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DISSECTING THE MECHANISMS OF NEUTROPHIL DYSREGULATION AND NEUROTOXICITY IN ALZHEIMER'S DISEASE

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Bruno Miguel dos Santos Lima

PhD thesis

Verona, 10 May 2022

Para ser grande, sê inteiro

Para ser grande, sê inteiro:
Nada teu exagera ou exclui.
Sê todo em cada coisa.
Põe quanto és
No mínimo que fazes.
Assim em cada lago a lua toda
Brilha, porque alta vive

(Ricardo Reis)

ABSTRACT

Alzheimer's disease (AD) constitutes the most common form of dementia worldwide and is characterized by the accumulation of extracellular senile plaques mediated by amyloid-β (Aβ), intracellular neurofibrillary tangles, synaptic degeneration and neuronal injury. It is considered an urgent social and economic problem due to the lack of effective therapeutic therapies or curative approaches. Until recent times the central nervous system was considered an immune-privileged site, still, the contribution of peripheral immune cells to brain functions highlighted their role in CNS physiology and disease. Neutrophils constitute the most abundant leukocyte population in human circulation and our group has identified a crucial role for these cells in the pathogenesis of AD. Other reports have then further supported this role, emphasizing peripheral neutrophil hyperactivation in AD, although the cause is still unclear. The recent discovery of gastrointestinal dysbiosis in neurodegeneration, including AD, and the subsequent pro-inflammatory response associated with increased circulating microbial products points to a broad systemic dysregulation of the immune system in AD patients. Still, **neutrophil dysregulation and its impact on peripheral and brain inflammation in AD are not well understood and were the focus of this doctoral thesis.** In the first part, we studied peripheral neutrophil dysregulation and characterized intestinal microbiota changes of 3xTg-AD mice and healthy wild-type (WT) mice. Our results showed a profound dysbiosis characterized by an increase in pro-inflammatory bacteria in 3xTg-AD mice. In addition, we showed that dysbiosis was accompanied by inflammatory changes in the intestinal tract, including a decrease in the number of mucus-producing cells and an increase in permeability of the intestinal tract. Moreover, we identified enteric nervous system (ENS) degeneration and neutrophil infiltration as early events in AD pathogenesis, suggesting a link between neutrophils and intestinal changes in AD mice. To further connect dysbiosis and peripheral inflammation, we performed a phenotype characterization of circulating neutrophils. The analysis of peripheral blood identified a persistent state of neutrophilia and, most importantly, an increase of immature neutrophils in the 3xTg-AD mice, suggesting the activation of "emergency granulopoiesis".

Interestingly, we showed a positive correlation between intestinal permeability and the levels of circulating neutrophils, further showing an **interplay between gut dysbiosis and granulopoiesis.** To further understand the role of neutrophil pathological changes in AD, in the second part of this project, we studied neutrophil dysfunction from a "central" point of view. Importantly, scRNAseq of brain-infiltrating neutrophils revealed genes associated with neutrophil degranulation, which was later confirmed using flow cytometry. scRNA-seq also identified *Ctse* as one of the most upregulated genes in infiltrating neutrophils, suggesting its role in the pathogenesis of AD. Interestingly, *in vitro* stimulation of both human and mouse neutrophils with Aβ led to an increase in the degranulation of neutrophils that was abolished when neutrophils were pretreated with a CTSE inhibitor. To address the potential of CTSE as a therapeutic target, we pre-treated neutrophils with a CTSE inhibitor and cocultured these cells with neurons observing a strong decrease in neuronal death when CTSE was blocked, pointing to CTSE as a key mediator of neutrophil neurotoxicity in AD. Overall, this thesis provides novel insights into neutrophil dysregulation in a mouse model of AD, suggesting that dysbiosis may contribute to peripheral neutrophil responses and neuroinflammation in AD. Furthermore, our data suggest that targeting neutrophil-dependent neurotoxic mechanisms may represent a novel and promising therapeutic strategy for AD.

ACKNOWLEDGEMENTS

We often hear that doing a PhD is an endless and torturous road. I don't know about the last one, for that road brought me happiness in a bunch of different ways. Still, I must agree on the endless part. I have started a chapter that didn't finish as the PhD did. Of course there were bumps on the road, but between unexpected results, protocols that just won't work and drama (it seems there is always drama, regardless of the country), we managed to get to the "end". Still, it wouldn't be fair not to mention some people that, willingly or not, took part in this journey with me.

Firstly, none would be possible without the trust and encouragement I received from Gabriela since day one. (Now we will go down memory lane, so feel free to skip it if you don't feel like it). The first-ever reply I got from Gabriela was: "Would be possible for you to Skype next week?" (zoom was still not a thing back then). I must admit I got anxious. What was supposed to be a doubt-answering email turned out to be the scheduling of the "interview" that made me fly away from home. For believing in me then, and equally importantly, throughout these years, thank you.

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ABBREVIATIONS

AD – Alzheimer's Disease

APOE – Apolipoprotein E

APP – Amyloid Precursor Protein

Aβ – Amyloid β

BBB – Blood-Brain Barrier

C/EBP – CCAAT-Enhancer-Binding Proteins

FAD – Familial Alzheimer's Disease

CD – Cluster of Differentiation

CNS – Central Nervous System

CSF – Cerebrospinal Fluid

CTSE – Cathepsin E

CXCL – C-X-C Motif Chemokine Ligand

CXCR – C-X-C Motif Chemokine Receptor

DEGs – Differentially Expressed Genes

ENS – Enteric Nervous System

FACS – Fluorescence-Activated Cell Sorting

FBS – Fetal Bovine Serum

G-CSF – Granulocyte-Colony Stimulating Factor

GM-CSF – Granulocyte-Macrophage Colony-Stimulating Factor

GO – Gene Ontology

HBSS – Hanks balanced salt solution

HSC – Hematopoietic Stem Cell

IL – Interleukin

LBP – Lipopolysaccharide-Binding Protein

LCN-2 – Lipocalin 2

LFA-1 – Lymphocyte Function-associated Antigen 1

LPS – Lipopolysaccharide

LRP-1 - Low-density lipoprotein-Related Protein-1

MCI – Mild Cognitive Impairment

MPO – Myeloperoxidase

MS – Multiple Sclerosis

NETs – Neutrophil Extracellular Traps

NFT – Neurofibrillary Tangles

PAMPs – Pathogen-Associated Molecular Patterns

PAS – Periodic Acid-Schiff

PCR – Polymerase Chain Reaction

PRR – Pattern Recognition Receptor

PS – Presenilin

ROS – Reactive Oxygen Species

RT – Room Temperature

SCFA – Short-Chain Fatty Acids

scRNA-seq – Single Cell RNA sequencing

SNARE – Soluble N-ethylmaleimide-sensitive factor Attachment protein Receptor

t-SNE – t-distributed stochastic neighbor embedding

TLR – Toll-Like Receptor

TNF – Tumor Necrosis Factor

VAMP – Vesicle-Associated Membrane Protein

VCAM-1 – vascular cell adhesion molecule 1

VLA-4 – Very Late Antigen 4

WT – Wild Type

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CHAPTER I

GENERAL INTRODUCTION

1.1 CURRENT KNOWLEDGE OF ALZHEIMER'S DISEASE

AD is the most common cause of dementia, accounting for 60-80 % of reported dementia cases. On top of that, the number of people suffering from dementia is estimated to be over 55 million as of 2021, with forecasts reaching 78 million by 2030. The estimated cost of care given to the high number of AD cases is over 250 billion dollars in the US alone, identifying AD as an urgent social and economic problem worldwide[1,2] . Even though it affects all ethnic groups, the disease has been reported to develop slightly more frequently in females than males[3]. Despite manifesting a wide range of signs early on, the symptoms that characterize AD consist of early memory loss and marked cognitive impairment. Indeed, patients in the early phases of AD, also known as mild cognitive impairment (MCI), begin presenting slight deficits in working and long-term memory and only a moderate impairment in short-term and episodic memory[4]. The appearance of MCI is classified as a sign of AD initiation, preceding irreversible mood alterations and loss of episodic memory function that is observed later on as the disease progresses[4]. Despite the clinical manifestations that characterize AD, the post-mortem analysis of the brain is still the ultimate factor in AD diagnosis[4]. While the exact mechanisms of AD development and pathogenesis remain elusive, two major neuropathological hallmarks have been associated with the disease. These include senile plaques, mainly composed of extracellular deposits of Aβ, and NFTs, consisting of intracellular aggregates of aberrantly phosphorylated tau protein (Figure 1). The deposition of these proteins is accompanied by neuronal degeneration, synaptic loss, dendritic and axonal changes, and inflammatory reactive lesions[5].

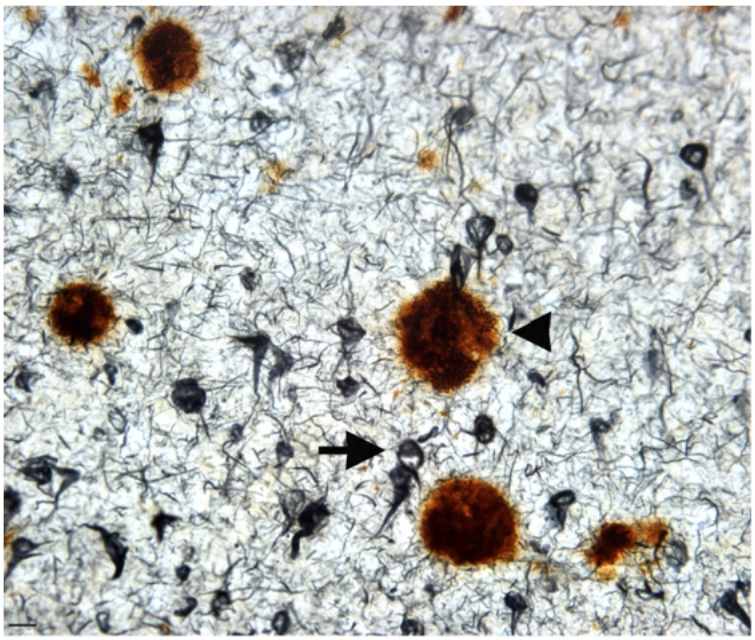

Figure 1. The neuropathologic hallmarks of Alzheimer's disease: senile plaques and NFTs*. In AD, widespread accumulation of extracellular beta-amyloid plaques is evident (arrowhead), together with the presence of NFTs along with neuropil threads, which are composed principally of modified and aggregated tau (arrow). (Adapted from Rohn T, 2013)*[251]

Even though AD pathology has been widely studied and provided clear evidence for brain alterations characteristic of AD, some questions remain to be elucidated. In fact, why some people develop AD is still unknown. Shortly after the discovery of amyloid precursor protein (APP), an autosomal dominant form of cerebral amyloid angiopathy was associated with its polymorphism. Since then, other familial AD (FAD) forms associated with intra-Aβ mutations have been pointed out, including E693K (Italian), E693G (Arctic), D694N (Iowa), A692G (Flemish) KM670/671NL Swedish V717I (London) and V717F (Indiana) (Reviewed in Kowalska 2003[6]). Interestingly, the Swedish double mutation at the β-secretase cleavage site results in a 6-8-fold increase in secreted Aβ peptide levels compared to WT APP. This increase in Aβ production is related to a shift from α-secretase towards β-secretase cleavage, as they directly compete for the APP substrate, leading to a shift towards amyloidogenic over the non-amyloidogenic processing of APP (discussed further ahead) [7]. In addition to missense mutations in APP that

increase deposition of Aβ, there have been familial mutations characterized in components of the processing machinery that can promote increased production of $A\beta_{1-42}$ peptides leading to aggressive early-onset forms of AD. These include presenilins (PS) [8] and neprilysin[9].

On the other side, sporadic or late-onset AD, the most prevalent form, is associated with age and environmental factors rather than genetic mutations[10]. Still, even though not considered a genetic disease, genetic abnormalities have also been described for the late-onset form of AD. Indeed, the diploidy for apolipoprotein E (APO-E) ε4 alleles seems to be one of the major genetic risk factors[11]. Nevertheless, consensus regarding sporadic AD still does not exist, considering that APOE4 variations can only be found in 50 % of the patients[12]. As a consequence, the cellular and molecular mechanisms underlying the etiology and progression of both disease forms, especially sporadic AD, are still poorly understood. In the past decades, some light has been shed on the neuropathophysiological mechanisms of AD. However, the currently available treatments and clinical trials show that no therapy can prevent, slow down, or cure AD[13]. Although reducing the amyloid burden in the brain, Aβ-directed strategies were proven to have secondary effects and to be ineffective in reverting the behavioral deficits in AD patients[14]. Moreover, although it is widely believed that brain pathology, neuroinflammation, neuronal and synaptic loss precede the initial signs of cognitive alterations in mild AD by at least 10-15 years[15], there is still no accurate and sensitive biomarker to detect AD initiation and/or progression. Therefore, as an attempt to have a readout of early neural dysfunction, new imaging techniques have been developed to detect overall changes in brain size, the integrity of brain networks, neural metabolism and Aβ deposition[16].

