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TESI DI DOTTORATO DI RICERCA

Role of leukocyte integrins in experimental models of Alzheimer's disease

S.S.D. MED/04 PATOLOGIA GENERALE

Supervisor: Prof.ssa Gabriela Constantin
Co-supervisor: Dott.ssa. Elena Zenaro

Dottoranda: Dott.ssa Gennj Piacentino

A mio figlio,

Fai dei figli.
Non sono un ostacolo,
come qualcuno proverà a farti credere.
I figli vengono a salvarti la vita
e a dare un significato
a tutto ciò che hai fatto e farai.

ABSTRACT

Alzheimer's disease (AD) is characterized by severe, progressive decline of cognition due to neuronal loss in brain regions involved in learning and memory. Two main pathophysiological hallmarks of AD are well characterized: amyloid beta (Aβ) plaques and neurofibrillary tangles (NFTs) of hyperphosphorylated tau protein. Significant evidence obtained over the last decade has shown that neuroinflammation is also associated with AD pathology. Furthermore, vascular leakage and endothelial activation were reported in AD brains, suggesting a role for vascular inflammation and leukocyte trafficking in the pathogenesis of AD. However, the inflammation mechanisms involved in AD pathogenesis remain largely unknown and a better understanding of the role of inflammation in AD may help to develop new therapeutic approaches to slow the progression of this disorder. Blood-derived leukocyte subpopulations, including lymphocytes, monocytes, and neutrophils, have been identified in the brains of patients with AD and in corresponding animal models, but their role in disease pathogenesis is unclear. We have recently reported an active inflammatory process taking place during AD, which includes up-regulation of adhesion molecules on cerebrovascular endothelium and leukocyte trans-endothelial migration into the brain of AD-like disease mice. Notably, neutrophil depletion during the early phase of disease led to an amelioration of cognitive deficits and neuropathological condition in mouse models of AD, suggesting their contribution to the pathology. Indeed, the inhibition of neutrophil function strongly reduced microglial activation and Aβ deposition, suggesting that neutrophils play a key role in disease progression.

The GOAL of the present study was to investigate the role of LFA-1 and VLA-4 integrins in the pathogenesis of AD-like disease. LFA-1, the predominant β2-integrin expressed on leukocytes, is known to play a key role in leukocyte adhesion on inflamed endothelium. Instead VLA-4, the predominant α4-integrin expressed on the surface of lymphocytes, is expressed only by approximately 3% of circulating neutrophils, but several studies demonstrated it represents an alternative pathway for neutrophil migration to inflammatory sites. Interestingly, in addition to neutrophil infiltration we found T cells infiltrating the brain in mouse models of AD at different time points of disease. Therefore, different leukocyte subpopulations migrate into the brain of 3xTg-AD mice, suggesting that neutrophils and T cells may play a role in disease evolution. We performed our experiments in 3xTg-AD mice, which reproduces AD-like cerebral amyloidosis and tangle pathology, and closely resemble the cognitive and behavioral alterations reported in human disease.

We first evaluated by two-photon microscopy the effect of monoclonal antibodies known to block the integrin LFA-1 on neutrophil thus affecting adhesion and extravasation in the central nervous system (CNS). Our results showed that LFA-1 integrin blockade prevents neutrophil adhesion, extravasation, and inhibits intraparenchymal motility in the brain of 3xTg-AD mice. In addition, we demonstrate that both oligomeric and fibrillary Aβ are able

to trigger rapid LFA-1- dependent neutrophil adhesion to its counterligand ICAM-1, with oligomeric Aβ preparation being more effective. Subsequently, we assessed the blockade of neutrophil trafficking by an anti-LFA–1 integrin antibody treatment in 3xTg-AD mice at early stages of the disease confirming that the integrin LFA-1 is fundamental for brain infiltration of neutrophils in AD-like mice. We next studied the effects of LFA-1 ablation on 3xTg-ADx*Itgal*$^{-/-}$ mice lacking LFA-1 integrin. We found that they show improved memory in cognitive tests compared to wild-type animals. Our results demonstrated a reduced of cognitive dysfunctions in 3xTg-ADx*Itgal*$^{-/-}$ compared to age-matched 3xTg-AD mice. These findings were supported by neuropathological data showing a lower density and activation state of microglia in the CA1 and DG and a reduction of Aβ deposition and tau hyperphosphorylation in 3xTg-ADx*Itgal*$^{-/-}$ compared to 3xTg-AD aged-matched controls. Taken together, these results suggest that the inhibition of neutrophil trafficking through the blockade of LFA-1 integrin may represent a new therapeutic strategy for AD.

In addition, our results showed that treated 3xTg-AD mice with an anti-α4 integrin antibody improved memory function compared to control treated mice in behavioral tests. Notably, restoration of cognitve function in mice that received anti-α4 treatment during early stages of disease was also maintained at late time points in aged animals, suggesting that therapeutic blockade of leukocyte adhesion during the early stages of disease has a long-term beneficial effect on cognition in older mice. In support of the results obtained in cognitive tests, neuropathological studies showed a reduction of amyloid beta deposition, tau hyperphosphorylated and microglial activation. Therefore, VLA-4 integrin may also play a key role in the induction of cognitive deficit and progression of AD-like disease.

In summary, the results obtained in the present study show that LFA-1 and VLA-4 integrins contribute to disease pathogenesis and may represent novel therapeutic targets in AD.

ABBREVIATIONS

3xTg-AD	*Triple transgenic Alzheimer's disease mouse with mutations in APPSWE, PS1M146V and TauP301L*
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*
CSF	*Cerebro- spinal fluid*
CTF	*C-terminal fragment*
CTFα	*83-residue carboxy-terminal fragment*
DAG	*Diacylglycerol*
EAE	*Experimental autoimmune encephalomyelitis*
ECM	*Extracellular matrix*
EOFAD	*Early onset familial Alzheimer's disease*
ER	*Endoplasmatic reticulum*
FAK	*Focal adhesion kinase*
fMLP	*N-formylmethionyl-leucyl-phenylalanine*
GEFs	*Guanine-nucleotide-exchange factors*
GPCRs	*G-protein-coupled receptors*

HEVs	*Venular endothelial cells*
HPK1	*Hematopoietic progenitor kinase 1*
I.P.	*Intraperitoneal*
ICAM-1	*Intercellular adhesion molecule-1*
IL	*Interleukin*
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*
MVB	*Mutivescicules body*
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*
TCA	*T cell Analysis program*
TIM	*T cell immunoglobulin and mucin domain*
TJs	*Tight junctions*
TMD	*Transmembrane domain*
TNFα	*Tumor necrotic factor α*
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*

CONTENTS

OVERVIEW OF THESIS SECTIONS

Sections 1 to 8 cover Alzheimer's disease definitions and features. Particularly, in Section 8, we give a description of inflammation and the molecular mechanisms controlling leukocyte recruitment under inflammatory conditions. Section 8.2 provides important information for the thesis, covering the structure, modulation and functions mediated by LFA-1 and VLA-4 integrins. In section 9, we describe neuroinflammation events that may represent a potential driving force in AD.

In Section 11, *Material and Methods*, we describe the methods and technologies used throughout this thesis, such as mice typization, isolation of brain leukocytes, flow cytometry analysis, two-photon microscopy, mouse treatment and behavioural assessments. A brief introduction to AD animal models is given in section 11.12. Figure 12 provides the strategy of our in vivo studies in mouse models of AD.
Section 12 shows the results obtained in all our experiments. The main data of the thesis can be summarized as follows:

- we observed that blocking monoclonal antibodies to LFA-1 integrin significantly inhibit neutrophil adhesion in brain vessels and extravasation into the CNS, suggesting a key role for this integrin in neutrophil recruitment in AD;
- we demonstrated that LFA-1 integrin has a key role in the induction of cognitive deficits and reduces microglia activation in mice with AD-like disease.
- we give a description of 3xTg-ADx*Itgal*$^{-/-}$ mice that we generated to better evaluate the effect of LFA-1 integrin blockade on disease. We reported that 3xTg-ADx*Itgal*$^{-/-}$ restores cognition at early and late stages of disease and it induces neuropathological changes.
- we demonstrate that the VLA-4 integrin antibody treatment could inhibit early pathogenesis and progression of AD in 3xTg-AD mice.

Finally, the *Discussion* (section 12) contains the main conclusions drawn from this project and possible directions for future research. The emerging role for leukocyte recruitments in CNS diseases provides insight into the mechanisms of brain damage during AD and may contribute to the development of novel therapeutic strategies. Our study suggests that LFA-1 and VLA-4 integrins mediate leukocyte trafficking into the SNC in AD models and may represent novel attractive therapeutic targets in AD. Section 13 contains all references cited in this thesis.

INTRODUCTION

1. ALZHEIMER'S DISEASE

In 1906 Dr. Alois Alzheimer, a German neurologist and psychiatrist, described the case of a 51-year-old woman, Auguste D., who had been admitted to a hospital 5 years earlier with a cluster of unusual symptoms, including problems with comprehension and memory, an inability to speak, disorientation, behavioral problems, and hallucinations. After her death, Dr. Alzheimer examined her brain tissue and described specific features: i.e. numerous globs of sticky proteins in the spaces between neurons called beta-amyloid (Aβ) plaques, and a tangled bundle of fibrils within neurons called neurofibrillary tangles (NFTs). That moment was crucial to establish the hallmarks of a new pathology, which nowadays is the most diffuse neurodegenerative disorder affecting more that affects only in the U.S. more than 48 million people and this number is set to increase in the future due to an increase in the life expectancy. It is expected that by 2050 approximately 88 million people in the U.S. will have AD, thus the number of people affected will double (Alzheimer's disease Facts and Figures, 2017). There are also indications that the incidence of illness double every 5 years after 65 years old (Querfurth H.W. & Laferla F.M., 2010). Because of the growing number of AD patients, the disease represents a category with high impact on social economy. In fact, the costs associated with the disease also put a heavy economic burden on society. It is estimated that total costs of care for individuals with AD by all payers in US were from $172 billion in 2010 to more than $1 trillion in 2050, with Medicare costs increasing more than 600 percent, from $88 billion today to $627 billion in 2050 (Washington, 2010). Taken together these data strongly suggest that AD is an urgent research priority, and the early identification of the disease and the possible intervention to slow its unrestrainable progression will be a benefit for individuals, families and Nations.

1.1 SIGNS AND SYMPTOMS OF AD

AD can affect different people in different ways, but the most common symptom pattern begins with gradually worsening difficulty in remembering new information. This phenomenon is due to the disruption of brain cells by Aβ and NFTs, in regions involved in forming new memories. The rate of progression varies greatly. On average, people with AD live 8 years, but some people may survive up to 20 years. 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. Brains of individuals with AD manifest a severe atrophy linked to

massive neuronal and synaptic loss, due to formation of Aβ plaques between nerve cells, but also of intracellular deposits of tau protein, called tangles.

1.2 DIAGNOSIS

Although AD apparently is different from other types of dementia, it is very difficult to differentiate between the onset of AD and other types of age-related cognitive decline from the early stages. Therefore, clinicians use a range of methods to diagnose what it is called "possible AD" (dementia that could be due to another condition) or "probable AD" (no other cause of dementia can be found).

Braak et al. guided an important study examining 83 brains coming from nondemented and demented individual autopsies, leding to the hallmark identification of six-stages of AD development (Braak H. and Braak E., 1991) (Fig. 1). The first two stages were characterized by an either mild or severe alteration of the transentorhinal layer Pre-α (transentorhinal stages I-II). The two forms of limbic stages (stages III- IV) were marked by a conspicuous affection of layer Pre-α in both transentorhinal region and proper entorhinal cortex. In addition, there was mild involvement of the first Ammon's horn sector. The hallmark of the isocortical stages (stages V-VI) was the virtual destruction of all isocortical association areas (Fig. 1). The cerebral cortex, inparticular the isocortex, is the predilection site for the deposition of Aβ. Then, the authors observed patient specific variation in size, shape and distribution pattern of the Aβ deposits and NFTs from and within architectonic units.

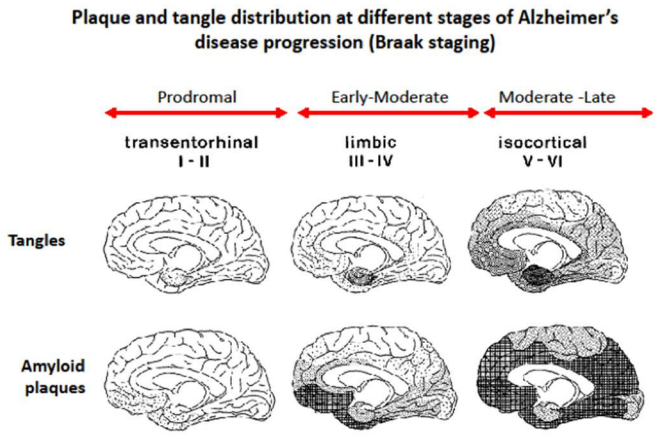

Figure 1. Neuropathological staging of AD-related changes.
H. Braak and E. Braak, 1991.

Additionally, in the Nun Study, a longitudinal study of aging and AD patients some authors have used the Braak method of staging AD's pathology in 130 women aging between 76-102 years. All the collected autopsy data showed a strong relationship between Braak stage and cognitive state (Riley K.P. et al., 2002). Much research is being done to identify the early changes, which may be useful in predicting dementia or AD. In 2011, the National Institute on Aging and the Alzheimer's Association recommended new diagnostic criteria and guidelines for AD. The new criteria and guideless update refine and broaden guideless published in 1984 by Alzheimer's Association and National Istitute of Neurological Disorders and Stroke (Scheltens P. et al., 2016). The 1984 criteria were based chiefly on a doctor's clinical judgment about the cause of a patient's symptoms, considering reports from the patient, family members and friends; results of cognitive testing; and general neurological assessment. The new criteria and guideless identify the three stages of AD: preclinical AD, mild cognitive impairment (MCI) and dementia. An early diagnosis results to be important because allow starting treatments in the first stages of AD and can help to preserve brain function.

1.2.1 PRECLINICAL AD

In the preclinical stage, AD develops in the entorhinal cortex, a brain region that is near the hippocampus and has direct connections to it. Healthy neurons in this region begin to work less efficiently, losing their ability to communicate, and ultimately die. This process gradually spreads to the hippocampus, the brain region that plays a major role in learning and is involved in converting short-term memories to long-term memories. Affected regions begin to atrophy. Ventricles, the cerebrospinal fluid (CSF) filled spaces inside the brain, begin to enlarge as the process continues. These brain changes may begin 10 to 20 years before any clinically detectable signs or symptoms of forgetfulness appear. A deeper knowledge of these early stages of the disease leads to more chances to develop new drugs or treatments that will slow or stop the disease triggering before significant impairment occurs.

1.2.2 MILD TO MODERATE AD

As AD spreads through the brain, there is an increase in Aβ and NFTs in the regions involved in memory, thinking and planning develop more. Hence, memory loss continues and changes in other cognitive abilities begin to emerge. AD patients develop problems with memory or thinking serious enough to interfere with work or social life. It's only when Aβ

and NFTs diffusion affects areas involved in speaking, understanding and sensing the body with respect to other surrounding objects, that people are first diagnosed with AD.

1.2.3 SEVERE AD

As severe stage is reached, Aβ and NFTs are widespread throughout the brain. Most areas of the brain have shrunk further, and ventricles have enlarged even more (Fig. 2). People become not self-sufficient in daily activities and start showing changes in personality and behavior, with efforts in recognizing friends and family members. At this stage of disease, patients are completely dependent on others for care.

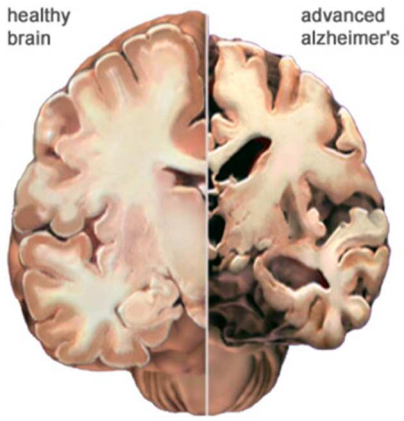

Figure 2. Differences in healthy and AD brain. [www.alz.org]

1.3 BIOMARKERS IN AD

A biomarker is a characteristic measurable and evaluable as an indicator of the disease progress. The CSF is considered the ideal source of biomarkers for AD because it is in contact with the cerebral tissue and it reflects its pathological changes. Besides, the sampling is simple and non-invasive and the measurement is precise. Indeed, the concentrations of several proteins in CSF reflect with good diagnostic accuracy the pathophysiological features of the disease. In particular, several studies have found that patients with AD have a reduction in CSF levels of the concentration of Aβ and a marked increase of total and phosphorylated tau (Forlenza O.V. et al., 2010). Magnetic Resonance Scans (MRI) and Positron Emission tomography (PET) are 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. et al., 2009). Structural MRI in AD can assess brain atrophy and changes in tissue characteristics through alterations in the radiofrequency signal emitted

(Figure 4). The earliest site of atrophy is the entorhinal cortex, followed by hippocampus, amygdala and parahippocampus. The distribution and the amount of cerebral atrophy are strictly correlated with cognitive deficits (Johnson K.A., et al., 2012). Instead, Positron Emission Tomography (PET) is used to detect Aβ plaques in the brain using the radiolabelled Pittsburgh Compound-B (PiB) that marks Aβ plaques (Johnson K.A., et al., 2013). A radiolabelled compound called PiB binds to Aβ plaques in the brain and it can be imaged using PET scans. Initial studies showed that people with AD take up more PiB in their brains than do cognitively healthy older people. However, high levels of PiB are found in some cognitively healthy people, suggesting that the damage from Aβ may already be underway. Data support the principle that the presence of Aβ plaques deposition alone, even in substantial quantities, is not sufficient to produce dementia, and abnormalities in Aβ deposition biomarkers precede clinical/cognitive symptoms (Stomrud E. et al., 2007). This principle is clearly illustrated by data from the individual (Fig. 3), who was cognitively normal with no evidence of atrophy on MRI, but had a highly abnormal PiB study. Furthermore, several tau pathology PET tracers have been recently developed and used in clinical studies for the diagnosis of AD (James O.G., et al., 2015). Tau-PET imaging represents a significant new advance for the field and it is hoped that the combination of tau positive and amyloid positive PET scans, along with the clinical presentation, may in the future move us closer to an affirmative diagnosis of AD.

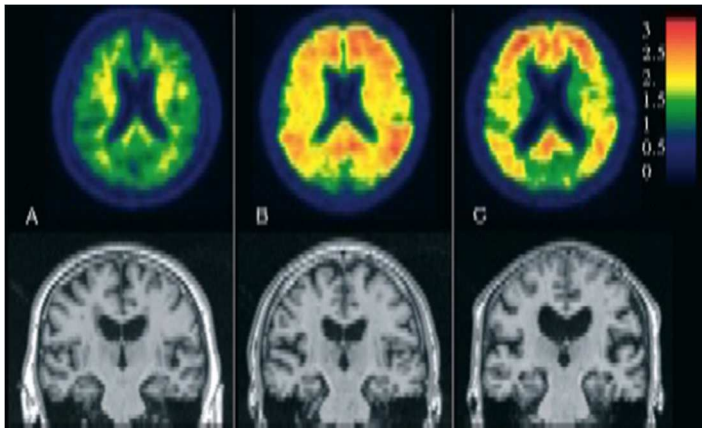

Figure 3. Illustration of biomarker Alzheimer's disease staging. [Jack C.R, Lancet Neurol, 2010]
Three elderly individuals are placed in order from left to right with a proposed biomarker staging scheme. (A) A cognitively normal individual with no evidence of Aβ on PET amyloid imaging with Pittsburgh Compound B (PiB) and no evidence of atrophy on MRI. (B) A cognitively normal individual who has no evidences of neurodegenerative atrophy on MRI, but has significant Aβ deposition on PET amyloid imaging. (C) An individual who has dementia and a clinical diagnosis of AD, a positive PET amyloid imaging study, and neurodegenerative atrophy on MRI.

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, accounting for less than 5% of total cases. It is diagnosed before age 65 with an autosomal dominant pattern of 19 inheritance, which means that one copy of the altered gene is sufficient to cause the disorder. Most cases of EOFAD are caused by gene mutations, found in one of three genes: amyloid precursor protein (APP), presenilin-1 (PSEN1) or presenilin-1 (PSEN2). When these genes are altered, large quantities of Aβ toxic protein fragments are produced in the brain and accumulate forming "clumps" called senile plaques (SP), which are characteristic of AD. LOAD is also known as "sporadic AD" because appears to be no genetic factor or family link involved. It is the most common form of the illness, affecting about 90% of sufferers. Neuropathologic hallmarks of AD are characterized by initial amyloidosis, due to extracellular deposition of Aβ toxic protein fragments (Aβ1-40 and Aβ1-42) and fibrils in SP; tauopathy, developed by abnormal intracellular accumulation of hyperphosphorylated tau proteins in NFTs and vascular deposition of Aβ resulting in cerebral amyloid angiopathy (CAA).

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. et al., 1987).

2.1.1 APP gene

The APP gene is located on the long arm of chromosome 21 at position 21.2, from base pair 27,252,860 to base pair 27,543,445 (bp 290,585).

APP is a single-transmembrane, receptor-like protein that is ubiquitously expressed in neural and non-neural cells (Haass C. & Selkoe D.J., 2007,). It is synthesized in the endoplasmatic reticulum, post-transcriptionally modified in the Golgi and transported to the cell surface via the secretory pathway (Sastre M. et al., 2008). Moreover, APP might also be endocytosed from the cell surface and processed in the endosomal-lysosomal pathway (Benzinger T.L., et al., 2000). Additionally, APP is cut by proteases to create smaller peptides, some of which are released outside the cell. More than 25 different mutations in the APP gene can cause EOFAD. These mutations are responsible for 10% to 15% of all early-onset cases of the disorder, which show between 45 and 65 years. The most common mutation is the replacement of the amino acid valine with the amino acid isoleucine at protein position 717 in the APP. Mutations in the APP gene increase the amount of Aβ peptide.

APP is coded on chromosome 21 and this explains why Down syndrome patients have an increased risk of developing AD and start suffering from AD symptoms in their late 30s (Farfara D., et al., 2008). In fact, Down syndrome patients have three copies of genes on chromosome 21 in each cell, including the APP gene, instead of the usual two copies. Although the connection between Down syndrome and AD is unclear, the production of excess Aβ peptide in cells may account for the increased risk.

2.1.2 APP PROCESSING

APP processing is considered a key event in the pathological cascade leading to AD (Hardy J., 1997). APP undergoes post-translational proteolytic processing, distinct in nonamyloidogenic and amyloidogenic pathway, where several sequential enzymes are involved. Normally about 90% of APP enters the non- amyloidogenic pathway, and 10% the amyloidogenic one, but these ratios can change due to mutations, environmental factors, as well as the age of the individual. In non-plaque-forming pathway α-secretase generates soluble amyloid protein, while β- and γ-secretases generate APP components with amyloidogenic features. The intermediates of Aβ aggregation from low molecular weight to higher molecular weight are respectively monomers, oligomers, protofibrils and fibrils. Fibril formation is a complex and nucleation-dependent process. The Aβ monomeric state does not appear to be neurotoxic; in contrast, oligomeric and profibril species are considered the cause of the cognitive impairment (LaFerla F.M., et al, 2007).

The non-amyloidogenic pathway

In the non-amyloidogenic pathway, APP is cleaved by α-secretase. This constitutive cleavage occurs in the interior of the Aβ peptide sequence, thereby precluding formation and deposition of the Aβ (Esch F.S., et al., 1990). A large soluble N-terminal fragment (sAPPα) is released into the lumen/extracellular space, leaving an 83-residue carboxy-terminal fragment (CTFα) in the membrane. CTFα is then digested by γ-secretase to yield a soluble N-terminal fragment (p3) and a membrane-bound C-terminal fragment (AICD, or APP intracellular domain). AICD is a short tail (approximately 50 amino acids) released into the cytoplasm after progressive e to γ cleavages by γ-secretase. AICD is targeted to the nucleus and is involved in activation signalling of transcription. Three related metalloproteases of ADAM (a disintegrin and metalloprotease) family, ADAM-9, ADAM-10 and ADAM-17, appear to exert α–secretase activity. A confirmation that ADAM-10 is involved in α–secretase activity came from studies in transgenic mice for human APP, where an over-expression of ADAM-10 showed increased secretion of the neurotrophic α- APPs, reduced formation of Aβ and prevention of their deposition in Aβ plaques (Postina R., et al., 2004). Another candidate with α–secretase activity is beta-site amyloid precursor protein-cleaving enzyme 2 (BACE2), which likewise cleaves within the Aβ domain and abrogates Aβ

formation (Tanzi R.E. & Bertram L., 2005). Therefore, ADAM-10 and BACE2 may become a promising therapeutic target to AD.

The amyloidogenic pathway

Some APP molecules that are not subjected to α–secretase proteolysis can be cleaved by β-secretase, also called beta-site amyloid precursor protein-cleaving enzyme 1 (BACE1), essential for initiating amyloidogenic processing. BACE1 cleaves APP at the N-terminal position of Aβ, leading to secretion of a smaller ectodomain derivative (βAPPs). This generates a 99-residue carboxy-terminal fragment (C99 or βCTF), which is inserted in 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 M.S., et al., 1999, Steiner H., et al., 2000) (Fig. 4). This complex is composed of PSEN1 or PSEN2, nicastrin, APH1 and PEN2 (Kimberly et al., 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., et al., 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., et al., 2001). 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., et al., 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*.

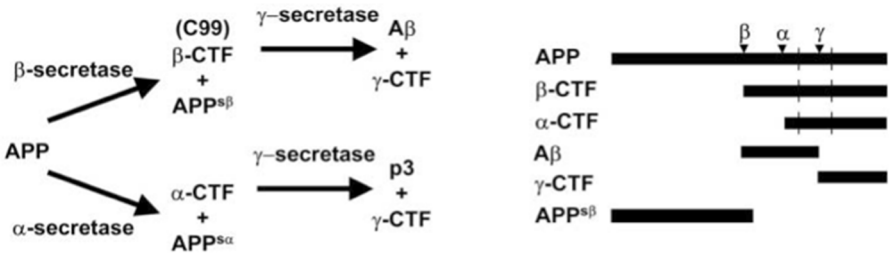

Figure 4. Neural activity controls formation of APP cleavage derivatives.
[Kamenetz, Neuron, 2003] Biochemical pathways leading to the formation of Aβ from APP. α and β cleavage of APP result in the production of a large, soluble ectodomain (APPs) and a membrane associated carboxy-terminal fragment (CTF). Cleavage of APP by α-secretase

precludes production of Aβ. γ-secretase cleavage of CTFs produce small peptides (Aβ and p3), which can be secreted and a truncated CTF (γ-CTF).

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). PSEN2 is a protein homologous to PSEN1. Several studies suppose that PSEN2 may act in synergy with PSEN1, in the function of γ-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. The late-onset form is probably related to variations in one or more genes in combination with lifestyle and environmental factors. Although a specific gene has not been identified as the cause of late-onset AD, the most influential genetic risk factor for LOAD is allelic variation in the apolipo-protein E (APOE) gene (Querfurth H.W. & Laferla F.M., 2010).

2.3. APOE4 GENE

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 A.M. & Holtzman D.M., 2000).
APOE has three common forms or alleles: ε2, ε3, and ε4. The ε2 form may provide some protection against AD, and ε3 is thought to play a neutral role. In particular, ε4 form is a known risk-factor gene for the common LOAD, and many studies are underway to clarify its impact. In fact, APOE4 promotes Aβ deposition and tau phosphorylation (Holtzam D., et al., 2000a; Holtzman D., et al., 2000b). 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.

3. NEUROPATHOLOGICAL HALLMARKS

3.1 Aβ DEPOSITION

Aβ deposition into SP has led to the development of the amyloid hypothesis of AD (Selkoe D.J., 2000). This hypothesis states that the amyloidogenic processing of APP ultimately results in Aβ deposition into Aβ plaques that initiates a cascade of events that culminate in neuronal dysfunction or death in AD. Gradual changes in the steady-state levels of Aβ protein in the brain are thought to initiate the amyloid cascade.

3.2 NEUROTOXIC FORM OF Aβ

Aβ principal forms are constituted by Aβ1-40 and Aβ1-42 peptides. These peptides form a variety of structures, including multiple monomer conformers, different types of oligomers, Aβ-derived diffusible ligands (ADDLs), protofibrils and fibrils (Fig. 5). In AD pathology, soluble Aβ undergoes a conformational change that renders it relatively insoluble, causing its deposition in brain parenchyma. Aβ peptide initially deposited into amorphous diffuse plaques, then becomes compacted into mature SP formed by fibrils displaying a β sheet conformation (Wisniewski H.M., et al., 1997). The structural relationships among these assemblies, as well as differences in the assembly processes of Aβ1-40 and Aβ1-42 are areas of active investigation (Klein W.L., et al., 2004). Of note, in some elderly individuals (high pathology controls, HPC) remain cognitively intact while showing high Aβ plaque loads, clearly demonstrating a poor correlation between Aβ plaques with pathological criteria used for AD diagnosis. Much of the fibrillar Aβ found in the neuritic plaques is the Aβ1-42 species, the slightly longer more hydrophobic form that, with its C-terminal Alanine and Isoleucine residues, aggregates more rapidly, therefore forming stable Aβ oligomers at an earlier time point (Burdick D. & Soreghan B., 1992). Moreover, Aβ1-42 tends to form stable

trimeric and/or tetrameric oligomers, whereas Aβ1-40 does not (Chen Y.R. & Glabe, C.G., 2006). The Aβ1-40 peptide is more frequently produced than Aβ1-42 by cells and probably by further cleavage of Aβ1-42 peptide. However, it is usually co-localized with Aβ1-42 in plaques. Early SP contain mainly Aβ1-42, whereas plaque maturation is associated with the progressive appearance of the shorter Aβ1-40 (Iwatsubo T., et al., 1994). The enhanced production of the Aβ1-42 peptide results from AD-causing mutations in APP and PSEN1 or PSEN2 (Scheuner D., et al., 1996).

