. Taking advantage from viral infection or autoimmune diseases it is possible to suppose that, once released, NETs could activate plasmacytoid dendritic cells and mediate the priming of T cells through TLR9. Adaptive immune system cells are encountered in the AD brain, and they may play a role in AD pathogenesis, but it is unclear whether NETs link the innate and adaptive immune responses in AD.

5.2 T CELLS IN AD

T lymphocytes are cells of the adaptive immune system originating from hematopoietic stem cells (HSCs) present in the fetal liver and later on during the evolution in the bone marrow. In these organs, HSC differentiate firstly in multipotent progenitors and then in lymphoid progenitors. A small portion of these immature cells migrate to the thymus, where differentiate into early thymic progenitors (ETP), which will become gradually reprogrammed into fully mature and functional T cells with a complete T cell receptor (TCR). Classically this process has been divided in four steps depending on the expression of the surface marker CD4 and CD8. Initially, the hematopoietic progenitor also called double negative (DN), does not express CD8 and CD4 on its surface (Ellmeier W, *Annu. Rev Immunol* 1999). Then, the successful assembly of the segments of TCR allows the formation of a pre-TCR complexes which without the recognition of self-peptide, induce the activation of CD4 and CD8 genes with the formation of

CD4/CD8 double-positive (DP) cells (Krangel MS**,** *Curr. Opin Immunol* 2009). These CD4/CD8 DP thymocytes express a complete antigen receptor (αβTCR) that can recognize self-peptides with the assistance of CD4/CD8 co-receptors. A small proportion of DP cell will be positively selected to become a CD4+ or CD8+ single-positive (SP) thymocytes that finally released into the periphery as naive T cells (Klein L**,** *Nat Rev Immunol* 2014). TCR has a key role in antigenic recognition. In particular, it cannot recognize antigens in their natural form, but it binds only to linear peptides that have been processed and presented by the Human Leukocyte Antigen-I (HLA-I) or HLA-2 present on the surface of Antigen Presenting Cells (APCs), such as dendritic cells, macrophages and B-lymphocytes. T-cells can be successfully activated only if 3 signals are present (Pennock ND, *AJP Adv Physiol Educ* 2013):

- Interaction between TCR and the peptide presented by the HLA molecule
- Presence of cytokines, fundamental to drive the subsequent clonal expansion
- Presence of the co-stimulatory receptors CD3 and CD4 or CD8

Thus, after maturation, T-lymphocytes are released into the blood and lymphatic vessels, where, under pathological conditions, can meet APCs undergoing activation. In particular, depending by the nature and the concentration of the antigen, the type of APC and the cytokines present in the microenvironment in that moment, T-cells could differentiate in two different classes: (1) Helper T-cells CD4 positive and (2) Cytotoxic T-cells CD8 positive (Pennock ND, *AJP Adv Physiol Educ* 2013). These two main classes are also divided in specific subpopulations: CD4+ T-cells are divided into naïve and memory populations, which can differentiate into Th1, Th2, Th9, Th17 and regulatory T-cells (Treg) subpopulations, whereas CD8+ T-cells can differentiate into $CD8^{high}$ and $CD8^{low}$ subpopulations, depending on the pattern of cytokines expressed by the cell in a given moment of its life.

5.2.1 CD4+ T CELLS IN AD

Naive CD4+ T cells may interact with APCs that express the antigen presented by HLA class II complexes thus leading to the activation of specific signaling pathways (Stockwin LH. *Immunology and Cell Biology* 2000). Four main distinct fates are

open with the generation of: Th1, Th2, Th17, and induced regulatory T (iTreg) cells (however, other Th lineages named Th9, Th22 and Tfh may exist) (Zhu J, *Blood* 2008) **(Fig. 12).** Each Th subset releases specific cytokines with either pro- or anti-inflammatory functions, survival or protective functions. Th1 are generated mainly following infections by intracellular bacteria and some viruses, they release interferon-gamma (IFN-γ), interleukin-2 (IL-2) and tumor necrosis factor-beta (TNF-β) which activate macrophages and are responsible for cell-mediated immunity and phagocyte-dependent protective responses. Th2 cells mediate host defense against extracellular parasites including helminths. They are important in the induction and persistence of asthma and other allergic diseases. Th2 cells produce IL-4, IL-5, IL-9, IL-10, IL-13, IL-25, and amphiregulin which mediate a strong antibody production, eosinophil activation, and inhibition of several macrophage functions, thus providing phagocyte-independent protective responses (Romagnani S, *Inflamm Bowel Dis* 1999). Th17 cells, so called because of their IL-17 release, play an important role in host defense against bacteria, and fungi (Raphael I, *Cytokine* 2015). Diversely, Treg secretes mainly IL-10, a cytokine with an immunosuppressive function that maintain the expression of FOXP3 transcription factor needed for suppressive function of Treg on other cell types (Murai M, *Nat. Immunol* 2009). Emerging studies highlighted the involvement of CD4+ T cells in neuroinflammation processes. Indeed, the increase permeability of the BBB during AD allows the infiltration of circulating immune cells from periphery into the brain, although the precise mechanisms are still matter of debate. McManus and coworkers studied the influence of a common infection (Bordetella pertussis) on AD pathology. Using APP/PS1 mice they showed that both Th1 and Th17 cells infiltrate from the blood into the brain during AD evolution releasing IFN-γ and IL-17, respectively. These phenomena into the brain lead to glial activation and Aβ accumulation exacerbating neuroinflammation (McManus RM, *Neurobiol Aging* 2014). Later, Browne et al. confirmed the above-mentioned findings. They observed that the injection of Th1 cells increase Aβ accumulation and microglial activation and impairs performance in Morris water maze test in 6 months old APP/PS1 mice (Browne TC, *J Immunol* 2013). These effects are attenuated treating mice with anti–IFN-γ Abs as the principal Th1 cytokine. In contrast to the effect of Th1 cells on APP/PS1 mice, transfer of Th2 cells or Th17 cells exerted no

effect in the spatial learning task or on either Aβ accumulation or Aβ plaque deposition (Browne TC, *J Immunol* 2013). These findings are coherent with previous demonstration that Aβ-specific Th1 cells enhance proinflammatory cytokine production, MHC class II and costimulatory molecule expression by Aβ-stimulated microglia, whereas Aβ-specific Th2 cells suppress cytokine production by glial cells (McQuillan K, *Brain Behav Immun* 2010). In 2016, an interesting publication highlighted the importance of Tregs in APP/PS1 mice, a mouse model of AD. Authors showed that the depletion of Tregs accelerates the onset of cognitive deficits due to reduced recruitment of microglia towards Aβ deposits. Tregs amplification through IL-2 treatment induced an enhance of Aβ plaque-associated microglia and improves cognitive functions in APP/PS1 mice (Dansokho C, *Brain* 2016). Accordingly, Baek H. et colleagues address Tregs function in 3xTg-AD mice, a model of both Aβ and tau pathology. They showed that transplantation of Tregs improved cognitive function and reduced deposition of Aβ plaques. As a confirmation, Tregs depletion at early stage of the disease (4 months of age) exacerbate spatial learning deficits, together with a higher deposition of Aβ plaques detected in the hippocampus compared to control mice (Baek H, *Oncotarget* 2016). Confirmation in that sense came also from studies on humans, where an overall decreased in the frequency of Treg was described in patients with mild Alzheimer's disease as compared to age-matched controls (Larbi A, *Adv Exp Med Biol* 2008).

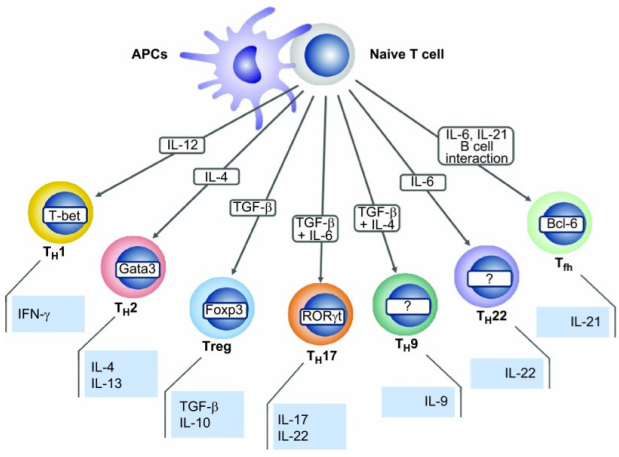


Figure 12. Different T helper cell subsets. Upon activation, naïve precursor T cell differentiates into effector T cell subsets, producing different cytokines and mediating distinct effector functions. Picture adapted from Wu RQ, *Int J Oral Sci* 2014).

5.2.2 CD8+ T CELLS IN AD

During an infection, naive CD8+ T cells recognize an antigen that is presented by HLA-I on APCs surface in lymph nodes and spleen. This event triggers CD8+ differentiation to effector cells, also called cytotoxic T lymphocytes (CTLs). (Zhang N, *Immunity* 2011). Once activated, they are ready to destroy infected or malignant cells using three distinct pathways, two of which involve direct cell–cell contacts between effector and target cells. The interaction induces the activation of FAS-FASL pathway or the release by CTLs of perforin and granzymes into the intercellular space. In both cases, the result is the apoptosis of target cells. The third mechanism is mediated by cytokines, such as IFN-γ and TNF-α which are produced and secreted subsequentially to the prolonged TCR stimulation. TNF-α binds its receptor on the target cells inducing apoptosis via the caspase pathway. Diversely, IFN-γ stimulates the presentation of endogenous peptides by MHC class I and increases FAS-mediated target-cell lysis (Hufford MM, *J Exp Med* 2011). A predominance of extravascular CD8+ T cells was primary observed in the brain of AD patients (Togo T, *J Neuroimmunol* 2002; Merlini M, *Neurodegener Dis* 2018). Furthermore, a higher proportion of activated CD8+ T cells was observed in the CSF of patients with an extensively relationship to AD-typical neuropsychological deficits (Lueg G, *Neurobiol Aging* 2015). This data from patients were also confirmed in AD-related mouse models. Indeed, Laurent C et al. reported the accumulation of CD8+ T cells in the hippocampus of THY-Tau22 mice, a model of hippocampal tau pathology associated with progressive cognitive deficits. Authors observed a high ratio of CD8+ T lymphocytes accumulating in the hippocampus of THY-Tau22 mice near an area exhibiting hyper-phosphorylation of tau species. Previous studies revealed an import role of microglia activation in the development/spreading of tau pathology and associated memory deficits (Yoshiyama Y, *Neuron* 2007; Gorlovoy P, *FASEB J* 2009; Bhaskar K, *Neuron* 2010; Asai H, *Nat Neurosci* 2015). Thus, to deeply understand the functional mechanism of recruitment of CD8+ T cells, they investigated microglia gene expression. An upregulation of CCL3, CCL4 and CCL5 was observed, and among them CCL3 was demonstrated to induce a CD8+ T cell infiltration in the hippocampus of tau transgenic mice. Interestingly, CD8+ lymphocyte infiltration

was also observed in the cortex of patients exhibiting frontotemporal dementia with P301L tau mutation. These data suggest that tau-triggered innate neuroinflammatory responses may promote CD8+ T cell infiltration into the brain of patients with AD (Laurent C, *Brain* 2017). In another publication using ArcAβ mice which develop only Aβ pathology, researchers showed an involvement of CD8+ T cell in AD pathogenesis. Indeed, flow cytometric analysis on whole brain homogenization revealed an increased number of CD8+ T cells at later time point of the disease (12 and 24 months of age) compared to control mice. In contrast, their results do not support a cytotoxic role for T cells in AD. Indeed, brain accumulating CD8+ T cells did not show a proliferative phenotype and neither display effector function (Ferretti MT, *Brain Behav Immun* 2016). Although nowadays the cause of the low CD8 T-cell proliferation and activation state in Aβ-burdened brains is not clear, one of the hypotheses is that Aβ may directly impair antigen presentation activity (Grant JL, *Sci Transl Med*. 2012; Ferretti MT, *Brain Behav Immun*). Based on the reports published so far on the role of CD8+ T cells in AD, it is suggested that these cells may be involved in tau pathology. However further experimental evidences are needed to understand their role in disease pathogenesis.

6. MECHANISMS FOR LEUKOCYTE RECRUITMENT IN THE BRAIN

Leukocyte recruitment to sites of inflammation is a key mechanism in the immune responses and consists of a multistep process regulated by adhesion molecules and chemoattractant **(Fig. 13)**. Leukocytes enter the CNS *via* three distinct routes: the first is from the blood to the parenchyma through the walls of parenchymal post-capillary venules; the second is from the blood to the subarachnoid space through the walls of meningeal vessels; and the third is from the blood to the CSF across the venule wall and then the stroma and epithelium of the choroid plexus. (Ransohoff RM, *Nat Rev Immunol* 2003). Under inflammatory conditions, the first two routes are used by leukocytes for CNS invasion, whereas the last route is considered the major site of CNS immunosurveillance under physiological conditions (Ransohoff RM, *Nat Rev Immunol* 2003;Engelhardt B, *Trends Immunol* 2005; Man SM, *Neurobiol Aging* 2007; Shechter R*, Nat Rev Immunol* 2013). Thus,

during several inflammatory diseases leukocyte recruitment represent a fundamental hallmark. Following brain invasion, they can induce, amplify and maintain inflammation, whereas the blockade of this process is critical for the resolution of the inflammatory processes. The leukocyte recruitment cascade is a sequence of adhesion and activation events in the site of inflammation that ends with the extravasation of leukocytes. The simplified classical four steps model involves: 1) rolling, mainly mediated by selectins; 2) chemokine-mediated activation; 3) arrest and 4) transmigration, mainly mediated by integrins (Ransohoff RM, *Nat Rev Immunol* 2003; Luster AD, *Nat Immunol* 2005). However, progress has been made in defining additional steps such as capture (or tethering), slow rolling, integrin-mediated leukocyte adhesion strengthening and spreading (post-binding phase of adhesion stabilization) and intravascular crawling (Ley K, *Nat Rev Immunol* 2007). The specificity of leukocyte migration is mediated by the expression patterns of cell adhesion molecules and chemokine receptors. Expression of high levels of specific cell adhesion molecules on endothelium is the consequence of an inflammatory condition in that specific district. Molecular specificity in the targeting of leukocytes at sites of inflammation is mediated by selectins, integrins, and immunoglobulin gene superfamily (Ig superfamily) proteins. The recruitment initiates with leukocyte tethering and rolling on the activated vascular endothelium. The primary capture is mainly mediated by selectins, a three-member family of highly conserved "C-type lectins" expressed on the surface of leukocytes and activated endothelial cells. The selectins are identified by capital letters: L for leucocyte (L-selectin), E for endothelial cell (E- selectin), and P for platelet and endothelial cell selectin (P-selectin). Some primary inflammatory cytokines released by activated leukocytes, induce up regulation of selectins and other adhesion molecules, chemokines, growth factor and lipid mediators (prostaglandins and nitrogen monoxide) amplifying leukocyte recruitment and their survival in the tissue. For instance, Tumor Necrosis Factor-α (TNF-α) and interleukin-1 (IL-1) stimulate the endothelial cells lining blood vessels to express the surface adhesion molecule P-selectin (Eppihimer MJ, *J Physiol Heart Circ Physiol.* 1997). Within few hours, a second surface adhesion molecule, E-selectin, is produced. L-selectin is expressed on most circulating leukocytes and is

the key receptor that initiates leukocyte capture events in high endothelial venules in secondary lymphoid tissues and at the peripheral sites of injury and inflammation (Rosen SD, *Annu Rev Immunol* 2004). L- and P-selectin are particularly efficient tethering molecules. P- and E- selectin are expressed in both acutely and chronically stimulated endothelial beds and are important determinants for neutrophil, monocyte, natural killer cell, eosinophil, effector T cell and B cell recruitment in most inflammatory processes. The interaction of selectins with their ligands enable leukocytes to roll along the inflamed vascular endothelial surface under condition of blood flow, thus allowing other molecules to interact with the "slowed" leukocytes and promoting their adhesion and transmigration into the tissue. Selectins bind sialyl-Lewis X-Like carbohydrate ligands presented by sialomucin-like surface molecules. P-selectin can bind PSGL-1 (P-Selectin Glycoprotien Ligand) which is constitutively expressed on all lymphocytes, monocytes, eosinophils, and neutrophils. Another P-selectin ligand is CD24, which appears to be important for tumor cell binding. L-selectin recognizes sulfated sialyl-Lewis X-like sugars in high endothelial venules and other ligands on inflamed endothelial cells as well as PSGL- 1 on adherent leukocytes. E-selectin can also interact with PSGL-1 and another sialyl-Lewis X-bearing glycoconjugates. The requirement for selectins in primary cell capture and rolling has also been confirmed by experiments using transgenic mice deficient for L-selectin, E-selectin, P- selectin, and for the prototypic ligand PSGL-1 (Mayadas TN, *Cell* 1993; Xia L, *J Clin Invest* 2002). Integrins also participate in rolling and mediate firm leukocyte adhesion. Integrins are a large family of heterodimeric transmembrane glycoproteins whose ligand-binding activity can be rapidly regulated by conformational changes as well as by transcriptional induction and redistribution from intracellular pools (Carman CV, *Curr Opin Cell Biol* 2003). Integrins consists of 2 bound subunits: α (120-170 kDa) and β subunits (90-100 kDa). One of the most important integrin is the αLβ2 (CD11a/CD18) integrin, also known as Lymphocyte Function- Associated Antigen-1 (LFA-1), which not only participates in rolling but also in the adhesion and arrest of leukocytes in lymphoid organs or in inflamed tissues by linking the Intercellular adhesion molecule-1 (ICAM1) and Intercellular adhesion molecule-2 (ICAM- 2) (Rossi B, *Journal of Leukocyte Biology* 2011). Another prominent

integrin expressed on leukocytes is the α4β1 integrin, the Very Late Antigen-4 (VLA-4, CD49d/CD29) that binds the vascular adhesion molecule-1 (VCAM-1), and it is also essential for leukocytes adhesion to vascular endothelium and leukocyte recruitment to the inflamed area (Luster AD, *Nat Immunol* 2005). On circulating leukocytes integrins are generally in a low affinity/avidity state and do not bind efficiently to their ligands expressed on endothelial cells. Rolling allows leukocytes to encounter activation factors, such as chemoattractants or chemokines, which bind to specific seven transmembrane receptors coupled to intracellular heterotrimeric Galphai proteins. Binding of chemokines to their respective receptors on the leukocyte surface leads to the so called "inside-out- signalling" rapidly up-regulating integrin avidity and/or affinity (Ley K, *Nat Rev Immunol* 2007). Once activated, integrins can interact with cell adhesion molecules from the immunoglobulins (Ig) superfamily expressed on endothelial cells. The vascular endothelium expresses molecules of the immunoglobulin superfamily which act as counter-receptors for leukocyte integrins. Two immunoglobulins particularly important in leukocytes recruitment are ICAM-1 and VCAM-1. ICAM-1 (or CD54) is a member of the Ig superfamily of adhesion molecules and contains 5 Ig like domains. It is one of the principal ligands for LFA-1 and Mac-1 integrins (Diamond MS, *Cell* 1991), although in the context of transmigration it seems that CD11a predominantly binds to ICAM-1, whereas CD11b is more promiscuous (Shang XZ, *Eur J Immunol* 1998). VCAM-1 (or CD106) contains six or seven Ig domains and is expressed on both large and small vessels only after the endothelial cells are stimulated by cytokines. The sustained expression of VCAM-1 lasts over 24 hours. Primarily, VCAM-1 is an endothelial ligand for VLA-4 and α4β7 integrins. VCAM-1 promotes the adhesion of lymphocytes, monocytes, eosinophils, and basophils. After slowing down their movement and arresting on endothelial cells, leukocytes pass through the crawling phase to find the optimal site of emigration that is usually different from the site of initial adhesion. There are regions defined "gates" for leukocytes transmigration with low matrix protein deposition in the venular basement membrane that facilitate their transmigration (Sanz MJ, *Eur J Immunol* 2012). The final step of the cascade is the transmigration through endothelial cells in inflamed tissue; this process involves migration of leukocytes

through two distinct barriers, namely the endothelial cell layer and the perivascular basement membrane. Of note, two ways of leukocyte diapedesis have been reported *in vivo* and *in vitro* models: the "paracellular way" that is the most prevalent type of extravasation processes, and the "transcellular route" for neutrophils and subsets of activated effector T cells (Ley K, *Nat Rev Immunol* 2007).

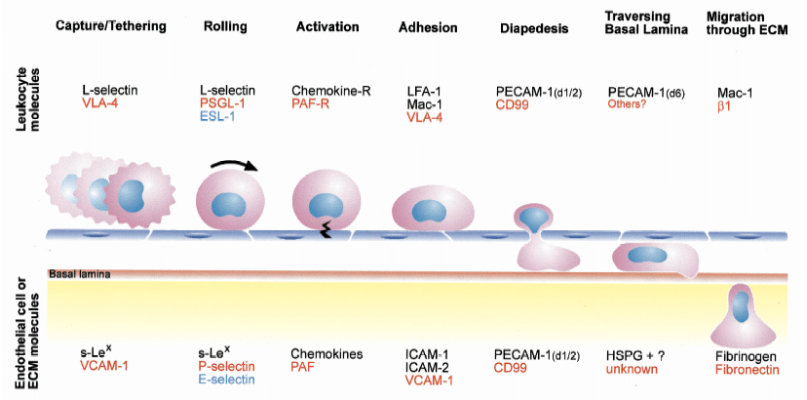


Figure 13. Representation of the sequential steps in leukocyte extravasation and the various adhesion molecules and receptors involved. Picture adapted from Calderwood JW. Book: Lymphocyte Homing to the Skin: Immunology, Immunopathology, and Therapeutic Perspectives 2005.

6.1 THE ROLE OF INTEGRINS IN LEUKOCYTE TRAFFICKING

Integrins exist as heterodimers on leukocyte membrane composed of one α and one β subunit. In humans, 18α and 8β subunits have been identified that in combination form at least 24 different heterodimers. Each subunit contains an extracellular domain involved in ligand binding, a single transmembrane domain, and a cytoplasmic domain, which regulates integrin function. The association of both subunits at N-terminal end forms the ligand- binding site, whereas the C-terminal region traverses the plasma membrane and interacts with cytoskeleton. Integrins are bi-directional signaling molecules and binding to their ligands results in intracellular signals and conversely, cellular signaling events can modulate their affinity for extracellular ligands. The most relevant integrins known to mediate leukocyte arrest belong to the β1 and β2 subfamilies. β2 integrins include 4 different heterodimers: Lymphocyte function-associated antigen 1 (LFA-1, CD11a/CD18), Macrophage-1-antigen (Mac-1, CD11b/CD18), p150,95 (CD11c/CD18), and CD11d/CD18. The most studied β2-integrin involved in leukocyte recruitment is

LFA-1, which participates in rolling interactions but predominantly mediates the firm adhesion/arrest of leukocytes in the blood vessels of lymphoid organs or at sites of inflammation by binding the Ig superfamily ligands ICAM-1 and ICAM-2 (CD102), expressed by the vascular endothelium (Luster AD, *Nat Immunol* 2005; Ley K, *Nat Rev Immunol* 2007). The integrin LFA-1 has a heterodimeric structure with two subunits: CD18 and CD11a, the first is common at all β2-integrins and the second is specific for LFA-1. Additionally, LFA- 1 contains an extracellular domain of 200 aminoacidic residues (I-domain) with modulatory activity. LFA-1 I-domain has a high homology with the A-domain of Von Willebrand Factor (vWFA) and cartilage matrix protein. LFA-1 is found in three distinct conformational states. The inactive low- affinity state shows a bent structure with the ligand-binding headpiece (αI domain) in close proximity to membrane-proximal stalk region. In the intermediate affinity state, LFA-1 extends the stalk regions shifting the molecule to the active higher-affinity conformation, where LFA-1 exhibits extended extracellular domain with the ligand-binding pocket for ICAM-1. Whereas, the most important β1-integrin expressed on leukocytes is Very Late Antigen-4 (VLA4, CD49d/CD29),which binds to its ligand VCAM-1, and is chiefly responsible for leukocyte adhesion to vascular endothelium and leukocyte recruitment to the inflamed area (Luster AD, *Nat Immunol* 2005; Ley K, *Nat Rev Immunol* 2007). VLA-4 has a heterodimeric structure and unlike any other integrin alpha subunit, the intact (150 kDa) alpha 4 subunit of VLA-4 can sometimes be cleaved into two noncovalently associated fragments (80 and 70 kDa). A major structural difference between VLA-4 and LFA-1 integrins is the presence of an additional "inserted" I-domain, which implies the difference in ligand binding kinetics. The binding of the fluorescent ligand to LFA-1 integrin was extremely slow without inside-out activation (at rest), compared to VLA-4 integrin. This suggests that an additional structural mechanism prevents rapid binding of the ligand to the resting LFA-1 integrin. For this blocking mechanism, LFA-1 integrin is not able to support cell rolling leading to the requirement for the selectin-mediated rolling step. On contrary, for VLA-4 integrin, the binding of the small fluorescent probe was not obstructed in its bent conformation. This physiological difference suggests that LFA-1/ICAM-1-mediated interactions will be more

difficult to establish. Accordingly, from a biological perspective seems that an additional protective mechanism for the binding of a ligand to the LFA-1 binding site indicates an additional "check" for adaptive immune responses, where immune cell interaction can directly lead to unwanted, or excessive immune activation resulting in cell and tissue damage. Therefore, VLA-4 integrin could be involved in innate antigen-independent immune responses, while the LFA-1 integrin in adaptive immunity (Chigaev A, *Front Immunol* 2012). Although the general mechanism that governs this conformational dependence of affinity has been laid out by structural and biophysical studies, a complete understanding of the intrinsic dynamics of the VLA-4 integrin is currently lacking. However, one major difference in leukocyte recruitment is that while LFA-1 integrin is mainly involved in leukocytes firm adhesion, VLA-4 integrin directly participates in leukocyte tethering and rolling on the endothelium. In addition, signaling from α4 integrins can stimulate β2 integrin-dependent leukocyte adhesion. Thus, leukocyte arrest and spreading can be mediated directly by VLA-4 ligation and indirectly via α4 regulation of β2 integrin-dependent adhesion (Kummer C, *Biochem Pharmacol* 2006). Unlike the β2-integrin-dependent recruitment pathway, which dominates under normal and acute inflammatory conditions, α4-integrin might play roles in mediating neutrophil recruitment in chronic inflammatory processes (Johnston B, I*mmunol Today* 1999). As a result, these two integrins have a different role in the immune system. It appears that VLA-4, representing an ancient integrin family expressed on leukocytes, is predominantly related to certain types of innate antigen-independent non-specific immune responses, where no significant role for LFA-1 is shown. LFA-1 is predominantly related to the signaling pathways, where antigen-dependent adaptive immunity plays a critical role. Starting from the emerging role of immune cells in AD progression, the study of the role of leukocyte recruitment could be an important step in the pathogenesis of AD, so it may represent a therapeutic target for AD. Nowadays, a complete effective drug for AD has not yet been found. The current treatments are prescribed for treatment of mild to moderate AD symptoms and slowdown progression of the disease, helping people with AD to maintain their mental functions. Unlike current treatments, a different and possible therapeutic approach may be turned towards other directions. The

blockade of leukocyte recruitment may lead to suppression of the immune responses and this approach has been successfully proved for the treatment of multiple sclerosis (MS), a chronic inflammatory, demyelinating disease of CNS and systemic lupus erithematosus (SLE). For example, Natalizumab (Tysabri®), the monoclonal humanized antibody against the integrin VLA-4, inhibits the adhesion and migration of lymphocytes in cerebral parenchyma, and consequently prevents the damage of nervous cells in MS patients (Mitroulis I, *Pharmacol Ther* 2015). Additionally, Efalizumab was largely use for the treatment of moderate-to-severe psoriasis able to bind and block the function LFA-1 and thus interfere with T-cell activation. Therefore, it can be hypothesized that a possible intervention aimed to block leukocyte recruitment may have beneficial effect also in AD.