Moreover, the measurement of Aβ and tau in fluids, like the cerebrospinal fluid (CSF) and the blood, are also currently used to help in diagnosing MCI and pre-symptomatic AD[17]. But, again, there is no specific cut-off universally recognized for the definitive prognosis of AD. It is, therefore, urgent to find better biomarkers to diagnose AD in its early preclinical stages (before significant brain damage occurs) and to develop new disease-modifying strategies for its treatment. In addition, it is imperative to better understand the cellular and molecular events that

underline the aging of the organism, as a whole, and of the brain in particular, and that prompt for the pathological alterations in AD. Some of the already known mechanisms associated with the pathophysiology of AD will be described in the next sections.

1.2 PATHOGENESIS OF ALZHEIMER'S DISEASE

Until now, the prevailing hypothesis behind the development of AD is characterized by three cardinal neuropathological features: 1) the accumulation of extracellular senile plaques, 2) intracellular NFTs, and 3) synaptic degeneration. These neuropathological changes occur mainly in the neocortex, hippocampus, and other subcortical regions necessary for cognitive function. Interestingly, the appearance of senile plaques and NFTs occurs several years before the initial clinical symptoms of the disease, suggesting their potential as biomarkers for AD prediction[18]. However, although the amyloid cascade is considered the main cause of the disease pathogenesis, all clinical and economic efforts aimed to decrease Aβ in the brains of AD patients have not yet allowed to find an effective cure[19]. To overcome this issue, in recent years researchers also studied the contribution of inflammatory and immune mechanisms in the progression of AD, and their potential as therapeutic targets[20]. Similarly, experimental data increasingly support the involvement of infections[21] and the evident gut dysbiosis in AD pathogenesis[22], pointing to a broader and immune-related pathogenesis.

1.2.1 Amyloid Plaques

The amyloid hypothesis, the prevalent theory of AD pathogenesis, suggests that the accumulation of pathological forms of Aβ produced by sequential cleavage of the APP in the brain is the main pathological feature of AD[23]. Under physiological conditions, APP is often found as a transmembranar protein expressed and present ubiquitously in different isoforms. Together with the sub-products of its proteolysis, these are closely involved in the regulation of brain cell adhesion, neuronal survival, and plastic processes such as long-term potentiation (LTP), axonal growth and pruning, and synaptic activity[24]. Regarding the cleavage of APP, two distinct membrane-anchored proteases can initially mediate the proteolytic process: α-secretase or β-secretase, also called β-site APP-cleaving enzyme, giving rise to the non-amyloidogenic or the amyloidogenic, respectively[25]. Afterward, the γ-secretase complex, constituted by different proteins including PS1 and PS2[25], comes into play

by acting on the sub-products of α- and β-secretase. The non-amyloidogenic proteolysis of APP is initiated by α-secretase and leads to the extracellular release of a large ectodomain, called APPsα, and a transmembrane 83-residue carboxy-terminal fragment (Figure 2). This transmembrane residue can then be further shortened by the γ-secretase complex resulting in the extracellular release of a P3 fragment and the APP intracellular domain[25]. Conversely, the amyloidogenic processing of APP starts with the initial action of β-secretase, which results in the release of APPsβ. The remaining membrane retained 99-residue carboxy-terminal fragment is able to bind to a docking site on the surface of the γ-secretase complex, where it can be cleaved at three different sites[25] (Figure 2). The last cleavage site, called γ-site, can occur after amino acids 38, 40 and 42, originating $A\beta_{1-38}$, $A\beta_{1-40}$ and $A\beta_{1-42}$, respectively[25]. This differential cleavage by γ-secretase is critical for the subsequent degree of aggregation of Aβ peptides, taking the higher propensity of $A\beta_{1-42}$ for aggregation[25].

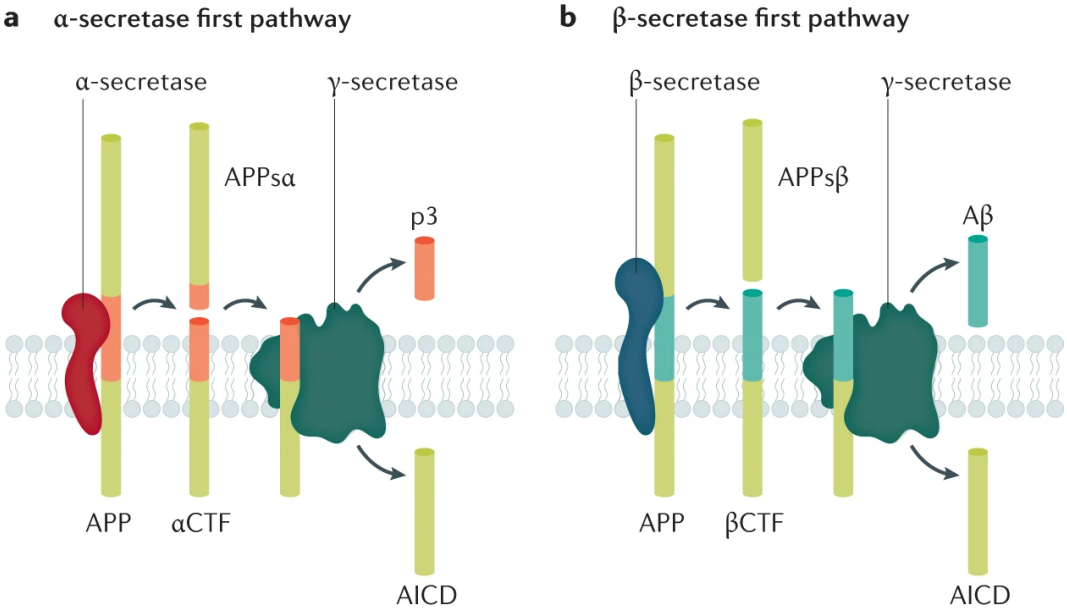

Figure 2. APP processing pathways. *In the non-β-amyloid pathway (a), the cleavage by α-secretase leads to the formation of APPsα and α carboxy-terminal fragment, which in turn can be further cleaved by γ-secretase resulting in extracellular peptide p3 and the intracellular fragment AICD. Aβ is formed in the 'amyloidogenic' pathway (b) by the cleavage of APP by β-secretase into APPsβ and β carboxy-terminal fragment, the latter being then cleaved by γ-secretase, producing Aβ and AICD (Adapted from Knopman et al, 2021)*[25].

Indeed, Aβ monomers undergo aggregation, leading to the formation of fibrils in three different phases: nucleation phase, elongation phase, and stationary phase (Figure 3) [26]. In the nucleation phase, monomeric precursors (folded or unfolded) form nuclei. Interestingly, the rate of dissociation is lower than that of monomer addition, leading to the fast aggregation of monomers to the nucleus. Oligomers can further associate to produce higher-order species, which can then be precursors of amyloid fibrils. During oligomerization, a nucleus is formed and is considered the most unstable species before rapid polymerization into amyloid fibrils. Then, each precursor undergoes a structural transformation during self-assembly to form β – strand-rich secondary structures, leading to the elongation phase. This elongation results in the exponential growth of fibrillar material; fibrils are dynamic and can release oligomers. Curiously, oligomers are considered to be the more pathogenic form of Aβ. In the last part of Aβ aggregation, fibrils also associate with each other, other proteins, and non-proteinaceous factors to form senile plaques characteristic of AD[26].

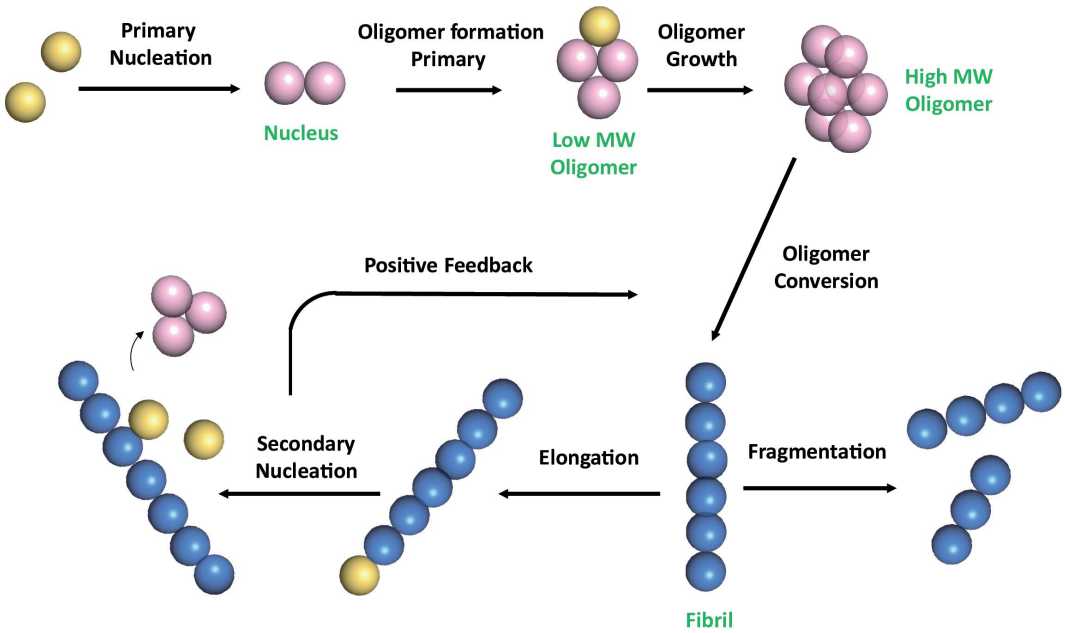

Figure 3. Schematic representation of Aβ aggregation cascade*. Aβ monomers first combine to form a nucleus through primary nucleation process. Addition of monomers to the nucleus results in the formation of oligomers that are transient soluble intermediates that further elongate into fibrils. Fibrils can be disrupted through monomer-independent processes, such as fragmentation, with a rate depending only upon the concentration of*

existing fibrils. Then, each precursor undergoes a structural transformation during self-assembly to form β – strand-rich secondary structures, leading to the elongation phase (Adapted from Pagano et al, 2020[27]).

1.2.2 Tau protein and Neurofibrillary tangles

Tau is a microtubule-associated neuronal protein, it is generated by neurons and is localized in the cell body and axons[28]. Under normal conditions, nerve growth factor increases tau expression during neuronal development, promoting the assembly of tubulin into microtubules and maintaining their stability. Still, Tau is believed to remain in a constant and dynamic balance with microtubules in physiological conditions, thanks to a correct equilibrium between phosphorylation and dephosphorylation processes (Figure 4) [29]. Experimental data show that in the AD brain, there is an hyperphosphorylation of tau, which leads to the aggregation of tau forms in neurons, resulting in the formation of NFTs. NFTs are structurally paired helical filaments composed of hyperphosphorylated tau proteins and neurofilaments. It is thought that NFTs with associated neurotoxic effects induce neuronal death contributing to dementia[30]. It has been suggested that many years before the appearance of clinical signs of AD[31] the deposition of both NFT and Aβ occurs within the neocortex, hippocampus, and other cognition–related subcortical structures. Increasing evidence shows that Aβ can be internalized or produced inside the cell, and this provides the opportunity for Aβ to facilitate NFT formation. On the other hand, disruption of tau formation can influence the production of Aβ plaques[32].

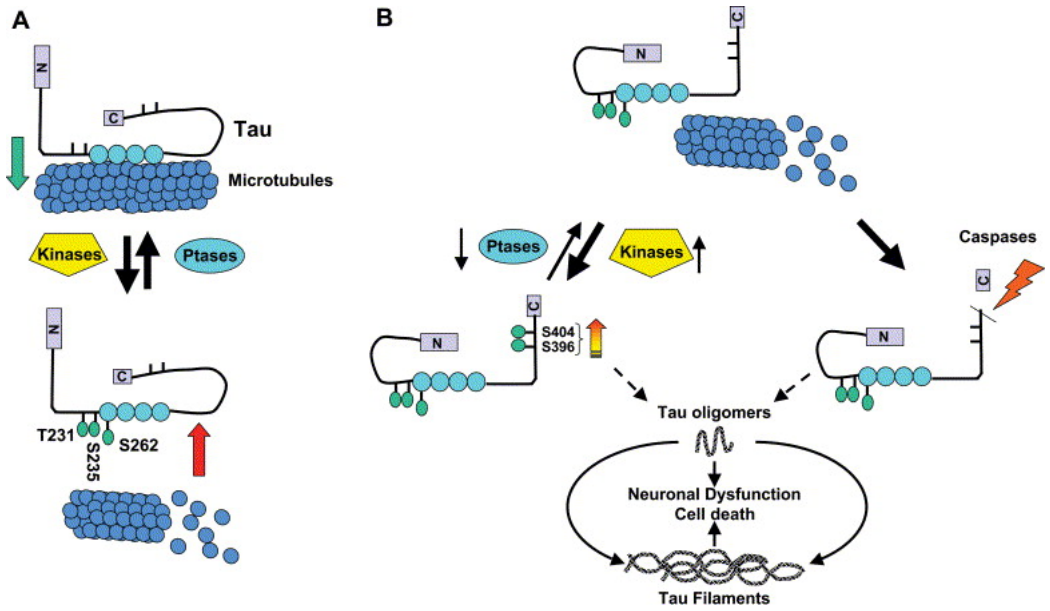

Figure 4. Diagram illustrating the role of tau phosphorylation in regulating tau function. *(A) Under physiological conditions, there is a balanced and dynamic change in tau phosphorylation, modulating tau's interactions with microtubules and allowing for normal neuronal function. (B) In a pathological state, dysregulation in the balance in the activities of specific phosphatases and kinases, results in increased rates of phosphorylation at critical microtubule regulatory sites, increasing the amount of "free" tau. These actions result in increased tau-tau interactions leading to the formation of tau filaments. (Adapted from Stoothoff et al, 2005[29])*