An increase in absolute Aβ1-42 levels, or at least an increase in the Aβ1-42/Aβ1-40 ratio, where Aβ1-42 can increase at the expense of Aβ1-40, seems to be sufficient to trigger the AD phenotype. Apart from the known neurotoxicity of the fibrillar form of Aβ, recently some studies have demonstrated that non-fibrillary structures, including oligomers, ADDLs and protofibrils are also neurotoxic (Dahlgren K.N., et al., 2002). In the contest of the human AD pathology, it was demonstrated that soluble Aβ oligomers detected in CSF showed correlation with the severity of the cognitive impairment (Santos A.N., et al., 2012). As confirmed by others, total levels of soluble Aβ correlate with cognitive decline even in the absence of detectable Aβ plaques (Dahlgren K.N., et al., 2002). Several studies show that Aβ oligomers are responsible of alterations in long-term depression (LTD) and long-term potentiation (LTP) while insoluble Aβ plaque cores from AD cortex did not impair LTP unless they were first solubilized to release Aβ dimers (Shankar G.M., et al, 2008). Thus, some authors 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 (Lih-Fen Lue et al., 1999).

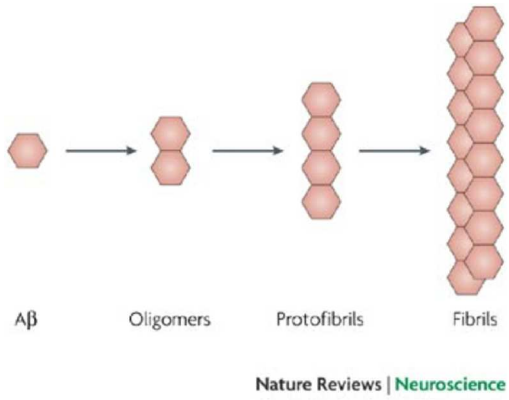

Figure 5. The amyloid cascade hypothesis.
[Haass and Selkoe, Nat Rev Mol Cell Biol, 2007]

3.3 THE INTRACELLULAR Aβ

Recent studies have documented the presence of intracellular Aβ in the human brain since first years of life, which increases in childhood, and stabilizes in the second decade of life,

remaining high throughout adulthood even in the healthy brain. The Aβ intracellular form has been described for the first time in a group of individuals with the age ranging from 38 to 83 years old with or without AD using an antibody against residues 17-24 of Aβ (LaFerla F.M., et al., 2007). Cytoplasmic inclusions of Aβ are often associated with the human muscle disease inclusion body myositis where it causes muscle degeneration, while the presence in the mouse model 3xTg-AD is still unclear. Aβ1-42 seems to be localized in the outer membrane of the multivescicules body (MVB) in AD patients, and often the Aβ-MVB is localized in the perinuclear region in APP/PS1 mice. The accumulation of Aβ in MVB is pathological and causes proteasome inhibition, calcium dyshomeostatis and even facilitates hyperphosphorylation of tau in 3xTg-AD mice (LaFerla F.M., et al., 2007). The presence of Aβ in mitochondria, organelles in which all subunits of γ-secretase have been located, and mitochondrial defects are described in culture cells (Pavlov P.F., et al., 2011). LaFerla F.M., et al. hypothesize that intracellular APP could be produced in endoplasmatic reticulum (ER) as other secretory proteins, or alternatively Aβ may be internalized by binding to its transporters or receptors such as the α7 nicotinic acetylcholine receptor, LDL receptor related protein (LRP-1), receptor for advanced glycation end products (RAGE) and APOE. The presence of intracellular Aβ at 4 months of age in 3xTg-AD mice may be a sign of early disease; in fact, it precedes the extracellular deposition evident at 6 months of age (LaFerla F.M., et al., 2007). Winton M.J., et al. tested a panel of antibody that distinguishes APP from Aβ cleaved peptides because of antibody cross-reactivity might cause discordant results. This study shows co-localization of signal between extreme C-terminal and N-terminal APP in 3xTg-AD mice deficient for β-secretase (3xTg-AD-BACE$^{-/-}$), a protease involved in production of Aβ. Therefore, authors conclude that the intracellular Aβ corresponds to the full-length of intracellular APP and not the cleaved Aβ (Winton M.J., et al., 2011). Various authors have published controversial results in the context of intracellular Aβ, therefore further investigations are needed to clarify their existence.

3.4 CLEARANCE of Aβ

Aβ levels can be elevated by enhanced production and/or reduced clearance. The steady-state level of Aβ depends on the balance between production and clearance. Dysfunction in Aβ clearance is crucial for the accumulation of Aβ in the brain. 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 A.E., et al., 2003; Carrano

A., et al., 2011; Erickson M.A. & Banks W.A., 2013). Several mechanisms have been proposed for Aβ clearance, including receptor-mediated Aβ transport across the BBB and enzyme-mediated Aβ degradation. The BBB regulates Aβ transport to and from the brain, using two main receptors, the LRP-1 and RAGE. LRP-1 and RAGE are multiple ligands cell surface receptors that mediate the clearance of many proteins in addition to Aβ. RAGE and LRP-1 play opposing roles: while LRP-1 appears to mediate the efflux of Aβ from the brain to the periphery, RAGE has been strongly implicated in Aβ influx back into the CNS (Deane R., et al., 2004). LRP-1 is expressed in reactive astrocytes and in brain capillary endothelium (Donahue J.E., et al., 2006). It can bind a variety of ligands: apoE, α2-macroglobulin (α2-M), and APP (Herz J., et al., 2003). Until recently, it has been assumed that Aβ could only bind LRP-1 indirectly, as part of a complex with the LRP-1 ligands apoE and α2M. However, recent data indicate that Aβ can be transported across the BBB and be cleared from the brain after directly binding to LRP-1. Dysfunction of LRP-1 leads to a reduced efflux of Aβ from brain and thus increased Aβ deposition (Shibata M., et al., 2000, Van Uden et al., 2002). Aβ levels negatively regulate LRP-1 expression, and this might explain the low activity of LRP-1 in brain microvessels in AD patients and mutant APP mouse models (Shibata M., et al., 2000).

Moreover, epidemiological studies demonstrated the link between LRP-1 and AD (Lambert J.C., et al., 1998). Accordingly, a recent report has shown that 1,25(OH)D3, the active form of vitamin D, has a neuroprotective effect during AD pathogenesis by inducing the clearance of Aβ, which is achieved by inducing LRP-1 expression and reducing the expression of RAGE in brain endothelial cells (Guo Y.X., et al., 2016). RAGE is a multiple ligand-receptor of the immunoglobulin superfamily of cell surface molecules and is expressed on endothelial and glial cells (Brett J., et al., 1993, Yan S.D., et al., 1996). RAGE is implicated in the development of the AD neurovascular disorder by mediating circulating Aβ transcytosis across the BBB. After BBB transport, circulating Aβ is taken up by neurons thus inducing cellular stress. The binding of Aβ to RAGE leads to the secretion of endothelin-1, a potent vasoconstrictor causing blood flow suppression (Deane R., et al., 2004). Accordingly, an impaired endothelium-mediated vasodilation is one of the markers of endothelium dysfunction leading to cerebrovascular disease characterizing AD (Zlokovic B.V., 2008). Down-regulation of RAGE can inhibit the influx of Aβ (Deane R., et al., 2003) while an excessive amount of Aβ leads to the up regulation of RAGE through a positive-feedback mechanism. During aging and in AD pathology, Aβ efflux is compromised and might exasperate cellular dysfunction because the increase of RAGE causes augmented

influx of Aβ (Shibata M., et al., 2000). 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. & Zlokovic B.V., 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 J.E., et al., 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 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 M.M., et al., 2007). In addition, some authors reported that endosomal compartment may be considered as a pathway for APP generation. They observed that uncleaved APP is internalized from the plasma membrane into retromer recycling endosomes by sortilin-related receptor 1 (SORL1). Some genetic variants of SORL1 correlate with the increased production of APP. Then, reduction of Aβ level and increased secretion of soluble APP oligomers correlate with alterations in the internalization of APP (LaFerla F.M., et al. 2007).

4. CEREBRAL AMYLOID ANGIOPATHY

Deposition of Aβ at cerebrovascular level is one of the AD hallmarks, mainly starting at early stages of the disease (de la Torre J.C., 2004, Prohovnik I., et al., 1988). This dysfunction is due to Aβ deposition also in the vascular walls of intracerebral and leptomeningeal vessels causing CAA (Glenner G.G. & Wong C.W., 1984). Thus, the neurodegeneration and vascular disorders act together to cause dementia (Snowdon D.A., et al., 1997). CAA is a common characteristic in AD patients, although many patients with CAA do not develop AD. Therefore, it is not clear the correlation existing between the distribution of brain CAA, SP and NFTs. The true incidence and prevalence of CAA are difficult to be specified, also because the pathologic diagnosis is typically obtained post-mortem. AD patients have increased incidence of vascular brain lesions (Snowdon D.A., et al., 1997), in fact numerous studies have demonstrated that CAA contributes to ischemic brain injury (Okazaki H., et al., 1979) and intracerebral hemorrhage (Itoh Y., et al., 1993). Aβ deposition damages the media and adventitia of cortical and leptomeningeal vessels, leading to thickening of the basal membrane, stenosis of the vessel lumen and fragmentation of the internal elastic lamina. These processes result in fibrinoid necrosis and microaneurysm formation, predisposing to hemorrhage. CAA is strongly associated with

aging and has been reported to be as high as 57% in case series of asymptomatic patients over 60 years (Yamada M., et al., 1987). The failure of Aβ elimination along with the perivascular pathways that serve as lymphatic drainage channels for the brain, probably causes CAA thus compromising the BBB integrity (Weller R.O., et al., 2008). BBB regulates solute exchange between blood plasma and brain interstitial fluid and is maintained in part by the presence of tight junctions (TJs) that restrict paracellular flux within brain microvessel endothelial cells (Kniesel U. & Wolburg, H., 2000; Zenaro E., et al., 2016). BBB impairment in CAA and AD patients is suggested by the detection of plasma proteins associated with Aβ plaques (Perlmutter L.S., et al., 1995, Wisniewski H.M., et al., 1997) and within AD brain parenchyma (Wisniewski H.M., et al., 1997, Zipser B.D., et al., 2007). In addition, increases in microvascular permeability associated with cerebrovascular Aβ deposits (Wisniewski H.M., et al., 1997) and the disruption of cerebral microvasculature endothelial TJs (Claudio L., 1996) have been directly observed in brain of AD patients (Carrano A., et al., 2011; Carrano A., et al., 2012). Aβ deposition in cerebral vessels is associated with pericyte and smooth muscle cell degeneration (Verbeek M.M. et al., 2000) and the direct treatment of acute hippocampal slices with Aβ1-42 oligomers increases the production of reactive oxygen species (ROS) by pericytes, which accelerates their loss (Veszelka S., et al., 2013). Impaired elimination and accumulation of soluble and insoluble Aβ peptide may underlie the pathogenesis of CAA and explain the link between CAA and AD. With progression of the disease, Aβ accumulation leads to vessel degeneration, capillary and arteriolar infiltration, and formation of dystrophic neuritic plaques. All data obtained by transgenic mice overproducing Aβ confined in the brain developed CAA (Herzig M.C., et al., 2006), which further supports the hypothesis that Aβ is entrapped in the perivascular pathways by which fluid and solutes drain from the brain (Weller R.O., et al., 2008). The major consequences arising from CAA are the weakening of arteries by deposits of Aβ in their walls, which tend to rupture and lead to intracerebral hemorrhage (Zhang-Nunes S.X., et al., 2006), the blockage of the Aβ perivascular drainage pathways that may be associated with accumulation of Aβ in the brain. Ultimately, the increased level of soluble Aβ correlates with cognitive decline in patients with AD (Lue L., et al., 1999). It is possible that drainage of other soluble metabolites from the brain may also be impeded in CAA. This would result in a loss of homeostasis in the neuronal extracellular environment that could contribute to cognitive decline in AD (Weller R.O., et al., 2008).

5. NEUROFIBRILLARY TANGLES

NFTs are one of the pathologic markers of AD and correlate with the degree of dementia (Arriagada P.V., et al., 1992). Neurofibrillary degeneration appears to be required for the clinical expression of the disease, in fact dementia or β-amyloidosis alone, in the absence of

neurofibrillary degeneration, does not produce the disease clinically (Iqbal K., et al., 2009). NFTs occur not only in AD, but also in other neurodegenerative diseases called Tauopathies (Lee V.M., et al., 2001). NFTs are formed by aggregates of the abnormal hyperphosphorylated protein Tau, belonging to the family of the microtubule-associated proteins (MAP) (Fig. 6). Tau is coded by single gene on chromosome 17 but is expressed in several molecular isoforms that are generated by alternative splicing of its mRNA (Himmler A., et al., 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) repetitive motives (Goedert M. et al., 1989).

Tau is involved in the stabilization of microtubules, which build up axonal cytoskeleton. In fact, this soluble protein binds to tubulin for facilitating its assembly into microtubules and stabilize their structure (Cleveland D.W., et al., 1977) (Fig. 6). Phosphorylation of Tau is regulated by addition or removal of phosphate residues at specific sites. Hyperphosphorylation of Tau renders the protein insoluble, decrease its affinity for microtubules and self-associates into paired helical filaments (PHF). Likewise, Aβ oligomers also intermediates of abnormal Tau molecules are cytotoxic (Khlistunova I., et al., 2006). Intracellular accumulation of PHF leads to formation of NFTs in pyramidal neurons. Therefore, microtubules appear destabilized and so depolymerize, compromising axoplasmic flow with consequent neuronal degeneration. Similarly, tau could trigger a significant decrease of transendothelial electrical resistance and an increase of endothelial permeability in a model of rat BBB (Kovac A., et al., 2009). These data were confirmed in a transgenic tauopathy mouse model showing correlation between dysfunction of BBB and the appearance of perivascular tau around major hippocampal blood vessels (Blair L.J., et al., 2015).

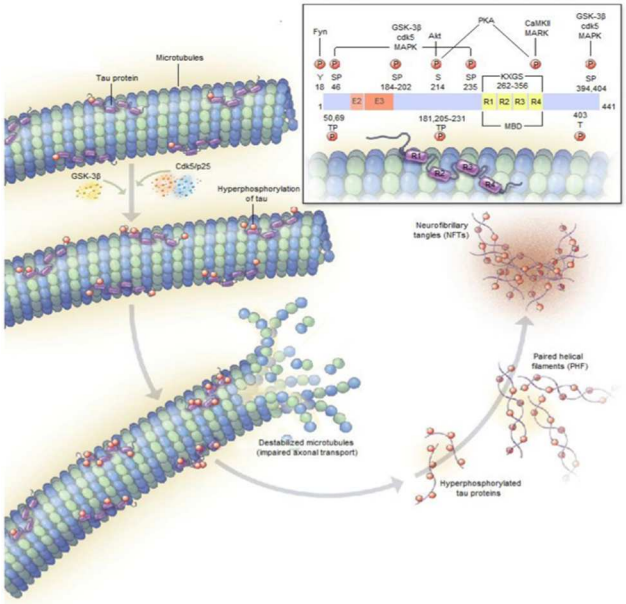

Figure 6. Formation of neurofibrillary tangles.
[Querfurth H.W. & Laferla F.M., N Engl J Med, 2010]

Several studies point out the relationship between neurofibrillary degeneration and β-amyloidosis. In fact, the Amyloid Cascade Hypothesis, one of the principal hypothesis on the etiopathogenesis of AD, states that the generation of Aβ is the primary pathological event preceding and driving Tau aggregation and finally leading to neuronal death and the development of dementia (Oddo S., et al., 2003). However, some authors demonstrated that in the absence of Aβ production, in 3xTg-AD mice deficient for BACE, tau pathology forms independently from Aβ peptide generation, thus refuting the initial hypothesis (Winton M.J., et al, 2011).

6. SYNAPTIC LOSS

Another important pathological hallmark is synaptic loss, an early and invariant feature of AD. There is a strong correlation between the extent of synapse loss and the severity of dementia; in fact, this loss involves particularly the dentate region of the hippocampus, thus promoting the memory impairment (Shankar G., Walsh D.M., 2009). This evidence is supported by the common knowledge that the synapses are the major sites for LTP and plasticity in the neuron. Recent studies have indeed 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 S.W., et al., 2007). These results have been obtained by using electron microscopy or immunohistochemical staining for synaptic markers that has documented significant decreases in synaptic density in the association cortices and hippocampus of AD brain (Hamos J.E., et al., 1989). A variety of mechanisms appear to contribute to synaptic dysfunction and have diverse consequences such as alterations in

synaptic proteins, membrane lipids, vesicular function and loss of plasticity (Coleman P., et al., 2004). The initial triggers may be Aβ plaques toxicity or disrupted intracellular transport of aggregated tau (Pereira C., et al., 2005). Indeed, in the intersinaptic 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 (Querfurth H.W. & Laferla F.M., 2010). The initial decrease in synapse number and density seems disproportionate to the loss of neuronal cell bodies suggesting that loss of synaptic endings may precede neuronal loss (Davies C.A., et al., 1987).

7. NEURON LOSS AND BRAIN ATROPHY

During the progression of AD pathology, the communication between neurons is inhibited but neurons can also be damage to the point that they cannot function properly and eventually die. As neurons die throughout the brain, affected regions begin to shrink in a process called brain atrophy. By the final stage of AD, damages are widespread and brain tissue has shrunk significantly. In according with amyloid cascade hypothesis, accumulation of fibrillar Aβ into plaques damages neurons through two different mechanisms. In a direct mechanism, Aβ interacts with membrane components and damages directly neurons, causing neuronal injury and synaptic dysfunction (Koh J.Y., et al., 1990). While in indirect mechanism, 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., et al., 1995).

8. INFLAMMATION

Inflammation is a relevant component of the innate immune response and it is the first line of defense of organisms to damage or injury, characterized by redness, heat, swelling, and pain. At first, it is characterized by dilation of capillaries to increase blood flow; then by microvascular structural changes and escape of plasma proteins from the bloodstream; and at last by leukocyte transmigration through endothelium and accumulation at the site of injury. The primary objective of inflammation is to localize and eradicate the irritant and repair the surrounding tissue. For the survival of the host, inflammation is a necessary and beneficial process. The phagocytes (monocytes, macrophages and neutrophils) are the main cells involved in the innate immune response. These cells, through patters recognition receptors (PRRs), bind to patterns molecular associates pathogens (PAMPs) and may internalize and kill the microorganisms. The other arm of immunity, called specific immunity, is

characterized by B and T lymphocytes participating in immune responses when the innate immune response is unable to eradicate a pathogen. The principal function of B cells is to produce and release antibodies against soluble antigens. On the contrary, T lymphocytes recognize foreign antigens that had to be first acquired and presented on major histocompatibility complex (MHC) molecules of an antigen-presenting cell (APC). B and T lymphocytes together assist innate immune cells and respond to whole microorganisms and to virtually any molecule that is 'foreign' to the host.

The inflammatory condition resulting from a trauma, ischemia-reperfusion injury or chemically induced injury typically occurs in the absence of any microorganisms and has therefore been termed "sterile inflammation" (de la Torre J.C., 2004). Some authors ascribe AD as a sterile inflammatory condition, suggesting his contribution to the pathogenesis of AD (Halle A., et al., 2008, Rock K.L., et al., 2010). This type of inflammation occurs by liberation of danger signal of non-microbial origin (DAMPs) and characterizes many neurodegenerative disorders, including AD, as previously reported (Halle A., et al., 2008, Rock K.L., et al., 2010). DAMPs may be intracellularly secreted in response to cell stress or passively released following sterile injury or cell death. The Aβ is considered a DAMP capable of activating a wide array of receptors expressed on immune cells (Halle A., et al., 2008, Rock K.L., et al., 2010). The inflammatory response induces the expression of vascular adhesion molecules, the mobilization of leukocytes in site of inflammation, and initiates a cascade of progressive interactions between leukocytes and others immune cells with the vascular vessels wall that precedes transmigration across the endothelial barrier and into the targeted tissue (Ley K., et al. 2007).

8.1 LEUKOCYTE RECRUITMENT

The leukocyte recruitment cascade is a sequence of adhesion and activation events in site of inflammation that ends with extravasation of the leukocyte, whereby the cell exerts its effects on the inflamed site. The simplified original four steps model involves: 1) rolling, mainly mediated by selectins; 2) chemokine-mediated activation; 3) arrest and 4) transmigration, mainly mediated by integrins (Ransohoff R.M., et al., 2003, Butcher and Picker, 1996, Luster A.D., et al., 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., et al., 2007) (Fig. 7). 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 the 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 the 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 M.J., et al, 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 S.D., et al., 2004). L- and P-selectin are particularly efficient tethering molecules. P- and E-selectin are rapidly 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 (McEver P.L.M., et al., 2002). 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. By contrast specific ligands for E-selectin are not yet known, however 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 T.N., et al., 1993, Xia L., et al., 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 C.V., et al., 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., et al., 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 A.D., et al., 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 Gi 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., et al., 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 et al., 1991), although in the context of transmigration it seems that CD11a predominantly binds to ICAM-1, whereas CD11b is more promiscuous (Shang X.Z. & Issekutz A.C., 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 M.J., et al, 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., et al., 2007).

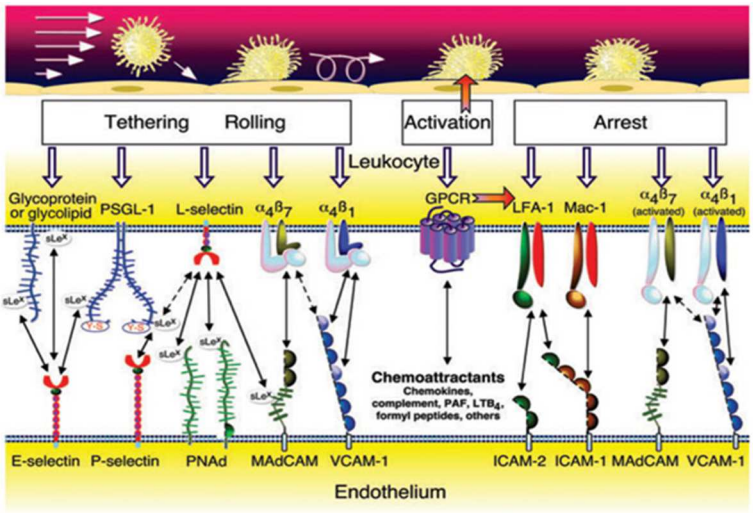

Figure 7. Molecular mechanisms controlling leukocyte adhesion cascade.
[Luster A.D., Nat Immunol, 2005]

8.2 INTEGRINS

On leukocyte membranes, integrins exist as heterodimers composed of one α and one β subunit. In humans, 18 α and 8 β 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 (Fig. 8). The most relevant integrins known to mediate leukocyte arrest belong to the β1 and β2 subfamilies. β2 integrins include 4 different heterodimers: LFA-1, 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 A.D., et al., 2005, Ley K., et al., 2007). The most important β1-integrin expressed on leukocytes is VLA-4, 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 A.D., et al., 2005, Ley K., et al., 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. Instead, the integrin VLA-4 lacks the alpha I-domain (Chigaev A., et al., 2007). The I-domain is constituted by a central β-sheet that is surrounded by α-helices called α/β Rossman fold, a structure common to intracellular enzymes. At the C-terminal end of the central β-sheet of α/β Rossman fold there is a conserved Asp-X-Ser-X-Ser motif termed the metal-ion-dependent adhesion site (MIDAS) used for binding divalent cations. The ligand binding is associated with $Mg2^{+}$-mediated coordination by residues within MIDAS of αL and by an acidic residue donated by the ligand. The presence of isoleucine in position 311 of CD11a called “socket for isoleucine” play a role in LFA-1 activation. The short cytoplasmic tail contains a conserved GFFKR motif that plays a role in heterodimerization and ligand affinity as so demonstrated by mutations in amino acids sequence that have effects on low affinity state conformation. The subunit CD18 contains a large extracellular domain of 676-685 residues, a single hydrophobic transmembrane region of 23 residues and short cytoplasmic tail of 46-48 residues. The extracellular region contains a cysteine-rich repeats so-called plexin semaphorin integrin (PSI) domain, an inserted I-like domain of 240 and 248 amino acids and a series of 4 cysteine-rich repeats that display a significant degree of similarity with the epidermal growth factor (EGF)-like domains. The CD18 cytoplasmic domain associate with cytoskeleton and is involved in endoplasmic reticulum retention, assembly, and transport to the plasma membrane of the mature LFA-1 (Zecchinon L., et al. 2006, Kallen J. et al. 1999, Crump M.P., et al. 2004). The head region is characterized by propeller and thigh domains of the α-subunit and the βA (also known as βI), hybrid and semaphorin integrin (PSI) domain of the β-subunit. The remaining C-terminal extracellular domains of the α- and β-subunit comprise two long ‘legs’ which are anchored in the plasmatic membrane.

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. & Sklar L.A., 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.

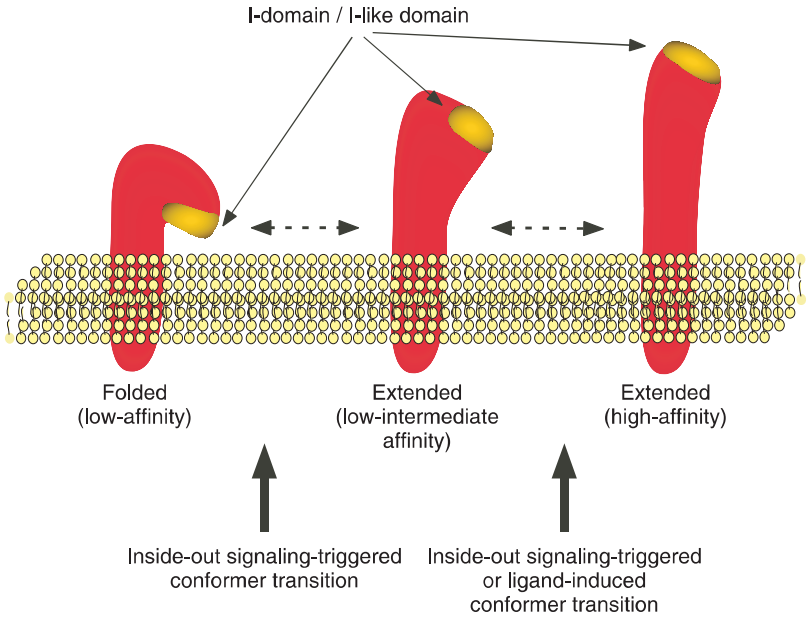

Figure 8. Integrin affinity triggering. The diagram shown the three different states (low, intermediate, and high affinity) for ICAM-1. The availability of the I-domain and I-like domain (in yellow), which are involved inligand binding with increasing affinity, increased with progressive extension of LFA-1 integrin. [Laudanna C., Bolomini-Vittori M., Wiley Interdiscip Rev Syst Biol Med. 2009].

However, integrins are expressed on the surface of a wide range of cell types in an inactive state to avoid inappropriate leukocyte adhesion and their activation is regulated by two mechanisms: binding affinity and valency of ligand binding (Ley K., et al., 2007). Higher affinity results from conformational changes, while the valency reflects the density of integrin heterodimers within the plasma membrane region involved in cell adhesion and can depend on the abundance of individual integrins and their lateral mobility (Ley K., et al., 2007). Crystallography and electron microscopy studies evidenced multiple distinct conformational states. In addition, the use of conformational sensitive antibodies can reconstruct a model of integrin conformational states.

The integrin conformation changes for LFA-1 are monitored using specific antibodies, such as Mab and 327C mAb that recognize the extended/open high affinity conformation of LFA-1 integrin (Constantin G., et al, 2000, Lefort C.T., & Ley K., 2012). Another study performed during human neutrophils rolling reported the partial extension of the epitopes in the β2 and αL subunits recognized by KIM127 and KNI-L16 mAb respectively.

Accordingly, three different states (low, intermediate, and high affinity) that differ in ligand binding affinity have been reported for LFA-1 integrin. The inactive low-affinity state shows a bent structure with the ligand-binding headpiece near membrane-proximal stalk region. In the intermediate affinity state LFA-1 start to extend the stalk regions shifting the molecule to the active higher-affinity conformation, where LFA-1 integrin exhibits extended extracellular domain with the ligand-binding pocket for ICAM-1.
Instead, changes in VLA-4 integrin affinity have been detected in real-time using a ligand-mimicking LDV-containing fluorescent small molecule (4-((N′-2-methylphenyl)ureido)-phenylacetyl-l-leucyl-l-aspartyl-l-valyl- l-prolyl-l-alanyl-l-alanyl-l-lysine-FITC (LDV-FITC)) (Chigaev A., et al., 2001). Interestingly, the discovery of several distinct signaling mechanisms indicates the conformational complexity of this non-I-domain-containing integrin (Chigaev A., et al., 2007). The bent and low affinity state is observed on resting cells where the ligand binding site is close to the membrane, and it prevents cell tethering and rolling. The high affinity state induces the slow accumulation of cell aggregates in suspension and the rolling phase. However, this state is not fully extended and consequently the ligand binding affinity remains low. Finally, the high affinity results in the rapid accumulation of cell aggregates in suspension (Chigaev A., et al., 2007, Chigaev A., et al., 2008) (Fig. 9).

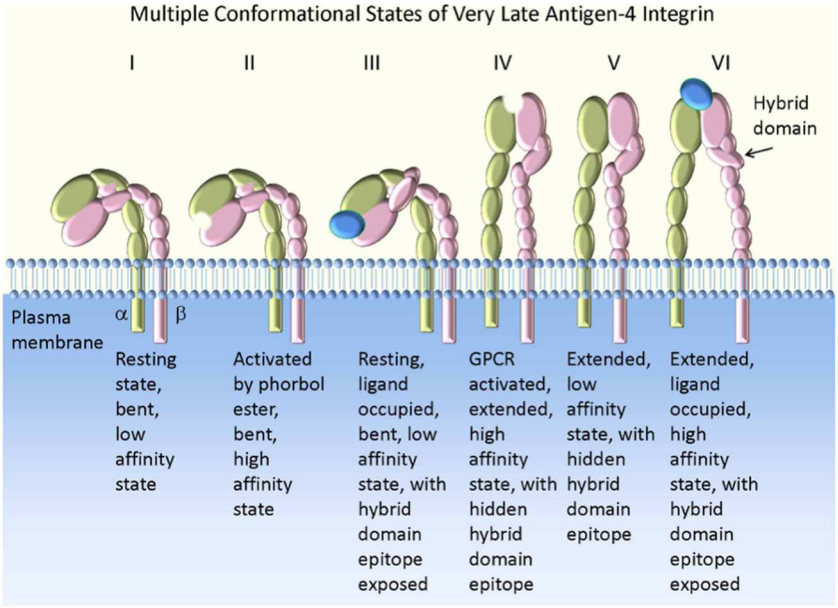

Figure 9. Model of VLA-4 integrin conformation and affinity. In suspension, this translates into rapid cell aggregation that reaches a steady-state intermediate between resting (I) and Gαi-coupled GPCR activated states (IV). [Chigaev A. and A. SklarJ L.A., Frontiers in Immunology 2012].