7. NEW IN VITRO MODELS TO STUDY DISEASE PATHOGENESIS

The recent failure of several clinical trials for AD therapy led scientists to reason on animal model limitations and on the necessity of new powerful tools. To this end, one possible avenue is the development of novel *in vitro* models able to recapitulate disease phenomena and discover the best targets to take forward into clinical trials. Several primary and immortalized cell lines are currently in use to study the pathogenesis of neurodegenerative diseases in two-dimensional (2D) and in three-dimensional (3D) cultures. Moreover, since the discover of new methods for the generation of induced pluripotent stem cell (iPSCs) from somatic cells (e.g. fibroblast) from both human healthy subjects and patients' enormous progresses were made (ref review). Thus, patients-derived iPSCs provided a source of cells that harbor specific genetic variants associated with unique pathogenesis in a definite microenvironment (Yagi T, *Hum Mol Genet* 2011; Israel MA, *Nature* 2012; Paquet D, *Nature* 2016). Initially, iPSCs culture were kept in a two-dimensional layer with the enormous disadvantage of creating an environment often homogeneous that poorly mimic the tissue complexity. Subsequently, new discoveries allowed the use of iPSCs to develop 3D culture models, named organoids (Lancaster MA, *Nat Prot* 2014; Clevers H, *Cell* 2016), that provide a physiological self-organization not induced by externally added growth factors or morphogens where cellular connections are preserved (Renner M, *EMBO J* 2017).

Organoids culture captured key aspects of real organs in a micrometric or millimetric scale, besides they can be successfully used to model human diseases and have a wide range of applications in basic research, drug discovery and regeneration medicine (Rossi G, *Nat Rev Genet* 2018).

7.1 STUDY OF ALZHEIMER'S DISEASE IN A DISH

Actually, one of the biggest limitation of the animal models which are largely used in AD research is that they are not fully able to model human AD pathology and may successfully mimic only the familiar form of AD, which is the rarest form of the disease (De Strooper B, *Cell* 2014; Henley DB, *Curr Med Res Opin* 2014). Based on these considerations, a new field to model AD using *in vitro* systems came out making possible new avenues for the discover of AD therapeutics approaches. Several researcher groups have reported the use of induced pluripotent stem cells (iPSCs) technology for AD modeling (Hu BY, *Nat Protoc* 2009; Yagi T, *Hum Mol Genet* 2011; Israel MA, *Nature* 2012). Yagi and colleagues firstly reported in 2011 the possibility to generate iPSCs from fibroblasts of familial Alzheimer's disease patients (FAD) harboring mutations in PSEN1 (A246E) and PSEN2 (N141I) and they demonstrated an increase in Aβ42 secretion along with an elevated Aβ42 to Aβ40 ratio in the iPSC-derived neurons as compared with those from non-AD controls (Yagi T, *Hum Mol Genet* 2011). In 2012 a new publication showed the possibility to reprogram primary fibroblasts from patients with both familial and sporadic forms of AD. They reported the presence of significantly higher levels of Aβ40 and active glycogen synthase kinase-3β (aGSK- 3β), along with hyperphosphorylation of tau. However, the relationship between these major biochemical markers of AD was found to be unclear (Israel MA, *Nature 2*012). Additionally, the treatment of purified neurons derived from these AD patients with β-secretase inhibitors significantly reduced p-tau and aGSK-3β levels, whereas the γ-secretase inhibitor showed no effects when compared to controls, suggesting a direct crosstalk between APP proteolytic processing in the activation of GSK-3β and phosphorylation of tau in human neurons (Israel MA, *Nature 2*012). Together with iPSCs technology, the development of efficient methods to modify the genome of living cells allowed Paquet and colleagues in 2016 to generate through

CRISPR/Cas9 technology knock-in human neurons which harbor heterozygous and homozygous FAD mutations (APP^{swe} and $PSEN1^{M146V}$) giving the possibility to efficiently insert specific changed sequences facilitating study of human disease (Paquet D, *Nature* 2016). Although, all these 2D cell cultures showed failures in the accurate reproduction of AD hallmarks. Aβ levels generated in these models may be insufficient for the formation of plaques and other downstream pathological features related to it, furthermore the short-term duration of neurons in culture may not be sufficient to replicate age-dependent pathogenic events typically seen in AD human brain (D'Avanzo C, *Bioessays* 2015). Most importantly, 2D cultures do not take in account other neuronal cells such us microglia and astrocytes that are reported to play a supporting role in AD pathogenesis (Nagele RG, *Neurobiol Aging* 2004; González-Reyes RE, *Front Mol Neurosci* 2017). These limitations led to the development of more accurate Alzheimer's 3D culture-based approaches. In this scenario, in 2014 was developed the first 3D *in vitro* model using an immortalized human cell line (ReN), recapitulating both senile plaques and NFTs not observed previously in most AD animal models or 2D in vitro models. ReN cells utilized in that models recapitulated multiple FAD mutations overexpressing both human APP (K670N/M671L, and V717I) and PSEN1 (ΔE9). The results demonstrated a high production levels of Aβ40 (~nine-fold), Aβ42 (~17-fold), and elevated Aβ42/Aβ40 (~five-fold) as compared with control ReN cells (Choi SH, *Nature* 2014; Choi SH, *US Neurol* 2015; Choi SH, *Mol Neurodegener* 2016). Interestingly, ReN mutated cells could differentiate in both neurons and glia cells representing a key aspect of Choi's model. Moreover, to avoid Aβ diffusion within the culture media, researchers used matrigel supports (D'Avanzo C, *Bioessays* 2015). The matrix was mainly composed by extracellular matrix proteins and it was able not only to limit Aβ diffusion but also supported cells growth simulating *in vivo* conditions (Choi SH, *Nature* 2014; Choi SH, *US Neurol* 2015; Choi SH, *Mol Neurodegener* 2016). Nowadays, emerging cerebral organoid cultures take advantages from self-organization capability of pluripotent stem cells or neural stem cells (e.g ReN) into structures that mimic the organ structure (Lancaster MA, *Nature* 2013; Jorfi M, *Adv Healthc Mater* 2017). Cerebral organoids (COs) represent the latest avenue for AD models *in vitro* permitting the generation of organized cortical-like structures

similar to human cortex. Human ReN cells were initially used to generated neurospheroids carrying FAD mutations inside microwells-based arrays, which displayed both Aβ plaques and p-tau after 8 weeks of culture (Jorfi M, *Adv Healthc Mater* 2017).Whereas, in 2016 was developed the first AD patient derived iPSC differentiated 3D culture model composed of neurospheroids derived from 5 patients with sporadic form of AD (Ochalek A, *Alzheimers Res Ther* 2017). Researchers cultured the cells using both traditional 2D and spheroid-based 3D formats and exposed them to BACE1 and γ-secretase inhibitor drugs observing different effects. They theorized that differences in individual genetic background (APOE genotypes) of the patients may be responsible for the variation in efficacy of the inhibitor (Lee HK, *PLoS ONE* 2016). Additionally, they observed that the reduction in Aβ levels after the treatment with inhibitors was lower in the 3D neurospheroids model compared with the 2D culture, possibly due to a reduction in the infiltration of drugs in the former. Few years later, was published an innovative work in which was described the elaboration of a more physiological model of AD brain organoids generated from iPSC from adult fibroblast of FAD and Down syndrome patients (Gonzalez C *Mol Psychiatry* 2018). The COs was highly reproducible and developed the two main pathological features of AD: Aβ and p-tau protein aggregates. Additionally, measurements of caspase-3 activation also indicated neuronal death in that AD brain organoid in a manner proportional to the accumulation of protein aggregates. However, some limitations still remain, and future improvement are needed. In Cos, the proportion of different nerve cells does not reflect human brain composition. Furthermore, they are mainly composed by neurons with a small portion of glial cells and the absence of oligodendrocytes. Finally, COs do not establish mature synaptic connections and lack vascularization site (e.g blood brain barrier) (Huch M, *Develop* 2017). All together these aspects clearly underling that this powerful technology needs to be improved to successfully recapitulate all the aspects of AD pathology. Despite the huge progresses made in these years, several aspects of these models need to be improved. In particular, by adding glia and immune cells, mimicking the blood stream and trying to develop standard protocols to decrease the variability between laboratories. For example, the development of new technological devices such as

microfluidic and brain-on-a-chip will revolutionize this field, enabling to mimic the blood stream and add complexity to the culture system.

MATERIALS AND METHODS

8. REAGENTS

The chemical reagents used in this study were: anti-CD4-VioBlue (Clone REA796, Miltenyi Biotec), Viobility™ 405/520 Fixable Dye (Miltenyi Biotec), anti-CD8a-VioBright-FITC (Clone: REA610, Miltenyi Biotec), anti-CD45-PE (Clone: REA737, Miltenyi Biotec), anti-CD3-PerCP-Vio700 (Clone: REA641 ,Miltenyi Biotec), anti-CD11b-PE-Vio770 (Clone: REA592, Miltenyi Biotec), anti-CD11a/CD18-FITC (Clone REA880, Miltenyi Biotec), 7-AAD Viability Staining Solution (Biolegend), anti CD3 (clone REA641), anti CD4 (clone REA769), anti-cd49d PE-Vio770 (clone R1-2, Miltenyi Biotec), Viobility™ Fixable Dyes (Miltenyi Biotec). We used non-conjugated anti-mouse Ionized calcium binding adaptor molecule-1 antibody (Iba-1) (Wako), anti-human Aβ (clone 6E10, Covance), anti-human total tau antibody (clone HT7) and anti-human phospho-tau (Thr231) antibody (clone AT180) (Thermo Fisher). Biotinylated secondary antibodies were purchased from Sigma-Aldrich.

9. ANIMALS

The transgenic AD animal models used were 3xTg-AD and LFA-1-deficient mice (*Itgal*$^{-/-}$) mice purchased from 'The Jackson Laboratory' (Sacramento, CA). 3xTg-AD mice were previously obtained by co-microinjecting two independent transgenes encoding human APPSwe and the human tauP301L (both under control of the mouse Thy1.2-regulatory element) into single-cell embryos harvested from homozygous mutant PS1M146V knock-in (PS1-KI) mice (Oddo S., et al., 2003). 3xTg-AD mice are of a mixed 129/C57BL6 genetic background. The Aβ plaques and NFTs deposition are detected in correlation with age; Aβ deposits initiate in the cortex and progress to the hippocampus, whereas tau pathology is first apparent in the hippocampus and then progresses to the cortex (Oddo S, *Neuron* 2003). The extracellular Aβ deposits start in frontal cortex at 6 months of age and become marked at 12 months of age in cortical regions and hippocampus. By 15 months, Aβ plaques are apparent in posterior cortical regions such as the occipital and parietal cortices, suggesting a related regional dependence to the Aβ deposits in

3xTg-AD mice. NFTs are evident at 12-18 months of age in hippocampus, thus suggesting that their formation may be influenced by generation of Aβ (Oddo S, *Neuron* 2003; Billings LM, *Neuron* 2005). The first memory deficits in 3xTg-AD mice are detectable at 4 months of age, such as deficits in long-term retention, and correlates with intracellular deposits of Aβ plaques in hippocampus and amygdala. Interestingly, this early cognitive dysfunction may be associated with early stages of MCI. At 6-months of age, these mice start presenting difficulties to retain the information from day by day. The continued accumulation of Aβ is likely to account for the continued decline of the cognitive phenotype to include short-term, as well as long-term, memory deficits (Billings LM, *Neuron* 2005). We also used LFA-1 knockout mouse (also called CD11a or *Itgal*$^{-/-}$ mutant mice) was generated by using a targeting vector containing neomycin resistance gene driven by the mouse RNA polymerase II promoter. The neomycin resistance gene was used to disrupt a 2.1 kb region containing exons 1 and 2. The construct was electroporated into 129s7/SvEvBrd-Hprt b-m2 derived AB2.1 embryonic stem (ES) cells. Correctly targeted ES cells were injected into recipient C57BL/6 blastocysts and chimeric male were mated with C57BL/6 female to obtain the LFA-1 mutant mice. Then, we crossed 3xTg-AD and *Itgal*$^{-/-}$ mice to obtain a transgenic mouse with all transgenes that characterized 3xTg-AD and LFA- 1$^{-/-}$ models (APPSwe, tauP301L, PS1M146V knock in and LFA-1 knockout). 3xTg-AD, 3xTg-AD*x*$Itgal^{-/-}$, WT and *Itgal*$^{-/-}$ mice were housed in pathogen-free climate-controlled facilities and allowed to have food and water ad libitum. Experiments in mice were approved by the board of the Interdepartmental Center of Experimental Research Service from the University of Verona and by the Italian National Institute of Health and followed the principles of the US National Institutes of Health Guide for the Use and Care of Laboratory Animals and the European Community Council directive (86/609/EEC).

10. ISOLATION OF BRAIN LEUKOCYTES AND FLOW-CYTOMETRY ANALYSIS

Mice were anesthetized and perfused through the left cardiac ventricle by injection of cold PBS. The brain was homogenized by gentleMACS™ Dissociator (Miltenyi Biotec) and digested with 20 U/ml of DNaseI and 1 mg/ml collagenase at 37°C for 45 min. Cells were isolated by passing the digested tissue through a 70-μm cell strainer, resuspended in 30% Percoll and loaded onto 70% Percoll. Tubes were then centrifuged at 1300 x g for 20 min at 4°C. Cells were removed from the interphase, washed and labelled with the following anti-mouse antibodies: anti-CD45-PE (clone REA737, Miltenyi Biotec), anti-CD11b-PE-Vio770 (clone REA592, Miltenyi Biotec), anti-Ly6g-APC-Vio770 (clone REA526, Miltenyi Biotec), anti-CD3-PerCP-Vio700 (clone REA641,Miltenyi Biotec), anti-CD8α-Viobright-FITC (clone REA601, Miltenyi Biotec) anti-CD4-Vioblue (clone REA796, Miltenyi Biotec). Cell viability was checked with Viobility 405/520 Fixable Dye. Labelled cells were acquired by flow cytometry with MACSQuant Analyzer (Miltenyi Biotec). Data were analyzed using FlowJo software.

11. MOUSE TREATMENT WITH ANTI-CD4 AND ANTI-CD8α DEPLETING ANTIBODIES

Anti-CD4 (Clone GK1.5, BioXCell) or an anti-CD8α (Clone 2.43, BioXcell) depleting antibody were used to induce a pharmacological depletion of CD4+ or CD8+ T-lymphocytes respectively. Anti-RAS antibody (Hybridoma Y13259) was used as control antibody and should not affect any mouse activity. The mAbs were diluted into sterile endotoxin-free PBS at concentration of 0,22mg/ml before each injection. The treatment was performed on 3xTg-AD mice and age-related sex matched controls at approximately 6 months of age. The treatment was continued for 4 weeks in 3xTg-AD mice until behavioral testing. Mice were selected in the bases of specific inclusion and exclusion criteria. For instance, mouse body weight (20-35g) was checked and mice with body weight higher than 40 g were excluded from the experiments. In particular, the medians of the weight of the female mice for each group were: anti-RAS= 27 g; Anti-CD4= 25.3 g ; anti-CD8α= 26 g. Thus, the median of the weight of the entire female group was 26 g (SD = 0.85). For the

male group the median of the weight for each group were: anti-RAS = 32 g; anti-CD4= 30.3 g; anti-CD8α= 32 g. Thus, the median of the weight of the entire male group was 32 g (SD = 0.88). Age and sex-matched animals were used for AD-like disease models and WT control mice. Animals with evident physical defects, such as loss of the whiskers or dwarfism and cutaneous defects (i.e. alopecia) were excluded from the experiment. Cellular depletion of CD8+ or CD4+ T-cells was confirmed by flow cytometry using a blood sample obtained from a mouse one day after the treatment.

12. MOUSE TREATMENT WITH AN ANTI-α4 INTEGRIN BLOCKING ANTIBODY

Anti-α4-integrins mAb (clone PS/2) was used to block leukocyte recruitment from peripheral circulation to inflammatory sites. Anti-human RAS mAb (hybridoma Y13259) was used as control antibody and should not affect any mouse activity. The mAbs were diluted into sterile endotoxin-free PBS at a concentration of 1mg/mL. mAbs were injected i.p. at a dose of 0,5 mg per mouse in the first treatment at approximately 22 weeks of age in 3xTg-AD mice. Then, mice were injected with 300 μg of mAbs i.p. every second day. The treatment was continued for approximately 4 weeks in 3xTg-AD mice until behavioral testing. Control mice were injected with endotoxin-free PBS. Mice were selected on the bases of the same inclusion and exclusion criteria described in the previous section.

13. BEHAVIOURAL ASSESSMENTS

Learning and memory capacity were assessed using Y maze and Contextual Fear Conditioning (CFC) tests. 8-12 mice (9-months of age) for treatment condition were tested in both tasks, approximately equal number of males and females were included in each group. The animals were housed with free access to water and food and were maintained on a 12 h light/dark cycle. All behavioural testing was performed during the light phase of this circadian cycle. To evaluate memory impairment following functional treatments is important to distinguish learning/memory deficits from general deficits in task performance. Therefore, pre-cognitive tests, such as Hindlimb clasping and Ledge test, are usually run before behavioural assessment as described

by Guyenet and colleagues (Guyenet SJ, *J Vis Exp* 2010). Y maze and CFC were carried out after four weeks from treatment to avoid possible variable like stress generating by continuer injections. Experiments were done blind with respect to the genotype of the mice. Behavioural tests were conducted as described by Imbimbo and colleagues (Imbimbo BP, *J Alzheimer's Dis* 2010). At the end of behavioural tests, mice were sacrificed for pathological analyses. Half of the brains were fast-frozen with dry ice for subsequent isolation of brain leukocytes. Instead, the second half of the brains were fixed in 4% PFA for 24 hours, transferred in 30% sucrose solution for one day and stored at 4°C.

13.1. LEDGE TEST

After the antibodies treatment, 3xTg-AD mice were tested with ledge test that is a direct measure of coordination, which is impaired in many other neurodegenerative disorders. The mouse is lift from the cage and placed on the cage's ledge. Then, the mouse is observed as it walks along the cage ledge and lowers itself into its cage. A wild-type mouse will typically walk along the ledge without losing its balance and will lower itself back into the cage gracefully, using its pawns. This is assigned a score of 0. If the mouse loses its footing while it is walking along the ledge, but otherwise appears coordinated, it receives a score of 1. If it does not effectively use its hind legs, or lands on its head rather than its pawns when descending into the cage, it receives a score of 2. If it falls off the ledge, or nearly so, while walking or attempting to lower itself, or shakes and refused to move at all despite encouragement, it receives a score of 3. Some mice require a gentle nudge to encourage them to walk along the ledge ore descend into the cage. The above procedure is performed four times and the score is averaged between the four tests.

13.2. HINDLIMB CLASPING

Hindlimb clasping is a marker of disease progression in several mouse models of neurodegeneration. To record the score, the tail is grasped near it base and mouse is lift away from all surrounding objects. The hindlimb position is observed for at least 10 seconds and the test is conduced three times. If the hindlimbs are consistently splayed outward, away from the abdomen, it is assigned a score of 0.

If one hindlimb is retracted toward the abdomen for more than 50% of the time suspended, the mouse receives a score of 1. If both hindlimbs are partially retracted toward the abdomen for more than 50% of the time suspended, the mouse receives a score of two. If its hindlimbs are entirely retracted and touching the abdomen for more than 50% of the time suspended, it receives a score of 3.

13.3 Y MAZE

Y Maze Spontaneous Alternation is a behavioural test used to evaluate, without training, reward or punishment, the willingness of rodents to explore new environments and to assess hippocampus- dependent spatial working memory, which is classified as short-term memory. Testing occurs in a Y- shaped maze with three grey opaque plastic arms at 120° angle from each other, extending from a central space **(Fig. 14)**. After the introduction to the center of the maze, the animal, naïve to the apparatus, was allowed to freely explore the maze for 8 minutes. Rodents typically prefer to investigate a new arm of the maze rather than returning to one that was previously visited. Thus, during the session, mouse should show a less tendency to enter in the recent visited arm. The sequence and the total number of arm entries were recorded to calculate the percentage of alternations. An entry occurs when all four limbs are within the arm. Each arm of the Y maze was arbitrary assigned as zone A, B or C. Alternation was defined as successive entries into the three arms in overlapping triple sets (e.g., ABC or ACB, but not ABA). The alternation percentage was calculated as the number of triads containing entries into all three arms divided for the maximum possible alternations, all multiply for 100.

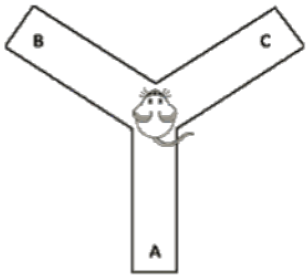


Figure 14. Representative image of the Y-Maze labyrinth.

13.4 MORRIS WATER MAZE

Morris Water Maze (MWM) is a test used to assess rodent spatial learning and memory. It relies on distal cues to navigate from start locations around the perimeter of an open swimming area to reach a hidden platform. Spatial learning is assessed through repeated trials and reference memory is determined by preference for the platform area during the probe trial when the platform is absent (Fridgeirsdottir GA, *Ups J Med Sci* 2014). MWM occurred in a white pool of 150 cm in diameter with 50 cm in height placed in a dark room. The platform was circular, 10 cm in diameter and it was submerged 1 cm above the surface. Two principal axes of the maze were designed, each line bisecting the maze perpendicular to one another to create an imaginary '+' **(Fig. 15)**. The end of each line demarcated four cues on the wall of the pool. Other cues were positioned on the wall of the room where the pool was. The platform was positioned in the middle of one of the quadrants and the pool was filled up with tap water, around 24°C to avoid mice hypothermia. Since stress is a negative influencing agent for MWM performance, a handling protocol was established to increase mice contact with the operator and with water. Two days before the beginning of the test, each animal was kept in the investigator hand for 5 minutes, allowing them to get confident with the manipulation. Subsequently, the day before the beginning of the test, mice were put for 30 seconds in a cage with 0,5 cm deep water (24°C) to get used to the contact with water. After 15 minutes, animals were put in the same cage with 8 cm deep water (24°C), where they swam for 30 seconds. After the handling, mice were trained on 7 consecutive days and their movements in the pool were recorded by the computer. Each training day consisted of three trials of 60 seconds, in which the entrance position of every trial occurred from a different quadrant of the pool (side or opposite to the platform). Between one trial and the other mice were dried and returned to their home cages. In the first day of training, called cued trial, the platform was not hidden and was pointed out by a flag. From the second to the sixth day of training, the platform was submerged and hidden through the addition of starch to the water. The last day the platform was removed, and the time spent in the target zone was analyzed.