1.2.3 Synaptic degeneration

The neuronal synapses are the functional units of neurotransmission in the brain and constitute an elaborate and complex network[33]. Signal transmission in the central nervous system (CNS) requires the presence of functional synapses, with arranged pre- and post-synaptic compartments. The presynaptic compartment is composed of all the structures for the formation, storage, and release of neurotransmitter-containing vesicles[34]. Thanks to the presence of an action potential, an increase of Ca^{2+} in the presynaptic terminal triggers synaptic vesicles to bind the presynaptic membrane and then neurotransmitters are released into the synaptic cleft[35]. Subsequently, neurotransmitters interact with receptors on the postsynaptic compartment and after the activation of different signaling pathways, the signal is transmitted further. Even though the exact pathway of synaptic loss

and dysfunction in diseases still requires further research, there is evidence that a reduction in synaptic density and activity is one of the earliest events connected to CNS disorders[36]. Post mortem studies in patients with AD have reported a reduction pre- and post-synaptic markers[37,38] indications of deficits at both sides of the synapse. Indeed, evidence of Aβ and tau-mediated regulation of synapse function has been reported. Accumulation of Aβ oligomers in the presynapse and the association of pathological tau with synaptic vesicles have also been observed in AD patients[39–44]. For instance, Aβ oligomers have been shown to interact with syntaxin 1 and inhibit vesicle fusion in vitro[45]. By interacting extracellularly with the presynaptic part, Aβ was shown to deplete phosphatidylinositol-4,5-bisphosphate in axons of cultured mouse hippocampal neurons, which in turn inhibits the release of neurotransmitters[46]. On the other side, pathological tau was described to bind to synaptic vesicles through synaptogyrin 3, leading to reduced synaptic vesicle mobility and neurotransmission[44,47]. Notably, in tau transgenic mice (ΔK280), the neuronal presynapse is described to be enlarged while synaptic vesicle density is decreased and synaptic transmission impaired[48,49]. Furthermore, hyperphosphorylated tau can also affect presynaptic function by regulating the location and length of the axon initial segment, where the action potential is firstly generated[49].

Another important factor of synapse dysfunction is related to glia-neuronal interactions. Neuroinflammation has been receiving increasing attention when it comes to CNS disorders, especially AD. In recent years, evidence for microglia and astrocyte activation and contribution to neuroinflammation has been published by many groups and the association between these events and synapse dysfunction has been proposed[50,51]. While most of these studies have been performed using *in vitro* culture models, these have provided important insights towards synapse loss in AD[51,52]. In fact, Recent data indicate interactions between astrocytes and microglia in developmental synaptic elimination as microglial secretion of complement molecules inducing early synapse loss in AD mouse models[52].

1.2.4 Neurodegeneration / Neuronal Injury

Neurons begin to die early in AD pathogenesis. Already in very mild phases of AD, estimates indicate that over half of neurons in layer II of the entorhinal cortex are lost[53]. The loss of neurons initiates when Aβ plaques and NFTs are not yet present, during the pre-clinical phase of AD[54]. Brain atrophy is considered a valid marker of AD-related neurodegeneration. Precisely, during the pathological process, the atrophy of the grey matter in AD was found in the medial temporal, frontal and parietal cortices, and in the final stages of the disease, the neuronal loss is so widespread that it affects the whole brain[55]. Although the cellular-molecular mechanism of neuronal death has not been determined, Aβ is considered one of the insults that can cause various types of toxic mechanisms favoring neuronal death. In fact, elevated concentration of Aβ triggers different cytotoxic processes, such as oxidative stress, increased levels of Ca^{2+} intracellularly and the activation of the complement system[56,57]. All these aspects, taken together, lead to the complexity of neuronal cells death in AD. It was found that the antioxidant enzyme activity in the AD brain is reduced and that the production of reactive oxygen species (ROS) by Aβ peptides can result in decreased membrane permeability and mechanisms of excitotoxicity by increased calcium influx[58,59]. In line with the increased oxidative state, it was demonstrated that Aβ causes the oxidation of low-density lipoprotein-related protein-1 (LRP-1), an essential protein involved in controlling the clearance of Aβ from the brain. This decreased activity in AD is thought to be a key mechanism behind the accumulation of the neurotoxic peptide in the brain[60]. Interestingly, it was found that Aβ leads to an increased expression and activity of ryanodine receptor 3 that subsequently causes an unbalance in intracellular levels of Ca^{2+} and neuronal degeneration[61]. Moreover, Aβ interacts also with N-Methyl-D-Aspartate, involved in the homeostasis of glutamate within neurons. The alteration of this homeostasis could induce toxicity and affect the plasticity of neurons[62]. Regarding the complement system, it is known that the activation of the classical and alternative pathways is induced by $Aβ_{40}$ and $Aβ_{42}$, leading to the production of C3a, C5a and the membrane attack complex[63]. As neurons in the AD

brain are not able to produce protecting molecules, it was found that the membrane attack complex affects their function and, consequently, leads to their death[64].

1.3 NEUROINFLAMMATION IN ALZHEIMER'S DISEASE

Despite the significant advances that have been made towards understanding AD pathogenesis, fundamental knowledge regarding the role of inflammation is yet to be elucidated. After two decades of the amyloid cascade hypotheses proposed by Hardy and Higgins[65], multiple lines of research still support Aβ aggregation as the critical step that initiates AD pathology. Cumulative data suggests however that neuroinflammation plays a prominent and early role in AD[66]. The amyloid cascade-inflammatory hypothesis proposes that Aβ induces an inflammatory response that is further enhanced by tau's presence[67]. The localized inflammatory response is driven principally by activated microglia, the resident immune cell of the CNS[68,69]. Microglial cells provide immune brain surveillance, microenvironment scanning, and maintenance of neuronal plasticity through the release of trophic factors. However, in AD, their continuous activation leads to detrimental effects. Indeed, microglia can be activated by Aβ oligomers and tau proteins, recognized as pathogen-associated molecular patterns (PAMPs), via cell-surface receptors. Its continuous activation by either Aβ or Tau, leads to a chronic inflammatory response, characterized by alteration of microglia morphology (microgliosis) (Figure 5). In addition, reports have also suggested a dysregulation of Aβ clearance mechanisms by microglia, leading to excessive accumulation of Aβ and neuronal debris. In addition, during neuroinflammation, activated microglial cells secrete interleukin (IL)-6 and activate kinases, such as cyclin-dependent kinase 5, p38-MAPK, and GSK3β further contributing to the formation of abnormal tau hyperphosphorylation[70].

Peripheral immune cells also play a crucial role in the onset and progression of the disease, infiltrating the CNS through a dysfunctional blood-brain barrier (BBB)[71]. Circulating leukocyte subpopulations were identified in the brains of patients with AD and its animal models. Although their role in the disease progression remains unclear, both the main cluster of differentiation (CD) 4+ and CD8+ T cells were found to adhere to the vascular endothelium and migrate into the parenchyma. Our unpublished data also reveal that lymphocytes play a role in AD progression: at early disease stages, we observe an imbalance between pathogenic and patrolling

$CD8^+$ T cells in the 3xTg-AD brain, a feature that becomes even more prominent at later disease stages. In addition to CD8, γδ T lymphocytes have also been described to play a pathological role in AD by increased production of IL-17 and disruption of the normal neuronal behavior[72]. Recently, we demonstrated that circulating CD4+ T cells and a significant proportion of blood CD8+ T cells expressed higher levels of the very late antigen 4 (VLA-4) (alpha4 beta1) integrin than WT animals, suggesting a role for this integrin in mediating the leukocyte recruitment from the periphery to the AD brain. Indeed, our recent studies demonstrated that blockade of alpha4 integrins leads to improved memory and reduced neuropathology in 3xTg-AD mice[73].

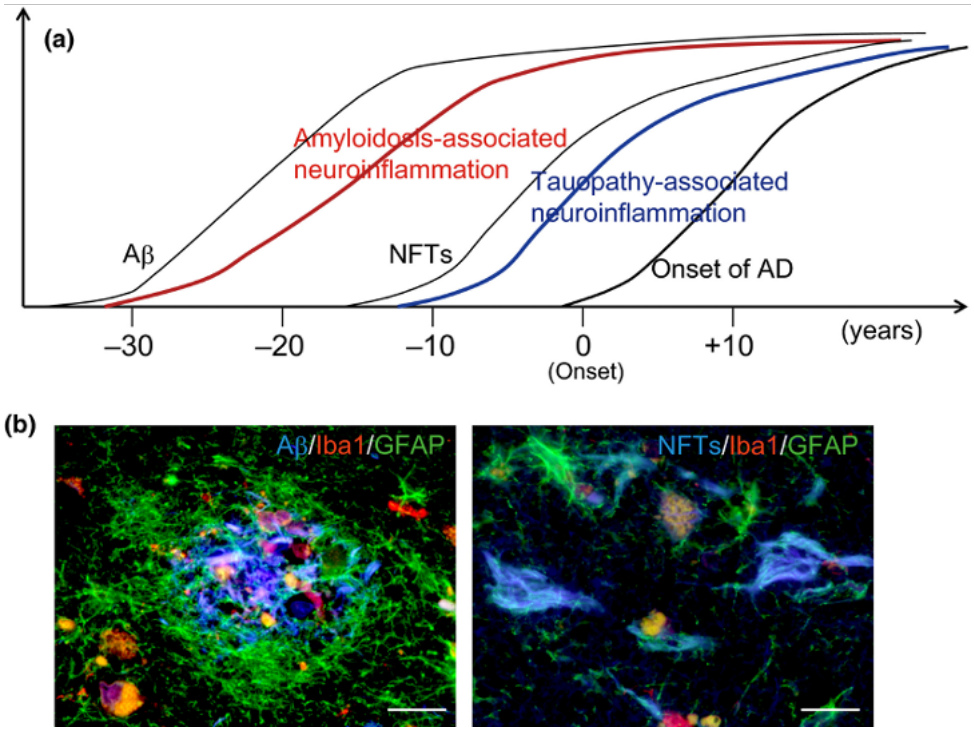

Figure 5. Neuroinflammation characteristics of AD*. a) Aβ deposition begins >25 years before the onset of AD and is followed by NFT formation. Both amyloid-associated and tauopathy-associated neuroinflammation might facilitate AD pathogenesis. (b) Immunohistochemical staining of astrocytes and microglia in the human AD brain. 1 ionized calcium binding adaptor molecule 1 (Iba1)-positive microgliosis shown in red and glial fibrillary acid protein-positive astrocytosis in green. Scale bar, 20 μm (Adapted from Saito et al, 2018)*[74].

Interestingly, in comparison to elderly subjects, plasma samples from AD patients show higher expression of soluble vascular cell adhesion molecule 1 (VCAM-1), the endothelial counter ligand of VLA-4, suggesting a role for VLA-4 and VCAM-1 adhesion molecules in lymphocyte migration during AD. Thus, VLA-4 and lymphocyte function-associated antigen 1 (LFA-1) integrins may represent key molecular pathways for leukocyte adhesion on brain endothelial cells thus mediating the migration into the CNS during AD. Neutrophils, the most abundant leukocytes in the human circulation, adhere to cerebral vessels and migrate into the AD brain. These cells are thought to be one of the most important players in AD pathogenesis[75]. They migrate into the parenchyma of mouse AD models at the onset of memory deficits, secreting IL-17 and producing neutrophil extracellular traps (NETs), which may harm endothelial and neural cells, contributing to AD pathogenesis and cognitive impairment. While it is clear that neutrophils play a role in the development of the disease, the clear mechanisms behind pathogenic behavior are yet to be determined. The current understanding of neutrophil involvement will however be discussed further ahead.