The mechanism of integrin conformational changes is adjusted by “inside-out” signaling, when chemoattractants cause modifications inside the cell that lead an “outside-in” signaling and allow the interaction of the integrin with its ligand (Heit B., et al., 2005).

The intracellular signaling comprises several inter-dependent pathways, including a) G-protein-coupled receptors (GPCRs), b) small guanosine triphosphate hydrolase enzymes (GTPase) (such as RapA1 and Rho family) and c) Talin-1. The G-protein βγ subunit of GPCR activates phospholipase C (PLC), which cleaves phosphatidylinositol 4-5-bisphosphate (PtdIns(4,5)P2) to produce inositol 1-4-5 triphosphate (InsP3) and diacylglycerol (DAG) with Ca2+ release from intracellular stores in the ER. However, the Ca2+ and DAG may trigger one or more guanine-nucleotide-exchange factors (GEFs) that activated GTPase. Pertussin toxin (PTx) completely inhibits the binding of LFA-1 to ICAM-1 thus confirms that the induction of high-affinity state is dependent on heterotrimeric Gαi-protein triggered "inside-out" signaling events (Constantin G., et al., 2000).

Regulation of the affinity of the β2 integrin LFA-1 by Rho signaling module was investigated in vitro studies. The small GTPases RhoA and Rac1 regulate the activation of LFA-1 by CXCL12 in a dose-dependent manner causing a rapid adhesion. Inhibition of RhoA signaling resulted in the blockade of LFA-1 integrin conformeric transition to low/intermediate as well as to high-affinity states. Inhibition of Rac1 function blocked rapid CXCL12- triggered LFA-1 transitions to low/intermediate and to high-affinity states. The Rho effectors Phospholipase D1 (PLD1) and phosphatidylinositol-4-phosfate 5-kinase isoform 1γ (PIP5KC) are critical to LFA-1 affinity modulation. PLD1 was rapidly activated in a dose-dependent manner by CXCL12 and its inhibition resulted in markedly reduced adhesion on ICAM-1. PIP5KC is a downstream effector of RhoA, Rac1 and PDL1. It is a conformer-selective regulator of LFA-1 affinity controlling triggering of LFA-1 to a high-affinity state, but no to low/intermediate-affinity state by CXCL12. Instead, CDC42 is a negative regulator of LFA-1 integrin and when constitutively active causes a block in LFA-1-mediated adhesion (Montresor A., et al, 2009; Bolomini-Vittori M., et al, 2009).

Talin-1, an anti-parallel homodimer involved in triggering integrin affinity upregulation, represents a shared regulator of all integrin classes expressed by leukocytes. Talin activity is regulated by activation of the small GTPase Ras-related protein 1 (Rap1), a known regulator of cell adhesion. RapA1 regulates rapid integrin-dependent adhesion either in the context of the LFA-1 integrin as well as VLA-4 integrin. Rap1-guanosine triphosphate (GTP)–interacting adapter molecule (RIAM) is a Rap1 effector molecule. GTP-Rap1–bound RIAM recruits talin to the plasma membrane by binding the rod domain and subsequently activates talin by binding the integrin-binding region on the talin head. Interestingly, knockdown of RIAM abrogated Rap1-induced adhesion to integrin ligands, suggesting a role in integrin LFA-1 inside-out signaling (Klapproth S., et al., 2015). Instead, the RIAM/talin complex contribution to integrin activation for the VLA-4 integrin was only partially affected by RIAM deficiency in leukocytes. Therefore, that β2 and α4-integrins use different RIAM-

dependent and -independent pathways to undergo activation by talin (Klapproth S., et al., 2015).

However, a pathway involving Rap GTPases and talin is critical for “inside-out” signaling but there are many unanswered questions. For example: the inactivation of Rap1 blocks SDF1α (CXLC12) stimulated LFA-1-mediated binding to ICAM1, but not binding of VLA-4-mediated binding to VCAM-1 in human T cells. Similarly, silencing of CalDAG-GEFI inhibits SDF1α and phorbol 12-myristate 13-acetate (PMA) stimulated adhesion to ICAM-1 but not VCAM-1. This data suggests that different pathways are used to activate LFA-1 integrin and VLA-4 integrin (Ghandour H., et al., 2007). The underlying reason for this integrin class-specific requirement of CalDAG-GEFI, Rap1 and RIAM is not clear. It is conceivable that different chemokines show specificity for activation of different integrins. In addition, different integrin classes localize to defined membrane compartments that are targeted by different signaling pathways and are therefore differently activated. One interesting membrane compartment, which operates independently of Rap1 and RIAM, could be the leukocyte microvilli. It has been shown that the integrin VLA-4 on T cells is enriched on microvilli during adhesion to VCAM-1 under flow conditions, whereas the integrin LFA-1 is enriched in non-microvillar compartments.

In addition, there are also other differences in the adaptive molecules that mediate the cascade of these two integrins as the hematopoietic Progenitor kinase 1 (HPK1) and the paxillin. A recent study identifies the HPK1 as specific regulator for LFA-1. HPK1 is critical for CXCL1-LFA-1-mediated neutrophil adhesion to ICAM-1 under flow conditions (Jakob S.M., et al., 2013). Instead, an unusual feature of α4 integrin signaling is the direct binding of a cytoplasmic adaptor protein, the paxillin, to the α4 cytoplasmic domain. This protein–protein interaction is regulated by trans-regulation by enhancing the activation of tyrosine kinases, focal adhesion kinase (FAK) and/or proline-rich tyrosine kinase-2 (Pyk2). The α4–paxillin interaction is required for the ability of α4-integrins to enhance migration (Kummer C., et al., 2006). Collectively, these data indicate multiple potential targets exist within these signaling pathways thus the integrin signaling might be a favorable therapeutic target for the treatment of chronic inflammatory disorders.

LFA-1 integrin is one of the most important and widely expressed integrin on leukocyte but its function partially overlaps with the integrin Mac-1. In *in vitro* studies have demonstrated that both LFA-1 and Mac-1 integrins are involved in the adhesion of neutrophils to endothelial cells and ICAM-1, but that adhesion through LFA-1 integrin overshadows the contribution from Mac-1 integrin (Ding Z.M., et al, 1999). In agreement, by intravital microscopy studies, other authors demonstrated the role of LFA-1 and Mac-1 in wild type (WT) and LFA-1 and Mac-1 deficient mice. These results delineate two different molecular

mechanisms for LFA-1 and Mac-1, with LFA-1-dependent adhesion followed by Mac-1-dependent crawling (Phillipson M., et al, 2006). All these data together underline that the integrin LFA-1 is mainly involved in neutrophil adhesion, which is an obligate step preceding extravasation, whereas Mac-1 play a main role in the crawling phase on endothelial cells.

VLA-4 integrin is most prominent integrin expressed on mononuclear leukocytes, but is also expressed on neutrophils (Johnston B. & Kubes P., 1999; Engelhardt B. & Ransohoff R.M., 2012). VLA-4 integrin mediates cells adhesion to VCAM-1 and is essential for embryogenesis, hematopoiesis, lymphocyte homing and the recruitment of leukocytes to sites of inflammation (Rose D.M., et al., 2002; Imai Y., et al., 2010).
Notably, mouse neutrophils in the blood show very low levels of VLA-4 integrin compared to bone murine marrow neutrophils, suggesting that VLA-4 integrin expression decreases during neutrophil maturation (Petty J.M., et al., 2009). In addition, human neutrophils do not constitutively express the VCAM-1 binding integrin VLA-4 or it is non-existent on the surface of resting neutrophils (Davenpeck K.L., et al. 1998). This has led to the general assumption that VLA-4 integrin is not involved in neutrophil recruitment or function. In contrast, several authors have demonstrated that neutrophils can express VLA-4 integrin by mediating adhesive interactions with endothelium and consequently, neutrophils can infiltrate numerous tissues independent of β2-integrin (Pereira S. et al. 2001, Reinhardt P.H., et al., 1997, Ibbotson G.C., et al., 2011; Taooka Y., et al., 1999; Neumann J., et al., 2015). However, one major difference in leukocyte recruitment is that while LFA-1 integrin is mainly involved in neutrophil 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., et al., 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 more chronic inflammatory processes (Johnston B. & Kubes P., 1999).

9. LEUKOCYTE TRAFFICKING IN AD

For the past decades, molecular mechanisms underlying AD pathology have been studied in exclusive relation by Aβ, considering mainly its effects on neuronal activity and functions (“the Amyloid Hypothesis”). However, most therapeutic efforts targeting Aβ have failed to show efficacy and none of numerous clinical investigations and drug developments has not even slow disease progression (Chiang K. & Koo E.H., 2014). This failure has raised many

doubts about the Amyloid Hypothesis and despite the Aβ plaque remains a well-established hallmark of AD pathology other aspects of pathology has been investigated.

Several evidences from basic research studies and clinical research suggest the inflammation and innate immune mechanism in the CNS enhance AD pathology (Rogers J. et al., 2008; Heneka M.T., et al., 2010). Interestingly, recent studies on the AD brain revealed that Aβ plaques are co-localized with a variety of inflammation-related proteins (complement factors, acute-phase proteins, pro-inflammatory cytokines) and clusters of activated microglia, suggesting a strong implication of endogenous brain immune responses in AD patients (Eikelenboom P., et al., 2006). In fact, glial cells (microglia and astrocytes) actively contribute to immune cell trafficking by integrating signals between the brain and the periphery. (Persidsky Y., et al., 1999; Hudson L.C., et al., 2005; Choi S.S. et al., 2014; Lécuyer M.A., et al., 2016; Rogers J., et al., 2008; Heneka M.T., et al., 2010). Microglial cells are involved in the active surveillance of the CNS and continuously scan the environment to detect pathogens or tissue damage (Yang J. et al., 2011). The activation of microglia induces an innate immune response dominated by the release of the pro-inflammatory cytokines and chemotactic factors that may act on circulating leukocytes (Heneka M.T., et al., 2015). However, microglial cells in AD pathogenesis seems to have a dual role: a protective role in clearing Aβ or a detrimental role in inducing an inflammatory state with the production of proinflammatory cytokine and chemokines (Hickman S.E., et al. 2008). In addition, also astrocytes have an important defensive function in brain by triggering a mechanism knows as astrogliosis, with which glial cells can repair and remodel damaged brain tissue (Li L., et al., 2008). Astrocytes are also important in regulating the turnover of the main excitatory neurotransmitter glutamate in the brain (Swanson R.A. et al., 2005). However, astrogliosis is associated also with cognitive decline in neurodegenerative diseases such as multiple sclerosis because it is correlated with neuroinflammation (Hostenbach S, et al., 2014). Indeed, also astrocytes activated by Aβ plaques are involved in the inflammation process in AD brain (Matos M., et al., 2008). Microglia and astrocytes are resident cells within the brain, which can be activated against injuries, however also circulating leukocytes invading the brain (such as monocytes, T cells and neutrophils) may contribute to wound healing but indeed may also cause tissue damage (Mcdonald B. & Kubes P., 2011).

Monocytes are the most widely-studied circulating immune system cells in AD and they migrate through the BBB into the AD brain in a CCR2-dependent manner (El Khoury J. et al., 2007; Naert G. & S., Rivest, 2013). The beneficial role of monocytes in AD relies on the clearance of Aβ, thus CCR-2 deficiency in the Tg2576 and APPSwe/PS1 mouse models of AD exacerbates amyloidosis and memory deficit (El Khoury J., et al., 2007; Naert G. & S., Rivest, 2011). Furthermore, APPSwe/PS1 mice are characterized by the defective production of CCR2+ monocytes leading to cognitive decline, the accumulation of soluble

Aβ and the disruption of synaptic activity (Naert G. & S., Rivest, 2012; Naert G. & S., Rivest, 2013). In agreement with these data, *in vivo* two-photon microscopy (TPM) studies indicate that patrolling monocytes are attracted to and crawl onto the luminal walls of Aβ+ veins and that their selective removal in APP/PS1 mice significantly increases the Aβ load in the cortex and hippocampus, suggesting that monocytes can naturally target and eliminate Aβ within the lumen of veins (Michaud J.P., et al., 2013). Despite these evidences, two studies have recently challenged the view that circulating monocytes help to clear Aβ in AD models. The replacement of brain-resident myeloid cells with circulating peripheral monocytes in mouse models of cerebral amyloidosis showed that monocyte repopulation does not modify the amyloid load, arguing against a long-term role of peripheral monocytes in Aβ clearance (Prokop S., et al., 2015; Varvel N.H., et al., 2015).

Interestingly, in addition to monocytic-derived cells also T cells were observed in association with Aβ deposited in leptomeningeal and cortical vessels, suggesting that Aβ angiopathy rather than Aβ plaques support T cell infiltration into the AD brain (Yamada M. et al., 1996). An increased frequency of T cells has been shown adherent to the vascular endothelium or migrated into the brain parenchyma in hippocampus and cortical regions of post-mortem AD subjects and AD-like mice (Itagaki S., et al., 1988; Rogers J. et al., 1988; Togo T., et al., 2002; Town T., et al., 2005; Ferretti M.T., et al., 2016).

Several studies found an altered frequency of CD4+ and CD8+ T cells in the peripheral circulation of AD patients in comparison to patients with other forms of dementia or age-matched controls (Tan et al., 2002; Town T., et al., 2005; Bonotis K., et al., 2008; Pellicanò M., et al., 2012; Lueg G., et al., 2015). In particular, a significant decrease in CD4+ T cell count was reported in AD patients of severe compared to mild-moderate stages of diesease and control patients (Bonotis K., et al., 2008; Pellicanò M., et al., 2012). In agreement, a report show augmented vulnerability to apoptosis in CD4+ T cells of AD subjects differently from normal elderly (Schindowski K., et al., 2006). Contrary, other reports showed either no differences in CD4+ T cells (Speciale L., et al., 2007) or the opposite situation, with total CD4+ T cells significantly increased in AD patients in comparison to healthy controls (Shalit F., et al., 1995; Lombardi V.R., et al., 1999; Richartz-Salzburger E., et al., 2007). Therefore, these data are inconsistent and do not allow drawing definite conclusions.

In the context of CD8+ T cells, a recent report showing higher numbers of activated CD8+ T cells in peripheral blood and in the CSF of MCI and mild AD patients, that seems to correlate with clinical AD markers (Lueg G., et al., 2015). Interestingly, these circulating CD8+ T cells in AD subjects were found to express the activation marker CD38 (Zhang R., et al., 2013), that is associated with migration into peripheral tissues and cytotoxic effector functions (Savarino A., et al., 2000). The inflammatory response stimulated by migrated T cells into the AD brain may activate microglia and astrocytes and may recruit other inflammatory cells that are potentially harmful to the CNS, thus exacerbating the

pathogenesis of AD. One of the major culprits in causing collateral damage during sterile inflammation is the neutrophil (Rock K.L. et al., 2010).

Polymorphonuclear leukocytes (PMNs), also called neutrophils, are most abundant population of cells in blood and represent the primary mediators of the innate immune response. They rapidly deployed to sites of inflammation, where perform a variety of antimicrobial functions such as degranulation and phagocytosis, to kill invading pathogens. The presence of neutrophils in human AD brains was also known in the past, but their specific role in the pathology was still everything to prove. Savage M.J., et al. showed by immunohistochemistry the distribution of cathepsin-G, a protease contained specifically in neutrophils, within the brain parenchyma as well as inside cerebral blood vessels of AD subjects and sometimes associated with Aβ deposits (Savage M.J., et al. 1994). Interestingly, the presence of CAP37, an inflammatory mediator expressed in neutrophils, was reported in blood vessels and in hippocampal vasculature in patients with AD (Grammas P., et al., 2000; Brock A.J., et al., 2015). Other reports investigate the neutrophils/lymphocytes ratio as an inflammatory marker, which levels in the blood are elevated in people with AD than healthy controls (Kuyumcu M.E., et al., 2012), and increase in function of age, but are weakly correlated with Aβ deposition (Rembach A., et al., 2014). A recent pioneering study of our laboratory reports neutrophil accumulation in the brain of AD animal models as well as AD subjects. We demonstrated that neutrophils can migrate in the brains of AD patients and they can induce cognitive dysfunction (Zenaro E., et al., 2015). For these reasons, neutrophils are under investigation in the attempt to treat AD (Zenaro E., et al., 2015).

Neutrophils are the most abundant type of white blood cells in mammals, and play an essential role in the innate immune system (Nathan C., 2006). They are one of the firstresponders of inflammatory cells to migrate toward the site of inflammation through the blood vessels, recruited by chemoattractant such as IL-8 and C5a in a process called chemotaxis. Neutrophils have an average diameter of 12–15 μm and a 12-h life span in circulation in non-activated condition. However, after activation and migration into tissues, they are able to survive for 1–2 days.

Neutrophils have three strategies to attack invaded microorganisms: phagocytosis, release of soluble antimicrobials (including granule proteins) and generation of neutrophil extracellular traps (NETs) (Brinkmann, V. et al, 2004; Hickey, M.J. et al, 2009). In phagocytosis the release of ROS is essential to degrade the pathogens in phagosomes. However, in excessive quantity they may damage host tissue and cells. As a result of digestion of pathogens (bacteria, fungi, and viruses), they often are phagocytosed by macrophages. In addition, the massive degranulation of neutrophils with release of their proteolytic enzymes may be another source of tissues damage. Neutrophils have four types of granules and each has specific proteolytic enzymes with a function correlated. These are: azurophilic (primary), specific (secondary), gelatinase (tertiary), and secretory granules. The main proteolytic enzymes contain in their granules are myeloperoxidase, serine proteases, α-defensins,

lysozyme, human neutrophil elastase (NHE), cathepsin G (CatG) lactoferrin, metalloproteinases (MMP-8 and −9 in particular), CD13 (aminopeptidase N) and CD16 (Fc gamma receptor III). (Yonggang Ma et al, 2013). In addition to their antimicrobial activity, the neutrophils play an essential role in non-infectious inflammation, innate immunity, present antigen though the MHC I and tissue remodeling. Recently, various authors reported that neutrophils release NETs decorated with proteins such as elastase and histones to entangle pathogens (Zawrotniak M. et al, 2013).

According to this study, the role of leucocytes recruitment could be an important step in the pathogenesis of AD, so it may represent a therapeutic target for AD. Nowadays, a real care for AD has not yet been found. The current treatments are prescribed for treatment of mild to moderate AD symptoms and slow down 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 successfuly proved for the treatment of multiple sclerosis (MS), a chronic inflammatory and demyelinating disease of CNS. Indeed, the humanized monoclonal antibody Natalizumab (Tysabri) blocks VLA-4 integrin by inhibiting the adhesion and migration of lymphocytes in cerebral parenchyma and, consequently, preventing the damage of nervous cells. Therefore, it can be hypothesized that a possible intervention aimed to block leukocyte recruitment may have beneficial effect also in AD.

MATERIALS AND METHODS

10. REAGENTS

The following rat-anti mouse monoclonal antibodies were purified from serum-free hybridoma media in our laboratory: anti-ICAM-1 (clone YN 1.1.7.4), anti-LFA–1 (clone TIB213 and clone KIM127), anti-VCAM–1 (clone MK 2.7), anti-E-Selectin (clone RME-1), anti-CD45 (clone 30G12), anti-RAS (clone Y13259) and anti-Gr–1 (clone RB6-8C5). The anti-LFA–1 antibody (clone 327A) was kindly provided by Dr. Kristine Kikly (Eli Lilly and Co.) and the anti-α–integrins mAb (PS/2) were purchased from Bioxcell. The chemical reagents are: mouse mAb anti-human to Aβ 1-16 (6E10) (Covance-Signet), rabbit mAb anti-mouse to Iba-1 (Wako), mouse mAb anti-human to total tau 159-163 (HT7) (Thermo Scientific), mouse mAb anti-human to phospho-tau to residue Thr231 (AT180) (Thermo Scientific). The following were purchased from commercial sources: anti-mouse P-selectin (clone CD62P) (BD Pharmingen), fluorescence-conjugated anti-mouse CD45 (clone 30F11.1), rat mAb anti-mouse to CD45 (RA3-6B2) (Biolegend); rat mAb anti mouse to CD11b (M1/70) (Biolegend); rat mAb anti mouse Gr-1–PE (Mlteny Biotech); rat mAb anti mouse to-Ly6G–FITC (1A8) (Biolegend); rat mAb anti- mouse CD4 (RM4-4) (Biolegend); rat anti mouse to-CD8 (53-6.7) (Biolegend); Biotinylated secondary antibodies and Avidin Texas Red were purchased from Vector Labs. CXCL12 were purchased from R&D systems. Dako mounting medium were purchased from DAKO. PTx and bocMLF were purchased from Tocris Bioscience. Cell trackers CMTPX, CMAC, and the 655-nm and 525-nm non-targeted Q-dots were obtained from Molecular probes.

11. ANIMALS

The transgenic AD animal models used are 5xFAD, 3xTg-AD and LFA-1-deficient mice (*Itgal*$^{-/-}$) mice purchased from 'The Jackson Laboratory' (Sacramento, CA).

5xFAD is a double transgenic APP/PSEN1 mouse model that co-expresses five familial AD mutations (FAD) under transcriptional control of the neuron-specific mouse Thy-1 promoter (Oakley H., et al., 2006; Ohno M., et al., 2006). 5xFAD represents one of the most early-onset and aggressive amyloid predominant mouse models. It starts to develop visible amyloid deposits as early as ~2 months of age consistent with their dramatically accelerated Aβ1-42 production. Later, these structures undergo a continuing process of sprouting/swelling and dystrophy, associated with the emergence and deposition of extracellular Aβ (Zhang X.M., et al., 2009). At 4- and 6-month-old 5xFAD mice show hippocampal dysfunctions, impaired contextual fear memory formation and a significant

remote memory dysfunction (Kimura, R. & Ohno, M., 2009). 5xFAD mouse is also one of the few currently available AD-like models known to develop neuron loss. 5xFAD mice were reported to develop neuron loss by 9 months of age at the cortex and subiculum (Oakley H., et al., 2006). As a consequence of the extensive pathology seen in 5xFAD mouse, this model develops different electrophysiological and behavioral impairments.

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., et al., 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., et al., 2003; Billings L.M., et al., 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 L.M., et al., 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 knockin and LFA-1 knockout).
5xFAD, 3xTg-AD, 3xTg-AD*x*Itgal$^{-/-}$, 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).

11.1 MICE TYPIZATION

The genetic backgrounds of 5xFAD mice were 50% C57Bl/6 and 50% B6/SJL. Transgenic lines were maintained by crossing heterozygous transgenic mice with B6/SJL F1 breeders. All transgenic mice used were heterozygotes with respect to the transgene, and non-transgenic littermates served as controls. In addition, we performed genotyping to distinguish 3xTg-ADx*Itgal*$^{-/-}$ homozygous from heterozygous mice by PCR analysis of APP and PSEN1 insertion and LFA-1 deletion. In addition, the PSEN1 PCR was followed by a step of restriction enzyme digestion with BstE II.

11.1.1 DNA EXTRACTION FROM TAIL BIOPSIES

Final part of mice tail was accurately cut in sterile condition and placed into polypropylene microfuge tube and then kept on ice. We performed DNA extraction using DirectPCR Lysis reagent (Viagen Biotech, Cat # 101-T, 101- T) containing freshly prepared 0,2-0,4 mg/ml of proteinsase K (P2308, 115K8614, Sigma-Aldrich, St.Louis, MO). We used 300 µl for 0,5 cm tail. Proteinsase K was stable in DirectPCR Lysis reagents for 24 hours. Proteinasi K was needed to degrade tissue proteins such as keratin. To obtain complete tissue lysis, tubes were incubated overnight at 55°C in agitation. After vigorous mechanical agitation, vials were incubated in water bath at 85°C for at least 45 min and mixed every 15 min. Tail were considered completely digested when at the bottom of vials were present only hairs and eventually bone residues. To eliminate tail residues tubes were centrifuged for 5-10 sec at maximum speed. Crude lysates were stored at -20°C for 1 years or at 4°C for 1 week without losing efficacy. 1µl of crude lysate was used for direct PCR reaction.

11.1.2 PCR

The PCR mix contained: 200 mM dNTPs (U1511, Promega, Madison, WI), 0.4 mM forward and reverse primers, 1.5 mM $MgCl^2$ (A351H, Promega, Madison, WI), 1.25 U GoTaq® Hot Start Polymerase (M5005, Promega, Madison, WI), 5X PCR Green GoTaq® Flexi Buffer

(M8911, Promega). 1µl of crude DNA lysate was used as template. Final PCR volume was 20 µl. PCR program consisted of an initial denaturation at 94°C for 4 min, followed by 35 cycles of 3 repeated steps: denaturation (94°C for 30 sec), annealing (54°C for APP, 65°C for PSEN1 and 58°C for LFA-1, for 30 sec) and extension (72°C for 30 sec) (GeneAmp® PCR System 9700). The following primers purchased from Life Technologies were used: 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 LFA-1 (5'CAC GGG TAG CCA ACG CTA TGT C 3'), FW LFA-1 (5' GCC CTG AAT GAA CTG CAG GAC GAC G 3'), RV LFA-1 ctrl (5' AGA AGC CAC CAT TTC CCT CT 3'), FW LFA-1 ctrl (5' AGC TGG AGT CCC AGT AGC AA 3'), RV PS1 (5' CAC ACG CAC ACT CTG ACA TGC ACA GGC 3'), FW PS1 (5' AGG CAG GAA GAT CAC GTG TTC AAG TAC 3'). A final extension at 72°C for 5 min ended the program. To estimate qualitatively the presence of APP, LFA-1 and PSEN1 PCR product of amplification, 5µl of PCR mixture were run on 1% agarose gel and visualized by Nancy staining (Sigma, Nancy-520). TrackItTM 100bp DNA Ladder (10488-058, Life Technologies) was used for sizing the double-stranded DNA from 100 to 1500 bp on the agarose gel.

11.1.3 RESTRICTION ENZYME DIGESTION

BstE II recognizes the DNA sequence: (5' G GTNAC C 3') and (3' C CANTG G 5'). BstE II restriction enzyme mix contained: BUFFER D 10X (60mM Tris- HCl pH 7.9, 1.5M NaCl, 60mM $MgCl^2$ and 10mM DTT), acetylated BSA 100X, 30 µl of PCR product, 10U of BstE II restriction enzyme and deionized water. The final volume of the mix is 50 µl. The solution was then incubated at 37°C for 3 hours. After incubation, 5µl of 10X loading buffer was added to proceed for the agarose gel analysis of DNA fragments obtained. The mixture was run on 1% agarose gel and visualized by Nancy staining (Sigma, Nancy- 520). TrackItTM 100bp DNA Ladder (10488-058, Life Technologies) was used for sizing the double-stranded DNA from 100 to 1500 bp on the agarose gel.

11.2 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. Mouse was 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 mouse was sedated, the abdomen was wet with ethanol. Mouse was 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. 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 (Fig. 14). A peristaltic pump (Minipuls3 Gilson) with a flow no higher than 0.5 ml/min is used to inject 25 ml with Ca^{2+}/Mg^{2+}(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 overnight at 4°C. Mouse organs were then post-fixed at 4°C overnight. 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.

11.3 IMMUNOFLUORESCENCE STAINING ON TISSUE SECTIONS

Frozen tissues correctly stored with OCT compound were cryo-sectioned in a cryostat. Brain coronal sections, if made at specific sites, allow similar areas to be examined, so that comparisons can be made between littermate controls and AD transgenic animals. Mouse brains were cut in coronal slices of 30-40 μm. Sections were collected and placed in 24-well plates containing 1 ml of PBS. Then, free floating sections were incubated in blocking buffer, corresponding to species for secondary mAb, for 1h at RT°C; then treated with primary antibody overnight at 4°C (40 μg/ml of anti-VCAM, 10 μg/ml of anti-ICAM, 10 μg/ml of anti-P-selectin, 5 μg/ml of anti-E-selectin, 5 μg/ml of anti-CD45, 5 μg/ml of anti-Gr1). Slices were incubated with biotinylated secondary mAb (7.5 μg/ml of rabbit anti-rat, T0226, VectorLAB) for 1h at RT°C, then washed with PBS. To reveal immunostaining, slices were treated with Avidin Texas Red (at 25 μg/ml, A2006, VectorLAB) for 1h at RT°C in the dark. After rinsing in PBS, sections were incubated with Dapi (at 1 μg/ml, D9542, Sigma-Aldrich, St.Louis, MO), for 8 min in the dark. Finally, brain portions were washed with PBS, transferred on glass slides and mounted with Dako (S3023 DAKO, Carpinteria, CA). Glass slices were kept at 4°C in the dark. Usage of this mounting medium help

reducing fading of immunofluorescence during microscopy acquisition. Slides were analysed by Tandem Confocal Scanning-SP5 (Leica, Germany).

11.4 ISOLATION OF BRAIN LEUKOYTES AND FLOW-CYTROMETRIC ANALYSIS

Mice were anesthetized and perfused through the left cardiac ventricle by injection of 35 ml of cold PBS. The brain was dissected, cut into small pieces and digested with DNaseI (20U/ml, Life Technologies) and collagenase (1mg/ml, Sigma) at 37°C for 45 min. Cells were isolated by passing the digested tissue through a cell strainer (70 μm), resuspended in 30% percoll and loaded onto 70% percoll. Then, tubes were centrifuged at 1300 xg for 20 min at 4°C. Cells were removed from the interphase, washed twice in PBS and resuspended in staining buffer for further analysis. Sensitive identification of various immune cell populations in a single sample was performed by antibody staining and flow cytometry with MACSQuant Analyzer (Miltenyi Biotec, Germany). The following anti-mouse antibodies were used: anti- CD45-Vioblue, anti-CD11b-APC Cy7, anti-Gr1-Viogreen, anti-Ly6G-FITC, anti-CD4-APC, anti-CD8-PE Cy7 and anti-γδTCR-PE (Miltenyi Biotec, Germany). Data were analysed using FlowJo software.