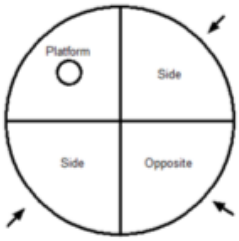


Figure 15. Morris Water Maze outline.

13.5 CONTEXTUAL FEAR CONDITIONING TEST

CFC is an associative learning task in which mice learn to associate a particular neutral Conditional Stimulus (CS; a tone) with an aversive Unconditional Stimulus (US; a mild electrical foot shock) and show Conditional Response (CR; freezing). After repeated pairings of CS and US, the animal learns to fear both the tone and training context. CFC is learned rapidly and is a useful test for neurobehaviour, genetic and pharmacological studies. It has been shown that both amygdala and hippocampus are key structures for the learning and retrieval of memories in this task. CFC occurred in 30 x 24 x 21 cm operant chambers (Ugo Basile). Each chamber was equipped with a stainless-steel rod floor through which a foot shock could be administrated, two stimulus lights, one house light and a solenoid, all controlled by ANYmaze computer software (Stoelting). The chambers were in a sound-isolated room in the presence of red light. Mice were trained and tested on two consecutive days. Training consisting of placing a subject in a chamber, illuminating stimulus and house lights and allowing exploration for two minutes. Afterwards, the 15 seconds of tone stimulus [2 Hz clicking via the solenoid; CS] co-terminated with 2 seconds foot shock [1.5 mAmp; US]. This stimuli pair was repeated two times, with presentation spaced 2 minutes apart. 30 seconds after the second shock, the mice were removed from the chamber. Before the first mouse and between mice the chambers were wiped clean with isopropyl alcohol and allowed to dry before testing. 24 hours later, mice were placed back to the same chamber in which training occurred (context) for the 5 minutes contextual conditioning test without tone or shock presentation and freezing behaviour was recorded by experimenter. Freezing was defined as lack of movement except that required for respiration. At the end of the 5 minutes contextual test, mice were

returned to their home cage. Approximately 2 hours later, freezing was recorded in a novel environment and in response to the cue. The novel environment consisted of modifications including an opaque Plexiglas divider diagonally bisecting the chamber, a Plexiglas floor and decreased illumination. Then the computer program recorded mouse movement for 3 minutes test without any stimulus presentation. The auditory cue (CS) was then presented for 3 minutes and again mouse movement was recorded. Freezing scores for each subject were expressed as a percentage for each portion of the test. Memory for the context (contextual memory) for each subject was obtained by subtracting the percentage of freezing in the novel environment from that in the context.

14. TISSUE PREPARATIONS FOR NEUROPATHOLOGY

Myocardial perfusion was used as a method to clean blood from circulation, allowing to conserve organs for future analysis. In fact, much better morphologic details were obtained when examining standardly fixed and processed tissue preparations. 4% paraformaldehyde (PFA) was commonly used for perfusion fixation, which renders good morphologic preparations. Mice were anaesthetized by intraperitoneal injection (i.p.) with phosphate buffered saline pH 7.4 (PBS) containing ketamine (5 mg/ml) and xylazine (1 mg/ml). Once mice were sedated, the abdomen was wet with ethanol. Mice were then placed with abdomen facing up and by using four small needles was secured on the four paws as wide as possible. Skin was grabbed with forceps at the level of the diaphragm, and cut to expose the liver, thus by cutting through the ribs the heart was easily accessible. A butterfly needle was placed into the left ventricle and immediately after the right atrium was cut so that blood flows out as the circulation is replaced. A peristaltic pump (Minipuls3 Gilson) with a flow no higher than 0.5 ml/min is used to inject 25 ml with Ca/Mg(1μM) and glucose PBS. If the perfusion was successful, tongue color become light pink, ears and tail veins were not visible, and the liver and the kidneys became blanch as the blood was replaced. After this was observed, the buffer solution was replaced with fixative (freshly made 4% PFA solution). We used approximately 25 ml of PFA for mouse. Once perfusion was finished, organs such as brain, spinal cord, lung, liver, kidney and spleen were removed and maintained

in ice-cold PFA solution 18h at 4°C. Mouse organs were then post-fixed at 4°C 18h. The day after, organs were rinsed with PBS for at least 30 min at RT°C and then transferred in 30% sucrose solution in PBS until they sank. Sucrose was used in order to cryoprotect and to prevent freeze artefact and loss of tissue architecture. Finally, organs were included in a cryo-embedding matrix such as optimum cutting temperature (OCT) (DDK Italia) and store at -80°C.

14.1 IMMUNOHISTOCHEMISTRY STAINING ON TISSUE SECTIONS WITH FREE- FLOATING METHOD

Brains were stored at 4°C and then included in a cryo-embedding matrix, such as OCT (CDK Italia) and then were cryo-sectioned in a cryostat. Brain coronal sections, if made at the same site, allow similar areas to be examined, so comparisons can be made between littermate controls and AD transgenic animals. Coronal sections of the entire brain may be examined to pick up small abnormalities. Examinations using special stains and immunohistochemistry will help to dissect out abnormalities better. Mouse brains were cut in coronal slices of 30 μm. Sections were collected and placed in 24-well plates containing 500 ml of PBS. Free-floating sections were incubated in blocking buffer for 1 hour at RT; then treated 18h at 4°C with the following primary antibodies: iba1, 6E10 and tau antibodies (HT7 and AT180). For the staining with 6E10 antibody, slices were pretreated with 70% formic acid for 10 minutes and then washed with PBS. For the staining with tau antibodies, slices were transferred to 10 mM sodium citrate buffer (pH 8.5) and preheated to 85°C in water bath and maintained in this solution for 30 minutes. After allowing the solution to cool to room temperature, the slices were washed with PBS. Sections were treated with blocking solution and then incubated with primary antibody in blocking solution 18h at 4°C. After washing with the blocking solution, we added 3% H_2O_2 for 10 minutes at RT to block endogenous peroxidase. Subsequently, brain slices were washed and incubated with a biotinylated secondary antibody (goat anti-rabbit for Iba1 antibody, Sigma-Aldrich; goat-anti-mouse for 6E10, HT7 and AT180) in blocking solution for 2 hours at RT. The immunoreactivity was visualized using the VECTASTAIN® ABC kit (Vector) for 30 minutes and Vector® NovaREDTM (Vector) as chromogen for 3 minutes at RT. In the end, brain portions were washed with water, transferred on glass slides

and mount with Eukitt® mounting medium (Sigma-Aldrich). Glass slides were kept at RT and acquired by AxioImagerZ2 microscope (Zeiss).

14.2 QUANTIFICATION OF MICROGLIA, Aβ LOAD AND TAU PATHOLOGY

The areas covered by Iba-1^{+} microglia, Aβ deposits, total tau and phospho-tau positive neurons were determined in coronal sections throughout the cortex and the hippocampus. Sections were taken from the anterior hippocampus through the bregma – 2.9 mm at an intersection interval of 500 µm (every fourth section) – in order to analyze the whole area of the cortex and the hippocampus. The sections were analyzed blindly with respect to the genotype and treatment using ImageJ v1.32j software.

15. ISOLATION OF NEURONAL CELLS

Dissociation of mouse brains from 3xTg-AD mice (10 days old) and WT mice was performed using the "Adult Brain Dissociation kit" (*Miltenyi Biotec*, 130-107-677) following the user manual with slight modifications, in combination with the gentleMACS Dissociator (*Miltenyi Biotec*). Mice were sacrificed by cervical dislocation and the brains isolated and cut into 15–20 pieces with a sterile and sharp razor blade. Two C Tubes (*Miltenyi Biotec*, 130-093-237) and a specific volume of enzyme mixes accordingly to the manufacture's instruction were used per brain. C tubes with tissue and enzymes mixes were placed on the gentle MACS Dissociator and brains were dissociated for 30 minutes at 37°C. Samples were collected at the bottom of the tube by brief centrifugation, re-suspended and applied to a MACS SmartStrainer, 70 µm, placed on a 50 ml tube. 10 ml of D-PBS was added. Subsequently, cells were re-suspended in cold PBS with the appropriated volume of debris removal solution (*Miltenyi Biotec*, 130-094-802). Cells suspension was transfer in a 15 mL tube and overlay with 4 ml of cold PBS. Centrifuge a 3000 xg per 10 minutes (ascending rate: 2; descending rate: 2). The bottom phase was collected and used for next steps. For the isolation of neurons and glial cells from the mixed brain cell suspension without debris, the Neuron Isolation Kit (*Miltenyi Biotec*, 130-115-389) and CD11b (microglia) microbeads

(*Miltenyi Biotec*, 130-093-634) were used. Indeed, the final brain suspension was re-suspended in 80 µl of buffer (PBS with $Ca^{2+}Mg^{2+}$ and 0.5% bovine serum albumin) and incubated with non- neuronal cell antibodies and CD11b microbeads for neurons and microglia cells respectively accordingly to company datasheet. The flow-through from microglia magnetic separation was used for astrocytes cultivation. Cells number was determined at the end of the separation procedures. Neurons were seeded in a 48 well plates pre-coated for 1 hour at 37° C with poly-D-lysin (0,1 mg/ml) and laminin (10 µg/ml) in PBS at the concentration of $5x10^5$ cell/ml in complete Neurobasal media (25 ml Neurobasal media, 0.5 ml B27, 1% GIn or Glutamax, 1% antibiotics). Astrocytes and microglia were seeded in a 96 well plate $1,5x10^6$ cell/mL and $1x10^6$ cell/mL respectively in DMEM-F12 with 10% FBS +1% GIn or Glutamax +1% antibiotics. The day after the isolation the media was changed for half and them every 3 days.

16. REAL TIME PCR ON ISOLATED NEURONS

One microgram total RNA, usually extracted by the RNeasy mini kit (Qiagen, Venlo, Limburg, Netherlands) from $2x10^6$ neutrophils or $2x10^6$ astrocytes was reverse transcribed for RT–qPCR analyses using the following gene-specific primer pairs (Life Technologies, Carlsbad, CA, USA):
RV APP (5’ CGG GGG TCT AGT TCT GCA T 3’), FW APP (5’ AGG ACT GAC CAC TCG ACC AG 3’), RV APP ctrl (5’ GTC AGT CGA GTG CAC AGT TT 3’), FW APP ctrl (5’ CAA ATG TTG CTT GTC TGG TG 3’), RV TAU (5’ TTG TCA TCG CTT CCA GTC C 3’), FW TAU (5’ TGA ACC AGG ATG GCT GAG 3’), RV TAU ctrl (5’ CTA GGC CAC AGA ATT GAA AGA TCT 3’) and FW TAU ctrl (5’GTA GGT GGA AAT TCT AGC ATC ATC C 3’). Data were calculated by Q-Gene software and expressed as mean normalized expression units after actin normalization.

17. IMMUNOBLOTS

Whole-cell proteins were obtained from protein-rich flow-through solutions after the first centrifugation step using the RNeasy mini kit (Qiagen) for total RNA extraction procedure. After an 18h incubation at −20°C, protein precipitates from

the flow-through solutions were washed with pre-chilled 100% ethanol, solubilized in Laemmli sample buffer. Samples (10–30 μg of proteins per lane) were subjected to electrophoresis on a 10% or 12% denaturing Tris/glycine sodium dodecyl sulfate polyacrylamide gel and then transferred to Hybond ECL nitrocellulose membrane (Amersham, Pharmacia Biotech). Membranes were blocked in 5% nonfat dry milk in TBS-Tween (TBS-T) for 1 h, followed by 18h incubation with the primary antibody diluted in 5% BSA in TBS-T. Membranes were then washed in TBS-T and incubated for 2 h with horseradish peroxidase (HRP)-conjugated anti-mouse or anti-rabbit IgG (1:4000, Amersham Pharmacia Biotech), diluted in 5% nonfat dry milk in TBS-T. Bound HRP-conjugated secondary antibody was detected with an ECL system (Millipore), according to the manufacturer's instructions. The following primary antibodies were used: anti-total tau monoclonal antibody (mouse, 1:500, Life Technologies Clone HT7), anti-amyloid beta monoclonal antibody (mouse, 1:500, Biolegend, Clone 6E10), and anti-actin monoclonal antibody (mouse, 1:10,000, Sigma). Band optical intensity of HT7 and 6e10 and GFAP proteins was determined using the LAS-3000 Imaging System from Fuji and normalized with that of actin.

18. IMMUNOFLUORESCENCE ON NEURONAL CELLS

Cells were plated on chamber slides after 15 days were washed with PBS and fixed with 4% paraformaldehyde over-night. The day after, slides were washed in PBS and blocked for 2 h in blocking buffer. The cells were incubated with primary antibodies 18h at 4 °C and then with the appropriate secondary conjugates. 4′,6-diami- dino-2-pheylindole (DAPI) was used for nuclear counterstaining. The following antibodies were used: rabbit anti-GFAP polyclonal primary antibody (ab7260 from Abcam), secondary antibody donkey Anti-Rabbit Alexa Fluor 594 (ab150076 from Abcam), mouse anti-MAP2 antibody (HM-2 ab11267 from Abcam), secondary antibody donkey Anti-Rabbit Alexa Fluor 594 (ab150076 from Abcam),secondary antibody Goat Anti-Mouse Alexa Fluor 488 (ab150113 from Abcam), rabbit anti-IBA1 polyclonal primary antibody (01919741 from Wako).The glass slides were mounted with Dako medium and ell interaction was checked through AxioImagerZ2 microscope (Zeiss) at 40x magnification.

19. GENERATION OF POLARIZED IL-17+ AND IFN-γ CD4+ T CELLS

CD4+ T cells were isolated from lymph nodes and spleen of control mice and 3xTg-AD mice (2 months of age) using CD4+ Isolation Kit from Miltenyi Biotec accordingly to the manufacture's instruction. Purity of isolated CD4 T cells was determined by cell surface staining and usually reached > 90% of pure CD4+ T cells. To obtained Th1 and Th1 cells we used a modified protocol by Domingues and colleagues (Domingues HS, *PLoS One* 2010). A 48 well plate was previously coated with anti-CD3 (10μg/ml) for 1 hour in the incubator. Then, $2x10^6$ of purified CD4+ T cells were cultured for 4 days in RPMI 1640 supplemented with 100 mM Na pyruvate, 200 mM in 0,85% NaCl solution ultraglutamine 1, 10000 U/ml penicillin, 10000 U/ml streptomycin and 10% fetal bovine serum (FBS). For Th1 and Th17 polarization the following cytokines and antibodies were further added in the culture. Th1: IL-12 (1 ng/ml), anti-IL-4 (10 μg/ml). Th17: TGF-β (5 ng/ml), IL-6 (20 ng/ml), IL-23 (2 ng/ml), anti-IL-4 (10 μg/ml), anti-IFN-γ (10 μg/ml). Th1 cells were supplemented with IL-2 (20 U/ml) and Th17 cells with IL-7 (10 ng/ml) at day 2 of culture.

19.1 DETECTION OF INTRACELLULAR AND EXTRACELLULAR CYTOKINES

For intracellular cytokines staining cells were stimulated 6 hours with Phorbol 12-myristate 13- acetate (PMA) (50 ng/ml), ionomycin (1 μg/ml) and brefeldin A (10 μg/ml). Permeabilized cells were labeled with FITC-conjugated rat anti-mouse IFN-γ and PE-conjugated rat anti-mouse IL-17. As control, cells were stimulated with the same conditions, but without brefeldin A. For extracellular staining, the following rat anti-mouse monoclonal antibodies were used: anti-CD3-PerCP-Vio700 (clone REA64, Miltenyi Biotec) and anti-CD4-VioBlue (Clone REA796, Miltenyi Biotec). Samples were collected and analyzed by FACS. The quantification of cd4 purity and cytokines production was made using using FlowJo software.

19.2 CO-CULTURES OF Th1 AND Th17 CELLS WITH GLIAL CELLS

After 4 culture days, Th1, Th17 and freshly isolated naïve CD4+ T cells were added on microglia and astrocytes. Supernatant of differentiated T cells was also used, whereas DMEM-F12 complete media was used as a control. The co-culture was maintained for 24 h, cells or supernatant were kept at 1:1 ratio with conventional glial media. After indicated time points, microglia and astrocytes were detached using Accutase™ enzyme (Sigma-Aldrich) for 5 minutes at 37°. Subsequentially, cells were collected for flow cytometry analysis.

19.3 DECTECTION OF ACTIVATION MARKERS ON GLIAL CELLS

For the evaluation of microglia and astrocytes activation obtained from AD and CTRL mice, the staining for flow cytometry was performed used the following antibodies: anti-CD45-PE (REA737, Miltenyi Biotec), anti-CD11b-PE-Vio770 (REA592, Miltenyi Biotec), anti-CD3-PerCP-Vio700 (REA641, Miltenyi Biotec), anti-MHCII Vioblue (M5/114.15.2, Miltenyi Biotec), anti-GLAST (ACSA-1, Miltenyi Biotec), anti-ACSA2 (REA 969, Miltenyi Biotec), anti-CD44 (REA664, Miltenyi Biotec), anti-MHCI (REA813, Miltenyi Biotec) anti-CD68 (REA835, Miltenyi Biotec). Cell viability was checked with Viobility 405/520 Fixable Dye. Labelled cells were acquired by flow cytometry with MACSQuant Analyzer (Miltenyi Biotec). Data were analyzed using FlowJo software.

20. IMMUNOFLUORESCENCE ON ASTROCYTES-CD4+ T CELLS CO-CULTURE

Cells were plated on chamber slides and then after 10 days were washed with PBS and fixed with 4% paraformaldehyde over-night. The day after washed in PBS and blocked for 2 h in blocking buffer. The cells were incubated with primary antibodies 18h at 4 °C and then with the appropriate secondary conjugates. 4′,6-diami- dino-2-pheylindole (DAPI) was used for nuclear counterstaining. The following antibodies were used: rabbit anti-GFAP polyclonal primary antibody (ab7260 from Abcam), secondary antibody donkey Anti-Rabbit Alexa Fluor 594 (ab150076 from Abcam) and Alexa 488 anti-CD4 (clone GK 1.5, Biolegend). The glass slides were

mounted with Dako medium and ell interaction was checked through AxioImagerZ2 microscope (Zeiss) at 40x magnification

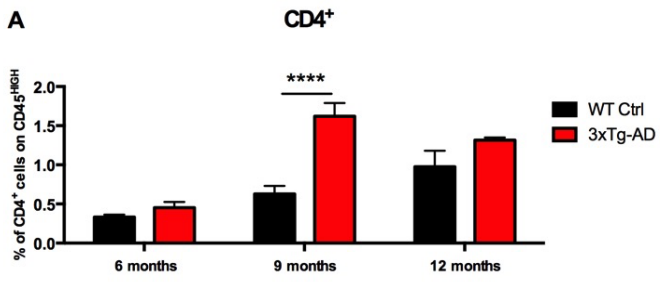
21. STATISTICAL ANALYSIS

All data were collated in Prism 6 (GraphPad Software) which was also used for statistical analysis. Where indicated, statistical analysis was performed using Mann–Whitney U test and paired *t*-test. Data are shown as mean ± standard deviation (SD) and mean ± standard error of mean (SEM). $P < 0.05$ was considered as statistically significant. Age and sex-matched 3xTg-AD and wild-type control mice were randomly assigned to each treatment. Data collection from flow cytometry experiments, behavioral tests and immunohistological analysis was performed in a blinded fashion.

RESULTS

22. LYMPHOCYTES INFILTRATE THE BRAIN OF 3xTg-AD MICE DURING DISEASE DEVELOPMENT

To assess T cell infiltration in the brain of wild-type and 3xTg-AD mice, we performed flow cytometry experiments on brain homogenates. In particular, to quantify the brain infiltrating lymphocytes, CD45+ cells were sub-gated by using CD3. Then, we evaluated the populations of cells expressing CD4 and CD8 in the CD3+ cells. We determined the accumulation of the lymphocytes at different disease stages: at 6 months of age when mice start to present memory deficit, and then progressively at later time-points (9 and 12 months) when the pathology and memory decline are more evident. Our results showed an increase of CD4+ T cells at 6 and 9 months of age in the brain of 3xTg-AD mice compared to control animals (**Fig. 16A)** reaching a significance at 9 months of age. Conversely, the number of CD8+ T cells migrating in the brain was similar in wild-type and 3xTg-AD mice at 6 months and progressively lower at 9 and 12 months of age (**Fig. 16B)**. Thus, our data suggest that the increased number of CD4+ T cells may correlate with AD and that distinct T cell sub-populations may have specific roles during disease progression. Also, we cannot exclude a role for CD8 T cells during disease pathogenesis especially during early phases as our analysis started at 6 months of age.



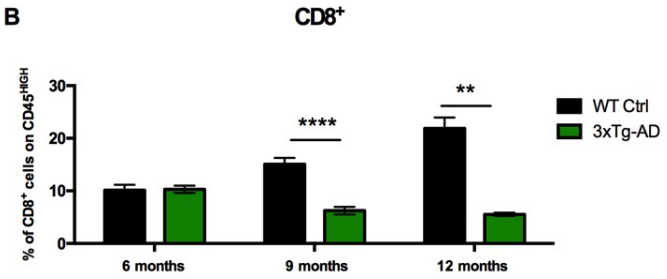


Figure 16. T cells accumulation in the brain of 3xTg-AD mice during different time-points of disease. Quantitative Flow-cytometry analysis of CD4+ **(A)** and CD8+ **(B)** accumulated in the brain of 3xTg-AD (red bars) mice at different months of age compared to wild-type control mice (black bars). Data are expressed as mean ± SEM. (**$P < 0.01$;****$P < 0.0001$; *Mann-Whitney U-test*). 8-10 animals/groups of the same age were analyzed.

22.1 T CELLS DEPLETION IN 3xTg-AD MICE: EXPERIMENTAL DESIGN

Next, we evaluated the effect of CD4+ and CD8+ T cells on the pathogenesis of AD-like disease by means of depleting antibodies. At the beginning of the treatment we checked the efficiency of T cells depletion in the peripheral circulation over time. A single anti-CD4 or anti-CD8α injection allowed the complete removal of T cells after 24 hours, as shown by FACS analysis on peripheral T cells in mice injected with either the antibodies, showing the specificity of the depletion compared to isotype control treated mice **(Fig. 17A, B).** T cell depletion was maintained also 48 hours after injection **(Fig. 17A, B).** Based on these results, we treated 3xTg-AD mice at 6 months of age, the time point in which T cells start to accumulate in the brain. The treatment was performed by an intra-peritoneal (i.p.) injection every other day for 4 weeks as described in "Materials and Methods" section.

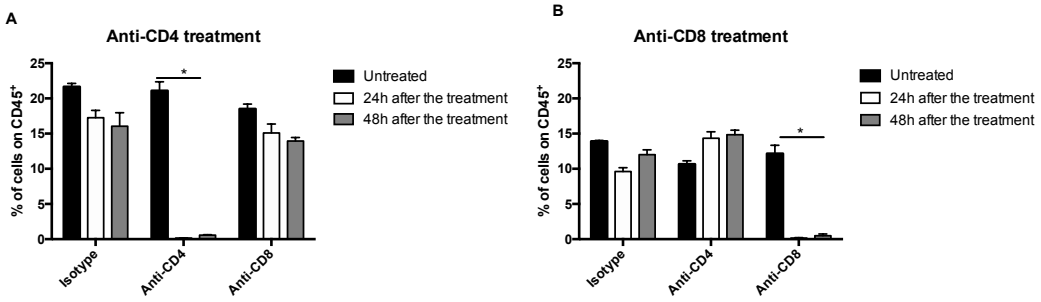


Figure 17. Anti-CD4 and anti-CD8α depletion efficiency. Quantitative flow-cytometry analysis on peripheral CD4+ and CD8+ T-cells from 3xTg-AD mice. The graphs confirmed the anti-CD4

(A) and anti-CD8α **(B)** depleting antibodies efficacy. The blood was obtained 24 and 48 hours after a single injection with depleting or isotype control mAbs. 3 animals/groups of the same age were analyzed. Values represent ± SEM in each group (*$P < 0.05$), *Paired t-test.*

22.2 CD4+ AND CD8+ DEPLETION DOES NOT AFFECT SHORT TERM MEMORY IN AD MICE IN THE Y MAZE TEST

At the end of the treatment, 7-month age-old mice were left untouched for 4 weeks before performing the behavioral tests at 8 months of age. This step allows minimizing the stress and fear mice due to frequent manipulation by experimenter, that could eventually confound the evaluation of memory impairment during behavioral tests. We firstly performed hindlimb clasping and ledge tests in order to verify that the acquisition of spatial working memory could be affected by processes other than learning/memory, such as locomotors activity, sensorimotor reactivity as well as general anxiety traits. In both tasks, 3xTg-AD treated with anti-CD4, anti-CD8α or isotype control mice did not perform significantly different compared to sex- and age-matched wild-type control mice. In fact, most mice examined when picked up by the tail and slowly lowered towards a horizontal surface extended all four paws in anticipation of contact with the ground. Likewise, most mice walked along the ledge without losing their balance and lowered themselves back into the cage quietly. Therefore, by assessing positive mouse motor coordination and no alterations in vestibular function, we could proceed with the evaluation of spatial memory performing the Y-maze test. The Y-maze test is a useful tool to evaluate spontaneous alternation in a Y shape maze without food deprivation or other aversive procedures. This test assesses rodent short-term memory related to intact prefrontal cortical functions and spatial reference which is related to hippocampal area (Kraeuter AK, *Methods Mol Biol* 2019). We recorded for 8 minutes the free movement of the mice and scored the entrances into the arms of the maze. Then, we calculated the total arm entries and the alternation score (percentage of alternation) in 3xTg-AD mice treated with either anti-CD4 or CD8α mAbs or isotype control antibody. We observed that the total number of arm entries during the session of the test was comparable among 3xTg-AD mice treated with depleting mAbs and isotype control antibody **(Fig. 18A).** These mice had a normal exploratory behavior and did not show any particular signs of motor deficit or anxiety. As mentioned above, we also calculated the percentage of spontaneous

alternation in the three cohorts of mice. Notably, the percentage of alternation showed no significant differences among the three groups of animals (**Fig. 18B)**, suggesting that T cells may not be involved in the formation of short-term memory events connected to spatial navigation.