1.4 BBB INVOLVEMENT IN ALZHEIMER'S DISEASE PATHOLOGY

Under physiological conditions, the peripheral milieu is physically separated from the brain parenchyma by well-defined cellular structures that compose the brain's barrier[76]. Besides preventing the free and uncontrolled circulation of molecules and cells from the blood into the brain, the brain barriers play a critical role, on one hand, in the uptake of small nutrients and proteins and, on the other hand, in the excretion of byproducts of brain metabolism, which is essential for brain development and homeostasis[76]. The BBB is formed by a monolayer of endothelial cells bound together by tight junctions[77]. The endothelial cells of the BBB are wrapped by the basement membrane and surrounded by pericytes and astrocytic endfeet. These, together with the branches of circulating surveying microglia and nearby neuronal dendritic and axonal processes, form the neurovascular unit[77]. The high vascularization of the brain parenchyma, and the cellular heterogeneity of the neurovascular unit, allow for rapid and plastic responses to alterations in the blood composition and cerebral blood flow[77]. Thus, most studies on the communication between the periphery and the CNS, particularly those that address neurodegenerative disorders, focus on the BBB. Consequently, changes in neural-vascular communication, which are often associated with neuropathological conditions, may lead to rapid and irreversible neuronal damage[78]. Curiously, pericytes, which contribute to the integrity and function of the BBB, were recently shown to greatly impact the progression of vascular damage and pathology in an AD transgenic mouse model[79]. Of interest, there is an increased accumulation and deposition of Aβ in the neurovascular unit, which often culminates in cerebral Aβ angiopathy, highly prevalent in the brains of AD patients[80]. It is thought that cerebral Aβ angiopathy results from the combination of both an increased influx of Aβ into the brain parenchyma, mediated mainly by increased expression of the receptor for advanced glycation end products by the endothelial cells of the BBB[81], and a decreased efflux of Aβ into the blood, which is largely influenced by the decreased expression of LRP1[82]. Of notice, it is believed that the overall decreased expression of LRP1 in the AD brain is mediated by the amyloidogenic cleavage of APP and increased levels of the APP intracellular domain, which, altogether,

strongly influence APO-E-dependent and -independent removal of soluble Aβ from the brain parenchyma[83,84]. Moreover, in AD transgenic mice, increased deposition of Aβ in the brain vasculature is associated with impaired endothelial cell function, decreased vascular density and vascular architectural changes, such as vessel distortion and constriction, even before plaque formation. Ultimately, it is believed that Aβ accumulation and Aβ plaque formation, in regions like the hippocampus and the cortex, lead to capillary degeneration[85,86]. This increased microvascular injury at the level of the BBB, observed in aging and seen aggravated in AD, may ultimately lead to shortages in oxygen supply, energy substrates, nutrient exchange and overall brain dysfunction. Interestingly, cardiovascular abnormalities associated with AD have been described, including coronary artery disease, atrial fibrillation, heart failure, vasculopathy, macro and microinfarcts, white matter hyperintensities, atherosclerosis, and hypertension[87]. All of these vascular features could lead to decreased cerebral blood flow and it is now understood that cerebrovascular dysfunction is a key feature of AD[88]. In fact, virtually in all AD patients, BBB dysfunction and breakdown occur early in disease, even before signs of neurodegeneration or cognitive impairment are evident[89]. In AD patients, blood-to-brain leakage quantification using magnetic resonance imaging has recently enabled the detection of subtle, regional changes in BBB permeability during normal aging and pathological conditions such as AD[90,91]. These studies have identified increases in BBB permeability in the hippocampus and dentate gyrus during normal aging, which is further augmented in patients with MCI preceding brain atrophy or detectable changes in Aβ and tau in the CSF[91]. Ultimately, these alterations contribute to irreversible brain damage and behavioral impairments[92].

1.5 NEUTROPHILS

Neutrophils are the most represented immune cell type in human peripheral blood and act as the first responding cells during sterile and microbial insults. They elicit powerful effector functions to erradicate foreign threats and play crucial roles in tissue remodeling[93]. Neutrophils are short-lived; they have an estimated half-life of 19 hours in humans[94,95]. Therefore, neutrophils must be constantly replenished; an impairment in their production and migration leads to neutropenia and life-threatening conditions[96]. It was believed that neutrophils consist of a homogeneous population. However, this view is rapidly changing thanks to increasing reports of neutrophil heterogeneity[97]. Neutrophils play a key role in host defense against bacterial, viral, and fungal infections, but the nature of their cytotoxic contents dictates that appropriate developmental and clearance mechanisms be in place to protect the host against unintended inflammatory injury. Neutrophil homeostasis is maintained through a careful balance of granulopoiesis, bone marrow storage and release, and migration into vascular compartments and peripheral tissues. Despite their relatively short life span, these intriguing cells not only are vital for pathogen elimination during early infection but also link innate and adaptive immune responses to promote the resolution of inflammation and wound healing.

1.5.1 Neutrophil development

Given its high abundance in human peripheral blood, the percentage of hematopoiesis that commits to the production of this cell is believed to reach 60 % in the bone marrow[98]. Despite the high abundance of granulocyte progenitors, the initiating factors that determine whether a Hematopoietic stem cell (HSC) differentiates into a myeloid or lymphoid progenitor are still unclear. Models to explain this phenomenon are contrasting. The classical or hierarchical model states that multilineage priming is functionally related to the ability of a cell to pre-determine its fate prior to its differentiation, after which the capacity to differentiate and give rise to another cell type is lost. In this model, HSCs have equal differentiation potential[99,100]. In contrast, the alternative model contends that myeloid and lymphoid progenitors have mixed lineage potential but possess transcriptional and functional heterogeneity[99]. In this case, cell fate is determined by the availability of differentiation factors, rather than a pre-determined lineage commitment[101]. In fact, recent studies have provided evidence supporting this last model. HSCs have been shown to directly differentiate in common myeloid progenitors (CMPs), megakaryocyte-erythrocyte progenitors and/or lymphoid-primed multipotent progenitor[102,103]. In this sense, some transcription factors have been suggested to play important roles in the commitment to the production of a specific cell type. *PU.1* and *Irf8* can induce CMPs to differentiate into monocytes and macrophages while CCAAT-enhancer-binding proteins (*C/EBP)-ε* and *Gfi-1* generate neutrophils and eosinophils[101,104] (Figure 6).

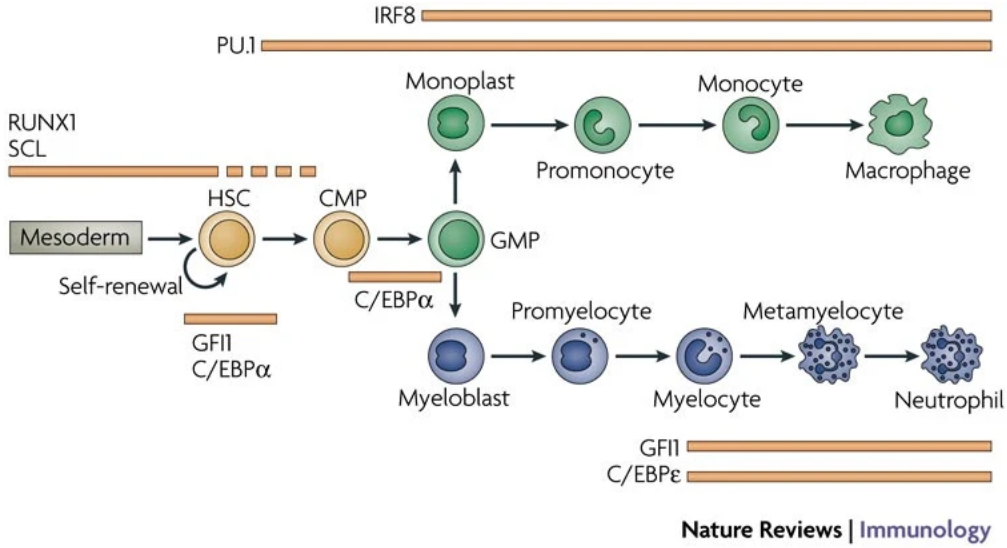

Figure 6. The differentiation of stem cells into monocyte and the neutrophil lineages. *Transcription factors are essential for the differentiation of HSCs. Runt-related transcription factor 1 and stem-cell leukemia factor are associated with the generation of HSCs, whereas Gf-1 and C/EBPα function in self-renewal of existing HSCs. C/EBPα is believed to play an important role in conferring the transition of CMPs into granulocyte-monocyte progentiors*[105]*. Gfi-1, and C/EBPε, are present in late-stage neutrophil production*[106] *while monocyte/macrophage production depends on PU.1 and IRF8. (Adapted from Paul et al 2015*[101]*)*

Interestingly, knockout of C/EBP-ε does not prevent initial differentiation towards neutrophil progenitors but rather its maturation and differentiation within the neutrophil compartment[101,107]. Therefore, neutrophil differentiation involves a complex interplay between transcription factors and modulators such as C/EBP-ε, PU.1, CCAAT displacement protein, Gfi-1, and retinoic acid receptor, placing granulopoiesis as a highly complex and heterogeneous process[101,108].

HSCs giving rise to neutrophils are situated in specialized niches in the trabecular regions near the endosteum or the interface between the bone and bone marrow[109]. In these specific sites, perivascular cells and osteoblasts express chemokines such as C-X-C Motif Chemokine Ligand (CXCL) 12, also called stromal-derived-factor-1), crucial for the retainment of neutrophils in the bone marrow[110]. As neutrophils develop into more mature stages, the expression of the CXCL12 receptor, C-X-C Motif Chemokine Receptor (CXCR) 4, on neutrophils decreases, making neutrophils less responsive to CXCL12 and leading to the release of neutrophils

from the bone marrow into the circulation[111]. Due to the high homeostatic turnover of neutrophils, a delicate balance between production, storage, release and migration processes must exist. Indeed, the regulation of neutrophil production has been described in terms of steady-state versus emergency granulopoiesis[112]. A continuous shift between the two states occurs and it is modulated by external stimuli[113]. The extent of each process is highly regulated by the strength and duration of the activating factor. In steady-state granulopoiesis, the ingestion of apoptotic neutrophils by tissue macrophages activates the transcription of C/EBP-α and factors of the LXR family, suppresses the production of proinflammatory cytokines and, in turn, lowers G-CSF levels[114]. Conversely, following microbial challenges, emergency granulopoiesis ensues and increases the release of both immature and mature neutrophil forms into the circulation[115]. In response to the presence of bacterial products, there is an induction of C/EBP-beta, accompanied by increases in the levels of inflammatory mediators such as IL-1β, tumor necrosis factor α (TNF-α), granulocyte-colony stimulating factor (G-CSF), and granulocyte-macrophage colony stimulating factor (GM-CSF), leading to a continuous production and release of neutrophils[112,116]. This process leads to clinical neutrophilia characterized by an increased proportion of immature forms. Recent evidence has emerged suggesting that pattern recognition receptors (PRRs) may unite the steady-state and emergency granulopoiesis pathways[117]. Direct or indirect activation of PRRs on hematopoietic stem and/or progenitor cells stimulates the proliferation and differentiation of neutrophils[118,119]. Alternatively, proinflammatory chemokines such as keratinocyte chemoattractant, macrophage inflammatory protein 2, G-CSF, and TNF-α can elicit the activation of NADPH oxidase to enhance ROS production by bone marrow myeloid cells[120]. ROS then act via paracrine mechanisms to trigger the oxidation and deactivation of phosphate and tensin homolog in resident myeloid cells, which leads to the upregulation of PtIns(3,4,5)P3 signaling, increased G-CSF production, and induction of emergency granulopoiesis[120].

1.5.2 Neutrophil granule formation

Granulopoiesis, or the formation of granules within the developing neutrophil, begins between the myeloblast and promyelocyte stages of development and proceeds over the subsequent 4 to 6 days[121,122]. Neutrophil granulopoiesis can be divided into two stages, with the first comprising neutrophil lineage determination and the second committed granulopoiesis[123]. While early neutrophil precursors, including myeloblasts, promyelocytes, and young myelocytes, retain proliferation capabilities, cells become committed to the neutrophil lineage during the transition between myelocytes and metamyelocytes, after which cell division stops[123]. Characterized by the stepwise emergence of granules and secretory vesicles during the maturation process, granulopoiesis begins with the appearance of azurophilic (primary) granules in myeloblasts and promyelocytes. This stage is then followed by the production of specific granules in myelocytes and metamyelocytes, during which the nucleus morphs into a kidney-shaped structure. Gelatinase granules are formed during the transition of metamyelocytes into band neutrophils, where the nucleus assumes a band-like shape. Neutrophil granulopoiesis is finalized with the development of ficolin-1 granules and secretory vesicles in already segmented cells, where the neutrophil acquires a characteristic segmented nucleus (Figure 7)[123,124]. No studies have definitively addressed whether neutrophils produce granules while still in bone marrow or when circulating.