11.5 NEUTROPHIL PREPARATION

Mouse neutrophils

We isolated bone-marrow neutrophils from femurs and tibias of WT control mice. Mice were sacrificed by cervical dislocation. On average, 10-20 x 10^6/mL neutrophils can be expected for mouse. Therefore, the number of sacrificed animals depends on the number of neutrophils necessary for the experiment. Femurs and tibias were cut out and muscles removed. We flushed the marrow cells from the bones with Ca^{2+}/Mg^{2+} free 0.1% BSA HBSS solution (GIBCO). Then, the cell suspension is mixed with 18G-needle syringe to disrupt cell clumps and centrifuged 1200 rpm for 10 min. Then, 3mL of 0,2% NaCl solution was added to cell pellet for hypotonic lysis of erythrocytes, and after 30 sec 7ml of 1,2% NaCl was added to restore the osmolarity. The cell suspension was filtered through a 70μm cell strainer (Falcon) to get rid of the clot and any bone remnants. Cell pellet obtained after centrifugation at 1200 rpm for 10 min was resuspended in 3ml of 45% Percoll solution and loaded on a Percoll discontinuous density gradient. Percoll gradient was prepared by slowly adding in a 15-mL tube the following solutions: 4ml of 81% Percoll at the bottom, 3 mL of 62% Percoll, 3 mL of 55% Percoll and 50% Percoll (Fig. 10). Then centrifugation was performed at 2700 rpm for 30 min without brake. Neutrophils were harvested at the 80%-

65% interface with a Pasteur pipette, washed twice with PBS, resuspended in RPMI and counted for use. More than 90% of the isolated cells were Gr-1 positive cells as assessed by flow cytometry analysis (data not shown).

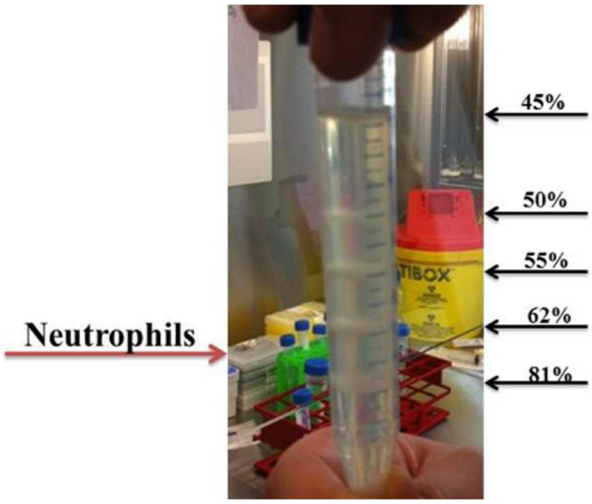

Figure 10. Percoll discontinuous density gradient.
The figure represents bone-marrow cells separated in Percoll discontinuous gradient in a 15-mL tube with the bands at 45%, 50%, 55%, 62% and 81%. Neutrophils were found at the 80%-65% interface.

Human neutrophils

Human PMNs were prepared from buffy coats of healthy volunteers by centrifugation through Ficoll Paque Plus (Amersham) at 2700 rpm for 30 minutes without brake. Patients provided their informed consent before samples were taken. Contaminating erythrocytes were removed by Dextran 500 (Amersham) sedimentation followed by 0.2% NaCl for hypotonic lysis of erythrocytes and after 50 seconds, 1.2% NaCl were added to restore the osmolarity. PMNs were washed with PBS and centrifugated at 1200 rpm for 5 minutes. Cell pellet was resuspended in RPMI and counted for the use in *in vitro* assays.

11.6 Aβ PREPARATION

Aβ (1–42) and Aβ (42–1) were purchased from Bachem AG. Oligomeric Aβ was prepared as reported previously (Kim et al., 2003). Briefly, after removal of hexafluoroisopropanol (Sigma), Aβ (1–42) was dissolved at 5 mM in DMSO and diluted to 100 μM in Dulbecco's Modified Eagle Medium, with nutrient mixture F-12 (DMEM/F12). After incubation for 24 h at 4°C, Aβ solution was centrifuged at 15,000 rpm for 10 min at 4°C and the supernatant, containing soluble oligomeric Aβ, was collected and quantitated by MicroBCA assay (Pierce, Rockford, IL). The same procedure was used for Aβ (42–1).

For the preparation of fibrillar Aβ, 5 mM Aβ 1-42 in DMSO was diluted to 200 μM, using 100 mM HEPES buffer, pH 7.5 and incubated for 1 week at 37°C. After aging, the Aβ sample was centrifuged at 15000 xg for 10 minutes at RT°C and the supernatant was removed. The pellet fraction was re-dissolved in HEPES buffer and quantitated by MicroBCA assay. The identity of fibrillar and oligomeric Aβ was evaluated by electrophoresis as previously described (Dahlgren K.N., et al., 2002). The identity fibrillar and oligomeric Aβ was evaluated by electrophoresis as previously described (Kim et al., 2003, Dahlgren K.N., et al., 2002). Briefly, 1–2 μg of Aβ peptides were diluted in lithium dodecyl sulfate sample buffer and loaded on precast 12% bis-Tris NuPAGE gel (Life Technologies) using MES running buffer. Proteins were transferred on polyvinylidene difluoride membrane (Millipore) using NuPAGE transfer buffer (Life Technologies). Both gel electrophoresis and protein blotting were made in non-reducing conditions. Membranes were blocked in 5% non-fat dry milk/TBSTween (TBS-T) for 1h, followed by incubation with 0,5 μg/ml of a mouse monoclonal Aβ antibody to residues 1–12, BAM-10 (A5213, Sigma, St. Louis, MO). Membranes were then washed in TBS-T and incubated with horseradish peroxidase-conjugated anti-mouse antibody (1:2000), and developed with the ECL system (Amersham). Western blot analysis of soluble oligomeric Aβ (1–42) preparation (24h at 4°C) reveals the presence of monomers, trimers and tetramers.

11.7 RAPID ADHESION ASSAYS

Eighteen-well glass slides were coated for 16 hours at 4°C with purified human ICAM-1 (3000 sites/mm^2). Neutrophils (10^5/well; $5x10^6$/mL in PBS containing 10% heat-inactivated FCS, $CaCl^2$ 1 mmol/L, $MgCl^2$ 1 mmol/L, pH 7.2; adhesion buffer) were added, incubated for 10 min at 37°C, and then stimulated by the addition of the agonists for 2 min before washing. Fixation was performed on ice in 1.5% glutaraldehyde for 60 min. Cell count was obtained by computer-assisted enumeration of cells bound in 0.2 mm^2, as previously described (Laudanna et al., 1996). When required cells were pre-incubated for 20 min with 100 μM of boc-MLF or 2h with 2 μg/ml of PTx at 37°C.

11.8 MEASUREMENT OF LFA-1 AFFINITY STATES

Human neutrophils re-suspended in standard adhesion buffer at $2x10^6$/mL were briefly pre-incubated with 10 μg/ml of monoclonal antibodies KIM127 (Robinson et al., 1992) to study the extended conformation epitope corresponding to an inter- mediate-affinity state of LFA-1 (Shimaoka et al., 2006, Stanley et al., 2008), or 327C (Lum et al., 2002) to study the high-affinity state of LFA-1. The cells were stimulated for 10 sec with 0.5 μmol/L of CXCL12,

0,1 μM N-Formylmethionine-leucyl-phenylalanine (fMLP), 20 μM Aβ1-42 or Aβ42-1 under stirring at 37°C. After rapid washing, cells were stained with FITC-conjugated secondary polyclonal antibody and analyzed by cytofluorimetric quantification.

11.9 TWO-PHOTON MICROSCOPY SURGICAL PREPARATION

"Thinned skull preparation" for long-term high-resolution imaging *in vivo* was performed as previously described (Zenaro E., et al., 2013; Pietronigro E.C., et al., 2016). Mice were deeply anesthetized and core body temperature was monitored and maintained using a regulated heating pad. The hair on the scalp was removed with an electric razor. The scalp was then sterilized with alcohol. An incision was made along the midline of the scalp to expose the skull overlying the cortical region of interest. Any fascia overlying the skull was scraped away with a scalpel blade. The skin and periosteum were removed. A 1 mm diameter region of skull was thinned using a high-speed micro drill and a stainless-steel burr. Drilling was halted every few seconds to prevent heating and bone dust is removed using a compressed air canister. Care was taken not to deflect the skull during drilling. Drilling continued until the fine vasculature of the dura madre was visible (Fig. 11). At this point, thinning continued by hand using a microsurgical blade. This process was repeated until image clarity is maximized. Animals showing any signs of damage, such as subdural or epidermal bleeding were discarded from the study. When imaging was complete, the wound margins of the scalp were sutured together using nylon suture. Mice were given a bolus of warm saline for rehydration and are allowed to recover from anesthesia on a water-circulating heating pad. Neutrophils were isolated from bone marrow and labeled with fluorescent cell trackers CMTPX or CMAC (Molecular Probes, Life Technologies). TPM studies were performed at 24-36h after intravenous injection of cells. To visualize blood vessels, 20 μL of 655-nm or 525-nm non-targeted Q-dots (Molecular Probes, Life Technologies) in 100 μL of PBS were injected intravenously before mice were anaesthetized using 1.5% isoflurane with a facemask.

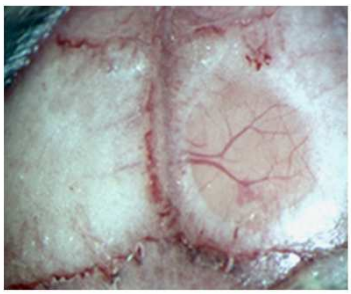

Figure 11. TPM study. Thinned skull preparation obtained by gentled scraping a little region of cranial skull on somatosensory cortex, until the fine vasculature of the dura madre was visible

11.10 TPM ACQUISITIONS AND DATA ANALYSIS

Time-lapse imaging was performed using a Tandem Confocal Scanning-SP5 (Leica). Each plane represents an image 525 μm by 525 μm (xy dimensions), and approximately 22-44 sequential planes were acquired at 2.5 μm increments in the z-dimension to obtain z-stacks. Z-stacks were acquired every approximately 32-63 seconds during time-lapse recordings. Image reconstruction, multidimensional rendering and manual cell tracking were done with Imaris software (Bitplane). Data were transferred and plotted in GraphPad Prism 5.0 (Sun Microsystems) for the creation of the graphs. The neutrophil movement analysis was performed by using functions of the T cell Analysis program (TCA; John Dempster, University of Strathclyde, Glasgow, Scotland).

11.11 MOUSE TREATMENT WITH LFA-1 OR α-INTEGRINS BLOCKING ANTIBODY

Anti-LFA–1 mAb (hybridoma TIB213) and an anti-α–integrins mAb (PS/2) was used to block leukocyte recruitment from peripheral circulation to inflammatory sites. Anti-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 specific inclusion and exclusion criteria. For instance, mouse body weight (20-40 gr) was checked and mice with body weight higher than 40 g were excluded from the experiments. Age and gender of AD-like disease models and WT control mice was matched. Mice with evident physical defects such as loss of the whiskers or dwarfism, and cutaneous defects (i.e alopecia) were excluded from the experiment. Moreover, to reach statistical significance a minimum number of 12-15 mice/experimental condition was used to study the effect of immune mechanisms inhibition in behavioural tests for cognitive functions (Zenaro E., et al., 2015). The experimental schedule is shown in Figure 12.

11.12 BEHAVIOURAL ASSESSMENT

Memory loss is the cardinal and one of the earliest clinical manifestations of AD. The animal behavior is usually evaluated with either operative or associative learning tasks. However, in the contest of AD pathology, where adult/old mice are usually tested, it is also important to distinguish learning/memory deficits from general deficits in task performance. Therefore, pre-cognitive tests such as “Hindlimb clasping” and “Ledge test” were run before behavioral assessment. Instead, learning and memory capacity were evaluated using Y-maze and Contextual Fear Conditioning (CFC) tests. 12-15 mice 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/food and were maintained on a 12-hour light/dark cycle. All behavioral testing was performed during the light phase of this circadian cycle. Experiments were made blind with respect to the genotype of the mice. All behavioral tests were performed as previously described (Sarter et al., 1988; Imbimbo et al., 2010). Y-maze and CFC tests were performed after one month of treatment, to avoid possible variable-like stress, generating by continuer injections (Fig. 12).

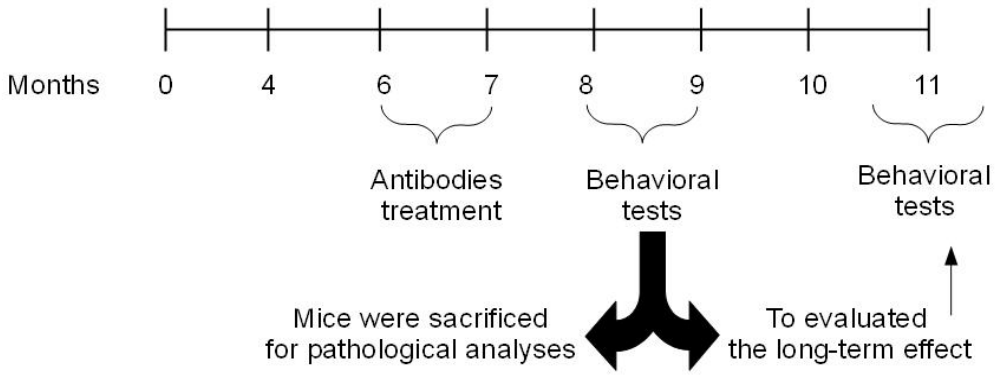

Figure 12. Experimental protocol of behavioral performances.
We treated 3xTg-AD mice with an anti-LFA–1 or anti-α-integrins antibody starting at 6 months of age (peak of maximum neutrophilic infiltration) for 4 weeks. An isotype antibody was used as control antibody and age-matched control mice were injectedwith endotoxin-free PBS. After the antibody treatment, 3xTg-AD and age-matched control mice were behaviorally tested at early stages of disease (at 8-9 months of age) or at later time-points to evaluated the long-term effect of the treatment (at 11-12 months of age). At the end of behavioral assessment, mice were sacrificed for neuropathological analyses.

11.12.1 HINDLIMB CLASPING

Hindlimb clasping is used as a marker of disease progression in many mouse models of neurodegeneration. To record the score, the tail was grasped near its base and mouse was lift away from all surrounding objects. The hindlimb position was observed for at least 10 sec and the test was conducted three times. If the hindlimbs were consistently splayed outward, away from the abdomen, a score of 0 was assigned. If one hindlimb was retracted toward the abdomen for more than 50% of the time suspended, it received a score of 1. If both hindlimbs were partially retracted toward the abdomen for more than 50% of the time suspended, it received a score of 2. If its hindlimbs were entirely retracted and were touching the abdomen for more than 50% of the time suspended, it received a score of 3.

11.12.2 LEDGE TEST

After the antibody treatment, 3xTg-AD mice were tested with ledge test. The ledge test was a direct measure of coordination, which is impaired in many other neurodegenerative disorders. The mouse was lift from the cage and placed on the cage's ledge. Mice will typically walk along the ledge and attempt to descend back into the cage. Then, the mouse was observed as it walked along the cage ledge and lowerd itself into the cage. A WT mouse typically walked along the ledge without losing its balance, and lowered itself back into the cage gracefully, using its paws. This was assigned as score of 0. If the mouse losed its footing while walking along the ledge, but otherwise appears coordinated, it received a score of 1. If it did not effectively use its hind legs, or lands on its head rather than its paws when descending into the cage, it received a score of 2. If it falled off the ledge, or nearly so, while walking or attempting to lower itself, or shacked and refused to move at all despite encouragement, it received a score of 3. Some mice required a gentle nudge to encourage them to walk along the ledge or descend into the cage. The above procedure was performed four times and the score was averaged between 4 tests.

11.12.3 Y-MAZE TEST

Y Maze Spontaneous Alternation is a behavioral 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 gray opaque plastic arms at a 120° angle from each other, extending from a central space (Fig. 13). After 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. So during the session, mouse should show a less tendency to enter in the recently visited arm. The sequence and the total number of arm entries were recorded to calculate the percentage of alternation. An entry occurs when all four limbs are within the arm. Each arm of the Y maze was arbitrarily 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 (number of triads containing entries into all three arms / maximum possible alternations) x 100. To diminish odor cues, the maze was cleaned with 70% ethanol solution prior to test each mouse.

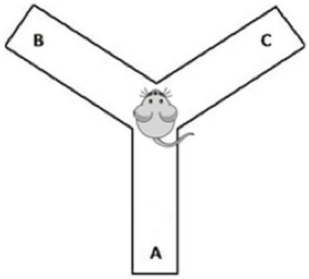

Figure 13. Representative image of Y-maze.

11.12.4 CONTEXTUAL FEAR CONDITIONING

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 (freezing). After repeated pairings of CS and US, the animal learns to fear both the tone and training context. Contextual Fear Conditioning is learned rapidly and is a useful test for neurobehavioral, genetic, and pharmacological studies. 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, Comerio, Italy). Each chamber was equipped with a stainless-steel rod floor through which a footshock could be administered, two stimulus lights, one house light, and a solenoid, all controlled by ANY-maze computer software (Stoelting, Wood Dale, Illinois) (Fig. 14a). The chambers were in a sound-isolated room in the presence of red light. Mice were trained and tested on 2 consecutive days (Fig14b). Training consisted of placing a subject in a chamber, illuminating stimulus and house lights, and allowing exploration for 2 min. Afterwards, the 15 sec tone stimulus [2 Hz clicking via the solenoid; conditioned stimulus (CS)] co-terminated with 2 sec footshock [1.5 mAmp; unconditioned stimulus (US)]. This stimuli pairing was repeated two times, with presentation spaced 2 min apart. 30sec 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 min contextual conditioning test without tone or shock presentation, and freezing behavior was recorded by the experimenter. Freezing was defined as lack of movement except that required for respiration. At the end of the 5 min 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. Mice were placed in a novel environment, the computer program recorded mouse movement during 3 min test without any stimulus presentation. The auditory cue (CS) was then presented for 3 min, 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 (Fig. 14b).

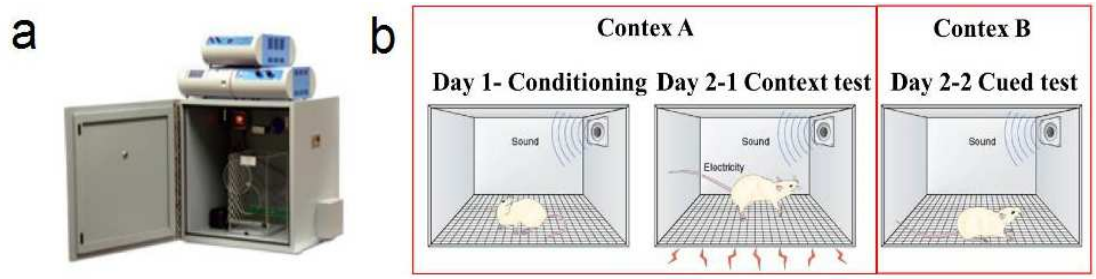

Figure 14. (a) CFC chamber [ugobasile.it]; (b) schematic representation of contextual fear conditioning procedure. Mice were trained and tested on 2 consecutive days. The first day mice were placed in the box and submitted to 3 rounds of tone stimulus [conditioned stimulus (CS)] co-terminated with unconditioned stimulus (US)](context A, day 1). In the first part of the second day mice were placed back to the same box in which training occurred without tone or shock presentation (context A, day-2). In the second part of same day, mice were placed in a novel environment (context B) and freezing was evaluated in response to the tone.

11.13 HISTOPATHOLOGICAL ANALYSIS

Brain sections of treated mice with anti-LFA–1 and anti-α–integrins antibodies and 3xTg-ADx*Itgal*$^{-/-}$ mice were cut at 30μm thickness in coronal portions. Aβ and tau/phosphorylated-tau staining required epitope retrieval with 70% formic acid for 20 min and 10mM sodium citrate buffer (pH 8.5) preheated to 85°C in water bath, respectively.

After epitope retrieval, brain sections were treated with 2% normal goat serum (Vector) and 0.4% Triton and then incubated with primary antibodies at 1μg/ml in blocking solution overnight at 4°C. The primary antibodies used are: for microglial staining the anti-mouse Ionized calcium binding adaptor molecule-1 antibody (Iba-1; Wako); for Aβ staining mouse anti-Aβ (6E10; Covance); for total tau staining mouse anti-tau HT7 (Thermo Scientific) and for total phosphorylated tau staining mouse anti-phospho-tau AT180 and AT8 (Thermo Scientific). After washing with PBS with 0.05% Tween20, we added 3% H_2O_2 for 10 min at RT°C to block endogenous peroxidase. Subsequently, brain slices were washed and incubated with a biotinylated secondary antibody at 10μg/ml (goat anti-rabbit for Iba-1 antibody, Sigma-Aldrich and goat anti-mouse for anti-Aβ 6E10, anti-tau HT7 and mouse anti-phospho-tau AT180 antibody) in blocking solution for 2h at RT°C. The immunoreactivity was visualized using the VECTASTAIN® ABC kit (Vector) for 30 min and Vector® NovaREDTM (Vector) as chromogen for 3 min at RT°C. Finally, brain portions were washed with PBS, transferred on glass slides and mounted with Eukitt® mounting medium (Sigma-Aldrich). Glass slides were kept at RT°C and acquired by LEICA fluorescence microscopy (DM6000B, Leica).

11.14 QUANTIFICATION OF MICROGLIA CELLS, AMYLOID LOAD AND HYPERPHOSPHORYLATED TAU PROTEIN

The numerical density (nr/μm^2) and area of Iba-1 immunoreactive microglia, Aβ plaques and hyperphosphorylated tau protein was determined in 4 non-consecutive coronal sections throughout the cortex and the dorsal hippocampus of treated 3xTg-AD, 3xTg-AD x *Itgal*$^{-/-}$ and age-matched controls. Sections were taken from the anterior hippocampus through the bregma −2.9mm at an intersection interval of 500 μm (every fourth section) to analyze the whole area of the cortex and the hippocampus. The specific areas analysed were: the parietal cortex, the dentate gyrus (DG), the CA1 area of hippocampus and the amygdala (Fig. 15). Iba-1 microglia cells were clearly labeled against a light background making them easy to be identified with an equal chance of being counted. Microglia cells, Aβ plaques and hyper-phosphorylated tau protein images were acquired with LEICA fluorescence microscopy (DM6000B, Leica) at magnification 20x with a resolution of 1392 x 1040 pixels (449x335 μm of area) and then counted with a computer-assisted imaging analysis (ImageJ 1.32j) software. To ensure consistency and reproducibility, samples were counted blindly.

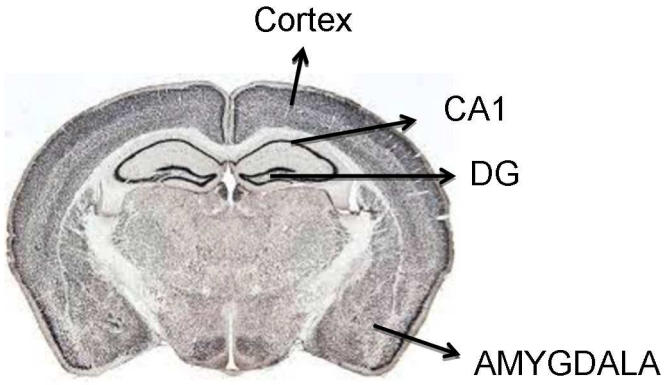

Figure 15. Coronal section of mouse brain, in which the main area investigated with immunohistochemistry are indicated: Cortex, CA1, DG and Amydgala.

STATISTICS

A two-tailed Mann-Whitney U-test was used for the statistical comparison of two samples. Multiple comparisons were carried out with Kruskall-Wallis test with the Bonferroni correction of P. For two-photon analysis, non-normally distributed data were presented as mean and compared using the Mann-Whitney U-test (two groups) or ANOVA followed by a suitable multiple comparison procedure (Dunn's or Dunnett's test).

12.RESULTS

12.1 EXPRESSION OF VASCULAR ADHESION MOLECULES IN 3xTg-AD MICE

Adhesion molecules are fundamental mediators of the leukocyte recruitment in sites of inflammation. Their expression on the surface of brain endothelium may be induced by pro-inflammatory cytokines, which can induce endothelial activation and amplification of the immune reactions. In the present study, we evaluated the expression of several adhesion molecules (ICAM-1, VCAM-1 and E-and P-selectin) by immunofluorescence staining in 3xTg-AD mice. At 4 months, we did not detect adhesion molecules expression in brain vessels except to some rare ICAM-1 positivity in post-capillary venules (data not shown). Surprisingly, we found an increased expression of all the adhesion molecules in the limbic system (hippocampus and amygdala) at 5-6 months of age in 3xTg-AD mice, compared to age-matched controls. Among integrin ligands, ICAM-1 was the most expressed adhesion molecule in 3xTg-AD mice. We found high level of expression of ICAM-1 in hippocampal blood vessels. Low levels of VCAM-1 expression was also found in the same brain districts. Among selectins, E-selectin was found expressed only in hippocampal vessels. We did not observe adhesion molecule expression in the brain of age-matched WT littermates (Fig. 16).

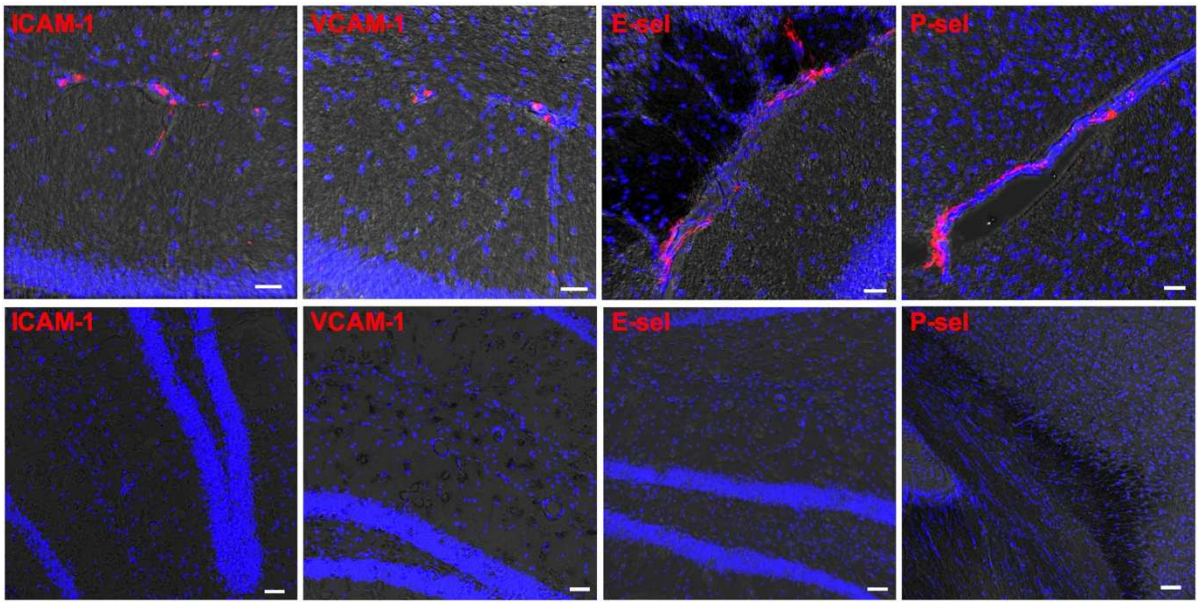

Figure 16. Expression of adhesion molecules in the brain of 3xTg-AD mice at the early stages of disease.
Confocal microscopy images show expression of endothelial integrin ligands (ICAM-1 and VCAM-1) and selectins (E- and P-selectin) in brain sections of 3xTg-AD mice (top panel) at early stages of disease and WT age-matched control mice (bottom panel). High expression of E- and P-selectin and moderate expression of ICAM-1 and VCAM-1 was found in hippocampal vessels in 3xTg-AD mice (top panel) at early stages of disease

compared to WT age-matched control mice (bottom panel). Nuclei are stained with Dapi in blue; adhesion molecules are stained with Avidin Texas Red. Scale bar: (a) 30 μm and (b) 50 μm.

12.2 LEUKOCYTE MIGRATION INTO THE BRAIN OF 3xTg-AD MICE

The results we described in the previous section suggest that the endothelial expression of ICAM-1, VCAM-1 and E- and P- selectin may mediate leukocyte adhesion in brain vessels and the subsequent migration of leukocytes in brain parenchyma. We also performed some confocal microscopy experiments to investigate the presence of leukocyte populations in 3xTg-AD mice at 5 and 6 months of age. The inflammatory cells were detected using antibodies towards specific antigens expressed on their surface.

To detect the presence of leukocytes, we used the following antibodies: anti-CD45 as a general leukocyte marker and anti-Gr1 for neutrophils. At 4 months, we did not detect any inflammatory infiltrates (data not shown). We observed that CD45+ leukocyte infiltration constituted principally of Gr1+ cells at 5-6 months of age in 3xTg-AD mice. These results are supported by the results obtained by Subramanian S. et al. showing an increase in Gr1+ granulocyte population in the brains of 5-6-month-old 3xTg-AD mice (Subramanian S., et al., 2010). Migrated leukocytes were localized in pial vessels, choroid plexus and hippocampus (Fig. 17). These data suggest that a consistent number of Gr1+ cells can migrate into the brain parenchyma at early stages of AD-like pathology. We did not observe any Gr1+ cell infiltrating the brain of age-matched WT littermates (data not shown). Of note, these results agree with the up-regulation of adhesion molecules on brain endothelium in 3xTg-AD mice, which might facilitate tethering, rolling and firm adhesion of neutrophils in postcapillary venules.