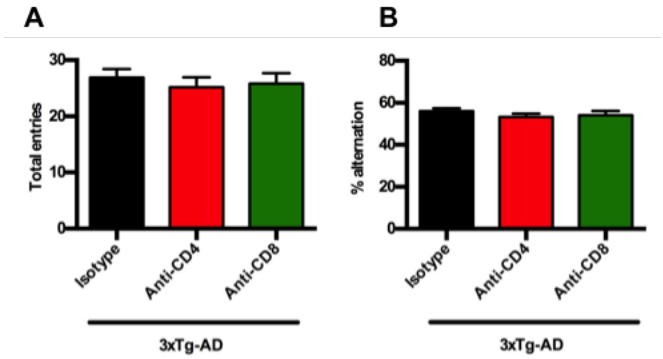


Figure 18. T cells in 3xTg-AD mice are not required for short-term memory in Y maze. (A). Total arm entries performed by the mice during the test. **(B)** Percentage of the alternation score obtained by the three cohorts of mice. Data derived from one representative experiment with 12-15 mice per condition. Values represent ± SEM in each group, *unpaired t-test.*

22.3 CD8+ T CELL DEPLETION IMPROVES LONG TERM SPATIAL MEMORY IN 3xTg-AD MICE

Morris Water Maze (MWM) is the prototypic paradigm for long-term spatial reference memory, in which mice are trained in four trials per day over a four-day period (training session), followed by a "probe" test at day 5. The test is performed to measure the capably of the mice to swim around the perimeter of an open swimming area to locate a submerged escape platform. Spatial learning is assessed across repeated training and reference memory and it is determined by the preference for the platform area when the platform is absent (Vorhees CV, *Nat Protoc* 2006). We performed the test on 3xTg-AD mice devoid of T cells to evaluate if this depletion could have an impact on the different cognitive processes and their underlying neural mechanisms. We recorded the movement of each mouse during the visible platform test in the training session to assess the working memory **(Fig. 19A, B)** and during the probe trial to assess the reference memory **(Fig. 19C)**. The "learning capabilities" of the mice can be evaluated by recording: i) the rate at which the time to find the platform (escape latency) decreases across training days, ii) the speed at which a mouse swim, and iii) the length and iv) directionality of the

swimming path. The data were combined values of four trials per day in mice of the same group. Time spent doing freezing/floating or thigmotaxis was considered as a parameter for the analysis. Indeed, freezing/floating behavior is considered as a state of inactivity without forwarding movement and the frequency of floaters in each group was statistically compared to determine whether or not the experimental manipulation significantly altered the frequency of non-performers compared with the WT. Differently, thigmotaxis represents the tendency to swim or float near the tank wall which indicates that the animal is not focusing on the task appropriately. Animals doing thigmotaxis were removed from the analysis. Our results showed that mice treated with anti-CD4 or anti-CD8α were more prone to learn during the four days of the training session compared with isotype control mice as indicated from the reduction of the time spent to find the platform during the four days of training **(Fig. 19A)**. Of note, 3xTg-AD mice treated with anti-CD8α compared to age- and sex-matched control mice reached the platform much faster as it is evident by the low latency to find the platform in the learning curve **(Fig. 19A)** and by the statistically significant reduction observed on the last day of training **(Fig. 19B)** suggesting a potential connection between the depletion of peripheral CD8 T cells and the rescue of spatial working memory related to the amelioration of hippocampal circuits. Additionally, the performance on probe tests was evaluated by percent time in the target quadrant on its own as well as in comparison to the other three quadrants. Our data showed that all examined groups spent more time in the quadrant where the platform was, highlighting the success of the test (**Fig. 19 C**). In particular, 3xTg-AD mice depleted of CD8 T cells spent more time than the other mice tested in the target zone **(Fig. 19C)**, underling the results obtained during the training phase and showing that CD8 T cell depletion improves memory suggesting a restoration of hippocampus, entorhinal cortex, and surrounding structures circuits necessary for allocentric navigation.

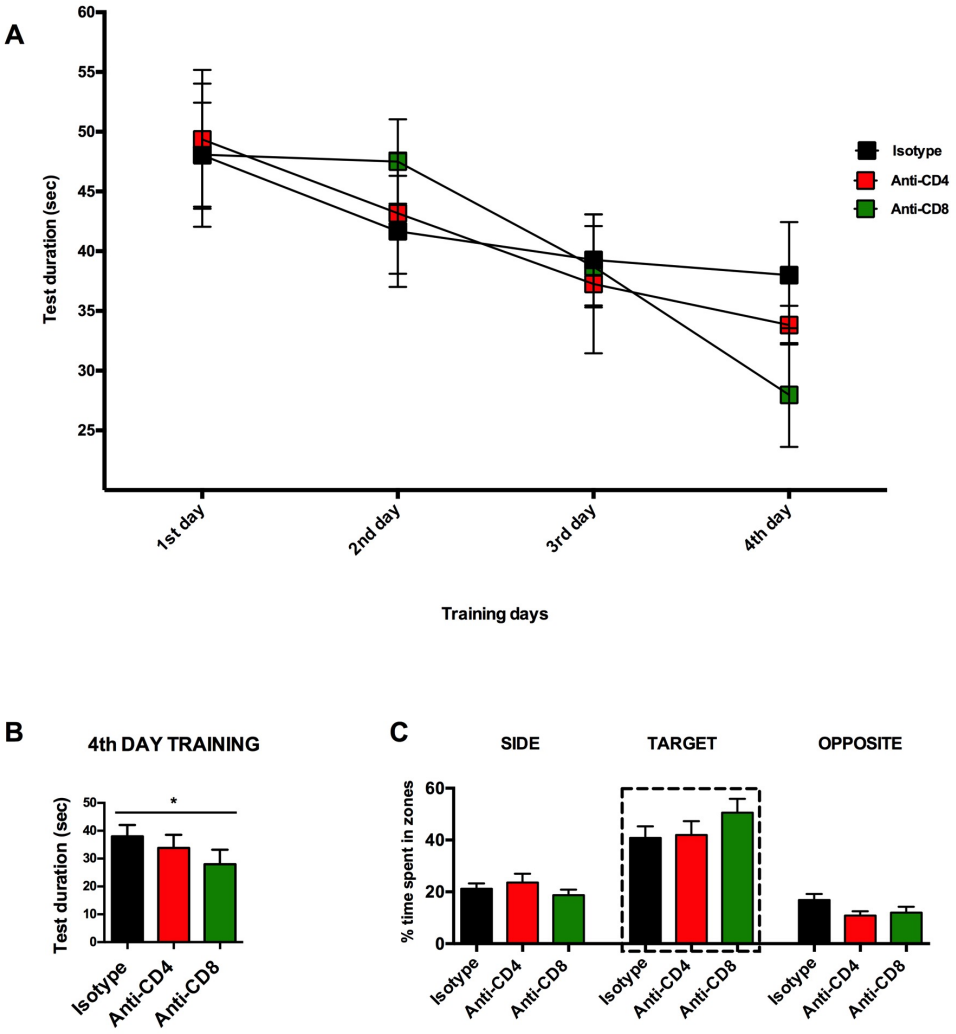


Figure 19. The depletion of CD8 improves cognitive performance in 3xTg-AD mice in Morris Water Maze test. (A) The graph shows the learning curve (test duration) during the four days of training in the three cohorts of 3xTg-AD mice. **(B)** Test duration at the fourth day of training. **(C)** Displays time spent in the target zone during the probe. The histograms show that mice treated with anti-CD8α spent more time in the target zone compared to anti-CD4 and isotype treated animals. Data derived from one representative experiment with 12-15 mice per condition. Values represent ± SEM in each group (*$P< 0.05$; *Mann-Whitney U-test)*

22.4 T CELL DEPLETION AMELIORATES FEAR RESPONSE IN 3xTg-AD MICE

Fear Conditioning (FC) is a type of classical conditioning that involves pairing an aversive or unconditioned stimulus (such as an electric shock) with a neutral or conditioned stimulus (either a context or a sound) **(Fig. 20A).** This results in the expression of the fear response in the presence of the context or stimulus alone. The neutral stimulus of the sound is then able to cause the fear behavior itself without the shock. This memory in the rodents is exemplified by the “freezing behavior”,

considered as the lack of any movement (complete immobility) except the one necessary for breathing. The freezing response to the neutral stimulus is mainly dependent on amygdala that coordinates the fear response in concert with the intact thalamus, hippocampus and the sensory cortex, all brain areas relevant for AD. In the most commonly used version of the fear conditioning paradigm, animals are placed in a conditioning cage, in which they hear an auditory sound, followed by a brief foot-shock (context A). Thus, the animal can associate the aversive stimulus with the context as well as with the tone. To test contextual fear conditioning, animals are placed in the novel conditioning cage (context B) and freezing scores for each subject are expressed as a percentage for each portion of the test **(Fig. 20 A)**. Interestingly, we observed a significant increase in the percentage of freezing in mice treated with anti-CD4 or anti-CD8α depleting antibodies compared with isotype control mice, suggesting an almost complete restoration of cognitive impairment **(Fig. 20B).** Collectively, our data indicate that CD4+ and CD8+ T cells play a role in the development of memory deficit in mice with AD-like pathology, suggesting that 3xTg-AD mice devoid of CD4 or CD8 T cells restore specific brain circuits indicating a potential role for T cells in the impairment of the "executive functions" characteristic of the late disease stages.

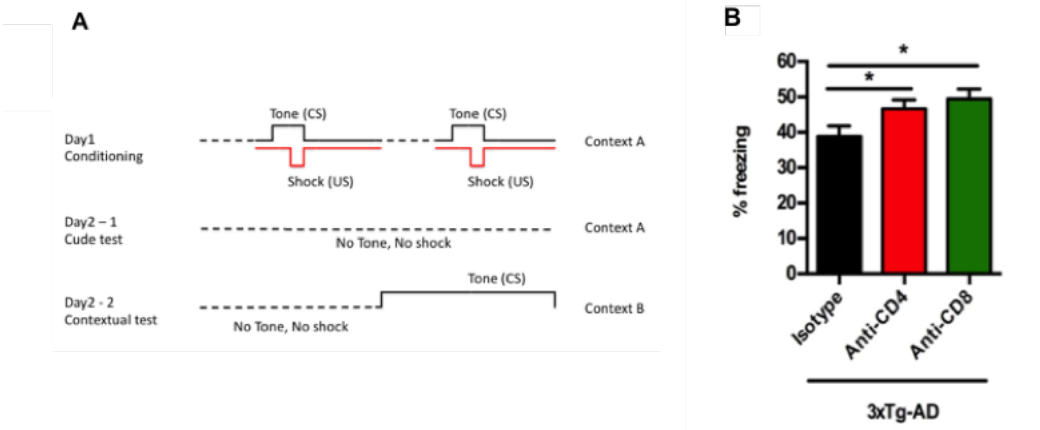


Figure 20. anti-CD4 and anti-CD8α treatment reduce cognitive deficit in contextual fear conditioning test. (A) Schematic illustration of the fear conditioning paradigm. **(B)** Mice treated with the depleting antibodies at 6 months of age showed an increased percentage of freezing compared with isotype control mice 1 month after treatment termination. Data derived from one representative experiment with 12-15 mice per condition. Values represent ± SEM in each group (*$P < 0.05$, *Mann-Whitney U-test*).

23. DEPLETION OF CD4+ AND CD8+ T-LYMPHOCYTES REDUCES NEUROPATHOLOGICAL HALLMARKS OF AD

After the termination of the behavioral tests, we investigated the effect of CD4 and CD8 depletion by immunohistochemical evaluation of brain samples of 3xTg-AD mice at 9 months of age (3 months after treatment start and 2 months after treatment termination). The immunohistological analysis of different brain areas allows the identification of subtle pathophysiologic changes in neuronal population that might be associated to behavioral changes. We quantified by immunohistochemistry the presence of Aβ accumulation, tau phosphorylation and microglial cell activation in different brain areas. In the earliest stages of AD-related pathologies, Aβ accumulation is mainly detectable intracellularly, temporally followed by phospho-tau accumulation and extracellular Aβ deposition. We particularly focused on cortical regions and the CA1 area of the hippocampus, known to be severely affected at earlier stages of disease in 3xTg-AD mice.

23.1 REDUCTION OF MICROGLIA ACTIVATION FOLLOWING T CELL DEPLETION IN AD MICE

Microglial cells are 'resident macrophages' normally present in the brain that continuously probe the CNS environment for signs of distress and migrate to sites of injury where they recruit and activate additional cells, proliferate, phagocytose, clear debris and reorganize the CNS parenchyma. The complex multistage activation process behind their reactivity include the transformation from a ramified to an amoeboid morphology by first retracting the microglia processes and then extending dynamic protrusions, followed by cellular locomotion. These brain-resident immune cells represent an important nexus between neurological and immunological activity in the CNS. During AD pathogenesis, microglial cells may also be responsible for lymphocyte recruitment from peripheral circulation to inflamed areas by triggering the expression of vascular cell adhesion molecules on endothelial cells and leading to BBB injury. In response to their ever-changing surroundings, microglia may display remarkable changes both in terms of morphology and numbers. Therefore, we labeled microglial cells and quantified microglia density and shape in 3xTg-AD mice devoid of CD4+ or CD8+ cells compared to isotype control mice. For the immunohistochemical analysis of

microglia we used an anti-Iba–1 antibody, a specific marker for microglia. Our aim was to evaluate the area and density occupied by Iba+ cells as known parameters to evaluate the activation state and proliferation profile, respectively. We observed a significant reduction in both the area and density of Iba1+ microglial cells in cerebral regions (cortex and hippocampus) of 3xTg-AD mice treated with T cell depleting antibody compared to age and sex-matched control mice **(Fig. 21A, B).** Our data support the hypothesis that both CD4+ and CD8+ T lymphocytes may play a role in the exacerbation of neuroinflammation observed in the brains of AD patients, suggesting that T cell infiltration can drive microglia activation in the brain of 3xTg-AD mice. Therefore, the blockade of T cell accumulation in the 3xTg-AD brain during the early phases of disease may also reduce microglia-induced neurodegeneration, allowing the preservation of cognitive functions in mice with AD-like disease.

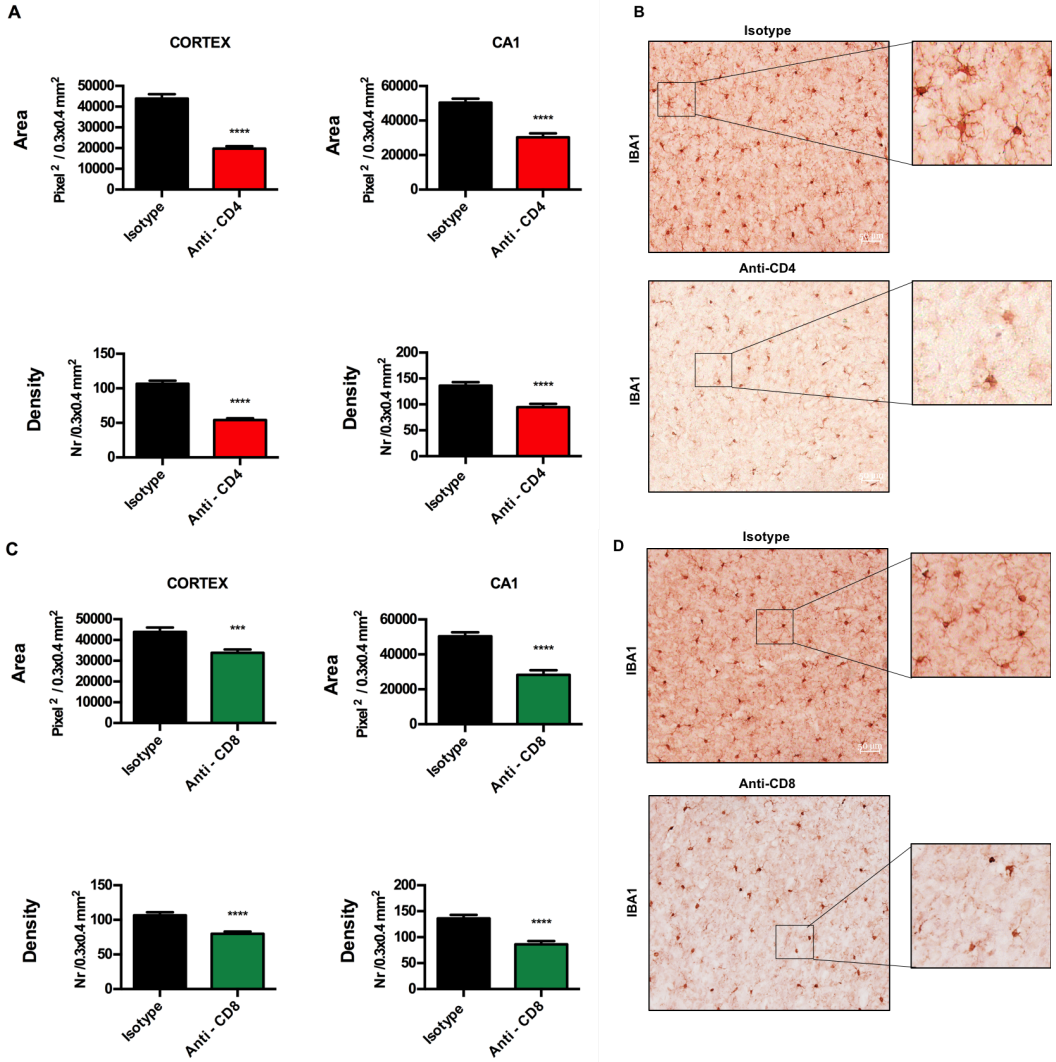


Figure 21. T cells depletion reduces microglial activation. (A, C) Quantitative analysis of Iba1 staining showing the density of Iba1 positive cells and the area occupied by cell soma in CA1 and cortex. Error bars indicate SEM (*** P <0.001; **** P <0.0001, *Mann-Whitney U-test*). **(B, D)** Representative images of Iba1 staining in cortical regions showing differences in the density and morphology of microglial cells in 3xTg-AD mice treated with anti-CD4 or anti-CD8α antibodies compared to 3xTg-AD mice treated with isotype control antibodies. 40X magnification on the right. Scale bar 50 µm. In all panels, n = 3 mice (2 F, 1 M) for the isotype group, and n = 3 mice (2 F, 1 M) for the anti-CD4 and anti-CD8α groups.

23.2 DEPLETION OF CD4+ AND CD8+ T CELLS AMELIORATES Aβ PATHOLOGY IN 3xTg-AD MICE

To quantify the accumulation of Aβ, we performed immunohistochemistry staining using 6E10 antibody, which recognizes amino acid residues 1-16 of Aβ protein. The analysis was performed in 3xTg-AD mice treated with anti-CD4 and anti-CD8α antibodies or with the isotype control antibodies at 9 months of age (2 months after treatment termination). The Aβ deposits are apparent in hippocampal neurons at 9 months of age, and then become more extensive in other brain areas during

disease progression. Notably, we observed a strong reduction of intraneuronal Aβ accumulation in 3xTg-AD mice devoid of T cells compared to mice treated with the isotype control antibody (**Fig. 22B, D, F, H**) in the hippocampus (CA1 and subiculum). Our data clearly showed a substantial amelioration of Aβ neuropathology following T cell depletion in 3xTg-AD, suggesting a role for T lymphocytes in the accumulation of Aβ. These observations were also confirmed by quantitative stereological analysis to determine the total area occupied by Aβ-positive neurons (expressed as pixel^2/total examined area) (**Fig. 22A, C, E, G**). These data confirmed our hypothesis on the detrimental role of T cells in AD pathology during established disease phase suggesting that the depletion of CD4+ and CD8+ T cells in 3xTg-AD mice may prevent exacerbation of inflammatory events thus reversing Aβ accumulation.

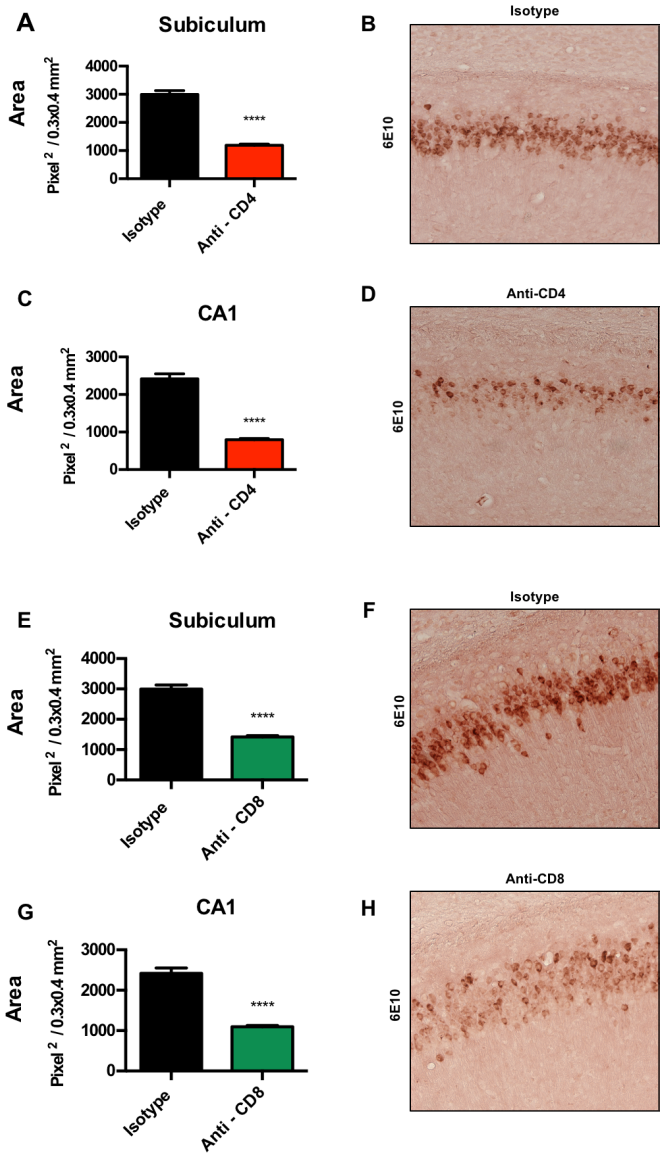


Figure 22. Treatment with anti-CD4 or anti-CD8α mAbs ameliorates Aβ pathology. (A-C-E-G) Quantitative analysis was performed in CA1 and subiculum to determine the area of cells accumulating Aβ following anti-CD4 or anti-CD8α treatments. Error bars indicate the SEM (**** *P* <0.0001, *Mann-Whitney U-test*). **(B, D, F, H)** Representative images of 6E10 monoclonal antibody staining in CA1 region of hippocampus revealing differential Aβ accumulation pattern in 3xTg-AD mice treated with anti-CD4 or anti-CD8α antibodies compared to 3xTg-AD mice treated with isotype control antibody (Isotype) in the CA1 and subiculum regions of hippocampus. Scale bar 10 µm. In all panels, n = 3 mice (2 F, 1 M) for the isotype group, and n = 3 mice (2 F, 1 M) for the anti-CD4 and anti-CD8α groups.

23.3 TAU PATHOLOGY CORRELATES WITH CD8+ T CELL INFILTRATION IN 3xTg-AD MICE

Since it has been shown that the abnormal morphologic entity in AD brains known as the neurofibrillary tangle is comprised primarily of tau, it has been proposed that

abnormalities of tau, directly or indirectly, play a central role in the pathogenesis of AD by progressively leading to a loss of fast axonal transport. To determine the effect of CD4+ and CD8+ depletion on tau pathology, we performed immunohistochemical staining for tau protein in 3xTg-AD mice devoid of T cells compared to control mice at 9 months of age. We used the HT7 antibody to detect total tau expression. As shown in **Figure 23,** 3xTg-AD mice treated either with anti-CD4, anti-CD8α or control antibody have comparable total tau levels in CA1 region of the hippocampus **(Fig. 23A, C)**. This allowed us to further evaluate the expression levels of the phosphorylated tau epitopes. Thus, we performed the immunohistochemical staining using AT180, an antibody which binds the protein tau at residue Thr231. 3xTg-AD mice treated with anti-CD8α antibody showed a significant reduction of tau phosphorylation in the CA1 region of hippocampus compared with control mice **(Fig. 23G, H).** Conversely, 3xTg-AD mice devoid of CD4 T cells did not show a reduction of tau pathology **(Fig. 23E, F)** compared to control littermates. Accordingly, a previously published study demonstrated a strong correlation between tau pathology and CD8+ T cell infiltration in a tau mouse model (THY-Tau22 mice). Moreover, evidence from *in vitro* studies and transgenic mouse tauopathy models suggest that tau may also promote BBB deterioration. Therefore, our data suggest that only CD8+ T cell infiltration in the brain of 3xTg-AD mice trigger events leading to tau phosphorylation thus exacerbating neuronal damage. Additionally, the depletion of CD8 T cells may reduce the level of tau hyperphosphorylation promoting BBB stabilization and integrity.