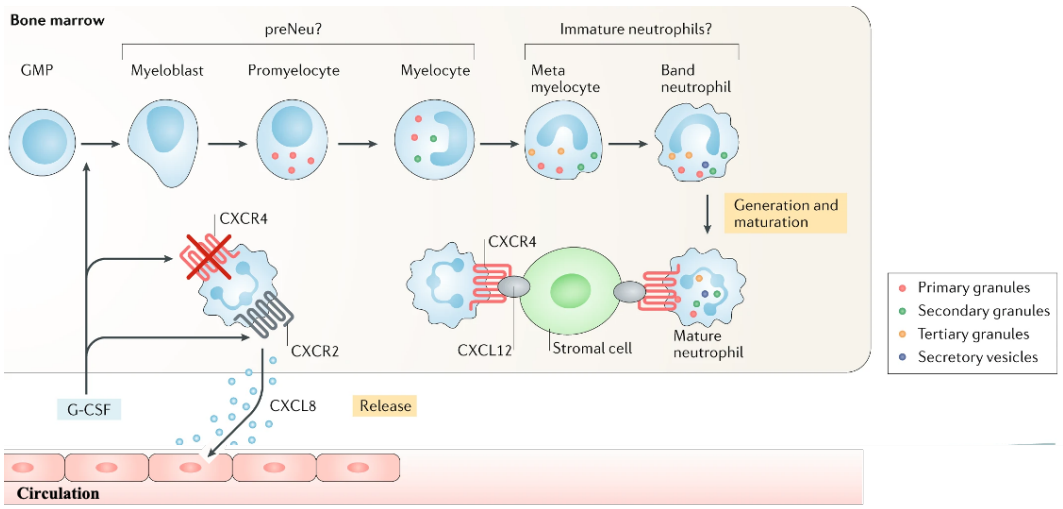

Figure 7. Generation and maturation of neutrophils occur in the bone marrow*. Neutrophil maturation is characterized by the formation of the different granules and secretory vesicles, along with nuclear segmentation. Granulopoiesis begins with the development of azurophilic granules in myeloblasts and early promyelocytes and ends after the creation of secretory vesicles in mature, segmented cells. (Adapted from Németh et al, 2020)*[125]

1.5.3 Neutrophil toxicity and the role of granule content

Four major classes of granules have been identified in both human and mouse neutrophils: azurophilic granules are characterized mainly by antimicrobicidal molecules; specific and gelatinase granules are used when moving from the circulation to tissues while still retaining some antimicrobial functions; and secretory vesicles are enriched in proteins required for neutrophil recruitment and extravasation[126].

Azurophilic granules. Azurophilic granules are the primary microbicidal granule in neutrophils characterized by the presence of several antimicrobial proteins: oxidant-producing enzymes such as myeloperoxidase (MPO), proteases such as cathepsin G, proteinase 3 and elastase, and membrane-permeabilizing proteins such as lysozyme[127]. Azurophilic granules demonstrate considerable heterogeneity in their size and shape, which is directly regulated by granular protein synthesis and packaging[128,129]. Even though azurophilic granules are packed with acidic hydrolases and microbicidal proteins, they are generally defined by their high content of MPO, which accounts for 5% of the neutrophil's total protein content[130]. MPO is typically released into phagolysosomes by the fusion of azurophilic granules and phagosomes. Phagolysosomes are vital neutrophil organelles, as they provide a confined location for toxic oxidative reactions designed to kill pathogens while protecting host tissue against harmful metabolites[131]. NADPH oxidase, localized on the phagolysosome membrane, mediates the production of ROS by the conversion of oxygen to superoxide. Superoxide dismutase then enables the conversion of O_2^- to hydroxydioxylic acid and hydrogen peroxide, both of which with low bactericidal activity[132]. MPO on the other hand catalyzes the oxidation reaction between hydrogen peroxide and chloride to form hypochlorous acid,

hydroxyl radicals, and chloramines, all of which are potent oxidants that contribute to the neutrophil's killing capabilities[133]. MPO can also bind to the surface of neutrophils and platelets via electrostatic carbohydrate-dependent mechanisms, thereby triggering proinflammatory functional activities[134,135]. The priming and activation of neutrophils by inflammatory mediators, such as Toll-like receptor (TLR) ligands, GM-CSF, TNF-α, and Ig/Fc receptor-mediated signaling, can liberate MPO extracellularly by both degranulation and cell death pathways[136,137]. Once in the extracellular space, MPO can bind to the plasma membrane via CD11b/CD18 receptors, provoking the degranulation of azurophilic and specific granule substances in a dose-dependent manner via the induction of tyrosine kinase, phosphatidylinositol 3-kinase, and calcium signaling pathways[138]. Additionally, azurophilic granules contain serine proteases, including proteinase 3, cathepsin G, elastase, and neutrophil serine protease 4, which display proteolytic enzymatic activity against extracellular matrix components such as elastin, fibronectin, vitronectin, laminin, and type IV collagen[122,139]. Excluding neutrophil serine protease 4, the serine proteases are potent substances that can also induce the activation of macrophages, lymphocytes, endothelial cells and platelets[122,139]. Specific functions of elastase include antimicrobial activity against Gram-negative bacteria. Both elastase and cathepsin G also have potent microbicidal activity against fungal species[140,141]. Other vital microbicidal peptides in the azurophilic granule include α-defensin, azurocidin, and bactericidal/permeability-increasing protein. α-Defensin, making up at least 5% of the protein content of neutrophils[142], has antimicrobial activity against a variety of microorganisms through the creation of multimeric transmembrane pores in the microbial outer membrane ([143]). Following extracellular exocytosis, α-defensins also induce the chemotaxis of monocytes[144], $CD4^+$ and $CD8^+$ T cells[145].

Specific granules. Specific (secondary) granules are rich in antibiotic substances that participate in neutrophil microbicidal activities either upon their mobilization within the phagosome or through their release into the extracellular milieu. Lactoferrin, a primary specific granule protein, has direct bacteriostatic and bactericidal activities against viruses, Gram-positive bacteria, Gram-negative bacilli, and fungi[146]. By sequestering iron in biological fluids, lactoferrin is also able

to disrupt and destabilize microbial cell membranes[147]. Additionally, lactoferrin can also play an important role in the resolution of inflammatory reactions. Lactoferrin can impair ROS production[148] and sequester LPS and CD14, thereby preventing the development of the proinflammatory pathway and tissue damage[149]. On the other hand, lactoferrin also modulates adaptive responses by speeding up the maturation of T-cell precursors into competent CD4+ T helper cells[150] and enhancing the differentiation of immature B cells into antigen-presenting cells[151]. Specific granule proteins also include other molecules that play important roles in the inflammatory response. Lipocalin-2 (LCN2) exhibits antimicrobial properties and works in coordination with lactoferrin by binding siderophores, limiting the availability of iron to interfere with microbial growth[152]. Due to its ability to bind N-formylmethionine-leucyl-phenylalanine in vitro, LCN-2 is also believed to bind lipophilic inflammatory mediators such as LPS, platelet-activating factor, and leukotriene B4[153]. Resistin, a proinflammatory cytokine that also localizes to the neutrophil's cell membrane, can limit the accumulation of neutrophils at sites of inflammation by inhibiting chemotaxis through the induction of NF-kB activity[154].

Gelatinase granules. Gelatinase (tertiary) granules carry several proteins required for neutrophil extravasation and migration, indeed they are mobilized when the neutrophil establishes primary rolling contact with the activated endothelium[155]. These granules contain matrix-degrading enzymes, such as gelatinase, and membrane receptors including CD11b/CD18, CD67, CD177, N-Formylmethionyl-leucyl-phenylalanine receptor, secretory carrier membrane proteins, and vesicle-associated membrane protein (VAMP) 2[123], which are important in the earliest phases of the neutrophil inflammatory responses and extravasation into inflamed tissues. Arginase 1, a key gelatinase protein, metabolizes arginine, reducing its availability as a substrate for nitric oxide synthase. This reaction leads to the diminished synthesis of nitric oxide, which is generally associated with endothelial dysfunction. By promoting the production of ornithine, arginase 1 also diminishes pro-inflammatory immune responses and fosters tissue regeneration[156].

Secretory vesicles. Not considered to be true neutrophil granules, secretory vesicles constitute an important reservoir of membrane-associated receptors, including CD10, CD11b/CD18, CD15, CD16, CD35, matrix metalloproteinase (MMP)-25,

secretory carrier membrane proteins, VAMP2, Natural resistance-associated macrophage protein 2, LFA-1, and macrophage 1 antigen, as well as actin, actin-binding proteins, and alkaline phosphatase that are required at the earliest phases of neutrophil-mediated inflammatory responses[157]. Secretory vesicles are significantly smaller than neutrophil granules and are the most exocytosed organelles in the neutrophils. Located throughout the cell's cytoplasm, these organelles contain cell membrane receptors that are vital for neutrophils to establish firm contact with the vascular endothelium, complete diapedesis into inflamed tissues, and undergo chemotaxis-directed migration within the inflamed areas[123].

1.5.4 Degranulation and exocytosis of neutrophil granules.

Exocytosis of neutrophil granules occurs hierarchically and is inversely related to granule production[158,159]. Consequently, low cellular stimulation/activation is sufficient to incite the release of secretory vesicles, while increasing stimulus strength is needed for the release of gelatinase, specific, and, finally, azurophilic granules[159]. Azurophilic granules are unique among the neutrophil granules not only because they require a strong agonist to promote the degranulation of their contents but also because the biggest percentage of primary granules are mobilized to phagosomes. Still, a small amount of their content can be exocytosed extracellularly[160]. Moreover, recently identified variations in soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) complexes explain these differences: whereas all neutrophil granules contain syntaxin 4 and SNARE complexes, specific and gelatinase granules present SNARE complexes with high concentrations of VAMP1, VAMP2, and 23-kDa synaptosome-associated protein 23. In contrast, azurophilic granules have increased levels of VAMP1 and VAMP7[160]. SNARE-interacting Sec1/Munc18 (SM) family members, particularly MUNC18-2 and MUNC18-3, also play critical roles in selective vesicular trafficking in neutrophils[161]. MUNC18-2 and MUNC18-3 are located near their respective SNARE-binding partners' syntaxin 3 and syntaxin 4. MUNC18-2 is preferentially associated with regulating azurophilic granular exocytosis, while MUNC18-3 regulates the degranulation of specific and gelatinase granules[161]. In summary, multiple mechanisms regulate neutrophil granular exocytosis. Specificity in exocytosis pathways provides important insights into neutrophil biology. Whereas specific and gelatinase, granules specialize in early inflammatory responses involving cell adhesion, diapedesis, and microorganism killing, azurophilic granules contain a wide array of damaging lytic enzymes and proteins with bactericidal activity that can be detrimental to surrounding tissue if secreted in an uncontrolled fashion[162]. All secretory vesicles fuse with the surface membrane during diapedesis, 40 % of gelatinase granules, 25 % of specific granules, and only 7 % of azurophil granules fuse with the surface membrane during this process[163]. Mobilization of secretory vesicles during diapedesis is fundamental considering that degranulation of their content furnishes the surface membrane with receptors

relevant for diapedesis without liberating anything but plasma proteins, thus causing no damage to the vascular endothelium[164]. Mobilization of gelatinase from gelatinase granules is also considered important in neutrophil mobilization, providing the needed molecules for the degradation of the basement membrane, otherwise impeding the further movement of neutrophils[165].

1.6 NEUTROPHILS IN ALZHEIMER'S DISEASE

A combination of experimental and clinical evidence, including our own research, suggests that immune system cells were found to migrate in the AD brain and play critical roles in neurodegeneration processes and cognitive decline. Moreover, leukocytes, including neutrophils, can be found in the proximity of Aβ plaques in the AD brain[75]. In order to perform their activities, neutrophils are highly adaptable cells due to their remarkable plasticity and high turnover. They can therefore adjust their phenotype and functions in response to various environmental stimuli, enhancing acute inflammatory responses. However, neutrophils often play a detrimental role when prolonged inflammation sustains, leading to chronic tissue damage and promoting pathological conditions such as autoimmunity and neurodegenerative diseases[166]. Emerging evidence reveals that neutrophils themselves are essential protagonists of AD pathogenesis at different levels. They release increased amounts of ROS, which in turn decreases levels of tissue inhibitors of metalloproteinase, thereby upregulating MMP-9 and increasing the BBB permeability through tight junction impairment (Sayed A. 2020). Work from our group performed on mouse models revealed that neutrophils infiltrate the brain before the onset of cognitive decline and peak at the onset of memory impairment, continuing their infiltration at later disease stages as well. These data suggest that neutrophils have a role in the induction of cognitive deficit and AD progression[75]. Additionally, we found that the integrin LFA-1 is necessary for neutrophil adhesion in brain vessels, and neutrophils from LFA-1 deficient mice are unable to adhere or crawl in blood vessels and thus cannot transmigrate in the brain parenchyma of AD mice. Accordingly, blocking the integrin LFA-1 and neutrophil adhesion has a therapeutic effect on mouse AD models, suggesting a prominent role for LFA-1 in neutrophil trafficking and AD pathogenesis[75]. Neutrophils were also found to contribute to brain damage by activating microglia, which in turn leads to the further activation of neutrophils and exacerbation of neuroinflammation. As shown in transgenic mice with AD-like disease, neutrophils produce intravascular NETs, suggesting these cells may promote the loss of BBB integrity and thus contribute to the pathogenesis of AD in humans[75].

Few studies have investigated the role of neutrophils in AD, although recent reports suggest their key role in AD pathogenesis[73,75,167,168]. Circulating neutrophils from AD subjects express higher levels of CD11b compared to healthy subjects, suggesting that adhesion and infiltration into the brain may correlate with the severity of the disease[169]. Recent clinical data revealing greater numbers of neutrophils, or a higher neutrophil to lymphocyte ratio associated with AD suggest that changes in the neutrophil population could be used as markers of AD-related peripheral inflammation[170,171]. Peripheral blood neutrophils in AD patients also produce larger quantities of ROS than healthy subjects, suggesting once more that neutrophils present a more activated state during AD[172]. In agreement with this evidence, a recent pilot study aimed to characterize the phenotype of human peripheral neutrophils at different stages of the disease, showing a neutrophil hyper-activation state in fast-decliner compared to slow-decliner patients, supporting the association of peripheral neutrophil phenotypes with AD cognitive decline[173].
Assuming the prominent role played by neutrophils during infection, their large variety of receptors for the recognition of PAMPs or damage-associated molecular patterns, and the proposed co-evolution of microbes and immune cells, it is not excluded that microbes influence multiple aspects of neutrophil biology. For instance, commensal microbes control the production of neutrophils and their phagocytosis capacity, increasing the opportunity that these factors also affect the aging of peripheral neutrophils[174].