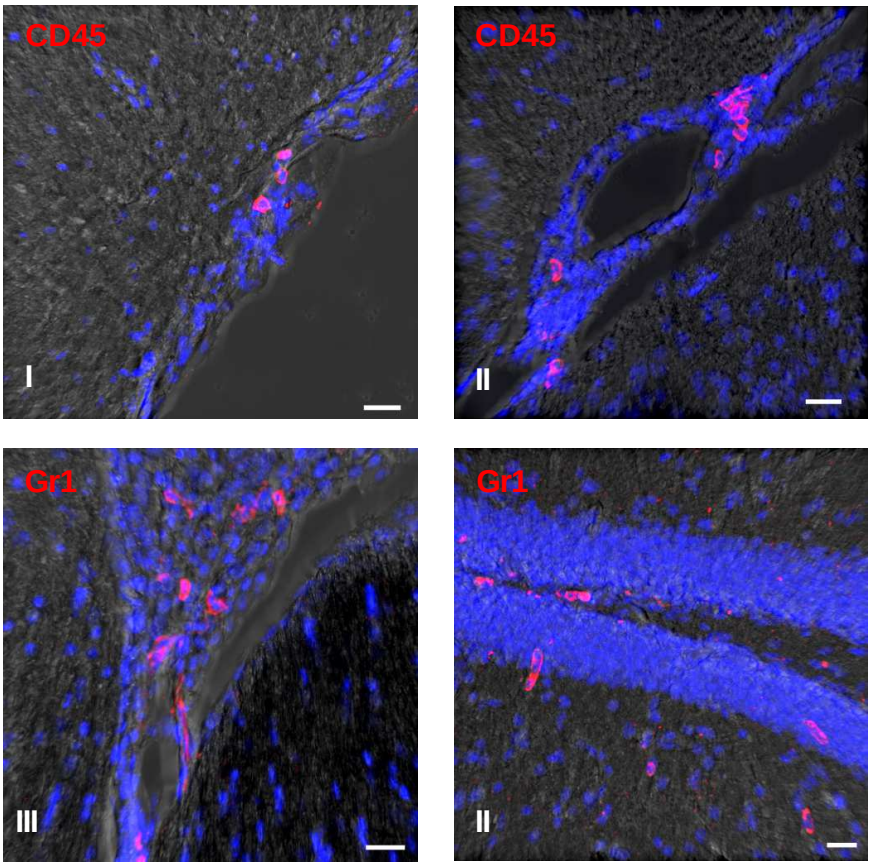

Figure 17. Inflammatory cells infiltrate the brain of 3xTg-AD mice during early disease.
Confocal microscopy images show the presence of CD45+ leukocytes (top panel), and Gr1+ cells (bottom panel) localized in (I) the choroid plexus, (II) the hippocampal blood vessels and (III) in the pial vessels in brain of 3xTg-AD mice. Scale bar: 20 μm Nuclei of the cells were labeled with Dapi in blue; CD45 and Gr1 staining was performed with Avidin Texas Red.

12.3 FLOW-CYTOMETRY ANALYSIS IN BRAINS OF 3xTg-AD MICE

Since the characterization of infiltrates in the mice brain, by histological approach, has the limitation of being a qualitative technique, we decided to extract the entire leukocyte infiltrating population. Then the leukocytes were immune-phenotyped and analyzed using flow cytometry to quantify the composition of the cell infiltrate. We quantified the accumulation of leukocytes from the brains of 3xTg-AD mice using flow cytometry.
To quantify the neutrophils population, CD45+ cells were sub-gated by using CD11b and Gr1 double gate allow the identification of three different populations: CD11b-/Gr1- cells (not shown), CD11b+/Gr1- cells (not shown) and CD11b+/Gr1+ cells (presumably granulocytes). For a more specific neutrophil labelling, Gr1 and Ly6G sub-gate was also performed during the analysis. The results showed that the number of

infiltrating neutrophils peaked in the early phases of the disease at 6 months of age in the 3xTg-AD mice and then gradually descended in the subsequent months (Fig. 18). No neutrophils were detected in the brains of the healthy controls (data not shown). Taken together these data suggest that neutrophils migrate into the brain at the early stage of the disease and continue to accumulate also during later phases of disease, suggesting they may play a role in chronic disease evolution.

Surprisingly we found also a high numbers of T cells infiltrating the brain of AD-like disease mice during all the progression of disease. T cell subsets showed different accumulation profiles during the progression of the disease. CD4+ T cells gradually increased showing a peak of accumulation during later phases of disease (Fig. 18). On the contrary, CD8+ T cells infiltrated the brain in the early phases of the disease with a wide peak of infiltration at 6-9 months of age and then gradually decreased during later stages of the disease (Fig. 18). WT age matched control mice did not show accumulation of infiltrating leukocyte in their brain (data not shown).

Taken together these data show that different leukocyte subtypes migrate into the brain of 3xTg-AD mice at different time-points of disease, suggesting that neutrophils and T cells may play different roles in disease evolution.

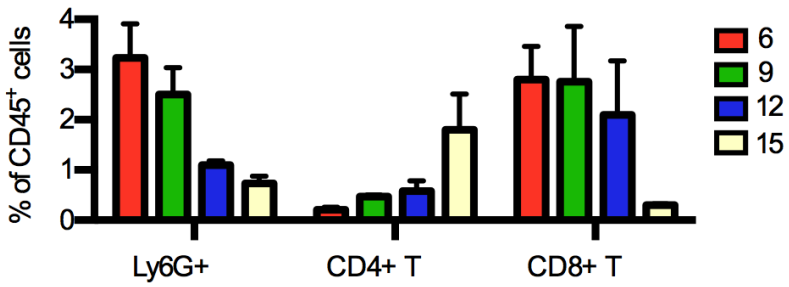

Figure 18. Leukocyte accumulation in 3xTg-AD mice during different time-points of disease.
Flow-cytometry quantitative analysis of leukocytes accumulated in the brain of 3xTg-AD mice at different months of age (6-9-12-15 months). 8-10 animals/group of same age were analysed. Error bars represent SEM.

12.4 CHARACTERIZATION OF Aβ-INDUCED ADHESION IN NEUTROPHILS

The results shown above suggest that the high expression of vascular adhesion molecules is associated with the presence of infiltrating leukocytes in the brain of 3xTg-AD mice at different months of age. To fully understand the mechanisms responsible for the neutrophil migration in the brain, we studied the effect of Aβ1-42 peptide on LFA-1 integrin-dependent

rapid adhesion assays. We prepared *in vitro* oligomeric and fibrillar Aβ1-42, characteristic of early and late-time points of disease respectively, to stimulate neutrophil adhesion in *in vitro*.

12.4.1 Aβ PREPARATION

Oligomeric and fibrillar Aβ were prepared as described in the *Materials and Methods section*. The preparations were run on native gel to verify the correct formation of Aβ. The native gel has revealed a progressive decrease of monomers, the formation of larger oligomers and a small quantity of insoluble aggregates that did not migrate from the well, presumably fibrillar Aβ (Fig. 19). The reverse Aβ peptide Aβ42-1 was used as negative control (data not shown).

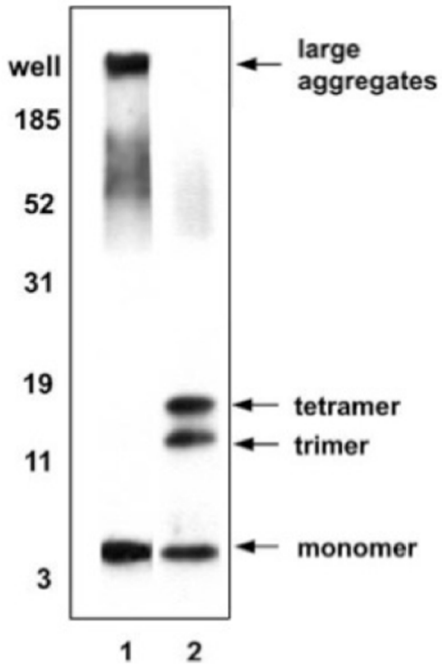

Figure 19. Representative Western blot of oligomeric and fibrillar preparations of Aβ (1–42).
Fibrillar (line1) and oligomeric (line 2) preparations of Aβ (1-42) were separated on 12% bis-Tris NuPAGE gel and probed with monoclonal antibody clone BAM-10 (recognizing residues 1-12 of Aβ). Relative molecular masses (Mr x 1000) are shown on the left side of gel lines.

12.4.2 Aβ INDUCES RAPID LFA-1 INTEGRIN DEPENDENT ADHESION

Neutrophils were extracted from bone-marrow as described in the *Materials and Methods section*. We have investigated the effect of Aβ1–42 on neutrophil LFA-1 integrin-dependent rapid adhesion (Fig. 20a). The results demonstrated that Aβ1–42 triggered a rapid neutrophils adhesion to ICAM-1 in a dose-dependent manner (Fig. 20b). In addition, our data showed that soluble the Aβ oligomer is a more effective trigger for the neutrophil integrin-dependent rapid adhesion if compared to fibrillary Aβ (Fig. 20c). The maximum effect was observed with a dose of 20 μM oligomeric Aβ. fMLP was used as positive control at the dosage 0.1 μM. The reverse Aβ peptide Aβ42-1 did not have any significant effect on LFA-1 dependent neutrophil adhesion (data not shown). Interestingly, one of the receptors for Aβ is a formyl-peptide chemotactic receptor (FPR) (Iribarren P., et al., 2005). To evaluate the contribution of this receptor on neutrophil adhesion, we inhibited its function using boc-MLF, i.e. an FPR antagonist. Our results clearly showed that LFA-1-dependent adhesion of neutrophils to ICAM-1 with both fMLP and Aβ1–42 was strongly blocked by the pre-treatment of the cells with boc-MLF (Fig. 21a). To exclude the possibility that the rapid integrin triggering was a consequence of a generic plasma membrane alteration due to the lipofilic nature of Aβ, we evaluated rapid adhesion in lymphocytes. These cells are known because of their lack of fMLP receptors and because they did not adhere to integrin ligands in the presence of Aβ (data not shown). In addition, to check whether GPCRs are responsible for Aβ-induced neutrophil adhesion, we treated the neutrophils with pertussin toxin (PTx) before performing the adhesion assays. The PTx, produced by Bordella pertussis, inhibits the α subunit of GPCRs and it is commonly used to investigate the involvement of GPCRs in biological assays. Our results demonstrated that neutrophil adhesion on ICAM-1 was abrogated by the pre-incubation of cells with PTx suggesting that both fMLP and Aβ1–42 stimulated adhesion is mediated by Gαi-coupled receptors (Fig. 21b).

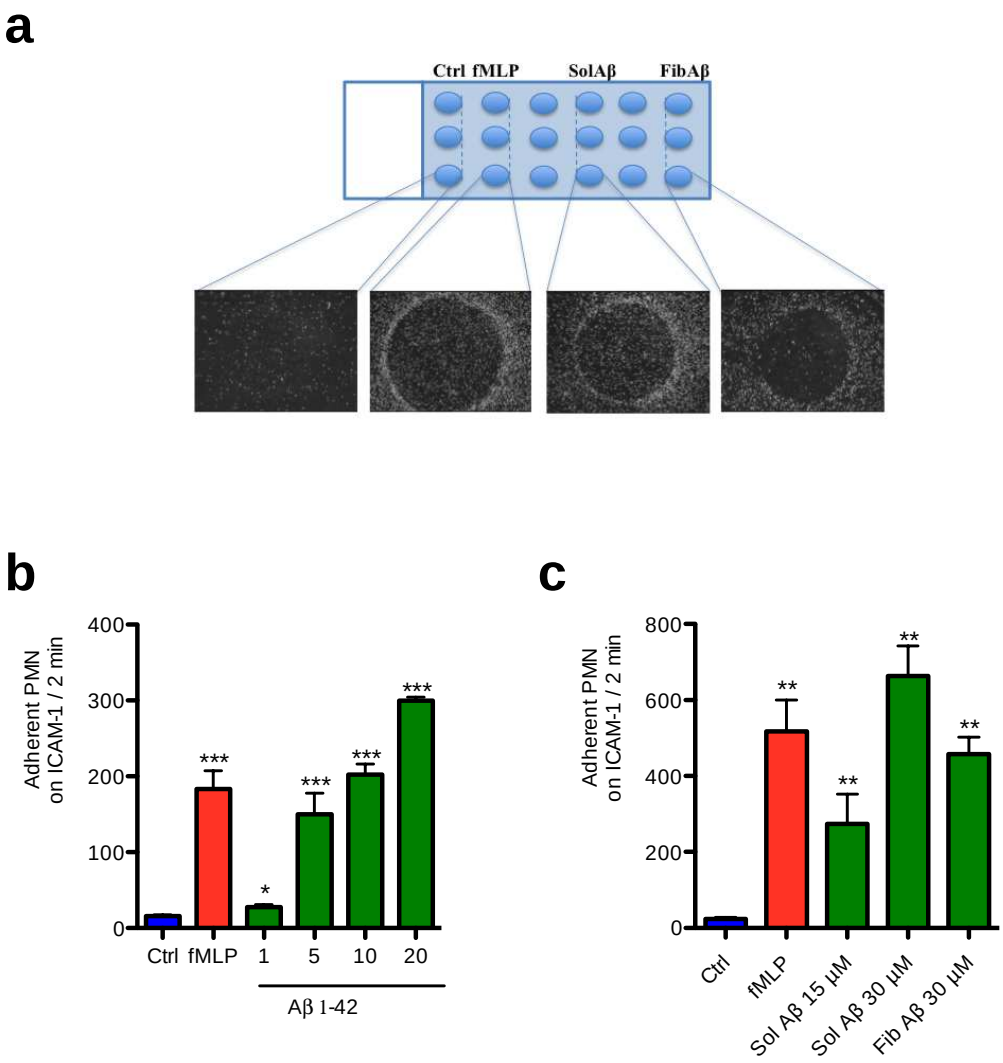

Figure 20. Oligomeric and fibrillar Aβ1-42 induce rapid neutrophil adhesion to ICAM-1. (a) Representative image of eighteen-well glass slides coated with human ICAM-1 as described in *Materials and Methods*. Samples were spotted in triplicate. (b) We coated 18-well glass slides with human ICAM-1 and mouse neutrophils were incubated for 2 min in buffer (Ctrl) or the stated concentrations of oligomeric Aβ (Aβ1-42). We used 1 µM fMLP as a positive control (fMLP). (c) Fibrillar Aβ1-42 (Fib Aβ) efficiently triggered the rapid adhesion of human neutrophils, but the oligomeric soluble form Aβ1-42 (sol Aβ) was twice as effective. Values represent mean counts of bound cells in a 0.2-mm^2 field in one representative experiment from a series of three independent experiments with similar results. Bars represent means ± SEM. (**P* <0.05; ***P* <0.005 ****P* <0.0005).

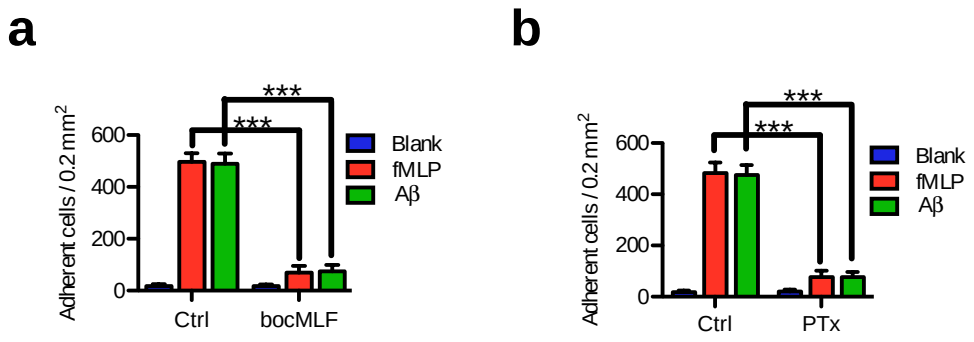

Figure 21. Rapid neutrophil adhesion to ICAM-1 triggered by Aβ is inhibited by bocMLF and PTx.
Eighteen-well glass slides were coated with human ICAM-1 as described in *Materials and Methods*. Human neutrophils were incubated for 2 minutes with buffer (Blank) or with oligomeric soluble Aβ1-42 (Aβ). 0,1 μM fMLP was used as a positive control (fMLP). Values are expressed as the mean counts of bound cells in 0.2mm^2 from three independent experiments in duplicate. Bars represent means ± SEM. (a) bocMLF inhibitor of FPR receptor and (b) PTx significantly inhibited neutrophil adhesion. (***P <0.0005).

12.4.3 Aβ1-42 OLIGOMERS TRIGGER LFA-1 INTEGRIN HIGH AFFINITY STATE IN HUMAN NEUTROPHILS

In vitro and *in vivo* studies have established that the leukocyte arrest is rapidly triggered by chemokines or other chemoattractants and is mediated by the binding of leukocyte integrins to immunoglobulin superfamily members, such as ICAM-1 and VCAM-1, expressed by endothelial cells (Ley K., et al., 2007). LFA-1 is one of the most relevant integrin involved with the neutrophil arrest, and classical chemoattractants and chemokines are the most powerful physiological activators of LFA-1–mediated adhesion *in vivo*. Ligation of specific heterotrimeric GPCRs through chemokines activates integrins by triggering a complex intracellular signaling network leading to the increase of both integrin affinity and valency. Inside-out signaling induces integrins to undergo a dramatic transition from a bent low-affinity conformation to extended intermediate- and high-affinity conformations, which lead to opening of the ligand-binding pocket (as described in paragraph 8.2) (Ley K., et al., 2007). We hypothesized that Aβ may trigger intermediate and high affinity state of LFA-1 integrin. So, we decided to investigate the effect of Aβ1-42 oligomers on human LFA-1 integrin using KIM127 and 327A conformer-specific antibodies for intermediate- and high-affinity state respectively. The data we obtained clearly confirmed the ability of Aβ1-42, but not scramble Aβ42-1 peptide, to trigger LFA-1 conformation to an intermediate- and high-affinity state (Fig. 22), demonstrating that Aβ enhances the propensity of LFA-1 integrin to bind its endothelial ligands and explaining the surprising capacity of neutrophils to perform arrest in areas with Aβ deposition *in vivo*.

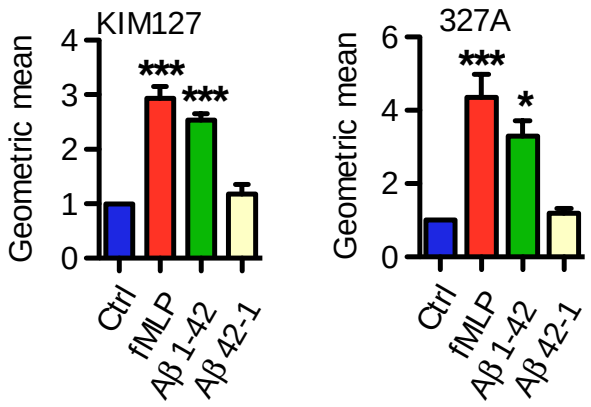

Figure 22. Soluble oligomeric Aβ1-42 trigger LFA-1 high affinity state in human neutrophils.
LFA-1 affinity states were detected with KIM127 mAb detecting low-intermediate affinity state or 327A mAb detecting high affinity state as described in *Materials and Methods*. Values represent geometric mean of fluorescence intensities measured by flow cytometry, normalized against the untreated control (Ctrl) condition for each experiment. Bars represent means ± SEM for three independent experiments. (**P* <0.05; ****P* <0.0005).

12.5 LFA-1 INTEGRIN IS FUNDAMENTAL FOR NEUTROPHIL INFILTRATION IN BRAIN PARENCHYMA

Neutrophil adhesion is largely mediated by β2-integrins and its extravasation is LFA-1–dependent (Ding Z.M., et al., 1999). Recently other authors have elegantly demonstrated, by TPM studies, that LFA-1 is the unique β2-integrins mediating the adhesion step of the leukocyte recruitment cascade (Phillipson M., et al., 2006). Therefore, we thought it would be significant to assess whether the ablation of LFA-1 integrin on neutrophils or the blockage of the molecule with an antibody prevented the cells to interact with blood vessels and to enter the brain parenchyma.
Our TPM video clearly demonstrated the success of this approach. Indeed, LFA-1 deficient neutrophils (*Itgal*$^{-/-}$) did not crawl/adhere on blood vessels (Fig. 23a) and did not accumulate in brain parenchyma (Fig. 23b) in 4 months old 5xFAD mice. Moreover, we demonstrated that LFA-1–blocking antibody interfere with the movement of already extravasated cells (Fig. 23c) and prevented further parenchyma invasion by neutrophils. After the injection of LFA-1 blocking antibody, neutrophil velocities were significantly slowed down as well as meandering index and motility coefficient (Fig. 23d), indicating a prominent role of LFA-1 integrin in neutrophil movement in brain parenchyma.

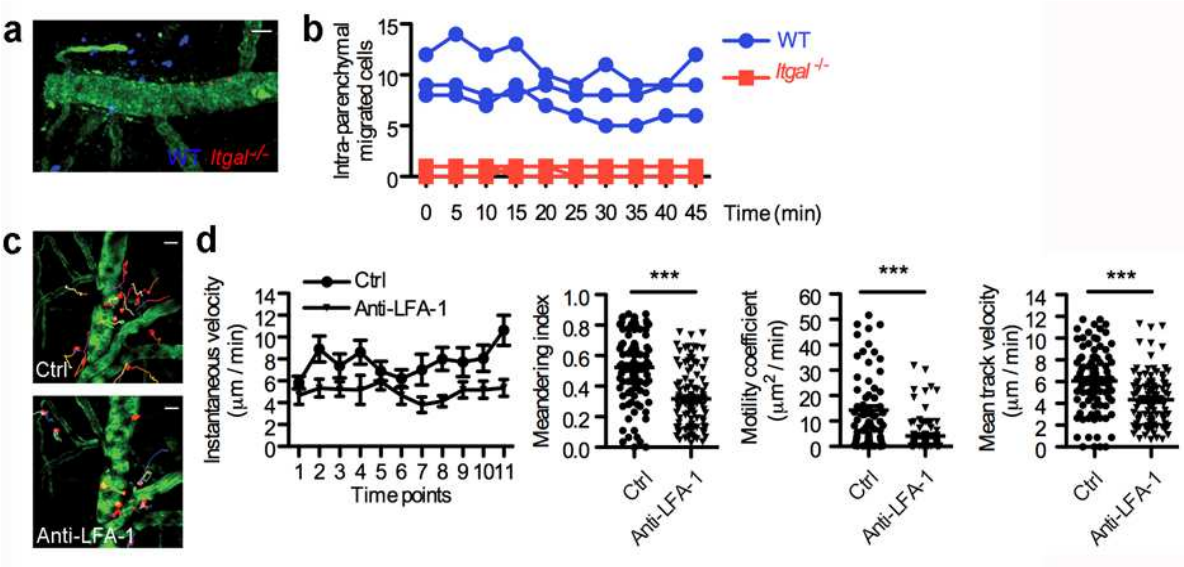

Figure 23. LFA-1 integrin is fundamental for neutrophil infiltration in brain parenchyma.

Blockade of integrin LFA-1 in TPM experiments lead to a diminished entrance and brain parenchyma accumulation, in 4 months old 5xFAD mice. Injection of 1:1 mixture of differentially labeled neutrophils isolated from bone marrow of WT and LFA1-deficient mice (*Itgal*$^{-/-}$). Blood cortical vessels are labeled in green, using 525-nm non-targeted Qdots, injection before image acquisition. (a) WT neutrophils (blue cells) invade brain parenchyma of 5xFAD mice, on the contrary *Itgal*$^{-/-}$ neutrophils (red cells) do not interact with brain vessels and are not able to infiltrate. Image from one representative experiment is shown, scale bar: 50 µm. (b) Quantification of neutrophil numbers with time during image acquisition clearly shows that *Itgal*$^{-/-}$ neutrophils are unable to infiltrate brain parenchyma. Neutrophils isolated from bone marrow of WT control mice, were labeled and injected in 5xFAD mice. 24 hours after cell injection images were acquired. 200 µg of mAb anti-LFA1 were injected into tail vein and images were acquired immediately; (c) images from one representative experiment are shown, scale bar: 30 µm. (d) Analysis of cell migration obtained with IMARIS software shows a significant difference in neutrophil movement after antibody injection, (***P <0.0005).

12.6 LFA-1 BLOCKADE RESCUES BEHAVIORAL IMPAIRMENT IN 3xTg-AD MICE AT EARLY STAGES OF DISEASE

Based on our TPM findings, showing that the *in vivo* blockade of LFA-1-mediated adhesion interferes with the neutrophil migration in the brain parenchyma, we asked whether the mouse treatment with LFA-1 blocking antibody might have benefit on AD pathology. Therefore, we decided to evaluate the effect of the blockade of LFA-1 integrin by treating 3xTg-AD mice with an anti-LFA1–1 integrin-specific mAb and to study the effect of LFA-1 genetic deficiency on AD-like disease. Then, we treated the 3xTg-AD mice with an anti-LFA1–1 antibody starting at 6 months of age. At the first day, the mice were injected i.p. with 500 µg of LFA-1 integrin-specific mAb (TIB213) and the treatment continued with 300

µg of antibody every other day, for 4 weeks. At the end of the treatment mice were left untouched for 4 weeks before performing the behavioral tests. This aspect allows minimizing the stress and fear induced in mice due to the frequent manipulation by experimenter that could eventually influence the results in behavioral tests. Finally, mice were tested for behavioral assessments, as described *Materials and Methods section* and in Fig. 12. Our results showed that the blockade of integrin LFA-1 at early stages of AD-like pathology inhibited the cognitive impairment as shown by the data obtained in the Y-maze (Fig. 24a) and CFC test (Fig. 24b). In fact, the treatment with anti-LFA1–1 antibody at early time-points of disease allowed the restoration of the memory impairment almost to WT situation in control mice (Fig. 24). Collectively, these experiments clearly showed that an intervention, designed to block the neutrophils recruitment in the brain of 3xTg-AD mice, at early time-points of the disease, allowed the recovery of the cognitive impairment and has neuroprotective effects.

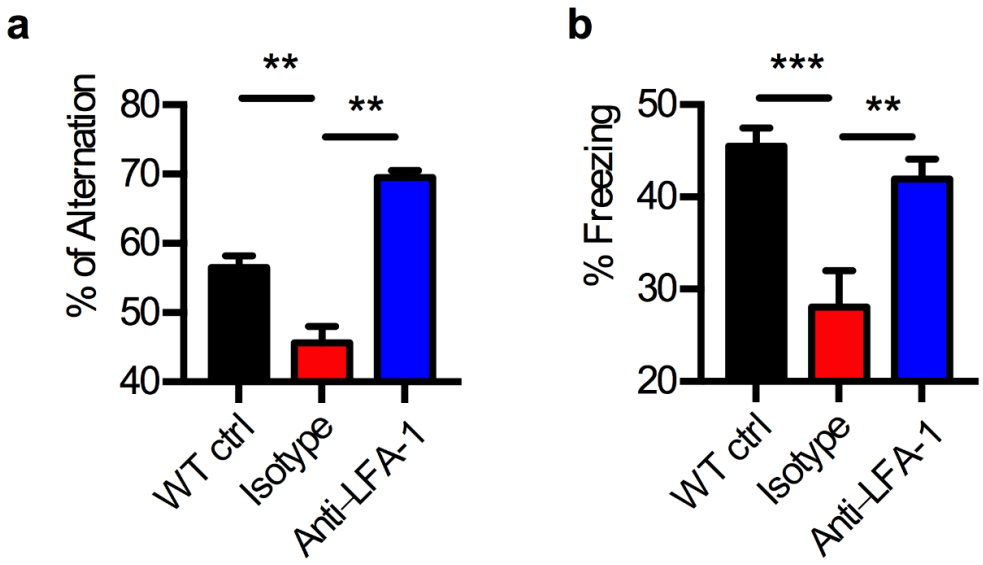

Figure 24. Anti-LFA–1 mAb inhibits cognitive deficits in behavioral tests.
LFA-1 integrin blockade was performed by treating mice with anti-LFA–1 antibody (Anti-LFA-1) for 4 weeks starting at 6 months of age in 3xTg-AD mice, as described *in Materials and Methods* and in Fig. 12. Control treatment was performed with an isotype control antibody (Isotype). WT age-matched control mice were treated with endotoxin-free PBS (WT ctrl). Mice were tested in behavioral paradigms after one month of treatment, to avoid possible variable-like stress, generating by mouse handling. (a) histogram shows the percent alternation performance in the Y-maze test in 3xTg-AD mice treated with anti-LFA–1 or isotype control antibodies. (b) histogram shows freezing response in 3xTg-AD mice treated with anti-LFA1–1 or isotype control antibodies. Values represent mean ± SEM of the data obtained from a representative experiment with 10-12 mice/group. (***P* <0.005; ****P* <0.0005).

12.7 THE LONG-TERM EFFECT OF LFA-1 BLOCKADE ON COGNITIVE FUNCTIONS IN 3xTg-AD MICE

3xTg-AD mice were treated with an anti-LFA–1 antibody at 6 months of age for 4 weeks. Control treatment was performed with an isotype control antibody, anti-RAS antibody. C57BL/6 mice were used as WT control mice. These mice were tested in behavioral paradigms 6 months after treatment termination, at 11-12 months of age, to assess if the restoration of the cognitive function was completely maintained also at later time points of disease. Surprisingly, we found that the temporarily blockade of LFA-1 integrin, during the early stages of the disease (6 months of age) improved the cognitive functions in 3xTg-AD mice at later time-points (11-12 months of age), as shown by the results obtained from the CFC test (Fig. 25).

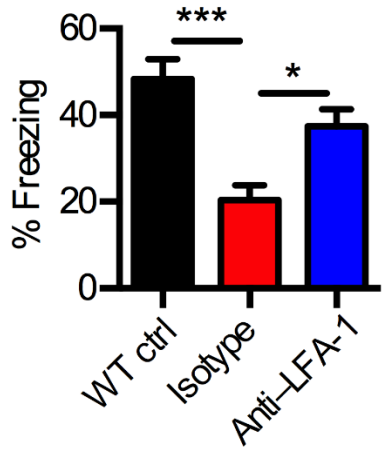

Figure 25. The early LFA-1 integrin blockade has long-term benefits.
LFA-1 integrin blockade was performed by treating mice with anti-LFA–1 antibody (Anti-LFA-1) for 4 weeks starting at 6 months of age in 3xTg-AD mice, as described *in Materials and Methods* and in Fig. 12. Control treatment was performed with an isotype control antibody (Isotype) . WT age-matched control mice were treated with endotoxin-free PBS (WT ctrl). Mice were tested in behavioral paradigms 6 months after treatment termination, at 11-12 months of age. The histogram shows freezing response in 3xTg-AD mice treated with anti-LFA1–1 or isotype control antibodies. Values represent mean ± SEM of the data obtained from a representative experiment with 10-12 mice/group. (**P* <0.05; ****P* <0.0005).