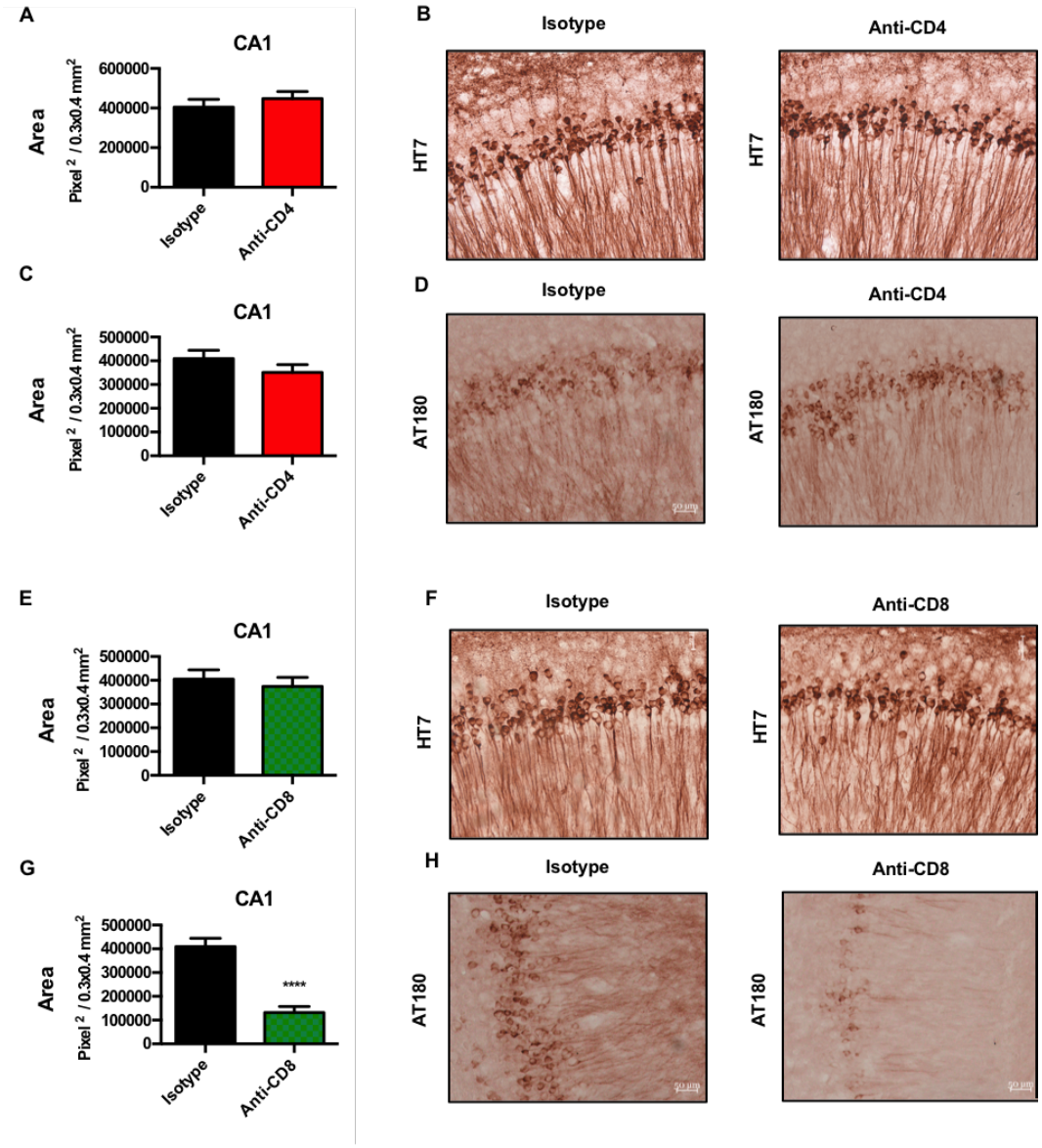


Figure 23. CD8 depletion reduces tau phosphorylation levels in 3xTg-AD brain. (A, C, E, G) Quantitative analysis showing the total area occupied by tau-expressing cells. HT7 and AT180 antibodies were used to detect total tau (**A, E**) and the Thr231 phospho-epitope of tau (**C, G**), respectively. Bar expresses mean ± SEM (**** *P*< 0.0001, *Mann-Whitney U-test*). **(B, F)** Representative images of total tau labeling in the CA1 area of hippocampus. Scale bar 10 µm. **(D, H)** Representative images showing phosphorylated tau in the CA1 hippocampal area. Scale bar 10 µm. In all panels, n = 3 mice (2 F, 1 M) for the isotype group, and n = 3 mice (2 F, 1 M) for the anti-CD4 and anti-CD8α groups.

24. TRAFFICKING MECHANISMS CONTROLLING T CELL MIGRATION DURING AD PATHOLOGY

In order to evaluate the mechanism leading to T cell recruitment in the brain of 3xTg-AD mice, we investigated the role of the VLA4 (CD49d/CD29, α4β1) and LFA-1(CD11a/CD18, αLβ2) integrins as adhesion receptors widely expressed on

immune cells and key mediator of multiple immune cell interactions. The expression of endothelial adhesion molecules is necessary for the recruitment of circulating leukocytes to sites of inflammation. We previously demonstrated that the integrin-ligands VCAM-1 and ICAM-1 were highly expressed in the hippocampus and cortex of 3xTg-AD mice at 6 months of age, compared to that in sex- and age-matched control animals (Zenaro E, *Nat Med 2*015). Therefore, we studied the involvement of VLA-4 and LFA-1, the major integrins expressed by T cells, during AD pathology in 3xTg-AD mice.

24.1. DIFFERENTIAL EXPRESSION OF α4 INTEGRIN ON PERIPHERAL LEUKOCYTES IN 3xTg-AD MICE

VCAM-1 is an important mediator of immune cell mediated rolling, adhesion and extravasation into inflamed tissues by binding to VLA-4 (α4β1 integrin) on leukocytes. We firstly checked the expression of the α4 integrin chain (CD49d) on blood leukocytes in 3xTg-AD mice compared to wild-type controls at early disease stages by flow cytometry. Our data revealed a progressive age-dependent increase of both the proportion of CD4+ T cells expressing α4 integrin, and the mean fluorescence intensity (MFI) representing α4 expression in 3xTg-AD mice compared to controls **(Fig. 24A).** CD8+ T cells also expressed CD49d, but the proportion of α4+ cells in AD mice was ~~s~~ignificantly lower compared to wild-type controls **(Fig. 24B),** suggesting that differently to CD4+ T, these cells may rely on different trafficking mechanisms in AD mice. We also did not observe differences in the CD49d MFI of CD8+ T cells between 3xTg-AD mice and controls at both 6 and 9 months of age. Collectively, our data suggest that α4 integrins may contribute to the recruitment of CD4+ T cells in the brain of 3xTg-AD mice at early disease stages, whereas other integrins may be responsible for the specific recruitment of CD8+ T cells.

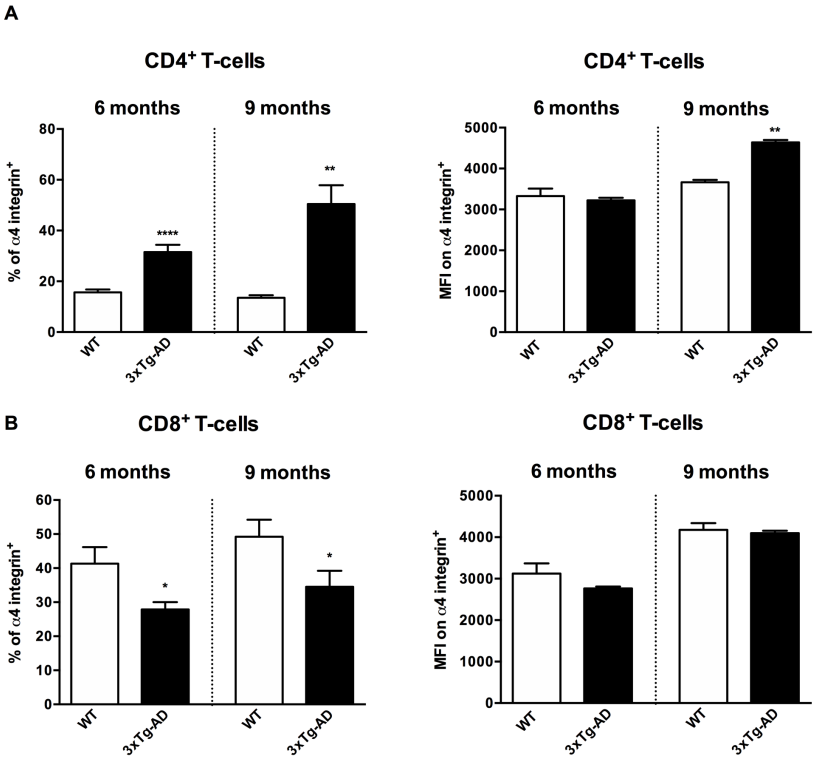


Figure 24. The expression of α4 integrin on circulating leukocytes in 3xTg-AD mice. Quantitative flow-cytometry analysis of peripheral **(A)** CD4+ T cells, **(B)** CD8+ T cells in 3xTg-AD (black bars) compared to wild-type control mice (white bars) at 6 and 9 months of age. For all cell populations, the graphs show the proportion of cells expressing α4 integrin and the mean fluorescence intensity (MFI) of normalized α4 integrin expression. Error bars represent SEM of 8–12 animals per group (*$P < 0.05$, **$P < 0.01$, **** $P< 0.0001$, *Mann-Whitney U-test)*

24.1.1 BLOCKING α4 INTEGRINS REDUCES LEUKOCYTE–ENDOTHELIAL INTERACTIONS IN BRAIN VENULES OF AD MICE

T cells and neutrophils have been previously shown to accumulate in the brain of several transgenic models of AD-like disease. To demonstrate that α4 integrins mediate the adhesion of leukocytes in the cerebral microcirculation of AD mice, we next performed epifluorescence intravital microscopy (EIVM) experiments using two types of recording and analysis systems to compare the cerebral microcirculation of 3xTg-AD mice and wild-type control animals aged 6 months. We first used a digital camera system (2.5 frames/s) to observe and record endogenous leukocyte adhesion within cerebral micro-vessels directly. Automatic high-throughput data analysis using Imaris software revealed a much greater number of rolling interactions in 3xTg-AD mice compared to wild-type controls **(Fig. 25A),** clearly demonstrating that the brain endothelium in AD mice is

activated to allow the adhesion of circulating leukocytes **(Fig. 25B).** Following the acquisition of baseline interactions in 3xTg-AD mice, we administered a single intravascular (*i.v.*) injection of a blocking antibody specific for α4 integrin and recorded endogenous leukocyte interactions 30 min later (**Fig. 25C**). Notably, automatic analysis demonstrated that blocking α4 integrin significantly reduced the percentage of interacting cells (traveling at velocities below V_{crit}) in 3xTg-AD mice (***$P < 0.001$) **(Fig. 25D).** No significant effect was observed following the administration of an isotype control antibody (data not shown).To confirm the results obtained by automatic EIVM, we repeated the analysis using a more sensitive analogue camera (25 frames/s) allowing the more accurate detection of adhesive interactions, followed by the manual analysis of the digitized movies **(Fig. 25A)**. This showed that inhibiting α4 integrin significantly reduced endogenous leukocyte rolling in 3xTg-AD mice (*$P < 0.05$), whereas no significant effect was observed after treatment with an isotype control antibody **(Fig. 25B).** As expected, fewer firmly adhering cells were observed after the blockade, but the number of the arresting cells was very low, and these data were not plotted or shown. Overall, our findings support the presence of vascular inflammation in AD mice and demonstrate that α4 integrins contribute to leukocyte–vascular interactions in cerebral vessels in mice with AD-like disease.

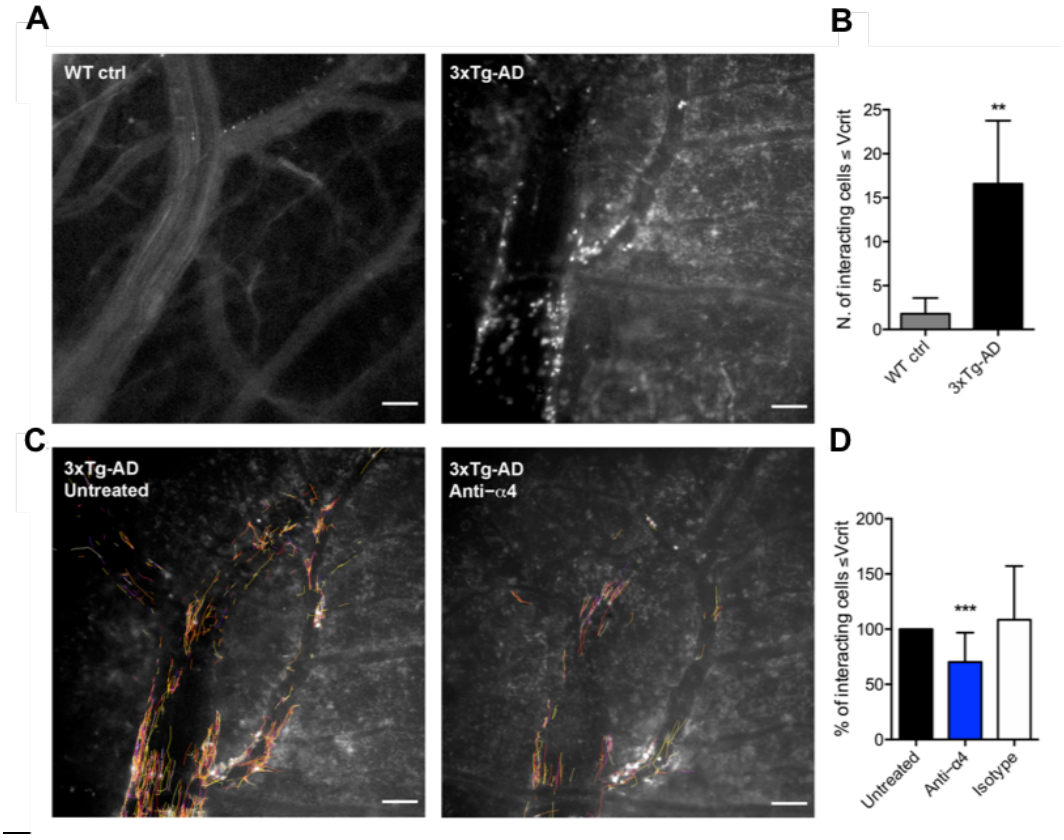


Figure 25. Increased leukocyte–endothelial interactions in the brains of AD mice and the effect of α4 integrin blockade. (**A**) Representative images from EIVM experiments using a digital camera to record endogenous leukocytes labeled with Rhodamine 6 G, and automatic cell tracking in cerebral blood vessels of wild-type control (WT ctrl) and 3xTg-AD mice at 6 months of age. Interacting cells were automatically tracked in the field of acquisition based on their centroid fluorescence intensity. Scale bar 100 µm. (**B**) Automatic Imaris analysis of endogenous leukocytes shows rare interacting cells in wild-type control (WT ctrl) mice compared to 3xTg-AD mice (**$P < 0.01$, Mann-Whitney *U*-test). (**C**) Representative images of leukocyte tracks in cerebral blood vessels of 3xTg-AD mice before (left panel, Untreated) and after (right panel, Anti-α4) i.v. injection into the lateral tail vein with a blocking antibody against α4 integrin. Scale bar 100 µm. (**D**) Automatic Imaris analysis of leukocyte tracks before (Untreated) and after 30 min of treatment with the α4 integrin-specific blocking antibody shows a significant reduction in the percentage of interacting cells in the treated mice compared to the before-treatment control, set at 100% (***$P < 0.001$, Mann-Whitney *U*-test), whereas no effect was observed with an isotype control antibody. Bars show the percentage of interacting cells in the same venule over the course of 1 min. At least three vessels per mouse were analyzed and three animals were tested for each condition. Data are expressed as means ± SD (n = 3 mice for all experimental conditions).

24.1.2 THERAPEUTIC BLOCKADE OF α4 INTEGRIN IMPROVES MEMORY IN 3xTg-AD MICE

The inhibition of VLA-4/VCAM-1 has been successfully used as a therapeutic approach to interfere with mononuclear cell recruitment in some inflammatory diseases (Yusuf-Makagiansar H, *Med Res Rev* 2002). Therefore, to evaluate the role

of α4 integrins on the memory impairment of 3xTg-AD mice, we administered an anti-α4 integrin antibody for 4 weeks to 6-month-old 3xTg-AD mice. As previously shown at 6 months of age 3xTg-AD mice start to accumulate CD4+ T cells in the brain **(Fig. 26A),** and interestingly this time-point corresponds to the initiation of cognitive impairment in this animal model. After treatment, mice were allowed to rest for another 4 weeks and were then tested for cognitive functions at approximately 8.5-9 months of age. In the Y-maze spontaneous alternation task and contextual fear-conditioning test, the therapeutic blockade of VLA-4 in 3xTg-AD mice restored memory compared to mice treated with an isotype control antibody (**Fig. 26A**). Cognitive functions in the treated mice were comparable to those in age-matched wild-type littermates in both tests, highlighting the efficacy of this early therapeutic intervention in AD mice. Blocking α4 integrins in wild-type mice had no effect on cognition, further supporting a role for lymphocytes adhesion in neuro-inflammation-mediated cognitive dysfunction in AD mice. Furthermore, 3xTg-AD mice treated with anti-α4 integrin antibody also showed a memory improvement in the MWM paradigm, i.e. a significant reduction of escape latency during the training period compared to mice treated with a control antibody (**Fig. 26B**). In addition, the therapeutic blockade achieved a statistically significant increase in platform location crossings by 3xTg-AD and significantly reduced the latency to find the platform location during the probe test compared to animals treated with an isotype control antibody (**Fig. 26C**). Collectively, these behavioural tests clearly show that interfering with leukocyte recruitment in the brains of 3xTg-AD mice significantly reduces cognitive impairment at early time points of the disease.

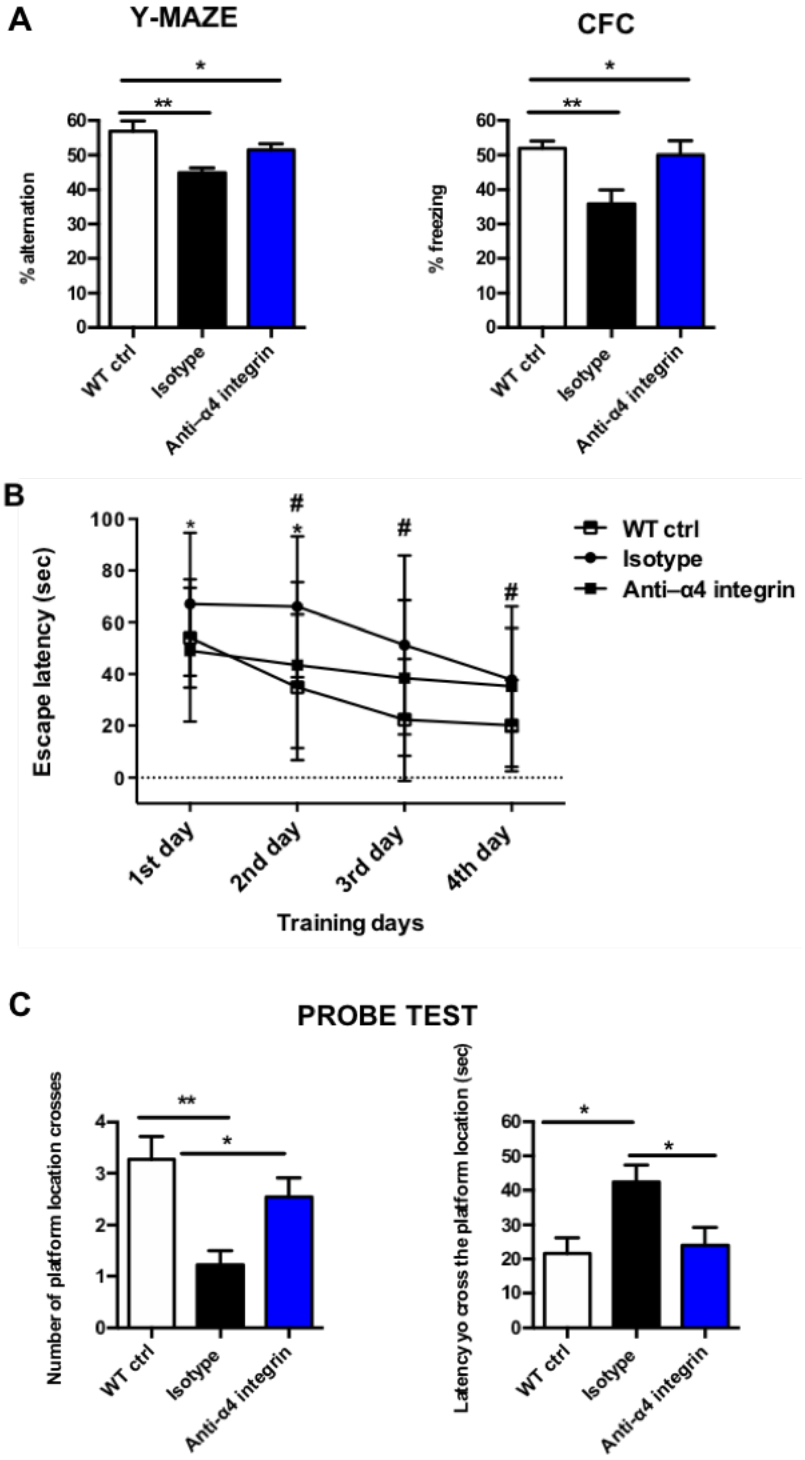


Figure 26. Blocking α4 integrin improves cognitive functions in 3xTg-AD mice. (**A**) Results showing the percentage of alternation in the Y-maze test (left) and the percentage of freezing from the contextual fear-conditioning (CFC) test (right) (n=8-10 mice/group, **P* <0.05, ***P* <0.01; Mann-Whitney *U*-test). (**B**) Evaluation of the escape latency to reach the hidden platform during training period during Morris water maze. Multiple t tests within the same day **P* <0.05 wild-type control (WT ctrl) versus isotype control; **P* <0.05 isotype control versus treatment group). **(C)** Number of platform location crossings and latency to cross platform location (**P* <0.05, ***P* <0.01; Mann-

Whitney *U*-test). Values represent mean ± SEM of the data obtained from a representative experiment with 10–15 mice/group.