1.7 INTESTINAL DYSBIOSIS IN ALZHEIMER'S DISEASE

In the past decade, the term gut-brain axis has received increasing attention especially when it comes to neurodegeneration and neuroinflammation. Indeed, the finding that several neurologic conditions are accompanied by microbiota dysregulation has further supported a strong link between the periphery and the brain[175]. Reports on both AD patients and animal models have described an alteration of the gut microbiota during disease. These microbiota studies have shown alterations in Firmicutes/Bacteroidetes ratio, a common indicator of dusbiosis[22]. Furthermore, in a group of patients with cognitive impairment and brain

amyloidosis, Cattaneo et al. showed an increased abundance of *Escherichia/Shigella*, a proinflammatory taxon and a decreased abundance of *E. rectale*, an anti-inflammatory taxon, indicating the intestinal pro-inflammatory response[176]. This has also been supported by studies using AD animal models where the authors have described similar findings[177–179]. Interestingly, the gut microbiota has been described to modulate CNS function both in homeostasis and pathology. This modulation of brain behavior by the gut microbiota has been associated with three main mechanisms: 1) bacteria-derived products including gamma-amino butyric acid, serotonin, histamine, and dopamine[180,181]. 2) neural transmission from the vagus nerve directly into the CNS[182]. 3) Immunomodulation of immune cell populations not only in the intestine tissue but also in distant tissues[183,184]. Interestingly, these mechanisms have been addressed and shown to be dysregulated in AD. The production of neuromodulators such as serotonin has been described to be associated with MCI and AD in a fecal metabolomics study. The authors have shown alterations in the tryptophan pathway, where a reduction of the serotonin precursor 5-HTP positively correlated with cognitive impairment in AD[185]. The vagus nerve constitutes an important direct communication between the gut and the CNS. Studies on the role of the vagus nerve as a CNS seeding pathway for alpha-synuclein in PD have opened a door for other neurologic disorders. In AD, vagus nerve stimulation has been described as a promising therapy to slow down disease progression in different clinical studies[186]. Furthermore, studies of gastrointestinal administration of aβ in mice have showed its propagation to the CNS, further supporting a possible route for gut-brain communication in AD[187]. Lastly, modulation of immune cell populations by the gut microbiota constitutes one important detrimental mechanism possibly responsible for AD pathogenesis. Given the involvement of the CNS-associated immune system in AD pathology, most studies addressing the immunomodulatory effect of the microbiota in AD were mainly focused on the microglia, without many studies addressing infiltrating leukocytes. Still, studies involving microbiota depletion using antibiotics or germ-free mice showed decreased microglial activation and interestingly, an amelioration of AD hallmarks such as amyloid deposition[179,188].

CHAPTER II

AIMS OF THE STUDY

A growing body of evidence has pointed out the gut microbiota as a key player in neurodegenerative disorders. Indeed, the gut-brain axis has been described to influence disease pathology in disorders such as Parkinson's Disease, Multiple sclerosis (MS) and amyotrophic lateral sclerosis[175]. Most of these studies, however, have only provided a small characterization of microbiota dysbiosis and microbial-derived products in patients, without focusing on the mechanisms of this dysregulation in disease development. In this thesis, we aimed at better understanding the intestinal inflammatory mechanisms contributing to neutrophil activation in AD. Recent evidence has pointed to a broad dysregulation of microbiota composition in both animal models and human AD patients[189], however, there is still a lack of knowledge regarding intestinal inflammation and how dysbiosis may contribute to systemic inflammation and cell activation. Thus, our **first aim** was to study intestinal dysbiosis and inflammation in the 3xTg-AD animal model of AD and establish a link between microbiota and neutrophil dysfunction in AD.

We have previously shown that neutrophils are increased in the AD brain. Also, by targeting neutrophil infiltration, either by blocking LFA-1 or by depleting neutrophils, we have seen an amelioration of the cognitive deficits, further suggesting a key role for these cells in neuropathological changes in AD. Still, the mechanisms behind neutrophil-mediated pathology have not been addressed yet. While cytotoxic NETs have been observed in AD, these are usually observed in the vasculature and few are observed in the brain parenchyma[190], suggesting its role in mediating BBB damage instead of direct neurotoxicity. Neutrophils, however, possess a wide array of lytic/damaging molecules that if released uncontrollably, can lead to cytotoxicity and tissue injury. In the light of these considerations, the **second aim** of this thesis was focused on understanding how CNS infiltrating neutrophils lead/contribute to neuronal damage by characterizing neutrophil phenotype and neutrophil-mediated neurotoxic mechanisms in AD.

CHAPTER III

MATERIALS AND METHODS

Mice

3xTg-AD mice (MMRRC stock no. 34830-JAX) and WT control B6129SF2/J (stock no. 101045) were purchased from the Jackson Laboratory. 3xTg-AD mouse expresses the human mutant APP and PS1M146V, associated with familial AD, and the TauP301L allele. Therefore, it develops both amyloid and tau pathologies. Animals were housed in pathogen-free and climate-controlled conditions, provided with food and water *ad libitum*. The experiments were conducted following the principles of the NIH Guide for the Use and Care of Laboratory Animals and the European Community Council.

Primary human cells

Human studies were approved by the University of Verona Ethics Committee. Neutrophils and lymphocytes were isolated from peripheral blood of healthy donors by discontinuous density Ficoll gradient separation. After erythrocyte sedimentation (4:1 ratio of blood to 4 % dextran), the neutrophil cell suspension was washed in PBS 1X and erythrocyte lysis was performed (0.2 % hypotonic NaCl solution followed by 1.2 % isotonic NaCl solution).

Primary murine neutrophils

Neutrophils were isolated from the bone marrow of 3 months of age WT mice. Tibias and femurs were surgically removed, and bone marrow cells were rapidly flushed out of the bones with Hank's Balanced Salt Solution (HBSS) 1X supplemented with 0.1 % Bovine Serum Albumin (BSA). After erythrocyte lysis (as explained in paragraph n2), cells were stratified onto discontinues Percoll gradient. After 30min of centrifugation, the neutrophil ring was collected.

Primary cultures of neurons

Brains isolated from new-born 3xTg-AD mice were dissociated using the Adult Brain Dissociation kit and a gentleMACS dissociator (Miltenyi Biotec) following the manufacturer’s nstructions. Cells were incubated with a non-neuronal cell biotin-antibody cocktail (Miltenyi Biotec) to isolate neurons. $2x10^5$ neurons were seeded in Neurobasal medium supplemented with B-27, 1% glutamine and 1%

penicillin/streptomycin in 48-well plates pre-coated with poly-D-lysine and laminin. After 3 days of culture, neutrophils were isolated as previously described and placed over neurons for 2 hours at a ratio of 1:1. Neurons were gently washed and stained with PI for dead cells for 5 minutes before acquisition.

Microbiota

Samples were collected, immediately frozen and stored at –80°C for microbiota analysis. Briefly, quantitative polymerase chain reaction (PCR) and targeted metagenomics sequencing (Illumina MiSeq V3) were used to profile the bacterial populations based on variable regions (V3-V4) of the 16S rDNA bacterial gene. For data analysis, DADA2 software was used, an open-source package that is able to model and correct Illumina-sequenced amplicon errors, thus allowing to discriminate between biological variation and amplicon sequencing errors. DADA2 generates as output a list of operational taxonomic units (OTUs, clusters of sequences that differ by less than a fixed dissimilarity threshold). The taxonomical assignment was performed using reference databases such as RDB, GreeneGenes or SILVA. We used different statistical analyses to assess the diversity between and among the sample groups using α and the β diversity that acts as a similarity score between populations. We also performed multivariate analysis such as PERMANOVA combined with phenotypic variables to assess the effect of the different factors (such as genotype and sex) on the community structure. The differential abundance was analyzed with Metastats (based on nonparametric t-test using permutation for non-sparse feature and exact feature test for sparse feature), LEFSe (couples the statistical analysis with the effect size estimation to achieve a robust biomarker discovery) and MetagenomeSeq (a novel normalization technique and a statistical model based on a zero-inflated Gaussian distribution that accounts for under-sampling). The main output was a taxonomic abundance matrix at several levels: kingdom, phylum, class, order, family and genus.

Intestine motility measurements

For measurement of total intestinal transit time, mice were individually housed in separate cages and were given an oral gavage of 6% carmine red (Sigma-Aldrich),

0.5% methylcellulose (Company) (prepared with sterile 0.9% NaCl). Individual starting time was registered at the moment of gavage and intestinal transit time was recorded when the first red fecal pellet was observed. Experiments were ended after 450 min.

Serum collection

Blood was collected from the retro-orbital plexus of anesthetized mice, using heparinized capillaries. The blood was allowed to rest for 1 hour and then centrifuged for 10 min at 2000rpm. The supernatant was collected into new Eppendorf and centrifuged again at 10000 rpm for 2 min. Serum was then collected and stored at -80ºC until further analysis.

Dextran FITC intestinal leakage

Fluorescein Dextran (Molecular weight 4000 Da, diluted in NaCl) was gavaged (250 mg/kg mouse). Five hours later, mouse blood samples were collected from the retro-orbital plexus of anesthetized mice and serum was obtained as previously described. Fluorescence intensity of the serum was measured on a fluorescent plate reader. To correctly quantify the amount of dextran in the samples, a standard curve was performed using ungavaged mouse serum for background control.

Intestinal histology

1cm pieces of intestine from Ileum and colon were gently cleaned of intestinal contents, embedded in optimal cutting temperature medium, frozen using liquid nitrogen and stored at -80ºC until sectioning. 10 μm sections were obtained using the cryostat and left to air-dry for 1 hour at room temperature (RT). For the histology of the intestine, sections were fixed with formalin 10% for 10 min and rehydrated with phosphate-buffered saline (PBS). Periodic acid-Schiff (PAS) staining was performed by applying 0.5% periodic acid for 5 min followed by a quick passage in distilled water. Schiff's reagent was then applied for 15 min and washed in running tap water for 1 min. For the counterstain, slides were incubated for 1 min in hematoxylin and washed with running tap water for 1 min. Slides were then dehydrated and mounted with Eukit mounting medium.

Tissue homogenates and cDNA synthesis

50 mg-pieces of tissue were cleaned of contents with PBS and snap-frozen using liquid nitrogen. 1.4mL of RLT lysis buffer (Qiagen) was added to a miltenyi M tube together with the frozen tissues and gentleMACS octo dissociator was used with a pre-set program for RNA processing to obtain tissue homogenates. 350 μL of homogenate were processed for RNA extraction using the RNeasy extraction kit (Qiagen) according to the manufacturer's instructions. A step of DNase treatment (Qiagen) was performed for 15 min at RT. The extracted RNA was then quantified using nanodrop. Only high purity samples (A260/280 and A260/230 higher than 2) were used. cDNA synthesis was performed using Superscript III (Invitrogen) according to the manufacturer's instructions. 1 μg of total RNA for each sample was used as starting material.

Quantitative PCR

Quantitative PCR was performed using power up SYBR green master mix 2X (Applied biosystems). 1 μL of cDNA was used as template in a final volume of 10uL. Primers for RNA expression analysis are listed below and were used at a final concentration of 300 nM. Data were normalized using GAPDH as an endogenous control. Relative changes in gene expression between 3xTg and WT samples were determined using the 2-ΔΔCt method. Levels of the target transcript were normalized to a GAPDH (ΔCt). For ΔΔCt values, additional subtractions were performed between 3xTg and WT ΔCt values. Final values were plotted as fold of change.

Intestinal preparations and muscularis externa dissection

Intestinal sections of 2-3 centimeters (Colon and Ileum) were dissected and promptly cleaned with PBS. The lower extremity of the intestine pieces was closed with a nylon string and filled with fixative (4% formaldehyde with 0.4 mM picric acid in PBS) using a blunt needle. When full, the upper extremity was also closed with a nylon string and left on fixative for 1 hour at RT. Afterward, the extremities

were cut and tissue was cleaned with PBS and kept at 4ºC. 0.5 centimeters of intestinal sections were cleaned of mesentery and opened lengthwise along the mesenteric border. The tissue was placed mucosa side down and the muscularis externa peeled off. The muscularis externa was then pinned on black silicon blocks.

Whole-mount immunofluorescence

Muscularis externa peels were incubated in blocking solution containing 2% BSA in PBS and 0.3% Triton X-100 overnight at 4'C. The tissue was incubated in primary antibodies diluted in the blocking solution for 48 h at 4 °C, washed three times with PBS (30 min each) and then placed in the secondary antibodies diluted in the incubation buffer for 2 h at RT. The tissue was washed three times with PBS (15 min each) and incubated with DAPI for 10 min. After two washes (10 min each), the muscularis externa peels were mounted using DABCO mounting media. Specifications of all the antibodies and fluorochromes can be found in table A1.