12.8 THE BLOCKADE OF LFA-1 INTEGRIN REDUCES MICROGLIAL ACTIVATION

Furthermore, significant reduction in the density of microglia Iba-1 positive cells, was detected in the CA1 and DG area of hippocampus in LFA-1–treated compared with isotype

control treated mice (Fig. 26a-b). Microglia cells showed different morphologies, indicating that modifications occurred in their activation state. Indeed, isotype control treated mice showed microglia with larger cell soma, suggesting an activated phenotype. Also, retraction and thickening of the processes is typical of highly activated microglia while, most of microglial cells in LFA-1–treated mice showed a small roundish soma and long processes, corresponding to a non-activated or intermediately activated cells (Figure 26c-d). Taken together, these results demonstrated a role for neutrophils in inflammatory brain activation and behavioral impairments in AD-like disease, suggesting that inhibition of neutrophil trafficking may represent a new therapeutic approach in AD.

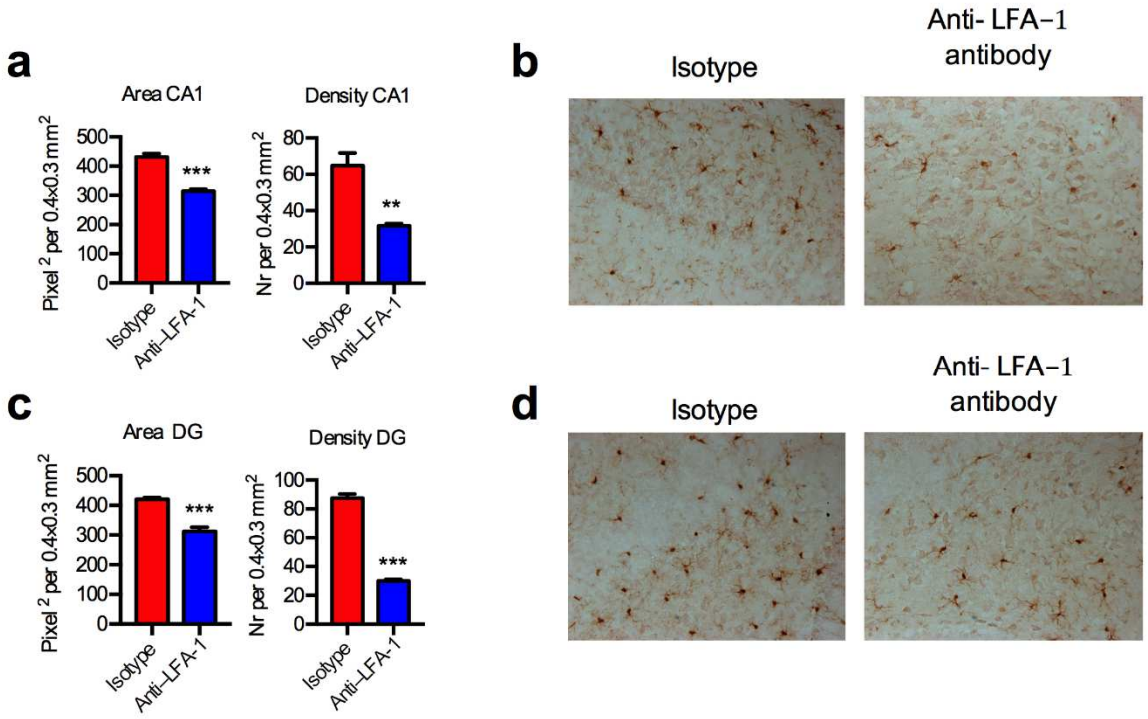

Figure 26. Blockade of LFA-1 integrin affects microglial cell activation in 3xTg-AD mice.

At the end of behavioral tests, mice were sacrificed for neuropathological analyses as described *in Materials and Methods* and Figure 12. Bar graph showing the density (nr/0.4x0.3mm^2) and area (Pixel^2 /0.4x0.3mm^2) of microglia cells in (a-b) the CA1 and (c-d) in DG of isotype control-treated (Isotype) compared to anti-LFA–1–treated 3xTg-AD mice (Anti-LFA-1). Bar is expressed as mean ± SEM (**P <0.005; ***P <0.0005). Representative images of Iba-1 microglia in (b) CA1 and (d) in DG: isotype control-treated 3xTg-AD mice show highly activated amoeboid cell morphology with large bodies and several cellular processes, 3xTg-AD mice treated with anti-LFA-1 antibody show a less activated microglia phenotype. Scale bars, 10 μm in c,d left panels. Higher magnifications are shown in the right panels; scale bars, 25 μm.

12.9 GENERATION OF 3xTg-AD x *Itgal*$^{-/-}$ MOUSE MODEL OF AD

In the present project, we also generated a 3xTg-AD x *Itgal*$^{-/-}$ AD animal model to investigate the effect of the LFA-1 genetic deficiency on the development of AD-like disease in 3xTg-AD mice. To this aim, we have crossed 3xTg-AD and *Itgal*$^{-/-}$ mice. The colony of homozygous 3xTg-AD x *Itgal*$^{-/-}$ was produced in two steps. In the first step, we obtained hemizygous 3xTg-AD x *Itgal*$^{-/-}$ mice. In the second step, we cross-breaded heterozygous 3xTg-AD x *Itgal*$^{-/-}$ to obtain homozygous mice for all transgenes. DNA was extracted from mouse's tail and we next performed PCR to evaluate the insertion of APP and PSEN1 and the deletion of LFA-1 transgenes as described in the *Materials and Methods section*. The insertion of APP transgene was evaluated by conventional PCR using primers (forward and reverse) annealing inside the APP transgene sequence. The deletion of LFA-1 gene was obtained using a targeting vector containing neomycin resistance gene used to disrupt the region containing exons 1 and 2 of LFA-1 gene. Therefore, we performed two conventional PCR: one was aimed at amplifying the WT LFA-1 gene and the other was aimed at amplifying the neomycin resistance gene. In homozygous WT control mice, we amplified only the WT LFA-1 gene, whereas in homozygous mutant mice we amplified only the construct. In hemizygous mice were present WT and mutant sequence of the LFA-1$^{-/-}$ transgene. We amplified a sequence outside the PSEN1 transgene insertion site (Fig. 27a). The sequence of PSEN1 transgene contains a specific cleavage site for BstE II restriction enzyme (Fig. 27b). Therefore, we performed an enzymatic digestion on PCR product to reveal the insertion of the PSEN1 transgene as described in the *Materials and Methods*. This digestion allowed us to identify not only the insertion of PSEN1 transgene, but also if the PSEN1 transgene is in the homozygous or heterozygous condition. The homozygous condition represents the incorporation of PSEN1 transgene in both alleles. The cleavage of BstE II produces respectively two fragments of 350 bp and 180 bp (Fig. 27b). We obtained only a band of 530 bp because the site of cleavage (within PSEN1 transgene) is not present and BstEII does not cut it (Fig 27c). Three bands were normally obtained in hemizygous situation: one of 530 bp corresponding to the WT allele and two bands of 350 bp and 180 bp respectively, deriving from the 3xTg-AD homozygote allele in which PSEN1 transgene PCR product is enzymatically cleaved (Fig. 27c). PCR products and restriction enzyme digestions were visualized by Nancy after run in 1% agarose gel as described in *Materials and Methods* (Fig. 28). We used C57BL/6J mice as negative control, as they do not carry the

transgenes (data not shown). 3xTg-AD and *Itgal*$^{-/-}$ mice were used as positive controls (Fig. 28).

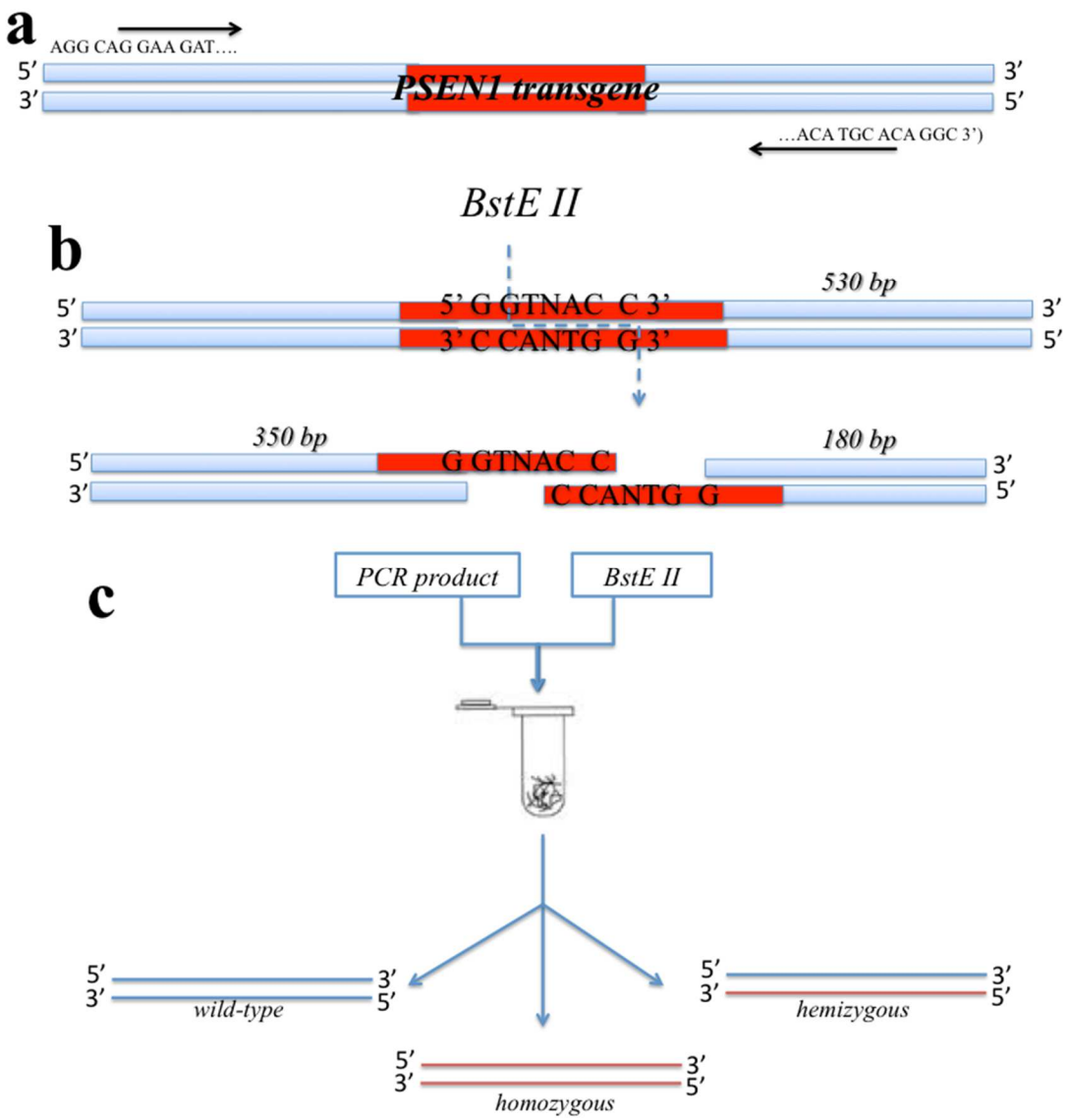

Figure 27. Schematic representation of sequence of PSEN1 transgene and enzymatic digestion procedure.
(a) figure a represents the amplified sequence with the PSEN1 transgene inserted within; (b) figure b represents the cleavage site of the restriction enzyme (BstE II) with two fragments generated of 350 bp and 180 bp respectively; (c) figure c represents the three possible results from enzymatic digestion of PCR product. The WT condition (left) has two sequences without the insertion of the PSEN1 transgene (line blue). In the homozygous condition (central) both sequences contain the PSEN1 transgene (line red). In the hemizygous condition (right) one sequence contains the insertion of the PSEN1 transgene (line red) whereas the other does not (line blue).

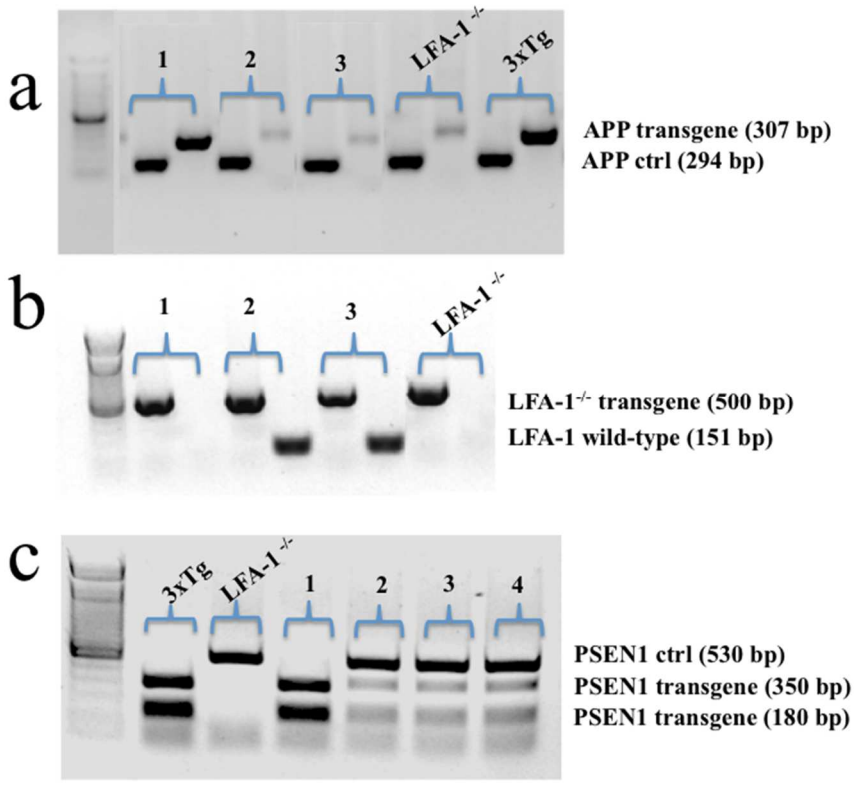

Figure 28. Agarose gel electrophoresis of amplified PCR fragments.
Representative images of agarose gels show PCR products of (a) APP and (b) *Itgal*$^{-/-}$ transgenes. (c) Figure in c shows the restriction reaction of PSEN1 PCR products. First line in all images designates marker. (a) samples 2 and 3 produce only the band of 294 bp (APP ctrl); sample 1 produces both bands of 307 bp (APP) and 294 bp (APP ctrl), therefore it is assessed as positive for APP transgene insertion. (b) sample 1 produces only the band of 500 bp and it is assessed as homozygous for *Itgal*$^{-/-}$; samples 2 and 3 produce both bands of 500 bp and 151 bp and they are assessed as hemizygous for *Itgal*$^{-/-}$ mice. (c) sample 1 produces both bands of 350 bp and 180 bp and it is assessed as homozygous for PSEN1 transgene; samples 2, 3 and 4 produce all three bands of 500 bp, 350bp and 151 bp and they are assessed as hemizygous for PSEN1 transgene.

12.10 LFA-1 DEFICIENCY IN 3xTg-AD MICE RESTORES COGNITIVE FUNCTIONS AT EARLY STAGES OF DISEASE

Billings L.M., et al. previously reported that the onset of the disturbances in 3xTg-AD mice start at 6-9 months of age and correlates with the accumulation of intraneuronal Aβ in the cortex and hippocampus. Aβ plaques and hyper-phosphorylated tau are not apparent at this age, suggesting that they contribute to cognitive dysfunction at later time points (Billings L.M., et al., 2005). Initially, we assessed 3xTg-AD*x*Itgal$^{-/-}$, 3xTg-AD and WT age-matched control mice in behavioral paradigms at 9 months of age (Fig. 29). As expected, in Y-maze (Fig. 29a) and CFC (Fig. 29b) tests, 3xTg-AD*x*Itgal$^{-/-}$ did not perform significantly worse when compared with WT age-matched control mice. As expected, a significantly decreased

alternation in Y-maze task was detectable in 3xTg-AD mice compared with WT age-matched control mice. Interestingly, 3xTg-ADx*Itgal*$^{-/-}$ mice did not show cognitive impairment and mice performed at comparable levels of WT age-matched control mice (Fig. 29a). In CFC test, WT age-matched control mice exhibited a robust freezing in response to the sound tone (Fig. 29b). In contrast, 3xTg-AD mice were significantly impaired. In fact, they were no longer able to associate the sound to the electric foot shock they received during the conditioning phase. Interestingly, the genetic deletion of the integrin LFA-1 in 3xTg-AD mice rescued memory impairment, indeed 3xTg-ADx*Itgal*$^{-/-}$ mice spend significantly more time freezing than age-matched 3xTg-AD littermates (Fig. 29b).

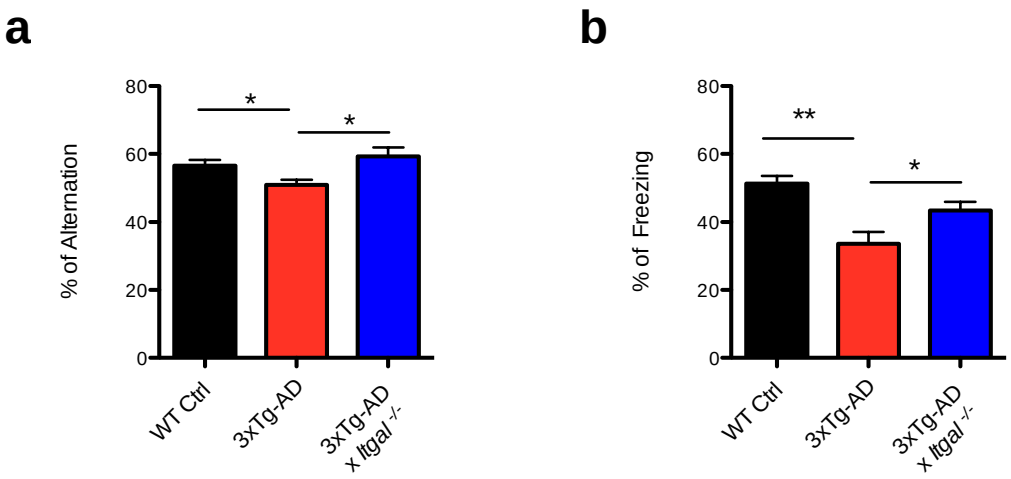

Figure 29. LFA-1 deficiency in 3xTg-AD mice induces normal cognitive performance in behavioral tests.
3xTg-AD, 3xTg-ADx*Itgal*$^{-/-}$ and WT control mice were tested in the Y-maze and CFC tests at 9 months of age. (a) histogram shows the of spontaneous alternation performance in the Y-maze test; (b) histogram shows a comparable freezing response between 3xTg-ADx*Itgal*$^{-/-}$ and WT age-matched control mice compared to 3xTg-AD mice. Data derived from one representative experiment with 8-12 mice per condition. Values represent mean ± SEM in each group (*P < 0.05; **P < 0.005).

12.11 LFA-1 DEFICIENCY IN 3xTg-AD MICE RESTORES COGNITION AT LATE STAGES OF AD

Next we explored the cognitive performance of 3xTg-ADx*Itgal*$^{-/-}$ mice at 12 months of age, to verify whether the amelioration in cognitive impairment obtained at 9 months of age was also maintained at later time-points. As expected, 3xTg-ADx*Itgal*$^{-/-}$ mice performed at comparable levels than WT control mice at 12 months of age in Y-maze (Fig. 30a) and CFC (Fig. 30b) tests. These experiments clearly confirmed the beneficial effect of LFA-1 integrin ablation on behavioral impairment on AD-like pathology. Therefore, we speculate that the

inhibition of neutrophil trafficking through the ablation of LFA-1 integrin was contributing to the cognitive function re-established in 3xTg-ADx*Itgal*$^{-/-}$ mice.

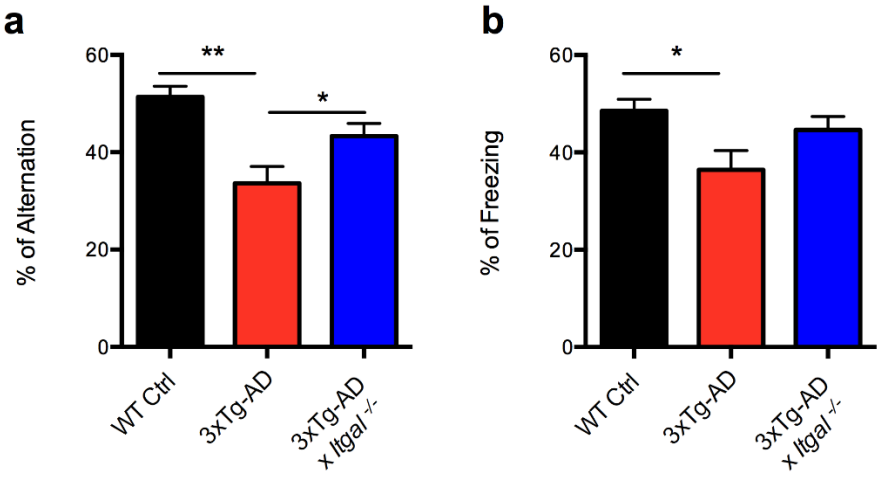

Figure 30. LFA-1 deficiency in 3xTg-AD mice has long-term beneficial effects in behavioral tests.
3xTg-AD, 3xTg-ADx*Itgal*$^{-/-}$ and WT age-matched control mice were tested in the Y-maze and CFC tests at 12 months of age. (a) histogram shows the of spontaneous alternation performance in the Y-maze test; (b) histogram shows a comparable freezing response between 3xTg-ADx*Itgal*$^{-/-}$ and WT age-matched control mice compared to 3xTg-AD mice. Data derived from one representative experiment with 8-12 mice per condition. Values represent mean ± SEM in each group (*P < 0.05; **P < 0.005).

12.12 EFFECT OF LFA-1 DEFICIENCY ON NEUROPATHOLOGICAL CHANGES IN 3xTg-AD MICE

In the present study, we quantified through immunohistochemistry analysis the presence of Aβ deposition, tau hyper-phosphorylation and microglial activation in different brain areas such as hippocampus and cortex of 3xTg-ADx*Itgal*$^{-/-}$ mice compared to age and sex-matched 3xTg-AD mice. C57BL/6 mice were used as control mice. Mice were sacrificed after behavioral assessment at 9, 12 and 20 months of age, and histological analysis was performed as described in *Materials and Methods*.

12.12.1 Aβ DEPOSITION

At 12-13 months of age only the intracellular presence of Aβ deposits were evident in 3xTg-AD mice, while extracellular deposition has not been detected, confirming a study reporting that the extracellular deposition begins to be apparent at ages later than 15 months (Mastrangelo et al., 2008). We documented a reduction of Aβ deposition in 3xTg-ADx*Itgal*$^{-}$

$^{/-}$ mice compared to 3xTg-AD mice in the cortical area (Fig. 31). The Aβ deposition highlighted by 6E10 staining in the cortex did not appreciable in 3xTg-ADx*Itgal*$^{-/-}$ mice, whereas age-matched 3xTg-AD mice showed a marked intracellular expression of Aβ in the same examined areas (Fig. 31). We performed a quantitative stereological analysis to determine the total area occupied by Aβ-positive neurons (pixel^2/total examined area). As it can be appreciated from the graphs, 3xTg-ADx*Itgal*$^{-/-}$ mice showed a reduction of Aβ deposition in cortical area (Fig. 31a). In fact, the values of both density and area occupied by Aβ-positive neurons reached a significant difference when compared to age and sex-matched 3xTg-AD mice. These data confirmed our hypothesis and agree with data previously obtained in 3xTg-AD mice treated with LFA-1 blocking antibody, showing a decreased deposition of Aβ in cortical neurons. The absence of the integrin LFA-1 in these mice might prevented neutrophil infiltration into the brain thus reversing Aβ deposition.

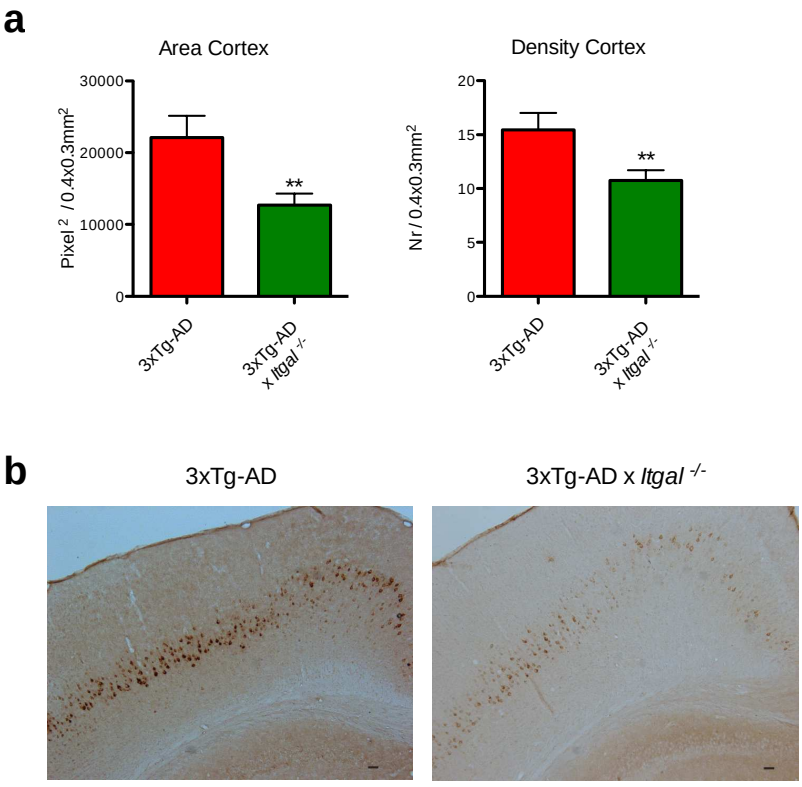

Figure 31. LFA-1 deficiency in 3xTg-AD ameliorates Aβ pathology.
(a) Unbiased quantitative analysis was performed in the cortex to quantify the area (left panel) and the density (right panel) of Aβ containing cells. Bar is expressed as mean ± SEM (**P <0.005). (b) Representative images of Aβ load in the cortex of (left) 3xTg-AD and (right) 3xTg-ADx*Itgal*$^{-/-}$. Scale bar: 50 μm.

12.12.2 TAU PROTEIN EXPRESSION

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 (Mastrangelo et al., 2008). Oddo S., et al. have previously shown that numerous phospho-tau epitopes are immunohistochemically detectable in the hippocampus and cerebral cortex of 3xTg-AD mice (Oddo S., et al., 2003). To determine the effect of LFA-1 ablation on tau pathology we performed immunohistochemical staining for tau protein in 3xTg-AD mice compared to 3xTg-ADx*Itgal*$^{-/-}$ mice.

We used the HT7 antibody to detect total tau expression. We did not detect any significant difference in total tau level in examined hippocampus (CA1 and DG) and cortical areas in 3xTg-ADx*Itgal*$^{-/-}$ compared to 3xTg-AD mice at 12 months of age (Fig. 32).

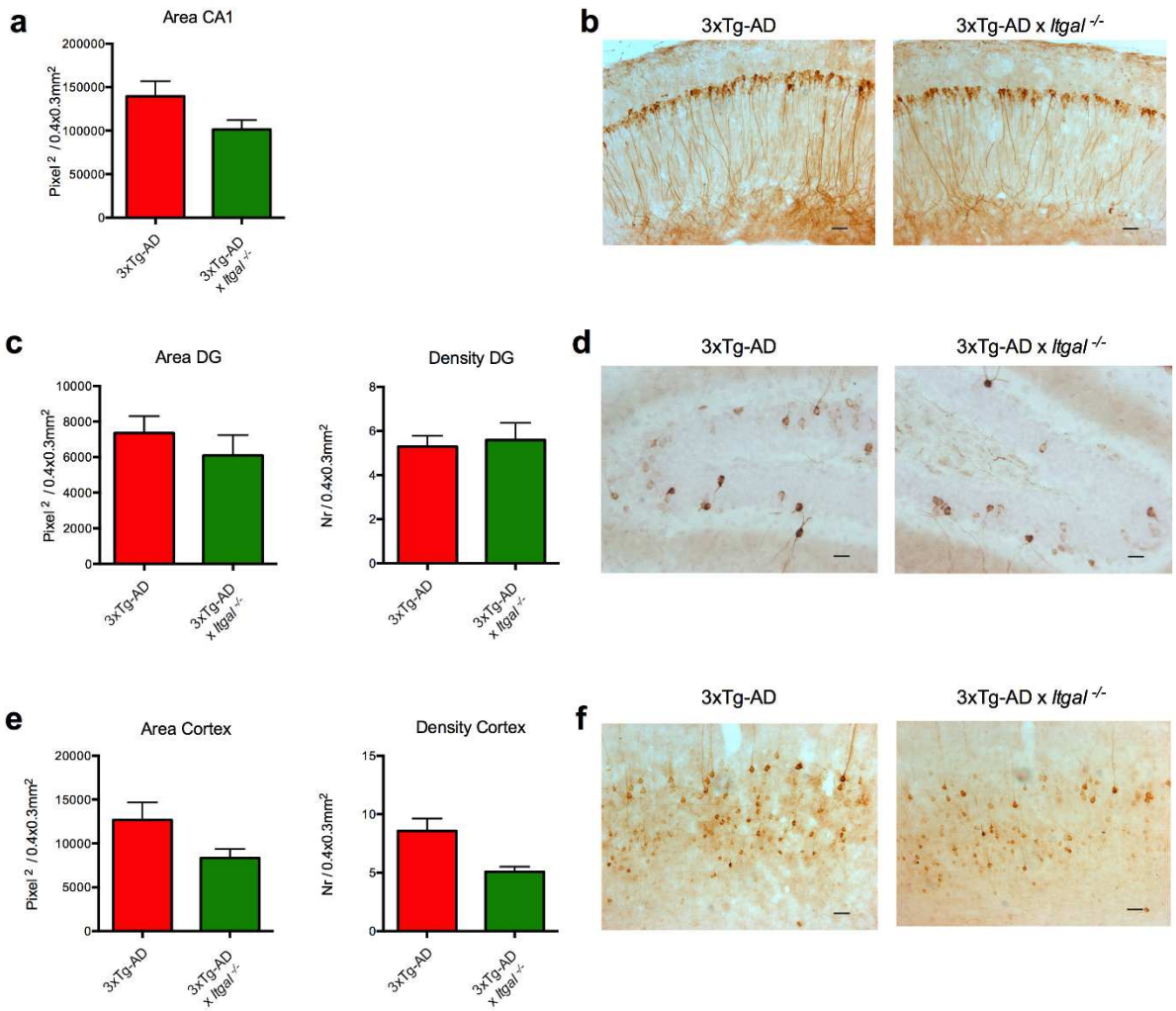

Figure 32. LFA-1 deficiency in 3xTg-AD mice does not affect total tau expression at 12

months of age.
Human tau detection was performed with HT7 antibody. Unbiased quantitative analysis was carried out on the brains of 3xTg-AD and 3xTg-AD*x**Itgal*$^{-/-}$ mice to quantify the area occupied in the (a) CA1 (c, left) DG and (e, left) cortex and the density in the (c, right) DG and (e, right) cortex of human tau positive cells. Bar is expressed as mean ± SEM. Immunohistochemical staining of total tau in the (a) CA1 (d) DG and (f) cortex are illustrated at 12 months of age. Scale bar: 50 μm.