24.1.3 THE α4 INTEGRIN-SPECIFIC ANTIBODY REDUCES NEUROPATHOLOGICAL CHANGES IN AD MICE

The beneficial effects of the therapeutic blockade shown by cognitive assessments in 3xTg-AD mice were also supported by neuropathological data. At 9 months of age we observed a reduction in microgliosis, assessed by Iba-1 staining, in mice treated with the α4 integrin-specific antibody than those receiving the isotype control **(Fig. 27A).** Microglial cells in the cortex of 3xTg-AD mice treated with anti-α4 integrin antibody showed a round cell bodies with few ramified processes, indicating a low activation state, whereas microglia in animals treated with the isotype control antibody presented wider and irregular cell bodies with highly ramified, compact and thickened processes indicating a high activation state **(Fig. 27A)**. The Aβ load was determined by staining with antibody 6E10, revealing less Aβ accumulation in the hippocampal CA1 region of 3xTg-AD mice treated with the therapeutic blockade compared to the isotype control group **(Fig. 27B).** The accumulation of tau protein in the hippocampus was comparable across all mouse groups **(Fig. 27C)** but the hyperphosphorylated variant, detected using antibody AT180, was less prevalent in the hippocampal CA1 region in mice treated with the α4 integrin-specific antibody than those receiving the isotype control **(Fig. 27D).** The overall amelioration of both neuropathological conditions and behaviour tests is partially in contrast with the results showed for the depletion of CD4+ T cells. However, this may be due to a more complex role for α4 integrins in the events of transmigration related to other key protagonists of innate and adaptive immunity (e.g monocytes, neutrophils or B cells). Taken together, our findings demonstrate that the therapeutic targeting of α4 integrins inhibits early disease development and limits the disease progression in 3xTg-AD mice

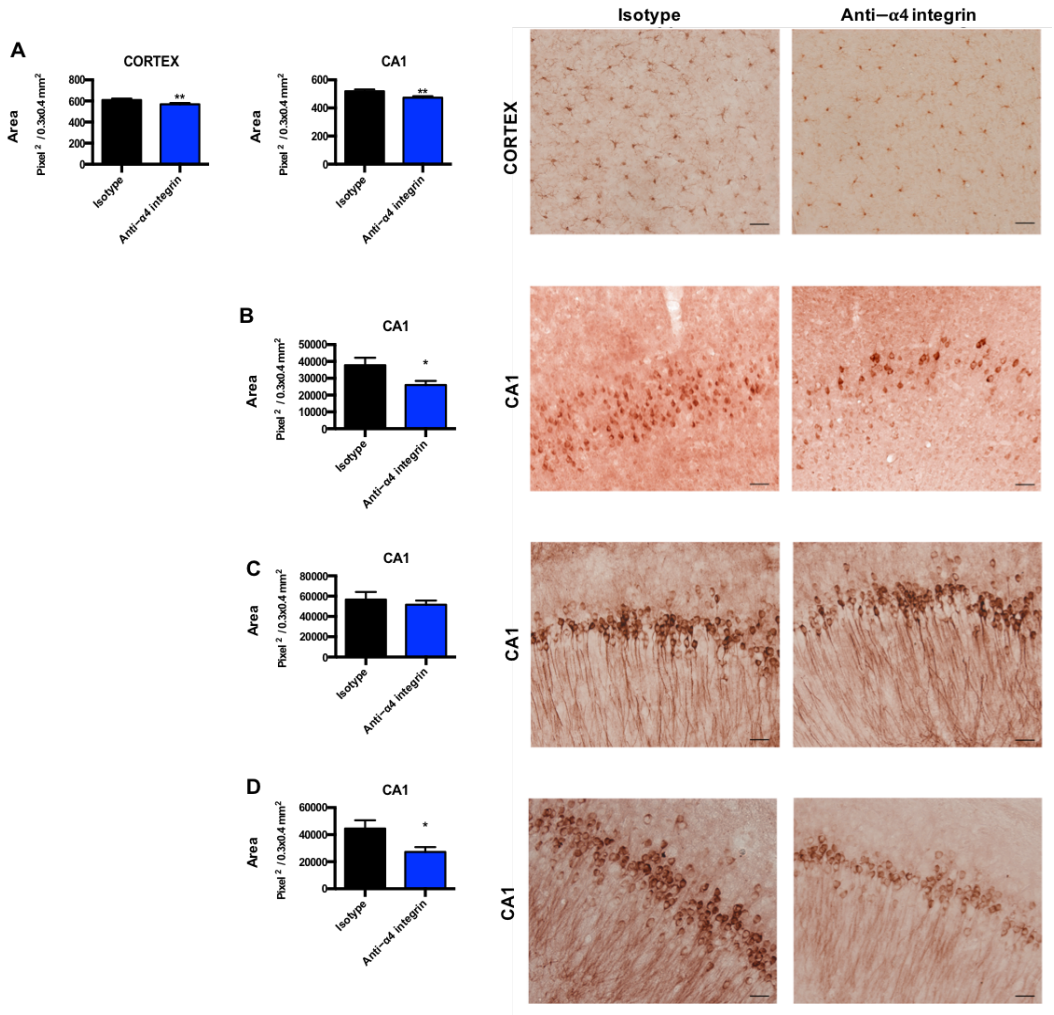


Figure 27. Inhibition of α4 integrins at the early stage of AD reduces microglial activation, Aβ load and hyperphosphorylated tau in 3xTg-AD mice. (**A**) The graphs show significant differences in microglial activation in the cortex and CA1 hippocampal region of treated mice and isotype controls. Error bars show SEM. Representative images show Iba-1 staining of microglia in the cortex of isotype controls (left) and the α4 integrin blockade (right). (**B**) Quantitative analysis of Aβ accumulation in the CA1 sub-field of hippocampus (left). Error bars show SEM. Representative images show Aβ deposition in the hippocampus of 3xTg-AD mice treated with isotype controls (left) and the α4 integrin blockade (right). (**C**) Quantitative analysis of total tau protein in the CA1. Representative images show staining for total tau in isotype controls (left) and the α4 integrin blockade (right). (**D**) Quantitative analysis of the area of $AT180^{+}$ cells in the CA1 of 3xTg-AD mice. Results are shown as mean ± SEM. Representative images show $AT180^{+}$ cells in isotype controls (left) and the α4 integrin blockade (right). $^{*}P$ <0.05, $^{**}P$ <0.01 Unpaired *t*-test with Welch's correction. Scale bar in all images 50 µm. In all panels, n = 3 mice (2 F, 1 M) for the isotype group, and n = 3 mice (2 F, 1 M) for the anti-α4 group.

24.2 LFA-1 INTEGRIN EXPRESSION IS INCREASED ON PERIPHERAL LEUKOCYTES IN AD MICE

Our data suggest that α4 integrins may contribute to the recruitment of CD4+ T cells in the brain of 3xTg-AD mice at early disease stages, whereas CD8+ T cells

may use other integrins for their specific recruitment to the inflamed brain. Therefore, we evaluated the expression of the integrin LFA-1 on the surface of circulating lymphocytes in 3xTg-AD mice during the disease progression. Although the role of LFA-1 has been already previously established by our group in the context of neutrophil trafficking in AD (Zenaro E, *Nat Med* 2015), we cannot rule out the hypothesis that this integrin may also be involved in the recruitment of other leukocyte populations. We collected blood samples from 3xTg-AD mice and their sex- and age-matched WT control mice. We identified by flow cytometry two distinct sub-populations in peripheral T cells characterized by different levels of LFA-1 expression intensity, named LFA-1^{high} and LFA-1^{int} **(Fig. 28A, B).** The analysis showed a significant increase in the frequency of both LFA-1^{high} CD4+ and LFA-1^{high} CD8+ T cells **(Fig. 28C, E)** in 3xTg-AD mice compared to control animals at early stage of the disease (6 and 9 months of age) and at later time-points (12 months of age). We observed that the frequency of LFA-1^{high} T cells significantly increases at advanced stage of the disease in 3xTg-AD mice compared to WT **(Fig. 28C, E).** Next, we checked the mean fluorescence intensity (MFI), and our data showed that the MFI of LFA-1^{high} CD4+ T cells increased in correlation with the disease progression **(Fig. 28D).** In contrast, peripheral LFA-1^{high} CD8+ T cells exhibited no difference in the MFI **(Fig. 28F).** Taken together, our results clearly show that in addition to neutrophils, also T cells express high levels of the integrin LFA-1 in the peripheral circulation of 3xTg-AD mice at early disease stages. We identified a specific subset of activated peripheral T-lymphocytes that expressed high levels of LFA-1, and the relative abundance of this subset is increasing during disease progression in both CD4+ and CD8+ T cells. Furthermore, our data suggested that the progressive increase of LFA-1 expression as MFI on CD4+ T cells may correlate with disease progression.

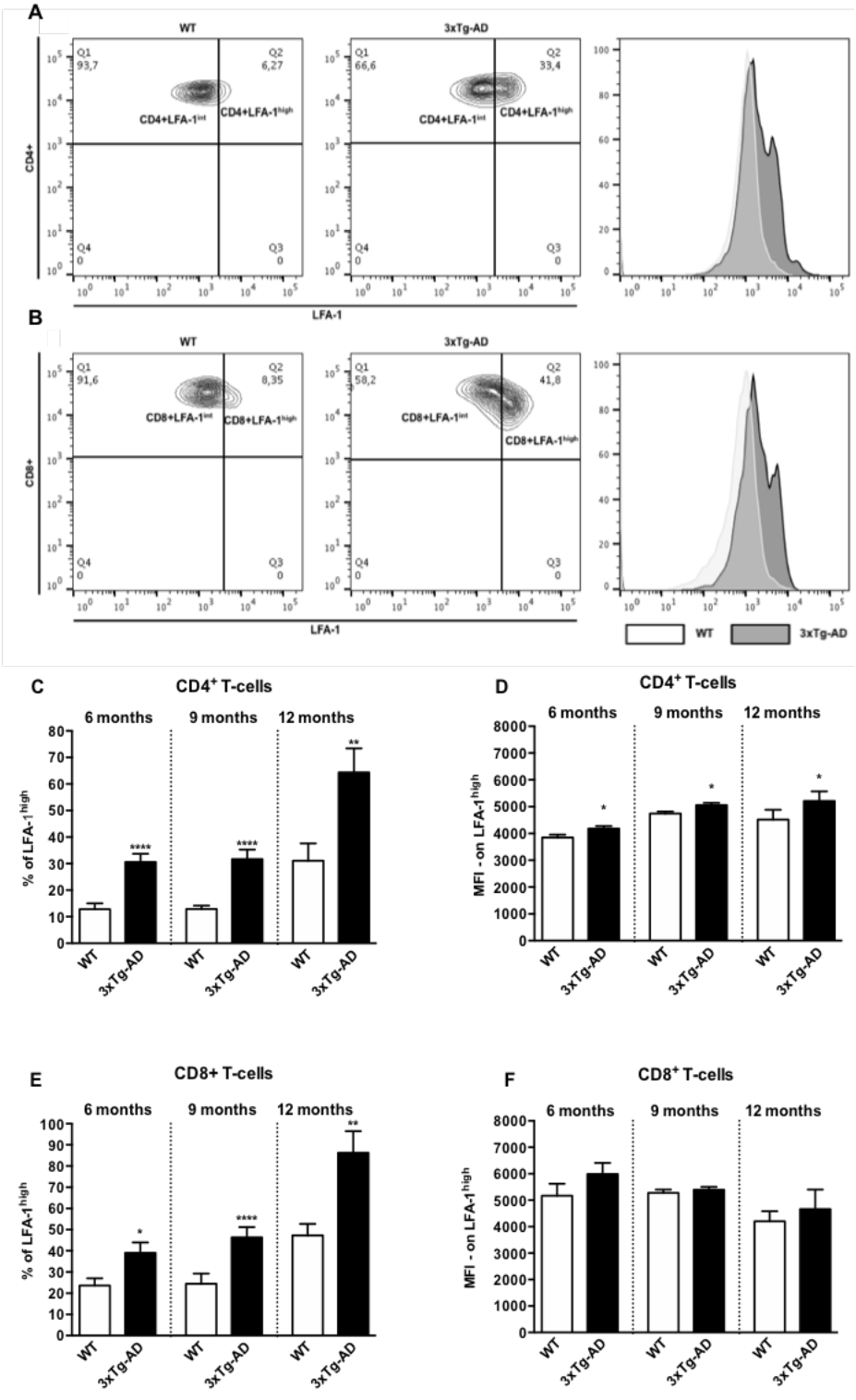


Figure 28. Differential expression of LFA-1 integrin on T-lymphocytes population. We evaluated the expression of the integrin LFA-1 (anti-CD11a/CD18) in CD4+ or CD8+ T cells. **(A, B)** Representative contour plots and related histogram of LFA-1^{high} expressing CD4+ and CD8+ T-cells isolated from the blood of 3xTg-AD mice and WT controls. **(C, E)** Plots represent the frequency of LFA-1^{high} cells as the percentage of positive cells in CD4+ or CD8+ populations **(D, F)** Geometric mean of mean fluorescence intensity (MFI) of LFA-1^{high} in indicated cell population.

Data are shown as mean ± SEM. (*P <0.05; **P <0.01, ***P < 0.001, ****P <0.0001, *Mann-Whitney U-test)*. 8-10 animals/groups of the same age were used.

24.2.1 LFA-1 INTEGRIN IS CRUCIAL FOR LEUKOCYTE INFILTRATION IN THE BRAIN DURING AD PATHOLOGY

We have previously generated a 3xTg-AD mouse model deficient for the integrin LFA-1 (3xTg-AD*x*Itgal$^{-/-}$). In order to evaluate the contribution of the integrin LFA-1 to the accumulation of T cells in the brain, we isolated brain infiltrating-leukocytes from 3xTg-AD*x*Itgal$^{-/-}$ mice at 6 and 9 months of age and, in comparison, age- and sex matched 3xTg-AD mice. As expected, our data confirmed the significant reduction of T cells infiltrating the brain of 3xTg-ADxItgal$^{-/-}$ when compared to 3xTg-AD mice **(Fig. 29A, B).** This blockade is particularly evident at both 6 and 9 months of age, where the genetic deletion of LFA1 highly reduces the brain infiltration of CD4+ T cells and more importantly of CD8+ T cells. These data suggest that the integrin LFA-1 plays a crucial role in the recruitment of all T cells.

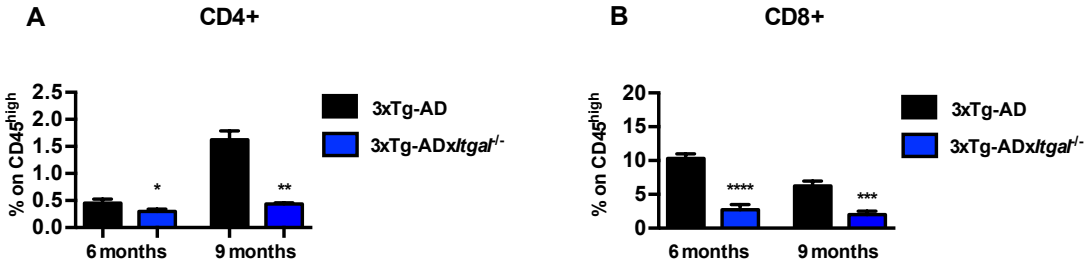


Figure 29. The integrin LFA-1 plays a role in the brain accumulation of T cells in 3xTg-AD mice. Quantitative flow-cytometry analysis of **(A)** CD4+ T cells and **(B)** CD8+ T cells accumulation in the brain of 3xTg-AD mice compared to 3xTg-ADxItgal$^{-/-}$ mice at 6 and 9 months of age. (*P <0.05, **P <0.01, ***P <0.001, ****P <0.0001, *Mann-Whitney U-test)*. A number of 8-10 were analyzed in each group. Error bars represent SEM.

24.2.2 LFA-1 DEFICIENCY AMELIORATES MEMORY IMPAIRMENT IN 3xTg-AD MICE

In order to evaluate spatial working memory performances, we assessed 3xTg-AD*x*Itgal$^{-/-}$ and 3xTg-AD age-matched control mice in behavioral paradigms at 9 months of age as described in materials and methods. In the Y-maze paradigm, 3xTg-AD*x*Itgal$^{-/-}$ mice performed significantly better when compared to age-and-

sex matched 3xTg-AD animals, as evident by their increased % of alternation **(Fig. 30A)**. We next performed the CFC behavioral test. The genetic deletion of LFA-1 integrin in 3xTg-AD mice also rescued associative memory impairment; in fact, 3xTg-ADx*Itgal*$^{-/-}$ mice spent significantly more time freezing than age-matched 3xTg-AD littermates **(Fig. 30B).** To further confirm the importance of LFA-1 integrin in hippocampus-dependent performances such as spatial navigation learning and long-term memory, we performed a third behavioral test, the MWM. Our results were in line with the previously described behavioral tests. Indeed, during the spatial probe test 3xTg-AD mice devoid of the integrin LFA-1 spent significantly more time in the target quadrant than 3xTg-AD control mice **(Fig. 30C)**. Additionally, the performance during the probe tests was evaluated by the number of entries in the proximity of the area on its own as well as in comparison to the other three quadrants. The probe represents the last part of the MWM test and during this phase the platform is removed, and each mouse is allowed to freely swim for 60s. Our results showed a statistically significant increase in the number of the entries in the "target zone proximity" in 3xTg-ADx*Itgal*$^{-/-}$ mice compared to 3xTg-AD mice suggesting an amelioration in the spatial memory **(Fig. 30D).** Collectively, these experiments clearly show that ablation of LFA-1 integrin contributes to the re-establishment of cognitive function in 3xTg-AD mice and that cognitive recovery could be due to a decrease in leukocyte recruitment into the brain.

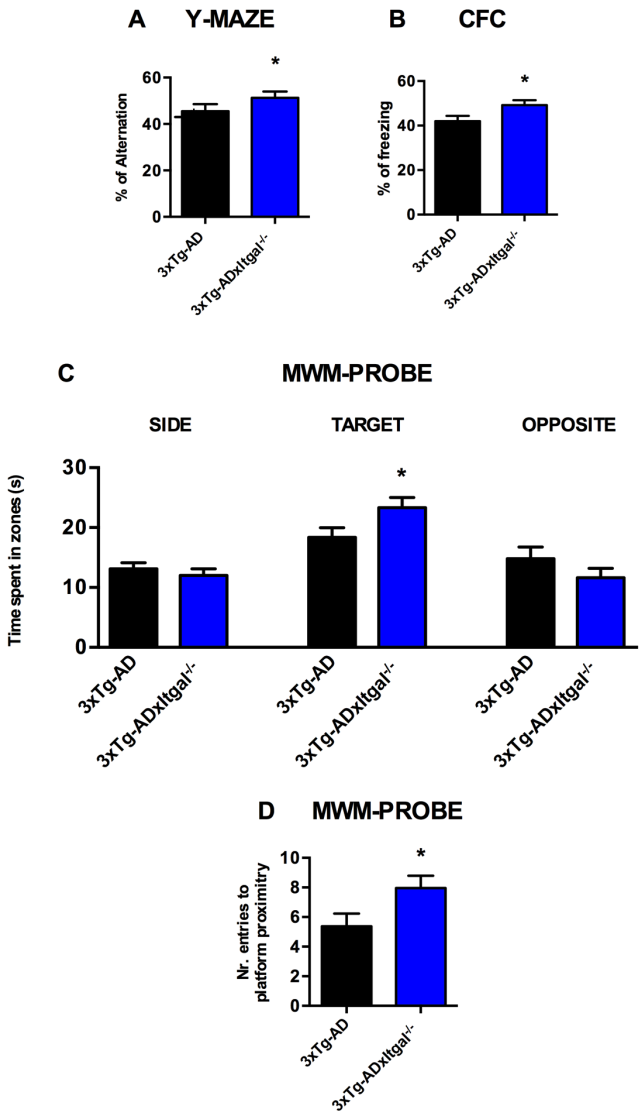


Figure 30. Deletion of LFA-1 integrin improves cognitive functions in 3xTg-AD mice. 3xTg-ADx*Itgal*$^{-/-}$ mice behavioral capabilities were tested at 9 months of age. (**A**) Histograms show the percentage of alternations performance in the Y-maze test and **(B)** the percentage of freezing response in CFC test. **(C)** Time spent during probe testing in different zones of the MWM. **(D)** The number of entries to the "target zone proximity" during probe testing. Error bars represent SEM (**P* < 0.05, *Mann-Whitney U-test*). A number of 10-15 mice/group were analyzed.

24.2.3 AD NEUROPATHOLOGY IS AMELIORATED IN 3xTg-ADxItgal$^{-/-}$ MICE

As the genetic ablation of LFA-1 integrin showed a reduction of cognitive impairment in 3xTg-AD mice, we therefore investigated whether these data were supported by neuropathological findings. At the end of the behavioral test, mice were sacrificed, and immunohistochemistry studies were performed. We previously demonstrated in 3xTg-ADx*Itgal*$^{-/-}$ mice a reduction of microgliosis, as assessed by

Iba-1 staining, compared to control mice (Zenaro E, *Nat Med 2*015). Here, we confirmed the data on microglia cells further increasing the number of animals analyzed. As showed in **Figure 31A, B,** microglia in 3xTg-ADx*Itgal*$^{-/-}$ mice showed a small roundish soma and long processes, corresponding to non-activated cell phenotype, in cortical and hippocampal (CA1) regions compared to 3xTg-AD mice. Additionally, we quantified Aβ deposition and tau hyper-phosphorylation in 3xTg-ADx*Itgal*$^{-/-}$ mice compared to age- and sex-matched 3xTg-AD mice. The accumulation of Aβ was examined by 6E10 staining. We performed a quantitative stereological analysis to determine the total area occupied by Aβ-positive neurons. Our data showed a reduction of Aβ accumulation in the hippocampus (CA1 and subiculum) of 3xTg-ADx*Itgal*$^{-/-}$ compared to 3xTg-AD mice **(Fig. 32A, B).** Finally, to determine the effect of the genetic ablation of the integrin LFA-1 on tau pathology we performed immunohistochemical staining for the protein tau in 3xTg-AD compared to 3xTg-ADx*Itgal*$^{-/-}$ mice. As previously described, we used the HT7 and AT180 antibody to detect the expression of total and phosphorylated tau, respectively. As expected, we did not detect any significant differences in total tau levels in examined hippocampal areas (CA1) **(Fig. 33A)**. However, the quantitative analysis of tau phosphorylation showed a decreased labeling in CA1 region of the hippocampus in 3xTg-ADx*Itgal*$^{-/-}$ compared to 3xTg-AD control mice **(Fig. 33B).** Collectively, these analyses clearly showed that the genetic deletion of the integrin LFA-1 may have neuroprotective effects during the progression of AD pathogenesis.

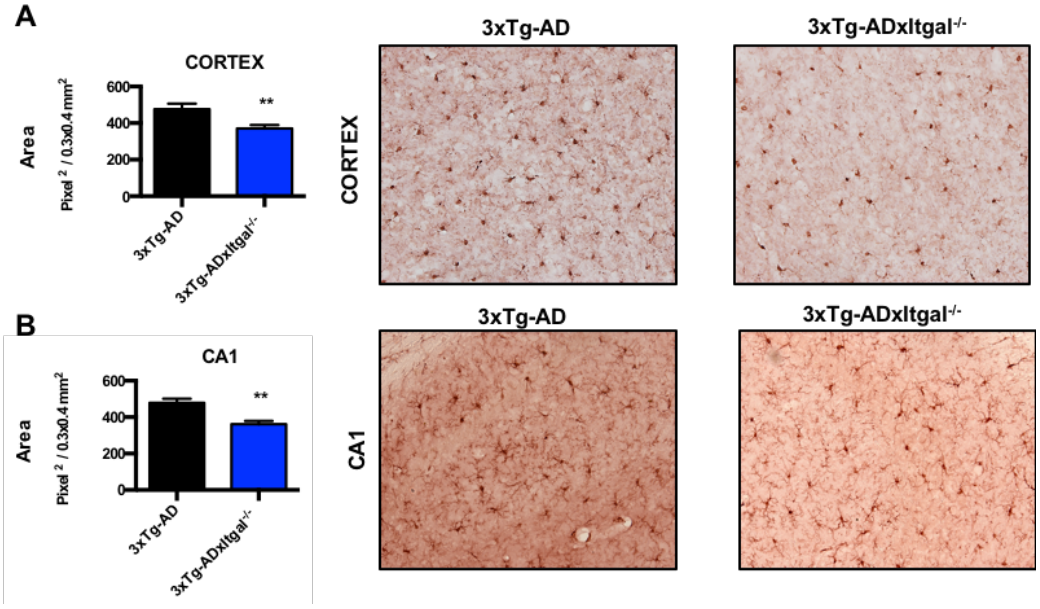


Figure 31. Morphological changes in microglial activation status are evident in AD-mice lacking the integrin LFA-1. (A) Quantitative analysis of microglia in cortical regions and **(B)** CA1 hippocampal region of 9 months old 3xTg-AD and 3xTg-AD*x*Itgal$^{-/-}$ mice. Results are shown as mean ± SEM. (**P <0.01, *Mann-Whitney U-test*). AD-mice deleted for LFA-1 integrin showed a less activated microglia phenotype. On the right, representative images stained with Iba-1 antibody. Scale bar 50 µm. In all panels, n = 3 mice (2 F, 1 M) for the 3xTg-AD group, and n = 3 mice (2 F, 1 M) for the 3xTg-AD*x*Itgal$^{-/-}$ group.

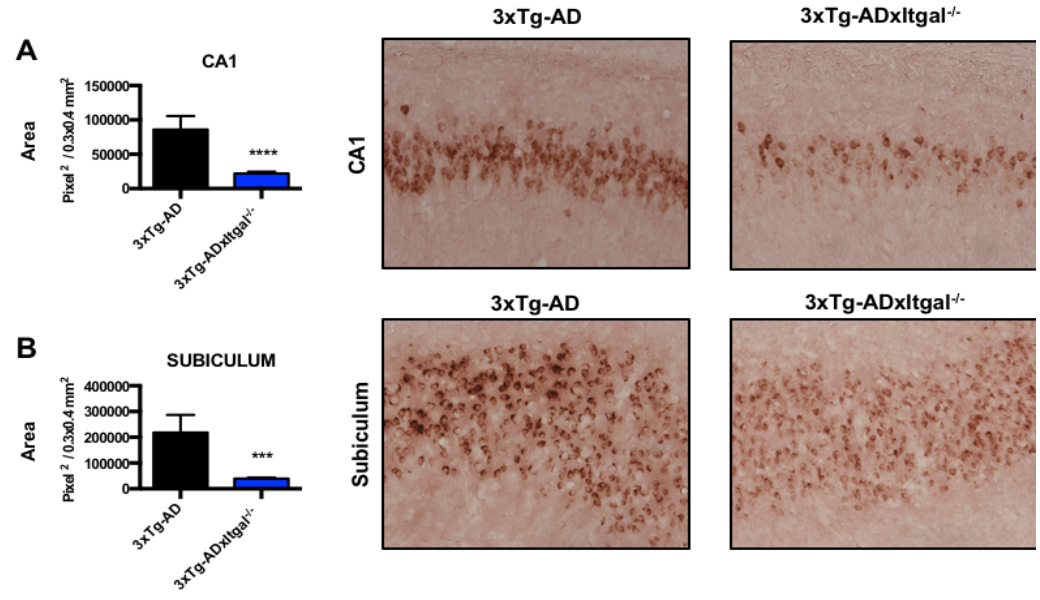


Figure 32: 3xTg-AD*x*Itgal$^{-/-}$ mice display a reduction in Aβ accumulation compared to age- and sex-matched 3xTg-AD mice. Quantification of brain sections of **(A)** CA1 and **(B)** subiculum areas of the hippocampus labeled with anti-Aβ antibody (6E10). Results are shown as mean ± SEM. (***P < 0.001, ****P <0.0001; *Mann-Whitney U-test*). Representative images are shown on the right. Scale bar: 50 µm. In all panels, n = 3 mice (2 F, 1 M) for the 3xTg-AD group, and n = 3 mice (2 F, 1 M) for the 3xTg-AD*x*Itgal$^{-/-}$ group.