Blood collection and isolation of leukocytes

Blood was collected from the retro-orbital plexus of anesthetized mice, using heparinized capillaries, and mixed with PBS 1X containing 1% (v/v) dextran and 10U/mL Heparin (in the ratio of 1:1). After incubation at RT for 1 h, the supernatant was collected and washed with PBS 1X and centrifuged at 300 g for 10 min; thus, the supernatant was discarded. The hemolysis was performed first by adding 3mL of NaCl 0,2% and gently shaking for 40 seconds; rapidly, 7mL of NaCl 1,2% were added in order to block the reaction. PBS 1X was added for washing and so centrifugation was performed at 300 g for 10 min. The supernatant was removed, and the pellet was resuspended in PBS 1X with 10% fetal bovine serum (FBS).

Lipopolysaccharide-binding protein quantification

The quantification of lipopolysaccharide-binding protein (LBP) was performed using the murine LBP ELISA kit from Hycult biotech according to the manufacturer's instructions. Briefly, samples were allowed to thaw, and all the reagents were allowed to reach RT. The dilutions of the samples used in the ELISA were 1:300, 1:600 and 1:1200 and the plate was read at 450 nm, with the wavelength

correction set at 570 nm. The LBP standard curve was set from 0.8ng/mL to 50 ng/mL and only the values within the standard curve were considered.

Flow cytometry analysis

Isolated cells were resuspended in 100µl of PBS 1X + 10% FBS. After that, 1µl of FcR Block (BD) was added to each sample at a final concentration of 3,3 µg/mL, leaving it at RT for 15 min. Cells were directly labeled for 25 min at 4℃ in the dark with anti-mouse antibodies purchased from commercial sources. PBS 1X + 10% FBS was used for washing, and a centrifugation was performed at 300 g for 10 min. The cells were then resuspended in fluorescence-activated cell sorting (FACS) buffer. In order to measure the vitality, 2 µl of 7-AAD per million cells were added 5 min before reading. The samples were acquired by flow cytometry using a BD LSRFortessaTM X-20 cell analyzer and data were analyzed using FlowJo software. Specifications of all the antibodies and fluorochromes can be found in table A1 while the gating strategy is depicted in Figures A1 and A3.

Cell treatment

2×10^5 of neutrophils, isolated as described above, were resuspended in 100 µL of HBSS with FBS. Then, cells were incubated with Cytochalasin B (for 15min at 37°C. Control and vehicle samples were treated with dimethyl Sulfoxide (Sigma-Aldrich d8418). The cells were then stimulated with 5 µM $A\beta_{1\text{-}42}$ for 30 min, washed and stained for flow cytometry analysis. In some cases, cells were pre-treated with Ritonavir for 30 min before stimulation or co-culture.

Viability assay

Vybrant™ DyeCycle™ Violet/SYTOX™ AADvanced™ Apoptosis Kit (Invitrogen) was used to identify viable cell fractions. 500.000 cells were resuspended in 100µL of probe mix (998µL of HBSS 1X, 1 µL of Vybrant™, and 1µL of SYTOX™) and incubated for 15min protecting from light. Directly after staining, cells were passed on a flow cytometer (MACSQuant Analyzer Miltenyi).

Intracardial perfusion

Mice were exposed to isoflurane inhalation, and once anesthetized, they were placed on a surgical support, flatting on their backs. After stretching and pinning the paws, skin and ribs were cut to make the heart accessible. The right atrium was incised, while a butterfly needle connected to a peristaltic pump (Minipuls3 GILSON®) was inserted in the left ventricle. The pump injected 25 mL of PBS 1X supplemented with 1 mM Ca^{2+}/Mg^{2+} (buffer solution) into the systemic circulation.

Isolation of brain infiltrating leukocytes

Mouse perfused brains were collected and homogenized by Gentle MACSTM Octo Dissociator (Miltenyi Biotec). The tissue was digested with 40 U/mL of DNaseI (Thermo Fisher) and 1 mg/mL collagenase (Sigma) at 37 °C for 45 min. After washing and centrifugation, the pellet was resuspended in 30 % Percoll. The cell suspension was filtered through a 70-µm cell strainer and loaded onto 70 % Percoll. Leukocytes were isolated at the interface of the discontinuous density gradient, washed and resuspended for further analysis.

Scrna-seq analysis of brain infiltrating leukocytes

Leukocytes were isolated from the brains of 6 month-old WT and 3xTg-AD mice, washed with PBS, and labeled with an anti CD45–BV480 antibody (Becton Dickinson). The cells were re-suspended in PBS supplemented with 10% fetal bovine serum (FBS), and viable CD45high cells were sorted on a FACSAria Fusion (Becton Dickinson). Both the 3xTg-AD and WT samples consisted of > 98% viable cells. Sorted cells were resuspended to a final concentration of 700 cells/µl and cDNA sequencing libraries were prepared using the 10′ Genomics Chromium Controller and the Chromium Single Cell 3′ GEM, Library and Gel Bead kit v3 (Pleasanton) following the manufacturer's instructions. Briefly, 10,000 live cells were loaded onto the Chromium Controller to recover 4000 single-cell gel-bead emulsions (GEMs) per inlet, uniquely barcoded. After cDNA synthesis, sequencing libraries were generated and final 10× library quality was assessed using the Fragment Analyzer High Sensitivity NGS kit (Agilent Technologies) before sequencing on the Illumina NextSeq500 platform, generating 75-bp paired-end

reads (28 bp read 1, 91 bp read 2) at a depth of 50,000 reads per cell, yielding a median per-library depth of 72,783 reads per cell.

Dimensionality reduction and clustering

The zinbwave package (v1.14.0)77 was used for dimensionality reduction, specifying K = 20 factors, using the 1000 most variable genes and accounting for the genotype effect. Clustering was carried out using the FindNeighbors and FindClusters functions of the Seurat package (v4.0.1)78-81, using the dimensionality-reduced matrix from zinbwave as input and using the smart local moving clustering algorithm (algorithm = 3; resolution = 0.5). To visualize the cells and the clusters in two dimensions, the t-distributed stochastic neighbor embedding (t-SNE) projection was computed, starting from the 20 zinbwave factors, using the runTSNE function of the scater package (v1.20.0)82.

Data normalization

Normalization factors were computed from the joint dataset using the computeSumFactors function of the scran (v1.20.0) package83, providing it with cell clusters obtained using the quickCluster function (parameters min.mean = 0.5, block = genotype). We then used the normalize function of the scater package to apply the normalization factors and obtain the log-normalized counts.

Cell classification, marker gene identification and enrichment analysis

Cell types were identified using the SingleR (v1.6.0) 84 package with the built-in reference database based on the Immunologic Genome Project, using fine labels. A filter was applied to remove genes that were found in less than 5% of the cells. Finally, we used the findMarkers function of the scran package to test for Differentially expressed genes (DEGs) between genotypes, within the population of neutrophils. DEGs were enriched using clusterProfiler (v4.0.0)85 as well as Gene Ontology (GO) biological processes terms database, to retrieve meaningful biological information about biological processes and pathways. GO analysis provided a list of terms involving the set of DEGs.

Statistical analysis

All data were represented using Prism 9 (GraphPad Software), which was also used for the statistical analysis. A two-tailed Student's *t*-test was used for the statistical comparison of two samples. One-way analysis of variance (ANOVA) followed by Dunnett's multiple comparisons test was used to determine differences in means among multiple comparisons. Quantitative data are shown as mean values ± standard error (SEM). $P < 0.05$ was considered statistically significant.

CHAPTER IV

RESULTS

4.1 GUT DYSBIOSIS INDUCES SYSTEMIC INFLAMMATION AND CONTRIBUTES TO PERIPHERAL NEUTROPHIL DYSREGULATION IN A MOUSE MODEL OF AD

4.1.1 3xTg-AD mice show intestinal dysbiosis at disease onset

Data from several neurodegenerative disorders point to a strong correlation between intestinal health and disease pathogenesis. Starting with PD and the description of the seeding of α-synuclein through the vagus nerve, more and more studies have addressed the intestinal influence also in disorders such as stroke, MS and AD[175,189]. Indeed, even though still in the first steps, research performed in AD patients and mouse models showed consistent intestinal dysbiosis[189]. Furthermore, reports showing increased levels of LPS in the circulation of AD patients provide evidence of microbial-derived products outside the borders of the intestinal tract supporting the role of intestinal dysfunction in AD[191]. To address this question, we used 16S rRNA gene sequencing to analyze and compare differences in the composition of gut microbiota between 3xTg-AD mice and age- and sex-matched healthy WT mice. We evaluated the microbiota composition in the small intestine of WT (female n = 7, male n = 7) and 3xTg-AD (female n = 8, male n = 8) mice at 6 months of age. This time point represents the onset of cognitive deficits and brain pathology in the transgenic AD-like mice[192]. Firstly, we evaluated the community composition of WT and 3xTg-AD mice by principal coordinates analysis (PCoA) plots of Bray–Curtis dissimilarity, a mathematical calculation of the major differences. The gut microbiota of 3xTg-AD mice was separated from that of WT (Figure 8a). Moreover, female and male 3xTg-AD mice clustered distinctly but closely and far away from that of the control WT mice, where instead, WT female and male clustered closely together (Figure 8b). These results indicate that the microbial community of 3xTg-AD mice is altered compared to WT mice, with slight differences between female and male.

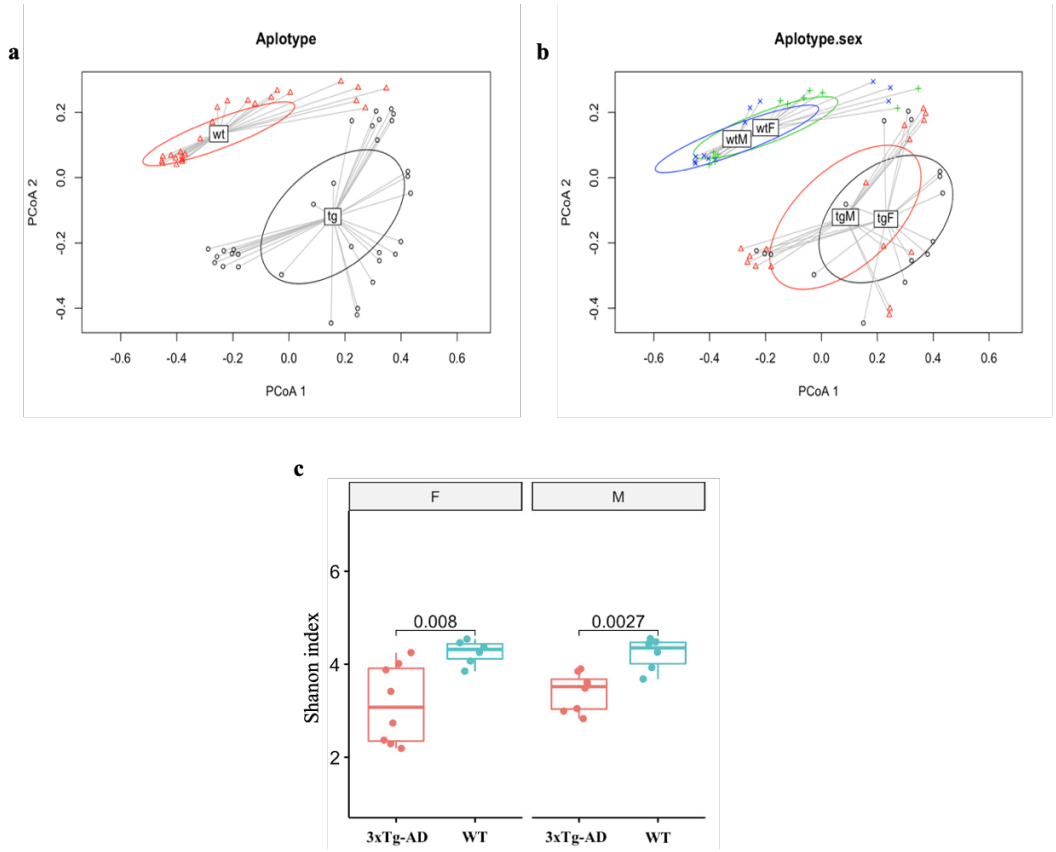

Figure 8. Microbiota analysis identifies differences between AD and WT mice. *Fecal samples were collected from the small intestine of 6 month-old animals. (a) Principal component analysis of microbiota in 3xTg-AD mice and WT mice shows a clear separation between AD and WT animals. (b) PCA also showed a slight difference between male and female mice in the 3xTg-AD group. Ellipses in both panels represent the spatial distribution of mice in each group. (c) α diversity of the microbiota in 3xTg-AD and and age- and WT control mice shows a clear reduction in the microbiota diversity (Shanon index).*

The α-diversity, on the other hand, refers to the diversity of bacteria or species within a community and is mainly related to the number of bacteria or species. Given that microbiota composition can drive sex-specific disease susceptibility[193], we then asked whether this was the case also in our model of AD-like disease. The cohort of 6-months-old 3xTg-AD mice compared to WT mice showed a significant lower α-diversity (Shannon index) in the gut microbiome in the small intestine (Figure 8c), suggesting a shift in microbial composition, with the disappearance of some microbial species. To further understand the dysbiosis observed in our mouse model, we further investigated the microbial composition in 3xTg-AD mice. The

microbial community structures displayed distinct differences between the two groups, and we observed important changes in the taxonomic profile at all levels. Particularly, we observed higher abundances of taxa known to cause pro-inflammatory conditions and a lower proportion of bacteria with the potential to synthesize short-chain fatty acids (SCFAs), known for their anti-inflammatory properties. An unbalanced gut microbiota often arises from a sustained increase in the abundance of the phylum *Proteobacteria*, and the natural gut flora normally contains only a minor proportion of this phylum shown to be related to inflammation[194] and an increased risk of dementia[195]. Indeed, our data showed that the gut microbiota of 3xTg-AD mice is characterized by an increase in the proinflammatory *Gamma-* and *Beta-proteobacteria*, whereas the class of *Clostridia, Deltaproteobacteria, Saccharimonas* and *Coriobacteriia* are significantly decreased (Figure 9).