The comparable levels of expression of total tau in 3xTg-AD*x**Itgal*$^{-/-}$ mice allowed us to further evaluate the expression of phospho-tau epitopes. Thus, we performed the immunohistochemical staining for AT180 and AT8 phospho-tau epitope in 3xTg-AD and 3xTg-AD*x**Itgal*$^{-/-}$ mice at 12 and 20 months of age, respectively.
We used AT180, an antibody which binds the protein tau at residue Thr231 and AT8, an antibody which binds the protein tau at residue Ser202/Thr205. 3xTg-AD mice showed tau hyperphosphorylation in the CA1 region of hippocampus, whereas 3xTg-AD*x**Itgal*$^{-/-}$ mice showed a significant reduction of tau phosphorylation in the same brain area at 12 months of age (Fig. 33a-b). The phosphorylation of tau was only detectable in the CA1 region of the hippocampus at 11-12 months of age in 3xTg-AD mice with the AT180 antibody, therefore we could not perform any evaluation of tau phosphorylation in cortical regions. In addition, the AT8 positivity was detectable at 20 months of age in hippocampus CA1 field in 3xTg-AD mice compared 3xTg-AD*x**Itgal*$^{-/-}$ mice (Fig. 33c-d). Oddo S., et al. reported that AT8 immunoreactivity could be observed at approximately 12 months of age (Oddo S., et al., 2003). However, we were not able to detect AT8 expression in 3xTg-AD*x**Itgal*$^{-/-}$ and 3xTg-AD mice until 20 months of age. These observations agree with what was reported by others showing that AT180-positive cells were not detectable until 26 months in cortical regions of 3xTg-AD mice (Mastrangelo et al., 2008).
These data clearly demonstrated a decreased phosphorylation of the protein tau in 3xTg-AD*x**Itgal*$^{-/-}$ mice when compared with 3xTg-AD mice, probably leading to a reduced tangle formation.

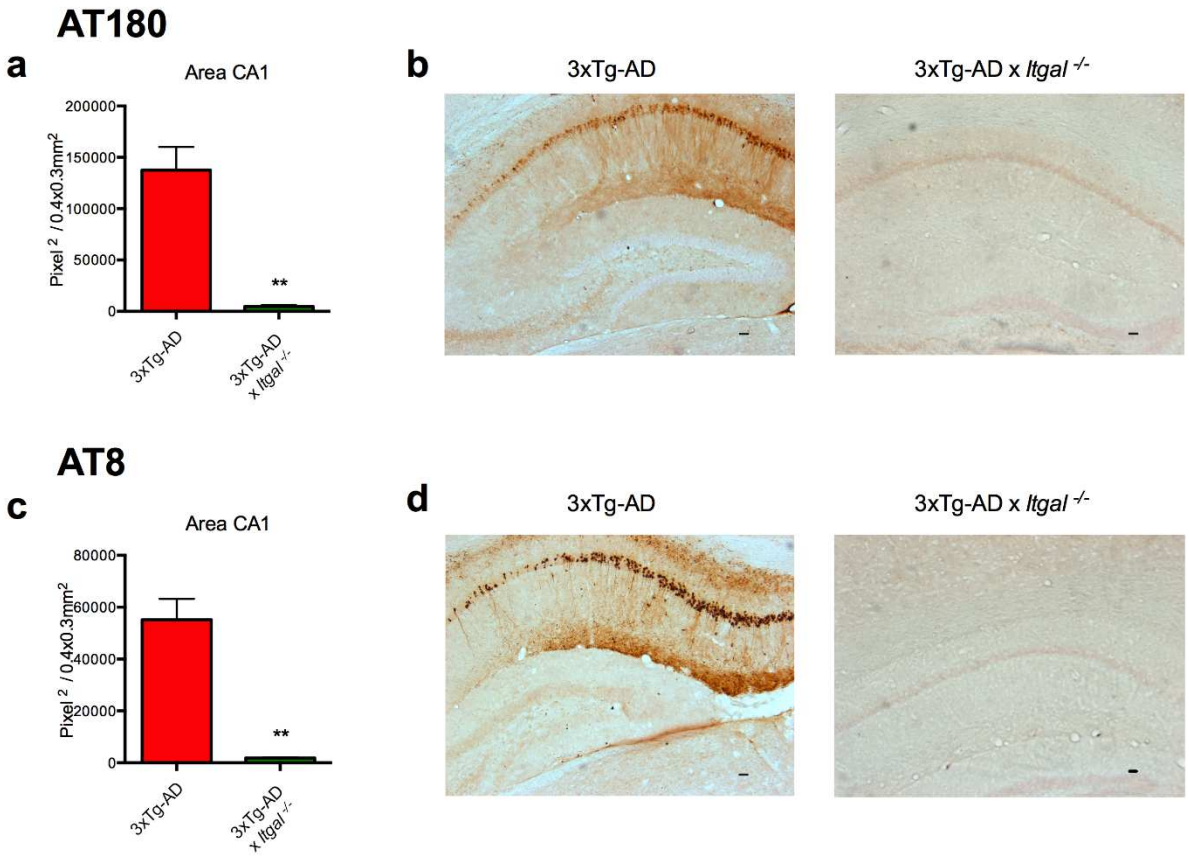

Figure 33. LFA-1 deficiency in 3xTg-AD induces a decreased phosphorylation of tau at 20 months of age.
(a) Bar graphs showing the area of AT180 positive cells in the CA1 of 3xTg-AD and 3xTg-AD*x Itgal*$^{-/-}$ mice. (b) Representative images clearly show a lack of AT180 expression in the 3xTg-AD*x Itgal*$^{-/-}$ mice compared to 3xTg-AD mice in the CA1 area of hippocampus at 12 months of age. (c) Bar graphs showing the area of AT8 positive cells in the CA1 area of hippocampus of 3xTg-AD and 3xTg-AD*x Itgal*$^{-/-}$. (d) Representative images clearly show a lack of AT8 expression in the 3xTg-AD*x Itgal*$^{-/-}$ mice compared to 3xTg-AD mice at 20 months of age. Bar is expressed as mean ± SEM (**P <0.005). Scale bar: 50 μm.

12.12.3 MICROGLIA ACTIVATION

Microglial cells play an important role in AD pathology as they appear to be activated in the brain of both AD patients and AD-like disease mice (El Khoury et al., 2007; Hickman S.E., et al., 2008; Krabbe G., et al., 2013; Zenaro E., et al., 2015). Therefore, we assessed their activation status in our 3xTg-AD*x Itgal*$^{-/-}$ mice compared to age and sex-matched 3xTg-AD mice. We revealed the microglia phenotype by staining the cells with an antibody against the protein Iba-1, a specific marker expressed both on resident as well as monocyte-derived microglia. Stainings were performed as described in the *Materials and Methods*. Our data showed that neutrophil depletion or the inhibition of neutrophil trafficking by blocking LFA-1 dramatically reduces microglial activation, suggesting that neutrophils play a role in

the microglial pathophysiology during AD (Zenaro E., et al., 2015). In the present study, we found a significant reduction in the area occupied by microglial cells in hippocampus CA1 field and DG in 3xTg-ADx*Itgal*$^{-/-}$ compared with 3xTg-AD mice at 12 months of age (Fig. 34). Of note, 3xTg-ADx*Itgal*$^{-/-}$ mice showed many resting microglial cells with a non-activated phenotype compared to activated microglia with enlarged cell bodies and thick processes present in 3xTg-AD mice of the same age. Indeed, 3xTg-AD mice showed larger microglia cells soma and retraction and thickening of processes typical of highly activated microglia, on the contrary most the microglial cells in 3xTg-ADx*Itgal*$^{-/-}$ mice showed a small roundish soma and long processes, corresponding to non-activated cells. The morphological differences clearly indicate changes in the activation state of microglia in the two experimental conditions. Interestingly, microglia phenotype in 3xTg-ADx*Itgal*$^{-/-}$ mice closely resembled the one of age-matched control mice (data not shown). Therefore, LFA-1 ablation in 3xTg-AD mice prevented the interconnectivity between neutrophils and microglial cells in the brain by reducing several feedback loops between these two phagocytes that amplify and sustain their activation.

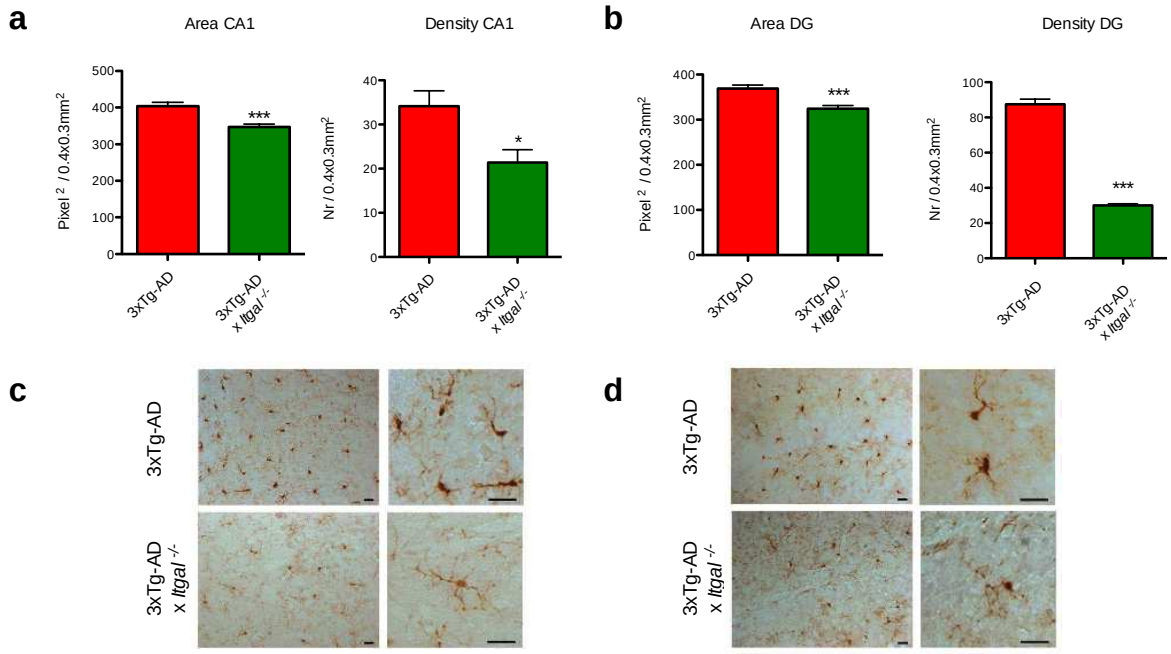

Figure 34. Microglial cell density, area and morphology diversity in 3xTg-ADx*Itgal*$^{-/-}$ and 3xTg-AD at 12 months of age.
Quantitative analysis was carried out on the brains of 3xTg-AD and 3xTg-ADx*Itgal*$^{-/-}$ mice to quantify microglia density and area occupied by cell soma in (a) CA1 and (b) DG area of ippocampus. Bar is expressed as mean ± SEM (*P <0.05; ***P <0.0005). Representative images of Iba-1 microglia (c) in CA1 and (d) in DG: 3xTg-AD mice show highly activated cell morphology with large bodies and several cellular processes, whereas 3xTg-AD mice

lacking the integrin LFA-1 show a less activated microglia phenotype. Scale bars, 10 μm in c and d panels. Higher magnifications are shown in the right panels; scale bars, 25 μm.

Overall our results demonstrated a role of LFA-1 integrin in neutrophil trafficking in the induction of behavioral impairment, Aβ deposition, tau phosphorylation and inflammatory brain activation in 3xTg-AD mice, suggesting that inhibition of LFA-1 integrin function may represent a new therapeutic approach in AD.

12.13 EFFECT OF ALPHA-4 INTEGRINS BLOCKADE IN 3xTg-AD MICE

Anti-α4 integrins antibodies have been used to block the integrin-mediated leukocytes migration to inflammatory sites and the homing to lymphoid tissues (Issekutz T.B. & Wykretowicz A., 1991; Issekutz and Wykretowicz, 1991; Johnston B., et al. 1996; Martin-Blondel G., et al., 2015). The interference with leukocyte adhesion by anti-α4 integrins antibodies showed the decreasing rolling interactions and arrest of neutrophils after status epilepticus in mouse brain (Fabene P.F., et al., 2008) and the inhibition of endothelial interaction and brain entry of neutrophils in animal models (Fleming J.C., et al., 2009; Kadioglu A., et al., 2011; Neumann J., et al., 2015). The use of anti-α integrins antibodies proved that this strategy is robust for the treatment of inflammatory pathologies through the blockade of leukocyte recruitment. Based on these evidences, we decided to evaluated the effect of α4-integrins antibody blockade on cognitive functions, Aβ deposition, tau phosphorylation and microglial activation in 3xTg-AD mice.

12.13.1 THE α4-INTEGRINS BLOCKADE RESCUES BEHAVIORAL IMPAIRMENT IN 3xTg-AD MICE AT EARLY STAGES OF DISEASE

We treated 3xTg-AD mice with an anti-α4–integrins antibody starting at 6 months of age. At day one, the mice were injected i.p. with 500 μg of α4–integrins-specific mAb (PS/2) and treatment continued with 300 μg of antibody every other day for 4 weeks. At the end of treatment, the mice were tested for behavioral assessment as described in *Materials and Methods* and in Fig. 12. Our results showed that the blockade of α4–integrins at early stages of AD-like pathology inhibits the cognitive impairment as shown by the data obtained in the Y-maze (Fig. 35a) and CFC (Fig. 35b) tests. In fact, the treatment with anti-α4–integrins antibody at early time-points of disease allowed the restoration of the memory impairment almost to WT situation in control mice (Fig. 35). Collectively, these experiments clearly

showed that an intervention aimed to block the leukocyte recruitment in the brain of 3xTg-AD mice at early time-points of disease allowed rescuing the cognitive impairment and have neuroprotective effects.

We hypothesized that the therapeutic efficacy of this treatment could be a consequence of the blockade of VLA-4/VCAM-1-mediated extravasation of neutrophils into the AD brain. For instance, VLA-4 integrin is expressed by activated lymphocytes that may also use VLA-4/VCAM-1 interactions to adhere in brain venues and subsequently migrate into the brain (Becker et al., 2001; Martin-Blondel G., et al., 2015). Therefore, we cannot exclude that the effects of the anti-α4 integrins treatment is due to the inhibition of the T cells infiltration.

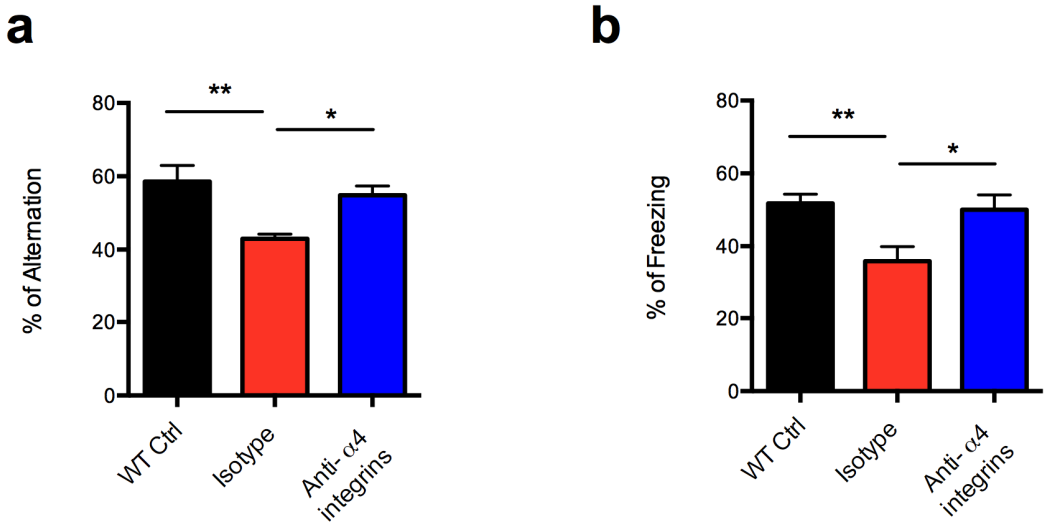

Figure 35. Anti-α4–integrins mAb inhibits cognitive deficits in behavioral tests.
α4–integrins blockade was performed by treating 3xTg-AD mice with anti-α4–integrins antibody (Anti-α4 integrins) for 4 weeks starting at 6 months of age in 3xTg-AD mice, as described *in Materials and Methods* and in Fig.12. As control, 3xTg-AD mice were treated with an isotype control antibody (Isotype). WT age-matched control mice were treated with endotoxin-free PBS (WT ctrl). (a) histogram shows the percent alternation performance in the Y-maze test. (b) histogram shows freezing response of mice in CFC test. Values represent mean ± SEM of the data obtained from a representative experiment with 10-12 mice/group. (*P <0.05; **P <0.005).

12.13.2 THE BLOCKADE OF α4-INTEGRINS HAS LONG-TERM BENEFICIAL EFFECTS

To evaluated the long-term effect of α4–integrins blockade, 3xTg-AD mice were treated with an anti-α4–integrins antibody at 6 months of age for 4 weeks and behaviorally test in CFC test 6 months after treatment termination (at 11-12 months of age) as described in *Materials and Methods* and in Fig. 12. Notably, the restoration of cognitive function in 3xTg-AD mice, treated during early stages of disease, was maintained also in aged animals.

This result suggested that the therapeutic blockade of the leukocyte adhesion during the early stages of the disease provides a long-term beneficial effect on cognition in older mice (Fig. 36).

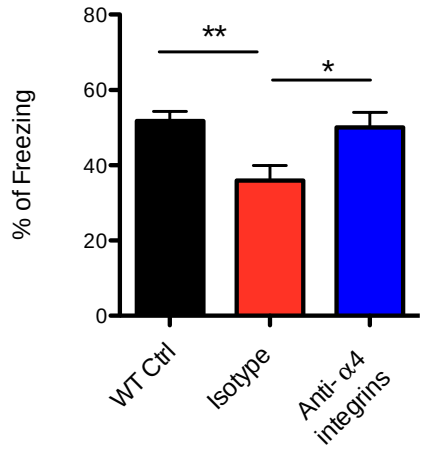

Figure 36. The early α4-integrins blockade has long-term benefits.
α4–integrins blockade were performed by treating mice with anti-α4–integrins antibody (Anti-α4–integrins) for 4 weeks starting at 6 months of age in 3xTg-AD mice, as described *in Materials and Methods* and in Fig.12. Control treatment was performed with an isotype control antibody (Isotype). WT age-matched control mice were treated with endotoxin-free PBS (WT ctrl). Mice were tested in behavioral paradigms 6 months after treatment termination, at 11-12 months of age. Histogram shows the percent alternation performance in the CFC test in 3xTg-AD mice treated with anti-α4–integrins or isotype control antibodies. Values represent mean ± SEM of the data obtained from a representative experiment with 10-12 mice/group. (*P <0.05; **P <0.005).

12.13.3 THE BLOCKADE OF α4-INTEGRINS INDUCES NEUROPATHOLOGICAL CHANGES

We next investigated the effect of α4-integrins blockade by immunohistochemical evaluation of brain samples of 3xTg-AD mice treated with anti-α4 integrins or isotype control antibodies at early stages of disease. The immunohistological analysis of different brain areas allowed 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. We observed a reduction of Aβ loading in 3xTg-AD mice treated with an anti-α4 integrins antibody in cortex, amygdala and subiculum at 12-13 months of age compared to 3xTg-AD mice treated with isotype control antibody (Fig. 37). In addition, the expression of total tau protein was investigated by immunohistochemistry staining with HT7 antibody in 3xTg-AD mice treated with isotype control antibody and anti-

α4 integrin antibody at 12-13 months of age. We did not detect any difference in total tau protein expression in CA1 region of hippocampus (Fig. 38a-b). However, we found a reduction of AT180 tau expression in CA1 region of hippocampus in anti-α4 integrins antibody treated 3xTg-AD mice compared to 3xTg-AD mice treated with isotype control antibody (Fig. 38c-d). Also, we observed a lower density and activation state of microglia in the cortex of 3xTg-AD mice, treated at early stages of disease with anti-α4 integrins antibody, compared to animals treated with an isotype control antibody, suggesting α4 blockade reduces neuroinflammation (Fig. 39). The area occupied by microglia cell soma was reduced, indicating a resting state of microglia cells in 3xTg-AD mice treated with an anti-α4 integrins antibody compared to mice treated with an isotype control antibody as shown in Fig. 39.

Taken together, these results showed that the anti-α4 integrin antibody treatment could inhibit early pathogenesis and progression of AD in 3xTg-AD mice. Thus, we speculated that VLA-4 integrin may represent a novel attractive therapeutic target in AD able to interfere with leukocyte subpopulations that contribute to disease pathogenesis.

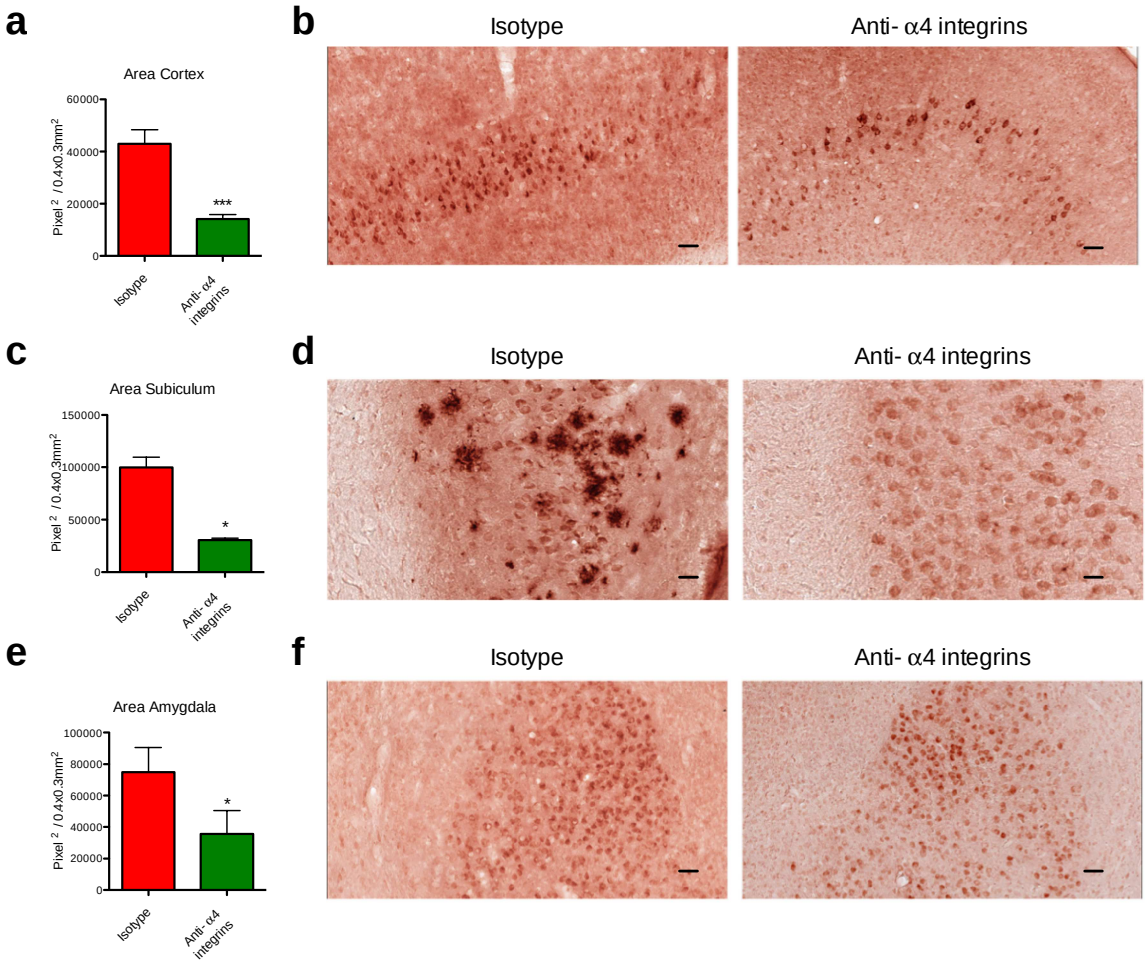

Figure 37. Anti-α4–integrins mAb treatment ameliorates Aβ pathology.
At the end of behavioral tests, mice were sacrificed for neuropathological analyses as described *in Materials and Methods* and in Figure 12. Quantitative analysis was carried out on the brains of 3xTg-AD mice treated with an α4–integrins antibody (Anti-α4–integrins) compared to 3xTg-AD mice treated with isotype control antibody (Isotype) to quantify the area of Aβ containing cells in (a) the cortex, (c) the subiculum and (e) the amygdala. Bar is expressed as mean ± SEM (*P <0.05; ***P <0.0005). Representative images show a reduction of Aβ deposition in (b) the cortex, (d) the subiculum and (f) amygdala of 3xTg-AD mice treated with an α4–integrins antibody compared to 3xTg-AD mice treated with isotype control antibody at 12-13 months of age. Scale bar: 50 μm.

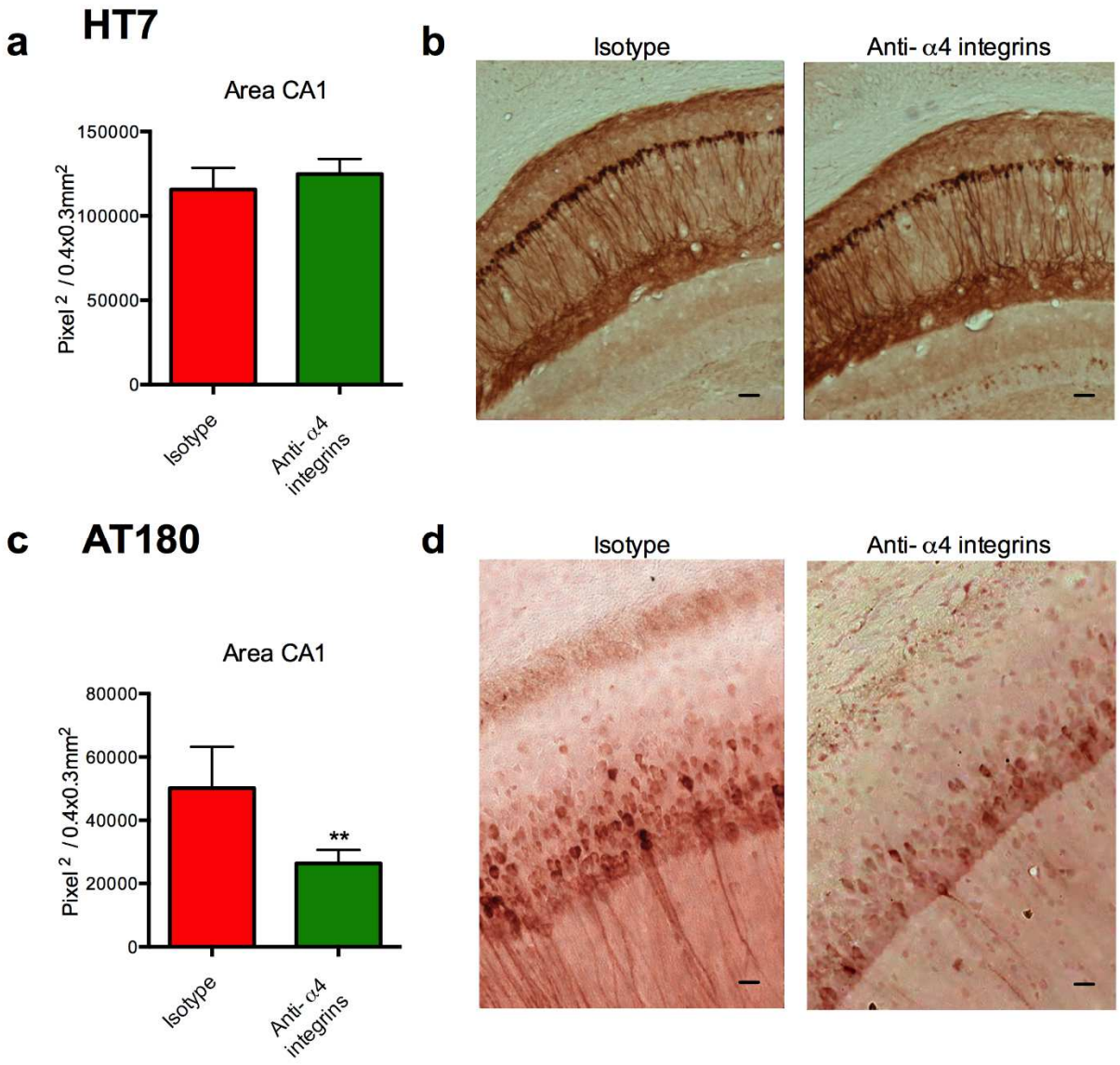

Figure 38. Anti-α4–integrin treatment affects tau phosphorylation.
At the end of behavioral tests, mice were sacrificed for neuropathological analyses as described *in Materials and Methods* and in Figure 12. Human tau detection was performed with HT7 antibody. (a-b) Anti-α4–integrins treatment does not affect total tau expression. (a) Unbiased quantitative analysis was performed in the CA1 to quantify the area of total tau protein. (b) Representative images show comparable levels of total tau expression in 3xTg-AD mice treated with an anti-α4–integrins antibody (Anti-α4–integrins) compared to 3xTg-AD mice treated with isotype control antibody (Isotype). (c-d) Tau phosphorylation was investigated by staining with AT180 antibody that detects the Thr231 phospho-epitope of tau protein. (c) Bar graph showing the area of AT180 positive cells in the CA1 of 3xTg-AD mice treated with an anti-α4–integrins antibody (Anti-α4–integrins) compared to 3xTg-AD mice treated with isotype control antibody (Isotype). Bar is expressed as mean ± SEM (**P <0.005). (d) Representative images clearly showing a reduction of AT180 expression in 3xTg-AD mice treated with an anti-α4–integrins antibody (Anti-α4–integrins) compared to 3xTg-AD mice treated with isotype control antibody (Isotype). Scale bar: 50 μm.