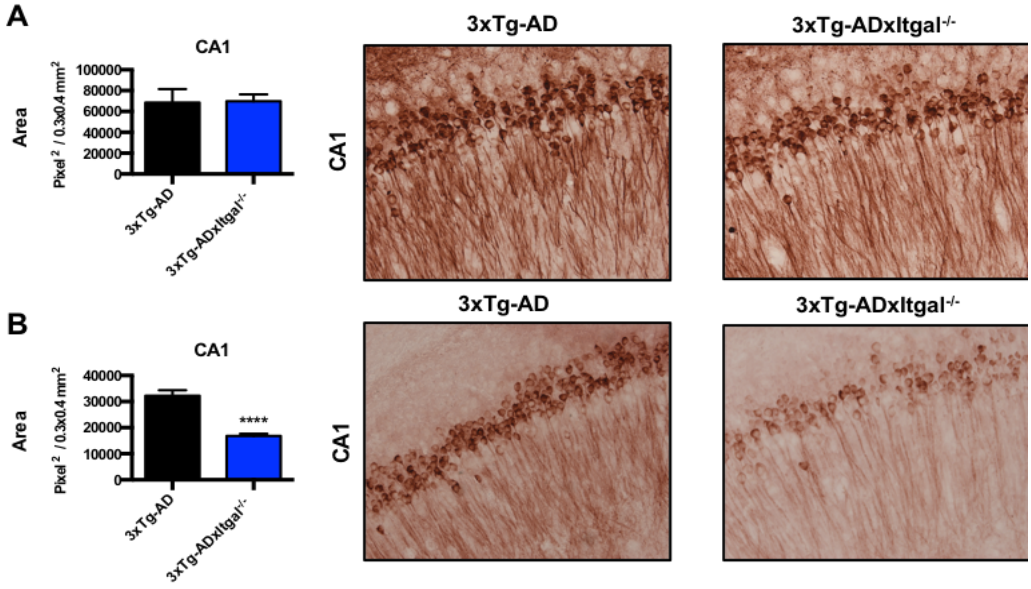


Figure 33. 3xTg-AD*Itgal*$^{-/-}$ mice displayed a reduction in tau phosphorylation levels. Coronal sections of 9-month-old 3xTg-AD*Itgal*$^{-/-}$ and 3xTg-AD animals were stained with human-specific **(A)** HT7 and (**B**) AT180 antibodies to assess total tau inclusions and phosphorylated tau, respectively. Quantitative analysis of proteins in CA1 region are shown on left panels. Results are shown as mean ± SEM. (****P <0.0001, *Mann-Whitney U-test*). On the right, representative images in CA1 are shown. Scale bar: 50 µm. In all panels, n = 3 mice (2 F, 1 M) for the 3xTg-AD group, and n = 3 mice (2 F, 1 M) for the 3xTg-AD*Itgal*$^{-/-}$ group.

25. AN *IN VITRO* MODEL FOR THE STUDY OF LYMPHOCYTE-CNS CELLS INTERACTIONS IN AD

25.1 MORPHOLOGY AND ORGANIZATION OF 3xTg-AD MICE DERIVED CNS CELLS

Alzheimer's disease transgenic mice have been used as a standard AD model for basic mechanistic studies and drug discovery. Recent advances in human stem cell and three-dimensional (3D) culture technologies made possible the generation of novel 3D neural cell culture models that recapitulate AD pathologies including robust Aβ deposition and Aβ-driven NFT-like tau pathology and discover the best targets to take forward into clinical trials. Since the invention of successful methods for the generation of induced pluripotent stem cells (iPSCs) from somatic cells, like fibroblast, from human healthy subjects and patient enormous progresses were made. Therefore, we started to generate an *in vitro* model using 3xTg-AD mice harboring human APP_{Swe} and human tau_{P301L} (both under control of the mouse Thy1.2 regulatory element) to isolate neuronal cells. The mouse Thy1.2 expression

cassette has been demonstrated to drive transgene expression predominantly to the CNS (Caroni P, *J Neurosci Methods* 1997) starting from day 7-10 of mouse growth. Unlike, a published method using cell line transfected with AD mutation, (Choi SH, *Nature* 2014) our aim was to develop a model that autonomously reproduce hallmarks of AD in order to evaluate intraparenchymal mechanisms associated with AD pathogenesis. At the day of isolation, we checked the purity of CNS cells by flow-cytometric analysis. The neuronal cell fraction showed a high purity (98.6–99.9%) and only a low percentage of contaminating non-neuronal cells with a viability rate for target cells of >90%. In order to confirm these data, two weeks after seeding neuronal cells were fixed with 4% PFA and stained for the expression of typical lineage markers by immunofluorescence staining. Indeed, we used CD11b, microtubule associated protein 2 (MAP2) and glial fibrillary acid protein (GFAP), as microglial, neuronal and astrocytic markers respectively. Our images clearly show that cells in *in-vitro* culture displayed the characteristic morphology of microglia, neurons and astrocytes. In particular, neurons showed differential stages of differentiation and prolonged axons touching nearby cells **(Fig. 34A)**. Microglia cells showed an elongated shape typical of an inactivated phonotype and activated amoeboid cells **(Fig. 34B).** Astrocytes formed a dense monolayer with heterogeneous cytoplasmic morphology. Some possessed broad, sheet-like cytoplasm, others gave rise to processes, some quite long, with frequent branching **(Fig. 34C).**

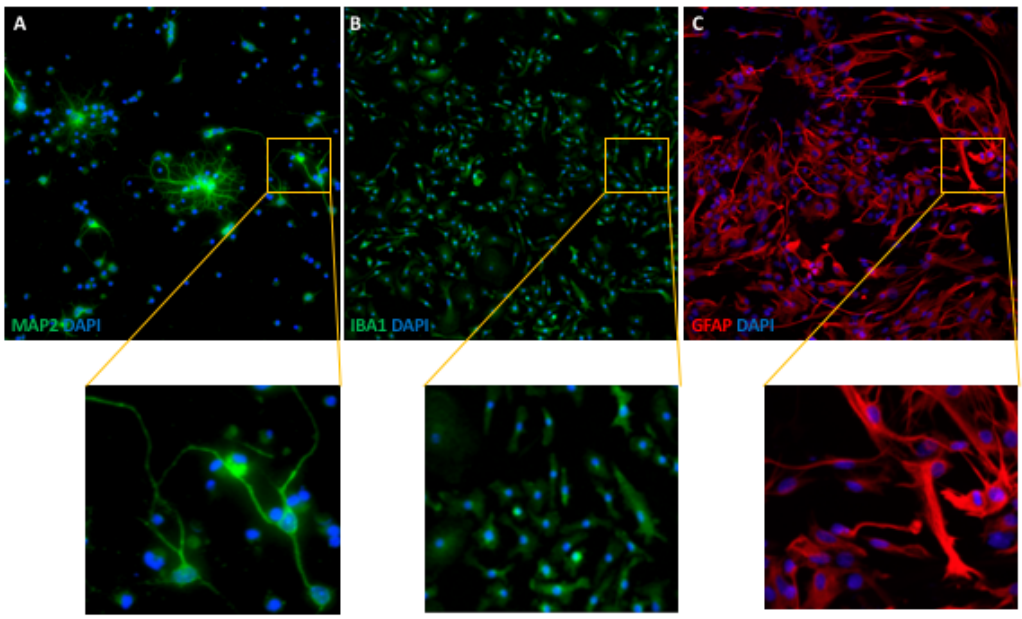


Figure 34. Representative epifluorescence images of CNS cells isolated from 12 days old 3xTg-AD mice. Confocal microscopy showing representative images of CNS cells. (**A**) MAP2 expressing neurons are shown in green; (**B**) Iba1+ microglia cells are shown in green. (**C**) GFAP+ astrocytes are stained in red. (**A**, **B**, **C**) Nuclei are stained with DAPI in blue. 40x magnification, Scale bar: 20 µm. Higher magnification of the quadrants is shown in the lower panels (40X).

25.2 CNS CELLS FROM 3xTg-AD MICE RECAPITULATE HALLMARKS OF AD IN VITRO

To further characterize our *in-vitro* model, we analysed the expression of tau and APP in cell culture by qPCR. qPCR array analysis showed an increase in the transcript of the transgenes (Tg) APPSwe and TAU*P301L in neurons from 3xTg-AD mice compared with neurons from WT mice (CTRL) kept in culture for 15 days **(Fig. 35A).** Interestingly, also astrocytes isolated from 3xTg-AD mice expressed the transcript of the transgene Tg (*APPSwe*) **(Fig. 35B).** Western blot (WB) analysis confirmed also the presence of the transduced protein; in fact, both neurons and astrocytes isolated from 3xTg-AD mice compared to WT mice (CTRL) showed higher expression of Aβ (Clone 6E10) **(Fig. 36A, B).** As expected, the expression of total tau (HT7) was found only in neurons but not in astrocytes from 3xTg-AD mice **(Fig. 36A, B).** Then, we checked on cell culture media whether the protein tau might be also released by neuronal cells isolated from 3xTg-AD mice and kept in culture for 21 days. Indeed, previous publications have shown that neurons need a

long time in culture to display extensive processes and release a soluble form of tau (Choi SH, *Nature* 2014; D'Avanzo C, *Bioessays* 2015). However, using the high sensible technology of Quanterix's Simoa assay, we detected a statistically significant increase of tau released in the supernatant of CNS cells derived from 3xTg-AD mice (4,9 pg/mL) compared to WT derived neurons (0,4 pg/mL) **(Fig. 37A)**. Together, our data showed that in our *in-vitro* conditions, both astrocytes and neurons kept for long time in culture display a stable phenotype. Besides, neuronal cells from 3xTg-AD mice displayed the genetic mutation typical of this strain making our model a useful tool to study AD-related mechanisms.

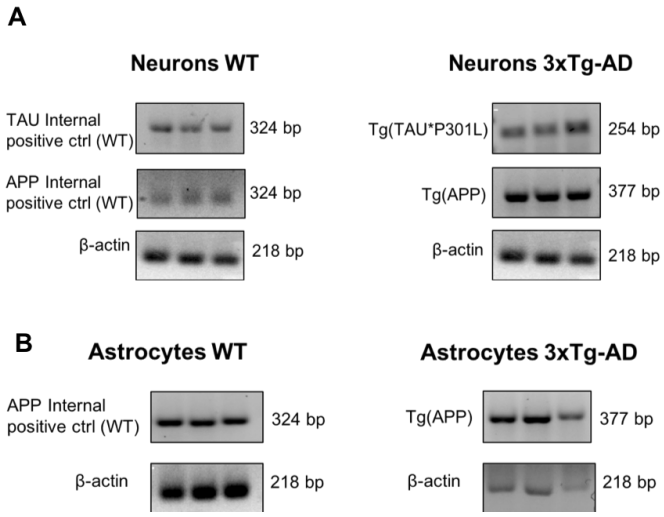


Figure 35. Comparison of mRNA transgene expression in neuronal culture. Representative qPCR data showing the mRNA expression of the transgenes *APPSwe (TgAPP)* and *TAU*P301L (TgTAU*P301L)* in **(A)** neurons and **(B)** astrocytes isolated from 3xTg-AD and B6129 control mice (WT).

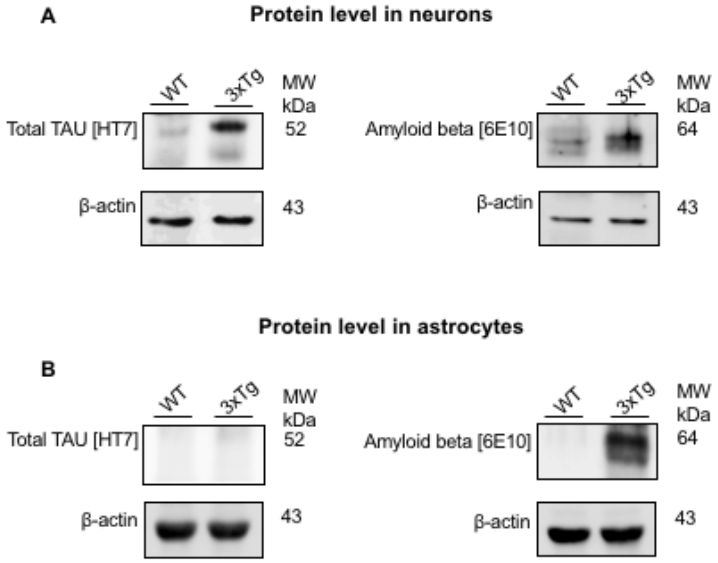


Figure 36. Comparison of mutated protein expression in neuronal cultures. Western blot analysis showing the protein expression of total tau (HT7) and Aβ (6E10) in 3xTg-AD-derived **(A)** neurons and **(B)** astrocytes compared to the ones isolated from WT control mice.

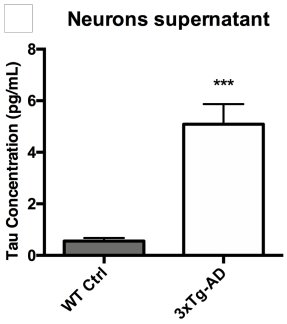


Figure 37. Quantification of Tau protein in the supernatant of neurons from 3xTg-AD and WT mice. Supernatant of neurons from 3xTg-AD and WT mice (Ctrl) maintained for 21 days in culture were tested for tau release. Analysis was performed using *unpaired t-test*, $***P < 0.001$. One representative experiment is shown.

25.3 CHARACTERIZATION OF Th1 AND Th17 CYTOKINES PRODUCTION

Based on the information collected by our *in-vivo* experiments performed on 3xTg-AD mice showing the capability of T cells to infiltrate and accumulate in the brain during AD pathogenesis, we decided to use our *AD in vitro* model to highlight mechanisms of interaction between lymphocytes and resident CNS cells. Indeed, activated T cells once in contact with brain resident cells can induce or amplify of neuroinflammatory events. We thus decided to focus our attention on Th1 and Th17 cells, as it was reported by others that these CD4+ T cell subsets infiltrate the brain during AD (Browne TC, *J Immunol* 2013). We isolated naive $CD4^+$ T cells from

WT mice and activated them *in-vitro* with a cytokine mixture in order to promote the establishment of effector T helper lineages, such as Th1 and Th17, expressing the hallmark cytokine IFN-γ and IL-17, respectively. For intracellular cytokines staining, cells were stimulated for 6 hours with Phorbol 12-myristate 13-acetate (PMA) (50 ng/ml), ionomycin (1 µg/ml) and brefeldin A (10 µg/ml). Permeabilized cells were labeled with proper antibodies to evaluate the intercellular IFN-γ and IL-17 accumulation. As control, cells were stimulated in the same conditions, but without brefeldin A. We obtained high numbers of pure Th1 and Th17 cells free of other contaminating cells. In Th1 cultures, we achieved more than 80% of pure T cells that produced IFN-γ, whereas in Th17 cultures 60% of cells produced IL-17 **(Fig. 38).**

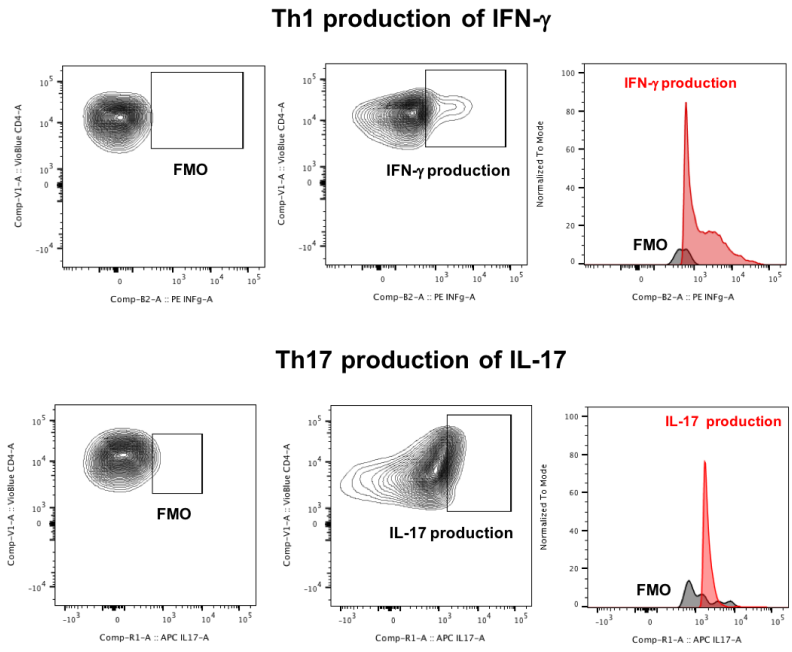


Figure 38. Intracellular staining of Th1 and Th17 cells. Representative dot-plot flow-cytometry panels showing the intracellular accumulation of IFN-γ and IL-17 in Th1 and Th17 cells.

25.4 POLARIZED Th1 AND Th17 CELLS TRIGGER MICROGLIA ACTIVATION

To determine whether IL-17+ Th17 and IFN-γ+ Th1 polarized cells can induce microglia activation, we co-cultures for 24 hours Th1 or Th17 cells with primary microglia isolated from 3xTg-AD and WT ctrl mice. Then, we evaluated by flow-cytometry the phenotype of microglial cells. CD11b positivity was used to detect

microglia and then cells were sub-gated by using CD68 and MHCII mAbs to evaluate their activation status. We observed that only Th1 cells co-cultured with AD-derived microglia triggered a significant upregulation of CD68 expression. On the contrary, no differences in CD68 levels were detected on AD-derived microglia cultivated with either Th17 cells or freshly isolated and unstimulated CD4+ T cells (naïve) **(Fig. 39A).** Additionally, both Th1 and Th17 cells were unable to trigger upregulation of CD68 expression in WT-derived microglial cells **(Fig. 39A).** Besides CD68, Th1 and Th17 cells triggered a significant upregulation of MHCII on AD-derived co-cultured microglial cells, reaching a higher statistical significance for Th1 cells **(Fig. 39B).** As reported for CD68, we observed no significant differences in MHCII expression in WT-derived microglia co-cultured with Th1 and Th17 cells **(Fig. 39B).** These results indicated that effector T helper cells can be involved in the enhancement of neuroinflammation in the brain during AD due to their interaction with microglia cells and subsequent activation of these cells.

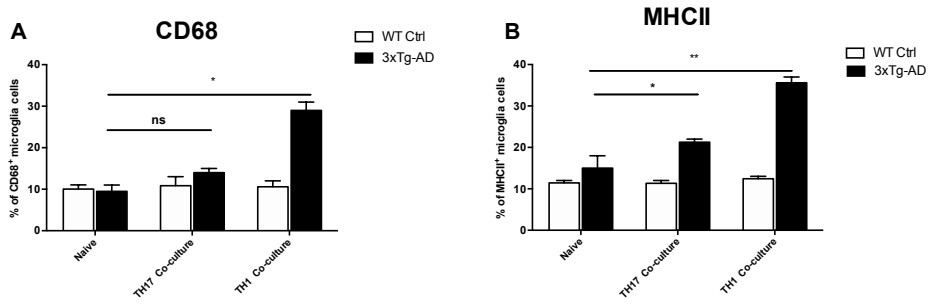


Figure 39. Effector T helper cells trigger expression of CD68 and MHCII on primary AD-derived microglial cells. Quantitative flow-cytometry analysis of primary microglia isolated from 3xTg-AD (black bars) and wild-type control mice (white bars). The graphs show the proportion of cells expressing CD68 and MHCII. Error bars represent SEM (*$P < 0.05$, **$P < 0.01$, Mann-Whitney *U*-test).

25.5 POLARIZED Th1 CELLS INDUCE CD44 EXPRESSION ON ASTROCYTES *IN VITRO*

To further evaluate the role of T helper cells in AD pathogenesis we co-cultured Th1 and Th17 cells with astrocytes isolated from 3xTg-AD and WT ctrl mice. Then, we evaluated by flow-cytometry the phenotype of astrocytes by staining co-cultured cells with CD44, an important astrocyte activation marker. CD44, which is a ubiquitous receptor expressed on the surface of many cells including astrocytes

(Underhill C, *J. Cell Sci,* 1992), can efficiently bind high and low molecular weight hyaluronan (HA). HA is one of the major glycosaminoglycans in the extracellular matrix in the brain and plays an important role in morphogenesis, remodeling and integrity of the CNS (Asher R, *J Neurosci Res* 1991). Moreover HA-CD44 interaction plays an important role in regulating astrocyte function (e.g., migration) following injury (Holtje M, *J. Neurochem* 2005). Our FACS results showed that IFN-γ producing Th1 cells triggered an increase of CD44 expression on astrocytes isolated from 3xTg-AD mice, whereas Th17 or naïve CD4+ T cells were unable to do so **(Fig. 40).** Of note, Th1, Th17 and naïve CD4+ T cells were unable to trigger upregulation of CD44 expression in WT-derived microglial cells. To determine whether CD44 expression is triggered by the direct interaction with Th1 cells, we stained primary astrocytes prepared from 3xTg-AD mice and polarized Th1 cells isolated from WT mice. We observed the presence of Th1 cells near area of astrocyte ramification, suggesting that CD44 expression on astrocytes could be correlated with a direct interaction between GFAP+ astrocytes and Th1 cells **(Fig. 41)**. Collectively, our data obtained in vitro suggest that the interactions between Th cells and AD-derived brain cells can trigger inflammatory events potentially contributing to the neuroinflammation during AD.

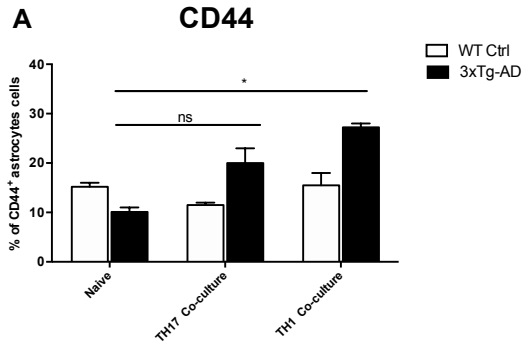


Figure 40. Th1 cells trigger CD44 expression on primary AD-derived astrocytes. Quantitative flow-cytometry analysis of primary astrocytes isolated from 3xTg-AD (black bars) compared to wild-type control mice (white bars). The graphs show the proportion of cells expressing CD44 receptor. Error bars represent SEM (*$P < 0.05$, Mann-Whitney *U*-test).

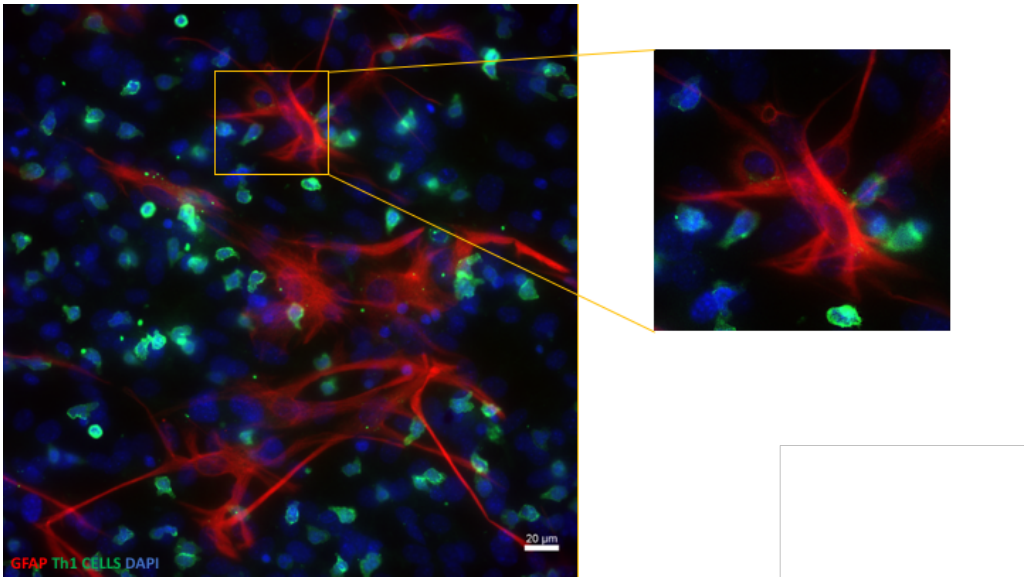


Figure 41. Polarized Th1 cells interact with AD-derived GFAP+ astrocytes. Representative confocal microscopy images of Th1 cells colocalizing with *in-vitro* cultivated astrocytes isolated from 3xTg-AD mice. Astrocytes are stained with Alexa Fluor 594 GFAP antibody (red), nuclei are stained with DAPI (blue), and Th1 cells are stained with Alexa Fluor 488 CD4 (green). Magnification of the quadrant is shown in the right-hand image: 40X.

DISCUSSION

Several studies on AD highlighted the presence of brain-infiltrating immune cells and explored their role in disease progression (Baik SH, *Neurobiol Aging* 2014; Zenaro E, *Nat Med* 2015; Hohsfield LA, *Exp Gerontol* 2015; Ferretti MT, *Brain Behav Immun* 2016; Sommer A, *Mol Neurodegener* 2017, Le Page A, *Exp Gerontol* 2018; Merlini M, *Neurodegener Dis* 2018). Innate and adaptive immune cells are found in the brain of human AD patients and related animal models. The presence and pathological role of neutrophils were clearly demonstrated in AD mouse models as shown by our lab as well as other groups (Baik SH, *Neurobiol Aging* 2014; Zenaro E, *Nat Med* 2015). Notably, we showed that neutrophils migrated into the AD-like disease brain and played an unexpected role in the induction of cognitive deficit and neuropathological changes (Zenaro E, *Nat Med* 2015). Moreover, our group has shown that interfering with LFA-1-dependent neutrophil adhesion improves cognitive functions, confirming the detrimental role of neutrophils and the potential of anti-integrin therapy in AD.

Although leukocyte trafficking during AD has not been investigated in great detail, several lines of evidences now support the hypothesis that both innate and acquired immune system cells adhere to vessels and migrate into the brains of AD patients and corresponding animal models (Zenaro E, *Nat Med* 2015; Cruz Hernández JC, *Nat Neurosci* 2019). Regarding the adaptive arm of the immune system, Ferretti et al. demonstrated increased numbers of infiltrating T-cells in the brain of AD transgenic mice (Ferretti MT, *Brain Behav Immun* 2016). Moreover, in the hippocampus and other limbic regions of AD patients, a predominance of cytotoxic T cells to T helper cells has been observed (Togo T, *J Neuroimmunol* 2002; Merlini M, *Neurodegener Dis* 2018), but their specific role is still a matter of discussion. Likewise, St-Amour et. al. recently showed an elevated level of IL-2, TNF-α, IL-17 and granulocyte-macrophage colony-stimulating factor (GM-CSF) in the serum of 3xTg-AD mice compared to their controls (St-Amour I, *Journal of Neuroinflammation* 2019) suggesting increased activation of T helper lymphocytes in the 3xTg-AD model. Furthermore, 9-month-old 3xTg-AD animals were shown to have increased serum levels of IL-2, TNF-α, IL-17, and GM-CSF compared with controls (St-Amour I, 2019 *J of Neuroinflamm* 2019). This cytokine profile in the

peripheral circulation suggests increased activation of blood helper T cells. The secretion of IL-2 is a critical and early landmark in the activation program of CD4+ T helper lymphocyte (Hagiwara E, *Cytokine*1995), and the cytokines IFN-γ/TNF-α and IL-17/GM-CSF are secreted respectively by the pro-inflammatory Th1 and Th17 cells (Romagnani S. *Ann Allergy Asthma Immunol* 2000; Mills KH. *Eur J Immunol* 2008; Korn T, *Annu Rev Immunol* 2009). In agreement with blood data sets, St-Amour et al. reported a rise of GM-CSF and IL-12, two cytokines associated to a Th1 immune response (St-Amour I, *J of Neuroinflammation* 2014), in the brain of 12-months old 3xTg-AD mice, suggesting the brain infiltration of CD4+ T helper lymphocyte.

Considering the potential role of T cells in AD pathogenesis, in the present PhD thesis we used several approaches in order to evaluate the contribution of these cells to the disease in 3xTg-AD mice, a mouse model of AD which develops both Aβ and tau pathology. Firstly, we characterized the phenotype of brain-infiltrating T cells during disease progression by flow cytometry, in order to understand how peripheral T lymphocytes may contribute to AD pathology at 6, 9 and 12 months of age in our mouse model. Indeed, our data showed a small but progressive age-dependent increase in CD4+ T helper cells accumulating in the brain of 3xTg-AD mice, reaching a peak of infiltration at 9 months of age. Differently, the frequency of CD8+ T cells was higher compared to T helper cells but their accumulation in the brain was inconsistent with disease progression. Thus, the quantitative differences among brain-infiltrating T cell subtypes in 3xTg-AD mice during disease progression suggests a distinct role for CD4+ and CD8+ T cells during different disease stages. Accordingly, Browne and colleagues reported a significant increase in the number of CD3+CD4+ T cells at 6 months of age in the brain of transgenic mice that overexpress APP and PS1 compared with WT mice (Browne TC, *J Immunol* 2013). Differently, Ferretti et al. demonstrated that the number of CD8+ but not CD4+ T cell was increased at later time point of the disease, reaching statistical significance at 24-months of age in ArcAβ mice, a transgenic mouse model developing only Aβ pathology (Ferretti MT, *Brain Behav Immun.* 2016). Collectively, these data suggest that the profile of T cells infiltration in the brain of

AD-like disease mice could be related to the phase of the disease, as well as with the mouse model of disease used in different reports.

We also evaluated the contribution of these cells to AD progression in 3xTg-AD mice transiently depleted of T cells. We administered monoclonal antibodies able to deplete CD4 or CD8 T cells from peripheral circulation to study their specific role in 3xTg-AD mice at early time-point of disease (6 months of age) and then performed behavioral assessment. At first, we carried out Y-maze, a hippocampus-dependent spatial working memory task. Several studies indicate that the hippocampus has a crucial role in tasks involving learning and remembering contexts (Holland P, *Curr Opin Neurobiol* 1999). Data collected following our Y-maze test showed no significant differences among 3xTg-AD mice devoid of T cells and control mice. In fact, all cohort of mice showed roughly comparable levels of alternation, thus indicating that T cells are not involved in the formation of short-term memory events connected to spatial navigation dictated by hippocampal functions. The Y-maze test investigates the spatial memory, one of the most widely studied forms of memory in animal models, although there are other forms of memory that must be taken into consideration to capture the full scope of memory loss that occurs during neurodegenerative states. For example, the associative and recognition memory are important to study, as those forms of memory loss often occur either in conjunction with or prior to, the loss of spatial memory in AD (Parra-Damas A, *Biol Psychiatry* 2017). The detection of familiar objects, a form of recognition memory, is known to deteriorate in early disease stages in AD (Laatu S, *Acta Neurol Scand* 2003). AD subjects may not recognize physical objects that they recently encountered, and in late stages of the disease those "objects" include those experienced regularly throughout life, including the faces of family members and friends. This so called "associative memory" has been suggested to be paired with working memory (Polcher A, *J Alzheimers Dis* 2017). Those types of associative and working memory, often called "executive functions", decline progressively in dementia (Baddeley AD, *Brain* 1991). Classical conditioning of the fear response in mice is a basic form of nondeclarative (nonconscious) memory that mediates both normal and pathological responses to aversive stimuli. Because fear conditioning critically depends on the amygdala, a medial temporal lobe

structure that frequently undergoes significant pathological changes early in the course of AD (Hamann S, *Neuropsychol* 2002), we further investigated this aspect in our 3xTg-AD mice devoid of CD4+ or CD8+ cells. Interestingly, our CFC test showed an increase in the fear response in both cohorts of 3xTg-AD mice treated with anti-CD4 or anti-CD8 depleting antibodies, indicating a potential role for T cells in the impairment of the "executive functions" characteristic of the late disease stages. Additionally, we performed Morris Water Maze (MWM) behavioral test in our cohort of animals to assess allocentric navigation, the ability to safely navigate through the environment using cues outside of the organism (distal cues), that depends on learning and remembering locations. In mice, this task involves the hippocampus, entorhinal cortex, and surrounding structures, whereas in humans this system encodes allocentric, semantic, and episodic memory. Apparently in AD occurs a break in the "mental frame syncing" between two kinds of allocentric representations, underpinned by damage to the hippocampus, which may significantly contribute to the deficit in episodic memory (Serino S*, Front Aging Neurosci* 2014). MWM represents the best way of assessing the allocentric navigation in mice. Interestingly, we found that our cohort of mice treated with anti-CD4 or anti-CD8 antibody were more prone to learn during the four days of the training session compared to 3xTg-AD mice treated with an isotype control antibody. In particular, a statistically significant reduction in the time to find the platform (escape latency) was observed in 3xTg-AD mice devoid of CD8 T cells, suggesting a potential contribution of these cells in the hippocampal circuits necessary for allocentric navigation.

After behavioral assessment, we performed immunohistochemical studies and evaluated the state of activation of microglial cells, accumulation of Aβ and tau phosphorylation. The transient early depletion of CD4 and CD8 T cells in 3xTg-AD mice showed a strong reduction of microgliosis and intraneuronal Aβ accumulation confirming a role for T cells in AD pathogenesis. Indeed, the beneficial effect we observed in behavioral tests may be partially due to a reduction of both microglia cell density and activation state in the hippocampus and cortex, which are known to play an important role in memory functions. In the initial steps of AD, microglia act as phagocytic cell facilitating the clearance of Aβ, whereas at

later disease stages, the chronic activation of microglia is accompanied by the diminished phagocytosis and the more abundant secretion of pro-inflammatory cytokines, leading to the accumulation of Aβ and the amplification of neuroinflammation (Hickman SE, *The J Neurosci* 2008; Krabbe G, *PLoS One* 2013). Therefore, the blockade of T cell accumulation in 3xTg-AD brain during the early phases of disease may also reduce events of microglia-induced neurodegeneration, allowing the preservation of cognitive functions in mice with AD-like disease.

Interestingly, comparing CD4 treated mice with sex- and age-matched isotype control mice, we observed no alterations in tau phosphorylation on Thr231 residue (AT180) in CA1 hippocampal area. Differently, the level of p-tau was significantly decreased after anti-CD8α treatment. Accordingly, a previous published study by Laurent et al. demonstrated a strong correlation between tau pathology and CD8+ T cell infiltration. Authors demonstrated that the development of tau pathology in THY-Tau22 mice at 12 months of age was associated with an early and progressive surge of microglia-produced CCL3, a chemokine contributing to brain recruitment of T cells. Indeed, among all leukocytes, CD8+ T cells were shown to specifically accumulate in the rostro-caudal axis area of the hippocampus, correlating with high levels of p-tau (Laurent C, *J Neurol* 2017) suggesting a connection between the infiltration of CD8+ T cell and the phosphorylation of the protein tau. Moreover, evidences from *in vitro* studies and transgenic mouse tauopathy models suggests that tau may also promote BBB deterioration (Vidal R, *Acta Neuropathol* 2000; Forman MS, *J. Neurosci* 2005; Kovac A, *J Alzheimers Dis* 2009). BBB dysfunction correlates with the appearance of perivascular tau around major hippocampal blood vessels (Blair LJ, *Acta Neuropathol Commun* 2015). Notably, when tau expression was suppressed, the integrity of the BBB was preserved, suggesting that the BBB can be stabilized in tauopathic brains by reducing tau levels (Blair LJ, *Acta Neuropathol Commun* 2015). Thus, reduce level of tau hyperphosphorylation as observed following anti-CD8 treatment may also promote BBB stabilization and integrity.

Our group has showed that the expression of E-selectin, P-selectin, VCAM-1 and ICAM-1 in brain vessels is significantly higher at early disease phases in 5xFAD

and 3xTg-AD mice compared to age-matched WT control mice, supporting the role of vascular inflammation in AD (Zenaro E, *Nat Med* 2015). Similarly, Ferretti et al. demonstrated an increased expression of VCAM-1 and ICAM-1 in the brains of 20–24-month-old Arc/SweAβ mice compared to WT littermates, further supporting a role for integrins and their counter-ligands during the entire AD progression (Ferretti MT, *Brain Behav Immun.* 2016). Firstly, we focused our attention on the integrin VLA-4, as main leukocyte integrin binding to the endothelial VCAM-1, known to contribute to T cell trafficking in the CNS (Engelhardt B, *Trends Immunol* 2012; Gorina R, *J Immunol* 2013). Our flow-cytometry data on peripheral lymphocytes showed a progressive age-dependent increase in the proportion of circulating CD4+ T cells expressing VLA-4, together with an increased mean fluorescence intensity (MFI), thus suggesting a higher activation status for peripheral CD4+ T cells during AD pathogenesis. VLA-4 was also expressed on circulating CD8+ T cells, but no differences were evident in numbers of cells or MFI between 3xTg-AD and wild-type mice. Hence, we speculate that VLA-4 may specifically control CD4+ T cell migration from the blood into the brain in AD mice. Based on this observation, we decided to block α4-integrins in order to interfere with CD4+ T cell recruitment into the brain. Indeed, this approach was successfully used in other brain inflammatory conditions such as multiple sclerosis, epilepsy and stroke (Yednock TA, *Nature* 1992; Baron JL, *J Exp Med* 1993; Fabene PF*, Nat Med* 2008; Neumann J, *Acta Neuropathol* 2015). We found that the therapeutic blockade of α4-integrins improves memory in cognitive test and reduces the neuropathological hallmarks of AD, including microglia activation, Aβ accumulation and tau hyperphosphorylation, supporting the beneficial effect of drugs targeting α4-integrins. Accordingly, previous data in APP/PS1 mice, a model of brain amyloidosis, showed that treatment with an anti-CD49d antibody reduces microgliosis, astrogliosis, CD4 immunoreactivity and synaptic changes, without altering the Aβ plaque load (Manocha G, *Curr Alzheimer Res* 2018). However, our observation on the reduction of intracellular Aβ accumulation during earlier stages of AD following α4-integrin treatment could be due to the use of a more complex model in which mice develop both Aβ and tau pathologies. Interestingly, treatments against the α4 chain of VLA-4 are already in the clinic. For example, natalizumab

is a humanized monoclonal antibody successfully used in patients with active relapsing–remitting multiple sclerosis and Chron's disease (Miller DH, *N Engl J Med* 2003; Ghosh S. *N Engl J Med* 2003) thus it can be rapidly translated to AD therapy.

Together with VLA-4, LFA-1 is a major integrin mediating firm adhesion during leukocyte recruitment cascade (Issekutz AC, *Immunol* 1992). LFA-1 is a β2-integrin expressed by all peripheral immune cells that, subsequently to its activation, mediates leukocyte sticking to the inflamed ICAM-1–expressing endothelium. Despite numerous evidences demonstrated the involvement of LFA-1 in T cell functions in several diseases, the involvement of LFA-1 in T cell recruitment in AD and its correlation with the neuropathological hallmarks of the disease have not been previously investigated. We therefore assessed the presence of a distinct subset of circulating CD4+ and CD8+ lymphocytes highly expressing LFA-1 in the blood of 3xTg-AD mice compared to controls, which increased during disease progression. Accordingly, high LFA-1 expression was described as being characteristic of memory T cells both in human and mouse cells (Sanders ME, *J Immunol* 1988; McNamara HA, *Sci Immunol* 2017). LFA-1 expression is also crucial for intratissue migration of effector and memory CD8+ T cells facilitating the entrance and local patrolling of target tissues such as liver and lungs (Thatte J, *Blood* 2003; McNamara HA, *Sci Immuno* 2017). LFA-1^{high} T lymphocytes, more frequently present in peripheral blood of 3xTg-AD mice compared to wild type mice, are considered a highly reactive T cell population (Hviid L, *Immunology* 1993), which may correlate with chronic peripheral inflammatory process taking place during AD. We also explored the brain accumulation of lymphocyte in 3xTg-ADx*Itgal*$^{-/-}$ mice, which were genetically depleted of the LFA-1 integrin. We showed a significant decrease in the recruitment of T cells at the onset of disease pathogenesis (6 months of age), which was maintained at 9 months of age, suggesting a sustained involvement of LFA-1 in T cell recruitment in 3xTg-AD mice. Interestingly, the effect of LFA-1 blockade was more prominent on CD8+ T cell, and several experimental evidence highlighted the involvement of LFA-1 signaling in functionality specifically associated to CD8+ cytotoxic T cells, such as contact dependent activation, proliferation and cell-mediated killing (Winter SS, *Br*

J Haemato 2001; Kandula S, *J Immunol* 2004; Semmrich M, *JEM* 2005; Meli AP, *Immunity* 2016). Apart from its function on brain-specific T cell recruitment, the absence of adhesion provided by LFA-1 on T cells was showed to induce an improper localization inside secondary lymphoid organs and altered binding to antigen presenting cells leading to an inefficient formation of immunological synapsis (Monks CRF, *Nature* 1998; Grakoui A, *Science* 1999; Kandula S, *J Immunol* 2004). Moreover, LFA-1 was showed to be critical in the reorganization of lymphocyte subsets within peripheral lymph nodes, and the blockade of LFA-1-ICAM-1 interaction leads to the exclusion of naive T cells from lymph nodes (Reisman NM, *Blood* 2011) meaning that 3xTg-AD LFA-1-deficient mice have more naive T cells in blood circulation compared to 3xTg-AD littermates. Consequently, low number of activated T cells in 3xTg-ADxItgal$^{-/-}$ blood stream could provide the beneficial effects in AD pathology which we observed in these mice. However, this speculation needs to be validated experimentally with a more specific flow-cytometry panel aimed at characterizing all the peripheral T cell subsets in the blood of 3xTg-AD mice in comparison to 3xTg-ADxItgal$^{-/-}$ mice.

As a consequence of the reduced immune cells infiltration into the brain, 3xTg-ADxItgal$^{-/-}$ mice displayed an improvement in both short- and long-term memory, and the neuropathological hallmarks of AD were also ameliorated in LFA-1 knock-out AD mice. Indeed, as mentioned above prolonged microglia activation may have a detrimental role in AD, and here we showed that the deletion of LFA-1 decreased neuroinflammation by reducing microglia activation. Microglia constitutively express LFA-1 in resting condition (Akyyama H, *J Neuroimmunol* 1990), but its expression has been demonstrated to increase in chronic inflammatory conditions like AD (Hailer NP, *Glia* 1996, Mizuno T, *Brain Res* 1999). Expression of LFA-1 on microglial cells is critical for effective antigen presentation and their interaction with neurons, thus the increase in LFA-1 expression might accelerate the neurotoxic and phagocytic activity which are typical features of activated microglia in AD (Rozumuller JM, *Neurosci* 1989; Shrikant P, *J Immunol* 1996; Mizuno T, *Brain Res* 1999). Accordingly, we hypothesized that the reduced neuroinflammation observed in 3xTg-ADxItgal$^{-/-}$ might be a result of two aspects: a decrease in lymphocytes capability to infiltrate the brain, and a reduction of microglial cell activation.

Moreover, the genetic ablation of LFA-1 in 3xTg-AD mice showed a strong reduction of Aβ accumulation and tau phosphorylation. Collectively these data confirm a positive outcome of the LFA-1 blockade in 3xTg-AD mice, consistent with our initial hypothesis that the inhibition of lymphocyte infiltration in the brain interferes with disease progression. LFA-1 blockade has been proven to be beneficial in several inflammatory diseases such as multiple sclerosis, inflammatory bone loss, psoriasis and inflammatory bowel disease (Mitroulis I, *Pharmacol er* 2015). As a result of these studies, LFA-1 targeting strategy has reached clinical trials. Indeed, it was previously demonstrated that the treatment of mouse models of arthritis with LFA-1 antagonist BMS-587101, effectively blocked inflammation and joint damage (Suchard SJ, *J Immunol* 2010). Moreover, the beneficial effect of LFA-1 blockade in inflammatory conditions has been well studied in case of psoriasis, in which Efalizumab, as an antibody against CD11a, interferes with LFA-1/ ICAM-1 interaction and disrupts extravasation of circulating T-cells in inflammatory skin and accordingly reduces histological signs of inflammation and the pathological hyperplasia characteristic of psoriasis plaques (Boehncke WH, *Biologics* 2007).

In line with other publications on the role of LFA-1 or VLA-4 in inflammatory diseases, here we provided evidence for the efficacy of anti-integrin treatments in AD, possibly due to the interruption of T cell trafficking into the brain. Although an important limitation of anti-integrin treatments is the induction of viral brain disease progressive multifocal leukoencephalopathy (PML) in patients with autoimmune disorders (Baldwin K, *Curr Opin Neurol* 2013), the fact that AD patients do not normally receive immunosuppressive therapy and the improved tests to detect patients at risk of PML may give to drugs that block VLA-4 or LFA-1 the possibility to represent a new therapeutic approach in AD with rapid translation into clinic.

To study the mechanisms controlling leukocyte-brain cell interactions more in detail, we established an in vitro co-culture model to investigate the interactions between primary AD-derived neuronal cells and T cells. Our data showed that the staining of microglia, neurons and astrocytes with CD11b, MAP2 and GFAP respectively, showed a high purity of our isolated primary cells. Moreover, RT-

PCR and WB confirmed that both neurons and astrocytes expressed the transcript and the proteins tau and Aβ. Besides, neurons kept for long time *in-vitro* revealed tau release and accumulation in the culture media, thus confirming the validity of our AD model. Based on our results obtained *in vivo* suggesting a detrimental role for T cells in AD pathogenesis, we used our *in-vitro* Alzheimer's model to collect new experimental evidence supporting these data. Several studies pointed out the interplay between pro-inflammatory Th1 and Th17 cells and intraparenchymal cells both in AD patients and related animal models. We produced Th17 and Th1 cells (IL-17 or IFN-γ producing cells, respectively) and co-cultured them with 3xTg-AD-derived microglia and astrocytes. Our data showed a higher activated phenotype in AD-derived microglia compared to WT-derived microglia, in line with previously published results (Murphy AC, *Brain Behav Immun* 2010). In fact, our data reached the statistical significance on enhancement in CD68 expression only following the co-culture with Th1-IFN-γ producing cells compared to both Th17-IL17 producing cells or naïve CD4+ T cells. Accordingly, it was demonstrated by others that an increased level of IFN-γ released by Th1 cells was associated to phenomena of microglia activation, Aβ deposition and it led to impaired cognitive functions in APP/PS1 mice (Browne TC, *J Immunol* 2013). Anti-IFN-γ antibody treatment in these mice led to a reduced disease severity, suggesting that IFN-γ has a neurotoxic effect in AD pathology probably mediated by activated microglia (Browne TC, *J Immunol* 2013). Confirming these data, AD transgenic mouse models lacking expression of the IFN-γ receptor type 1 showed a significant decrease in Aβ deposition as well as microglial activation (Browne TC, *J Immunol* 2013).

Microglial cells are the major histocompatibility complex (MHC) class II-expressing antigen presenting cells (APCs) in the brain parenchyma. The expression of MHCII is low under homeostatic conditions in the brain, but it can be rapidly increased upon microglia activation (Wyss-Coray T, *Neuron* 2002). Therefore, during AD, microglia may be primed by the CNS inflammatory milieu and can present antigens on MHC class II molecules for reactivation and modulation of migrating effector CD4+ T cells. In agreement with this hypothesis, we observed a higher frequency of MHCII+ cells on AD-derived microglia after Th1 or Th17 co-culture, whereas no differences in percentage of MHCII+ cells

were detected on microglia isolated from WT mice following co-culture with activated T cells. Together, our data suggest that Th1/Th17 cells may activate primed microglia towards a more inflammatory phenotype during AD.

Astrocytes are also involved in numerous pathophysiological brain functions and may be activated during inflammatory responses (Farina C, *Trends Immunol* 2007). In order to evaluate astrocyte activation following effector T cell challenge, we explored astrocyte expression of CD44, the major surface hyaluronan receptor implicated in intercellular and cell-matrix adhesion, cell migration and signaling. Interestingly, the expression of CD44 in the CNS was previously found restricted to astrocytes in the white matter (Quackenbush EJ, *Biochem. J* 1985; Girgrah, N, *Neuroreport* 1991), and its expression is upregulated by astrocytes in the AD brain (Akiyama H, *Brain Res* 1993). In our co-cultures, we observed an increase in the frequency of CD44+ cells on AD-derived astrocytes co-cultured with Th1 cells. Interestingly, this upregulation was specific for the Th1 subset, and no changes were observed on CD44 frequency in the presence of either Th17 cells or naïve unstimulated CD4+ T cells. Our results are in line with previous studies conducted on primary cultures of astrocytes showing up-regulation of both the CD44 transcript level and surface protein expression following stimulation with IFN-γ (Haegel H, *J Cell Biol* 1993). Moreover, McQuillan and colleagues generated Aβ-specific effector T helper lineages and demonstrated that astrocytes can act as APC for Aβ-specific Th1 and Th17 cells *in-vitro* (McQuillan K, *Brain Behav Immun* 2010). Our findings suggest that the recruitment of Th1 cells within the brain during AD could promote astrocyte activation. As a result, reactive astrocytes could act as APC promoting subsequent events of T cell activation and proliferation, thus establishing a loop circuit of neuroinflammation. In agreement, studies in EAE mice showed that the expression of CD44 was increased at sites of contact between T cells and astrocyte processes (Haegel H, *J Cell Biol* 1993). Likewise, our immunofluorescence staining indicated a close interaction between astrocytes (GFAP+ cells) and Th1 cells suggesting a role for CD44 in homotypic adhesion. Together, our in vitro studies point to T cells as potential astrocyte activators establishing a driving force during AD pathogenesis.

The results obtained during the development of this project suggest that the phenotype of T cells infiltrating the CNS depends on the stage of AD-like disease, and future experimental evidences are needed to better clarify the specific involvement of T cell subpopulations in AD. Our study provides novel insights on the detrimental function of these peripheral immune cells in AD and demonstrates that anti-integrin therapies may be useful to delay the progression of AD. Finally, we suggest the usage of *in-vitro* systems to better characterize the intraparenchymal interplay between peripheral leukocytes and CNS cells and identify new cellular and molecular mechanisms contributing to the pathogenesis of this devastating neurodegenerative disease.

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