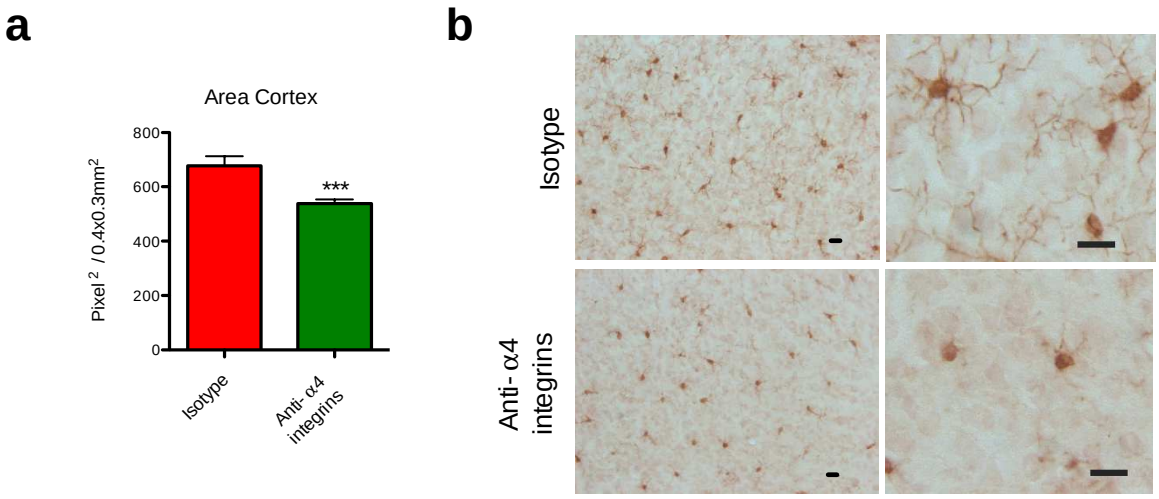

Figure 39. Anti-α4–integrins treatment reduces microglial activation in 3xTg-AD mice. At the end of behavioral tests, mice were sacrificed for neuropathological analyses as described *in Materials and Methods* and in Figure 12. (a) Bar graph showing microglia density (nr/0.4x0.3mm^2) in the cortex of isotype control-treated (Isotype) compared to α4-integrins–treated (Anti-α4 integrins) 3xTg-AD mice . Bar is expressed as mean ± SEM (***P <0.0005). (b) Representative images of Iba-1 microglia in the cortex: isotype control-treated 3xTg-AD mice (Isotype) show highly activated amoeboid cell morphology with large soma and several thick cellular processes, conversely 3xTg-AD mice treated with anti-α4–integrin antibody (Anti-α4 integrins) show a less activated microglia phenotype. Scale bars, 50 μm in left panels. Higher magnifications are shown in the right panels; scale bars, 25 μm.

13. DISCUSSION

The main neuropathological hallmarks of AD are extracellular beta amyloid (Aβ) peptides deposition, and neurofibrillary tangles (NFTs) formation, which are composed of hyperphosphorylated tau protein (Kidd M., 1963; Wisniewski T., Frangione B., 1992; Heneka M.T., et al., 2015). The pathogenesis of AD also involves chronic brain inflammation, synaptic loss and neuronal loss, that lead to cerebral atrophy (Wyss-Coray T. & Rogers J., 2012; Heppner F.L., et al., 2015).

The BBB is a highly specialized endothelial cell membrane that operates within the neurovascular unit (NVU), which includes clusters of glial cells, neurons and pericytes. The NVU controls BBB permeability and cerebral blood flow, and maintains the chemical composition of the brain interstitial fluid, which is required to maintain functional neuronal circuits (Zlokovic B.V., 2011). Vascular deposition of Aβ in the intracerebral and leptomeningeal vessels, also known as CAA, is associated with degeneration of smooth muscle cells, pericytes, endothelial and BBB breakdown (Roher A.E., et al., 2003; Carrano A., et al., 2011; Erickson M.A. & Banks W.A., 2013; Zenaro E., et al., 2016). In addition, Aβ deposition in the vasculature alters the expression of tight junctions (TJs) proteins and changes mechanical properties of the endothelial membranes thus favoring the transmigration of immune cells (Giri R., et al., 2002; Gonzalez-Velasquez F.J. & Moss M.A., 2008; Yang X., et al., 2010). Vascular inflammation and a dysfunctional blood-brain-barrier (BBB) have been implicated in the pathogenesis of AD suggesting that vascular pathology, hemodynamic changes and peripheral leukocytes may be involved in the initiation and progression of AD.

Several studies showed that the peripheral innate and adaptive immune cells, including monocytes, neutrophils, T cells and B cells can enter the CNS when the BBB is impaired during CNS diseases such as Parkinson's disease (PD), multiple sclerosis (MS) and AD (Theodore S., et al., 2008; Korn T., 2008; Brochard V., et al., 2009; Bhat R. & Steinman L., 2009; Goverman J., 2009; Chung Y.C., et al. 2010; Popescu B.F., & Lucchinetti C.F., 2012; Zenaro E., et al., 2015). In PD, several studies showed the presence of various peripheral immune cells, such as T cells, B cells and macrophages in the midbrain of PD patients and also in the brain regions in the toxin-based models of PD, suggesting that penetration of immune cells plays an important role in the degeneration of death of dopaminergic neurons in PD (Theodore S., et al., 2008; Brochard V., et al., 2009; Chung Y.C., et al. 2010). In MS, autoimmune disease of the CNS characterized by demyelination, several studies showed that infiltration of autoreactive T cells, which are targeted against antigens of the myelin sheath, is a critical event in the pathogenesis (Korn T.,, 2008; Bhat R. & Steinman L., 2009; Goverman J., 2009; Popescu B.F., & Lucchinetti C.F., 2012; Simmonset al., 2013). In AD,

Zenaro E., et al. reported the presence of neutrophils and their role in the induction of neuro-pathological changes and memory deficits associated with AD in 5xFAD and 3xTg-AD mice (Zenaro E., et al., 2015). Neutrophils are the first line of defense in our innate or non-specific immune system and are rapidly deployed to sites of inflammation, where they perform a variety of antimicrobial functions such as degranulation and phagocytosis, to kill invading pathogens. They are highly reactive cells that release ROS, enzymes, neutrophil extracellular traps (NETs) and cytokines and thus their inappropriate activation can cause long-term collateral tissue damage. Zenaro E., et al., found neutrophils within the brain parenchyma, especially in the cortex, hippocampus and in zones adjacent to vascular Aβ deposits and rich in Aβ plaques in 3xTg-AD and 5xFAD mice. Importantly, the accumulation of elevated numbers of neutrophils has been observed in human cortical brain samples from AD subjects compared to controls of the same age. Once the neutrophils infiltrate into the brain, they may contribute to neuronal damage causing cognitive decline in AD. In addition, soluble Aβ can generate ROS in mouse and human neutrophils. The production of ROS, carried out by neutrophils, can induce neuronal damage and release of structures composed of chromatin DNA, histones and granular proteins into the extracellular environment, known as NETs (Kolaczkowska E. & Kubes, P.P., 2013). Interestingly, they found intravascular and intra-parenchymal NETs in mouse models of AD, but also in cortical brain samples from human subjects with AD. These results suggest that NETs cause BBB damage and neuronal death and, also, that it may represent a neutrophil-dependent disease mechanism in AD. The deleterious role of neutrophils in the induction of cognitive dysfunctions in animal models was revealed by treating AD mice with monoclonal antibody to deplete neutrophils at the early stage of disease. In this case, it was possible to observe how the neuro-pathological hallmarks of AD and memory deficits are considerably reduced. In addition, the neutrophil depletion had a beneficial long-term effect in older animals, indicating that neutrophils have a potential role in the development of chronic disease (Zenaro E., et al., 2015). However, the role of circulating immune system cells in AD-related brain damage is still unclear (Wyss-Coray T., 2006; Schwartz M., et al., 2013; Baik S.H., et al., 2014; Zenaro E., et al., 2015).

Leukocyte trafficking into the brain parenchyma involves three distinct routes. The first path begins in the blood and reach the parenchyma through the walls of the parenchymal post-capillary venules; the second path goes from the blood to the subarachnoid space through the walls of the meningeal vessels; finally, the last path starts in the blood and reach the CSF across the venule wall and then the stroma and epithelium of the choroid plexus (Ransohoff R.M., et al., 2003). Under inflammatory conditions, the first two routes are used by leukocytes for CNS invasion, while the last route is considered the major site of CNS immunosurveillance under physiological conditions (Ransohoff R.M., et al., 2003; Engelhardt B.& Ransohoff R.M., 2005; Man S.M., et al., 2007; Shechter R., et al., 2013).

However, the leukocytes extravasation can be mediated by the release of pro-inflammatory mediators from activated glial cells. Interestingly, glial cells and neurons are source of various cytokines and chemokines (Ambrosini E., et al., 2004; Carpentier P.A., et al., 2005; Biber K., et al., 2008; Choi S.S., et al., 2014; Liu C., et al., 2014), thus they play a crucial role in immune response initiation and maintenance during several neuroinflammatory diseases. In particular, increased levels of CXCL8/IL-8 and monocyte chemotactic protein-1 (CCL2/MCP-1) were found in the CSF of MCI subjects and AD patients, and have been correlated with age and neuropathological changes during disease progression (Galimberti D., et al., 2003; Galimberti D., et al., 2006; Weeraratna A.T., et al., 2007; Galimberti D., et al., 2008; Li M., et al., 2009). In addition, cultured microglia from the adult human brain stimulated with Aβ strongly up-regulated the gene for CXCL8/IL-8 and monocyte chemotactic protein-1 CCL2/MCP-1 (Walker D.G., et al., 2001), two potent chemoattractants respectively for neutrophils and monocytes. The CCL2 overproduction by micorglia cells may induce monocyte recruitment from the blood to the brain, and their differentiation, thus enhancing neuroinflammation and AD pathogenesis (Naert G. & Rivest S., 2011, 2012, 2013).

Leukocytes extravasation is generally considered the initial step of inflammatory responses and it is composed of several steps mediated by interaction between selectins and integrin ligands, expressed on vascular endothelial cells, with their counter-ligands expressed on leukocyte surfaces. During inflammation, leukocyte–vascular interactions in CNS venules are mediated predominantly by endothelial P selectin and E-selectin and their mucin ligands PSGL-1 and TIM-1, as well as leukocyte integrins that include α4β1 (also known as very late antigen 4, VLA 4) which bind VCAM-1, and leukocyte integrins αLβ2 (LFA-1) and αMβ2 (Mac-1) which bind ICAM-1 and ICAM-2 (Ley K., et al., 2007; Rossi B., et al., 2011; Angiari S. et al., 2014; Angiari S., & Constantin G., 2014).
The expression of vascular adhesion molecules that mediate the leukocyte trafficking is undetectable or minimal under physiological conditions, with P-selectin, E-selectin and ICAM-1 immunoreactivity detected in pial and choroid plexus venules in the normal brain (Kivisäkk P., et al., 2003). However, during brain inflammation, the expression of endothelial adhesion molecules strongly increases, and their soluble forms are released into the circulation to provide a biomarker of endothelial dysfunction and vascular inflammation (Bö L., et al., 1996; Jander S., et al., 1996; Stins M.F., et al., 1997; Staykova M., et al., 2000; Garton K.J., et al., 2006; Alvarez J.I., et al., 2011; Rossi B., et al., 2011). Several studies have reported the presence of ICAM-1 and VCAM-1 in AD body fluids, including serum and the CSF, thus suggesting a role for leukocyte trafficking mechanisms in the pathogenesis of AD (Engelhart M.J., et al., 2004; Rentzos M., et al., 2005; Nielsen H.M., et al., 2007; Zuliani G., et al., 2008; Davinelli S., et al., 2011; Doecke J.D., et al., 2012; Huang C.W., et al., 2015). Interestingly, soluble ICAM-1 level was included among the 18 plasma

proteins that can be used to classify blinded samples from AD and control subjects with 90% accuracy, and to identify patients that progress from mild cognitive impairment to AD within 2-6 years (Ray S., et al., 2007). In addition, higher levels of soluble VCAM-1 correlate with more advanced dementia, including poor short-term memory and visuospatial functions, and with changes in white matter hyperintensities observed during MRI (Huang C.W., et al., 2015). Similarly, these evidences suggest the use of soluble VCAM-1 and ICAM-1 as a biomarker for cognitive decline during AD. However, their role as blood-based markers of AD require further validation role because it is unclear if these systemic alterations are an early or late pathological events.
Recently, Zenaro E., et al. showed that the expression of E-selectin, P-selectin, VCAM-1 and ICAM-1 was significantly higher in 4-month-old 5xFAD mice than in age-matched WT control mice, supporting the role of vascular inflammation in AD (Zenaro E., et al., 2015). Similarly, in the present study we observed up-regulation of ICAM-1 in choroid plexus and in hippocampal blood vessels, if compared to other adhesion molecules in 3xTg-AD mice at early stages of disease. In addition, VCAM-1 was also found in the same brain districts although to a lesser extent. In agreement with our observations a recent study performed in Arc/SweAβ mice also have shown increased expression of VCAM-1 and ICAM-1 in the brains of 20–24-month-old mice compared to WT littermates, further supporting a role for integrins and their ligands in AD (Ferretti M.T. et al., 2016).

Important our previous findings demonstrated the direct presence of neutrophils in AD brain and their deleterious role in the induction of neuropathological changes and memory deficits in 5xFAD and 3xTg-AD mice (Zenaro E., et al., 2015). Based on these results we decided to interfere with leukocyte recruitment in the AD-like mice by blocking the adhesion mechanisms controlling leukocyte–endothelial interactions. In particular, we studied the LFA-1 integrin which is the main β2-integrin mediating the firm adhesion step of the leukocyte recruitment cascade (Issekutz A.C. & Issekutz T.B., 1992; Ding Z.M., et al., 1999; Phillipson M., et al., 2006). In the first step, we assessed whether the ablation of LFA-1 integrin on neutrophils or the LFA-1 blockade with an antibody may prevent the interaction between the neutrophils and the blood vessels before entering in the brain parenchyma by TPM studies. After, we studied the effect of LFA-1 integrin blockade in AD animal models for its potential therapeutic application in AD.
In addition, we focused our attention also on the VLA-4 integrin that contributes to T cell trafficking in the CNS (Engelhardt B. & Ransohoff R.M., 2012; Gorina R., et al., 2014). In fact, in the present study we quantified the accumulation of leukocytes from the brains of 3xTg-AD mice compared to sex- and age -matched WT control mice using flow cytometry. Interestingly, the results showed a high numbers of infiltrating T cells, such as CD4+, CD8+ and T cells at different time-points of the disease in 3xTg-AD mice as well as the neutrophil infiltration.

Our data demonstrated that LFA-1 integrin controls intravascular adhesion, but also intraparenchymal migration of neutrophils in transgenic mice with AD-like disease (Zenaro E., et al., 2015). Most LFA-1 deficient neutrophils did not slow down enough to adhere and crawl on blood vessels and did not accumulate in brain parenchyma in in vivo studies. Our findings agree with the results obtained by Li W., et al. that show how the blockade of LFA-1 integrin prevented neutrophil adherence to endothelium and extravasation in heart grafts (Li et W., al., 2012). Notably, we observed a significant difference in the movement of already extravasated cells after injection of LFA-1–blocking antibody, suggesting that LFA-1 integrin has a key role not only in the neutrophil adhesion on the endothelium inside brain venules, but also in the intraparenchymal motility in the AD brain (Lämmermann T., et al., 2013). Importantly, Zenaro E. et al. found a higher number of neutrophils infiltrating at early stages of disease when 3xTg-AD mice show the first memory dysfuncitons. In addition, the appearance of the cognitive impairment coincides with the presence of soluble oligomers of Aβ in human AD pathology (Oddo S., et al, 2003, Santos A.N., et al, 2012, Zenaro E., et al., 2015). Then, we investigated the effect of Aβ1-42 peptide on LFA-1 integrin-dependent rapid adhesion assays. Our data showed that soluble Aβ oligomers are more effective than fibrillar Aβ to trigger LFA-1–dependent adhesion of neutrophils, through the FPR engagement, on mouse neutrophils in *in vitro*, proving a possible mechanisms responsible for the neutrophil migration in the brain. In addition, we demonstrated that Aβ oligomers trigger LFA-1 transitions from low-intermediate state to high-affinity state thus enhancing the propensity to bind ligand efficiently. These results suggested that Aβ soluble oligomers activating neutrophil integrins might be responsible for the spreading and adhesion of neutrophils on blood vessels in AD-like mice. Therefore, the LFA-1–mediated neutrophil adhesion may represent a new therapeutic approach in AD.

For a potential therapeutic application of anti-adhesion therapy in AD, we studied the effect of LFA-1 integrin blockade in 3xTg-AD mice with cognitive deficits during early stages of disease using a blocking anti-LFA–1 integrin antibody. We clearly showed a recovery in cognitive deficits of anti-LFA–1 antibody-treated 3xTg-AD mice compared with control antibody-treated 3xTg-AD mice. In addition, the blockade of the LFA-1 integrin during the early stages of disease in 3xTg-AD mice had long-term beneficial effects, allowing a preservation of the cognitive function in old animals. We confirmed the impact of LFA-1 integrin on AD-like disease in 3xTg-AD x *Itgal*$^{-/-}$ mice by demonstrating the positive outcome in cognitive functions at early and late stages of disease. The beneficial effect that we observed in anti-LFA–1 antibody-treated 3xTg-AD mice and in 3xTg-AD x *Itgal*$^{-/-}$ mice might be partially due to a reduction of microglia cells density and their activated state in the hippocampus, especially in the DG and CA1 field, regions that play an important role in memory function. In fact, microglia accumulation in senile plauqes is an integral part of the disease process in AD and it is widely accepted that microglia-mediated neuroinflammatory

responses have a role in AD–associated neurodegeneration (Wyss-Coray T., 2006; El Khoury J. & Luster A.D., 2008). Microglia may initially play a protective role in AD by 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 (El Khoury J., et al., 2007; Hickman S.E., et al., 2008; Krabbe G., et al., 2013; Heneka M.T., et al., 2015). Therefore, the blockade of LFA-1–mediated neutrophil adhesion during the early phases of the disease might also inhibit microglia-induced neurodegeneration, allowing the preservation of cognitive functions in AD-like mice.
In addition, our immunohistochemical analysis demonstrated a clear reduction of Aβ load in the cortex of 3xTg-ADx*Itgal*$^{-/-}$ mice compared to 3xTg-AD mice. We found a reduction in the amount of AT8 (Ser202/Thr205) and AT180 (Thr231) hyper-phosphorylated tau in the hippocampus areas (CA1 and DG) of 3xTg-ADx*Itgal*$^{-/-}$ compared to 3xTg-AD mice, but without modifications in the quantity of total tau protein. In this context, several studies demonstrated that Aβ and tau play a role in the NVU dysfunctions, exacerbating the vascular inflammatory response and neurodegenerative process (Kovac A., et al., 2009; Erickson M.A. & Banks W.A., 2013; Zenaro E., et al., 2016). Aβ deposition is also observed in the cerebrovasculature, and is usually described as CAA (Jellinger K.A., 2002; Viswanathan A. & Greenberg S.M., 2011) and leads to pro-inflammatory and cytotoxic events that contributes to the accelerated BBB permeability in the AD brain (Roher A.E., et al., 2003; Carrano A., et al., 2011; Erickson M.A. & Banks W.A., 2013). Likewise, the appearance of perivascular tau around major hippocampal blood vessels contribute to the disruption of the BBB (Blair L.J., et al., 2015). Both tau and Aβ may therefore promote the loss of BBB integrity, exacerbating the neurodegenerative process and associated inflammatory responses. Therefore, our results demonstrated that the blockade of neutrophils infiltration through the blocking LFA-1 integrin may have a therapeutic effect on the progression of the pathology by reducing Aβ accumulation, tau phosphorylation and microglial activation in 3xTg-AD mice. These data, together with TPM analysis, provide consistent evidence on the key role of LFA-1 integrin in the pathogenesis of AD-like disease.

Although the role of T cells in the pathogenesis of AD is unclear, evidences suggest that activated T cells may play a detrimental role in disease evolution (Togo T., et al., 2002; Ferretti M.T., et al., 2016; Zenaro E., et al., 2016). T cells are a subtype of the lymphocyte family that include also natural killer (NK) cells, and B cells. They have a central role in cell-mediated immunity, but their activity normally decreases with advancing age (Linton B.P., et al., 1996). The involvement of T-cells in AD has been studied and it was first described by Lombardi et al. in 1999 when they immunophenotyped T cells from AD patients and healthy age-matched controls, founding evidence of a higher presence of different subpopulations of T cells in the blood of AD patients (Lombardi V.R., et al., 1999).

Furthermore, MCI and mild AD patients contained a greater number of activated CD4+ and CD8+ T cells in the CSF, with the proportion of activated CD8+ T cells showing the highest increase, supporting the hypothesis that activated T cells migrate from the blood into the brain during AD (Lueg G., et al., 2015). Similarly, T cells also infiltrate into the brains of APP/PS1 mice, and may secrete IFNγ or IL-17 (Browne T.C., et al., 2013), suggesting that the release of these cytokines could accelerate AD neuropathology. In addition, recent studies suggest that regulatory T (Treg) cells, which suppress adaptive T cell responses and T cell activation, play a beneficial role in AD models during the early phase of the disease, by slowing disease progression and modulating microglial responses to Aβ deposition (Dansokho C., et al., 2016). More recently, lymphocyte-depleted transgenic models of AD lacking T- and NK cells contribute to better understanding of the involvement of lymphocytes in AD. In fact, these experimental mice showed an increase in Aβ deposits and a severe gliosis and neuroinflammation (Marsh S.E. et al., 2016).

The role of T cell populations at different AD stages therefore remains to be determined, and future studies should aim to clarify the role of these cells in AD pathology. In addition, the mechanisms controlling T cell recruitment in the AD brain are not well understood. However, several evidences show that the brain amyloidosis promotes T cell migration into the brain and the expression of vascular adhesion molecules in brain vessels (Ferretti M.T., et al., 2016). Notably, Aβ1-42- induces the release of TNFα by microglial cells, which in turn promotes major histocompatibility complex I expression on the brain endothelium followed by the transendothelial migration of T cells in *in vitro* study (Yang Y-M., et al., 2013). Accordingly, T cells together with macrophages and monocytes were observed in leptomeningeal and cortical vessels, associated with CAA, suggesting that Aβ and CAA favor T cell migration into the AD brain (Yamada M., et al., 1996). Furthermore, circulating T cells in AD patients also overexpress CXCR2, a chemokine receptor that is widely expressed on immune system cells that may promote their transendothelial migration (Liu Y.J., et al., 2010). The inflammatory response stimulated by T cells that have migrated into the AD brain may activate microglia and astrocytes and may recruit other inflammatory cells that are potentially harmful to the CNS, thus exacerbating the pathogenesis of AD. In fact, activated T cells could contribute to the delayed cell death of PMNs. Indeed, some *in vitro* experiments demonstrated that T cell-derived cytokines, particularly IFN-γ, extend the lifespan of PMN in a functionally active state (Klebanoff S.J., et al., 1992). So, T cells and neutrophils may cooperate to have deleterious effects on the progression of the disease.

However, it is not clear how circulating T cells penetrate the BBB and infiltrate the AD cerebral parenchyma, or how local inflammatory milieu influences T cell migration or survival resulting in the accumulation of T cells in the brain. It is important to determine whether T cell trafficking is involved in the pathogenesis of AD or is solely an epiphenomenon, and whether T cells are beneficial or deleterious in AD.

Our data on the expression of VCAM-1 in blood vessels in transgenic animal models of AD suggested that it may interact with VLA-4 integrin expressed by several leukocyte subpopulations. VLA-4 integrin is expressed by activated lymphocytes and its blockade has been shown to have therapeutic effect in numerous experimental models of inflammatory diseases including experimental autoimmune encephalomyelitis, the animal model of multiple sclerosis (Luster A.D., et al., 2005). However, recently the anti-VLA-4 treatment improved stroke outcome mainly by blocking the entry of neutrophils, but not T-cells, in a mouse model of stroke (Neumann J. et al., 2015). Indeed, the VLA-4 integrin is expressed by approximately 3% of circulating neutrophils in both humans and mice and previous studies demonstrated it represents an alternative pathway for neutrophils migration to sites of inflammation (Johnston B. & Kubes P., 1999; Massena S., et al., 2015). VLA-4 integrin expressed on neutrophils can mediate cellular responses such as tight adhesion, spreading, sustained respiratory burst, and specific granule release. In addition, neutrophils could migrate across purified VCAM-1 or through activated endothelial monolayers (Taooka Y., et al. 1999; Pereira S., et al.2001).

Our promissing data show that α4-integrin blockade rescues memory impairment in 3xTg-AD mice, suggesting that VLA-4 integrin could represent a novel attractive therapeutic target in AD pathology. Interestingly, the restoration of cognitive functions was maintained also in aged animals in 3xTg-AD mice treated during early stages of disease. We also performed immunohistochemical labelling of microglial cells to identify their degree of activation and evaluated the accumulation of Aβ and tau phosphorylation. The results showed a reduction of microgliosis in the cortex of 3xTg-AD mice treated with an anti-α4 integrins antibody compared to animals treated with an isotype control antibody. Moreover, we found a reduction of Aβ load in the cortex, amygdala and subiculum and a reduction of AT180 hyper-phosphorylated tau protein in the CA1 region of the hippocampus in 3xTg-AD mice treated with an anti-α4 integrins antibody compared to animals treated with an isotype control antibody at 12-13 months of age. These data confirmed the therapeutic potential of α4 integrin blockade in 3xTg-AD mice, consistent with our initial hypothesis that interfering with leukocyte infiltration can ameliorate AD disease progression.

Notably, preclinical and clinical therapeutic applications of antibodies that target leukocyte integrins in various inflammatory disorders confirmed the validity of their utilization as key therapeutic targets (Yonekawa K., et al., 2005; Mitroulis I., et al., 2014; Ley K., et al., 2016). Integrins are probably the most important class of cell-adhesion receptors to be targetted for a therapeutic approach (Shimaoka M., et al.2003; Mitroulis I., et al., 2015; Williamson et al., 2015). In effect, anti-integrin therapy has been tested in humans in several pathologies, especially autoimmune diseases. The usage of Natalizumab, a recombinat humanized monoclonal IgG4 antibody against the α4 chain of integrin VLA-4, is emblematic for anti-integrin therapies. Natalizumab strongly blocks leukocyte trafficking

across the BBB into the CNS by blocking the interaction between VLA4-integrin and VCAM-1. Initial clinical studies performed in 2003 have shown that Natalizumab, is effective in patients with Crohn's disease as well as those with as and multiple sclerosis (MS) (Ghosh S., et al. 2003). Subsequently Natalizumab was approved in 2004 by the U.S. Food and Drug Administration and the European Medicines Agency as monotherapy for highly active relapsing–remitting MS. However, in 2005 the drug was temporary withdrawn form the market due to the occurrence (in >1:1000 patients) of an exceedingly rare virus-induced neurodegenerative process, progressive multifocal leukoencephalopathy (PML) (Van Assche G., et al. 2005; Kleinschmidt-DeMasters B.K., et al., 2005; Langer-Gould A., et al., 2005). However, natalizumab represents the most potent drug tested for the treatment of MS as demonstrated by a phase III clinical trial and was then reintroduced as a second-line therapy for relapsing-remitting MS (Steinman L., et al., 2005; Polman C.H., et al., 2006; L., Steinman et al., 2009). Other agents have been developed to block α4 such as small molecules that may be administered orally with lower safety concerns, representing further therapeutic opportunities for inflammatory diseases.

LFA-1 integrin was effectively targeted in psoriasis by the humanized anti-αL antibody efalizumab (Lebwohl M., et al. 2003; Frampton J.E., et al., 2009). Efalizumab was withdrawn in 2009 after several PML cases occurred (occurrence 1:1000) as well as natalizumab treatment or other immunomodulatory therapies including rituximab and belatacept (Major E.O., et al., 2010; Klintmalm G.B., et al. 2014). The generation of new integrin antagonists based on structure-based drug design has been proposed to interfere with LFA-1-ICAM-1 interaction opening new therapeutic possibilities to interfere with integrin function (Shimaoka M., et al., 2003).

In conclusion, the role of circulating immune system cells in AD-related brain damage is poorly understood, and future studies are needed to determine how specific populations of cells representing the innate and adaptive immunity systems promote the cognitive deficit and neuropathological changes in AD. In this sense, our study revealed novel insights on the role of circulating immune cells in AD highlighting the role of leukocyte recruitment. Considering the results obtained with natalizumab and efalizumab that proved the concept that blockade of leukocyte trafficking has therapeutic effects in humans we hypothesize that the anti-integrin therapies may be useful also to prevent and delay the progression of this neurodegenerative disease.

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