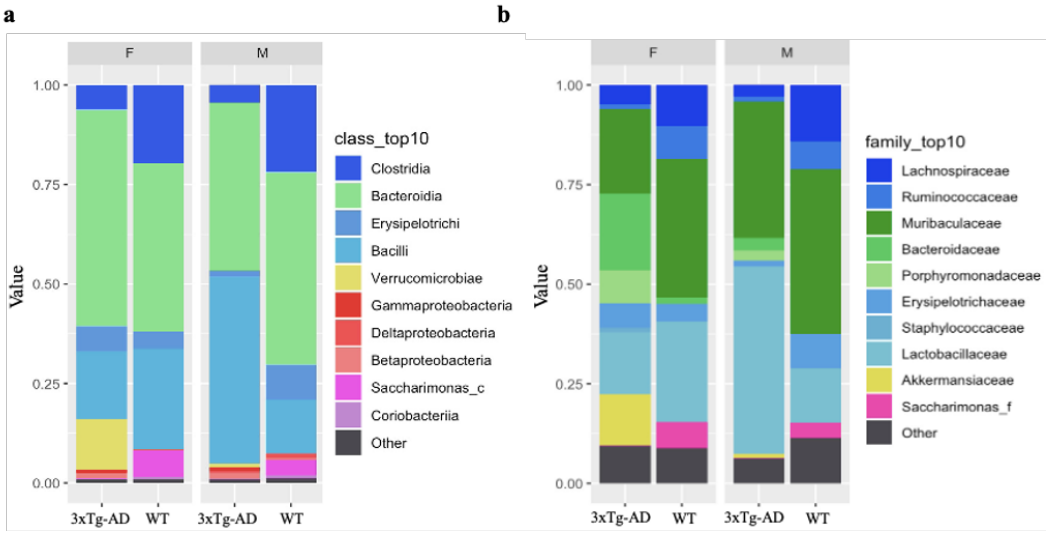

Figure 9. Taxonomic analysis of the intestinal microbiota in 3xTg-AD and age- and sex-matched WT control mice. *Phylogenetic composition of intestinal microbiota analyzed by (a) class and (b) family, showing intestinal dysbiosis in the 3xTg-AD mouse model.*

4.1.2 Intestinal inflammation and structural changes in the ileum of 3xTg-AD mice

The gastrointestinal epithelium is exposed to a broad range of challenges such as food components, bacterial metabolites or toxins, and it might also be compromised by aging or varying microbial compositions[196–198]. A compromised barrier may culminate in a 'leaky gut'[199], allowing the intrusion of bacteria, their metabolites or food components into the gut wall, and therefore contributing to the development of inflammatory disorders. Considering the intestinal dysbiosis observed in our animal model of AD, we next sought to characterize the inflammatory status of the intestine in 3xTg-AD mice. As expected, we observed a decrease in the number of mucus-producing cells and an increase in the size of the intestinal mucosa, described as hyperplasia (Figure 10), both classic markers of intestinal inflammation.

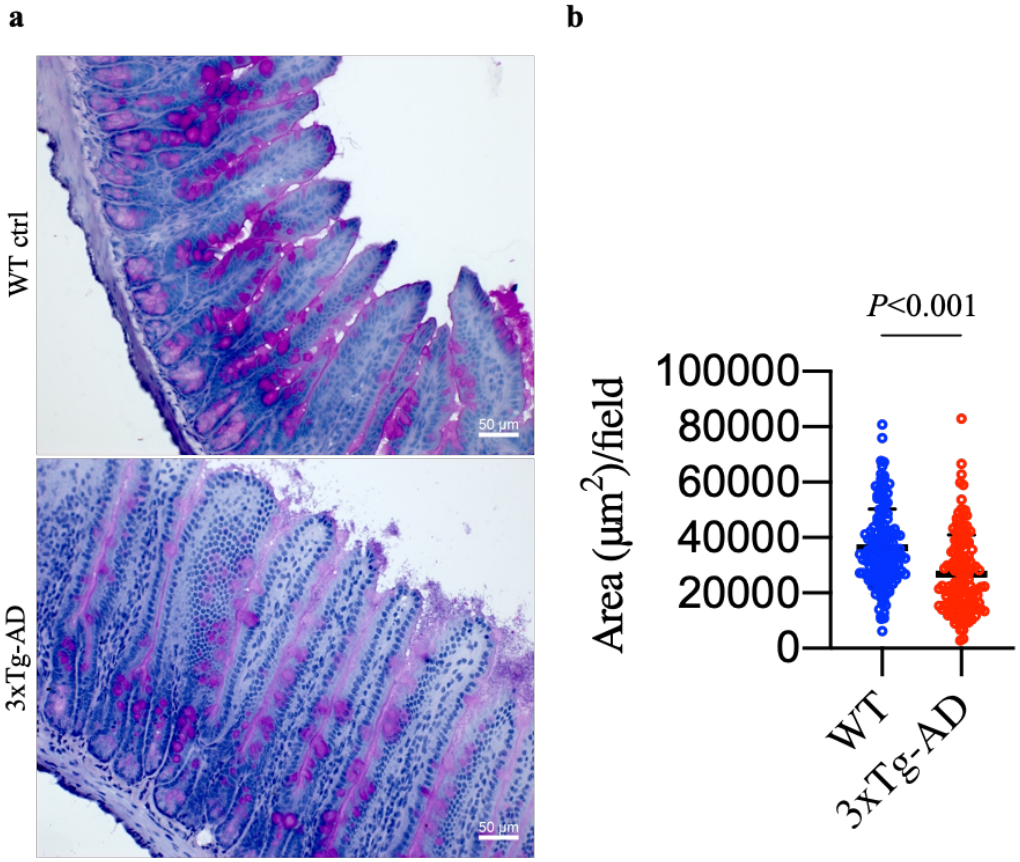

Figure 10. Histopathologic changes in the small intestine of 3xTg-AD mice*. Segments of small intestine from male mice were emersed in OCT and flash frozen. 10 µm sections were used for PAS staining. Representative images of control and 3xTg-Ad mice are shown in (a). (b) Total mucin area (pink) was quantified using ImageJ. Unpaired t-test was used for statistical analysis.*

Considering the decreased mucus production, and therefore reduction of the protective mucus layer of the intestine of our 3xTg-AD mice, we quantitatively evaluated the gut barrier permeability by an *in vivo* non-invasive method, the FITC-dextran translocation. Interestingly, 3xTg-AD mice showed an early gastrointestinal permeability barrier defect compared to age- and sex-matched healthy WT mice (Figure 11a). Intestinal histology has been widely used for the quantification of intestinal inflammation. Still, recent reports have shown that the modulation of pro-inflammatory mediators can be used to assess even low levels of intestinal inflammation. Along with the dysregulation of the intestinal epithelium, we therefore evaluated the presence of fecal LCN (lipocalin)-2 as previous studies have shown that at low levels of inflammation, LCN-2 is found slightly elevated, suggesting its use as a sensitive biomarker[200,201]. Accordingly, we found an increased amount of LCN-2 in the feces of 3xTg-AD mice already at 6 months of age in comparison to WT mice (Figure 11b). Considering the inflammatory state found in the intestine of 3xTg-AD mice and the data on circulating LPS in AD patients, we measured the levels of LBP as an indirect indicator of LPS levels. Accordingly, we found an increase of LBP in the serum of 3xTg-AD mice at 6 months of age, supporting the translocation of bacteria and/or bacterial products from the gastrointestinal tract to the circulation (Figure 11c). Together, these data suggest an early dysregulation in gastrointestinal barrier integrity and a strong intestinal inflammatory state, thus supporting the imbalanced condition of the microbiota in the pre-clinical phase of AD-like disease.

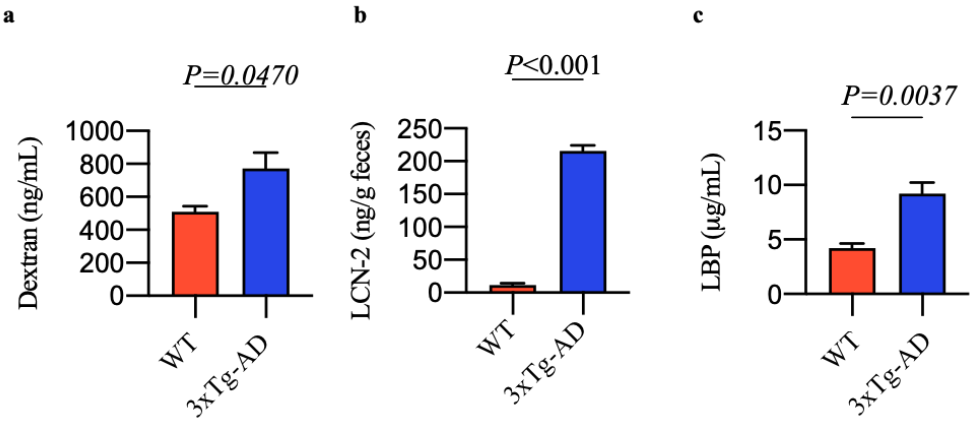

Figure 11. Intestinal dysbiosis is associated with increased permeability and markers of intestinal inflammation*. (a) FITC-dextran 4kD was administered by gavage and 4 hours later gut leakage was assessed by measuring the fluorescein signal in serum. (b) Fecal pellets were resuspended in PBS/Tween 20 0.5% (v/v) and LCN-2 was quantified by ELISA. (c) LBP was quantified in serum samples by ELISA. Unpaired t-test was used.*

4.1.3 3xTg-AD mice show features of enteric nervous system degeneration

The Enteric Nervous System (ENS) innervates the digestive tract, from the esophagus to the anal sphincter, and is constituted by neurons and glial cells[202]. ENS is part of the peripheral nervous system in the gastrointestinal tract and works independently from the CNS[203]. The ENS in humans contains over 200 million neurons, organized in the myenteric and submucosal plexuses[203]. The structural arrangement of enteric ganglia is comparable to that of the brain[204]. The submucosal plexus is located between the mucosa and the circular muscle layer, and it is responsible for regulating secretion. The myenteric plexus is located between the circular and longitudinal muscle layers and plays a major role in the coordination of motility[205]. The gut senses nutrients and generates electrical signals and this process occurs via individual enterochromaffin cells along the gut axis, that bridge the mucosal barrier and are in contact with both the CNS via the vagal nerve and the local ENS via enteric glial cells and intrinsic neural fibers. This intricate network was termed the gut connectome[206]. Using whole-mount immunofluorescence, we evaluated the state of the neuronal network in distal ileum myenteric plexus. In line with a previous report[207], we found a decrease in the neuronal network in the distal ileum also in our AD model (Figure 12), suggesting

a strong ENS impairment in 3xTg-AD mice. The loss of neurons and/or glia in disease is, in general, accompanied by disturbed gastrointestinal function, including delayed gastric emptying and slow transit constipation. These functions are regulated by the ENS and are therefore viewed as a sign of ENS complications[208]. Accordingly, we evaluated the gastro-intestinal motility in our cohort of mice. The total intestinal transit time was measured by oral administration of carmine red. 3xTg-AD mice showed a statistically significant increase in intestinal transit time compared to WT control mice (Figure 12a). Furthermore, we measured the water content in the feces of 3xTg-AD animals at 6 months of age and found a significantly decreased amount (Figure 12b), further confirming the transit constipation in AD-like mice[209]. To better understand changes associated with the ENS, we obtained a small portion of the ileum and proceeded with RNA extraction for quantitative PCR. Interestingly, even though very preliminary, we found a decrease in several neuronal markers including acetylcholinesterase *(Ache)* and tyrosine hydroxylase *(Th)*, two enzymes involved in the breakdown of acetylcholine and in the synthesis of dopamine and norepinephrine, respectively, the β-tubulin isoform III (*Tubb3),* and the RNA-binding protein ELAV4 (*Elavl4),* involved in neuronal development and function (Figure 12c, d, f and g, respectively). These data suggest a broad degeneration of the ENS in the 3xTg-AD mice already at early phases of the disease.

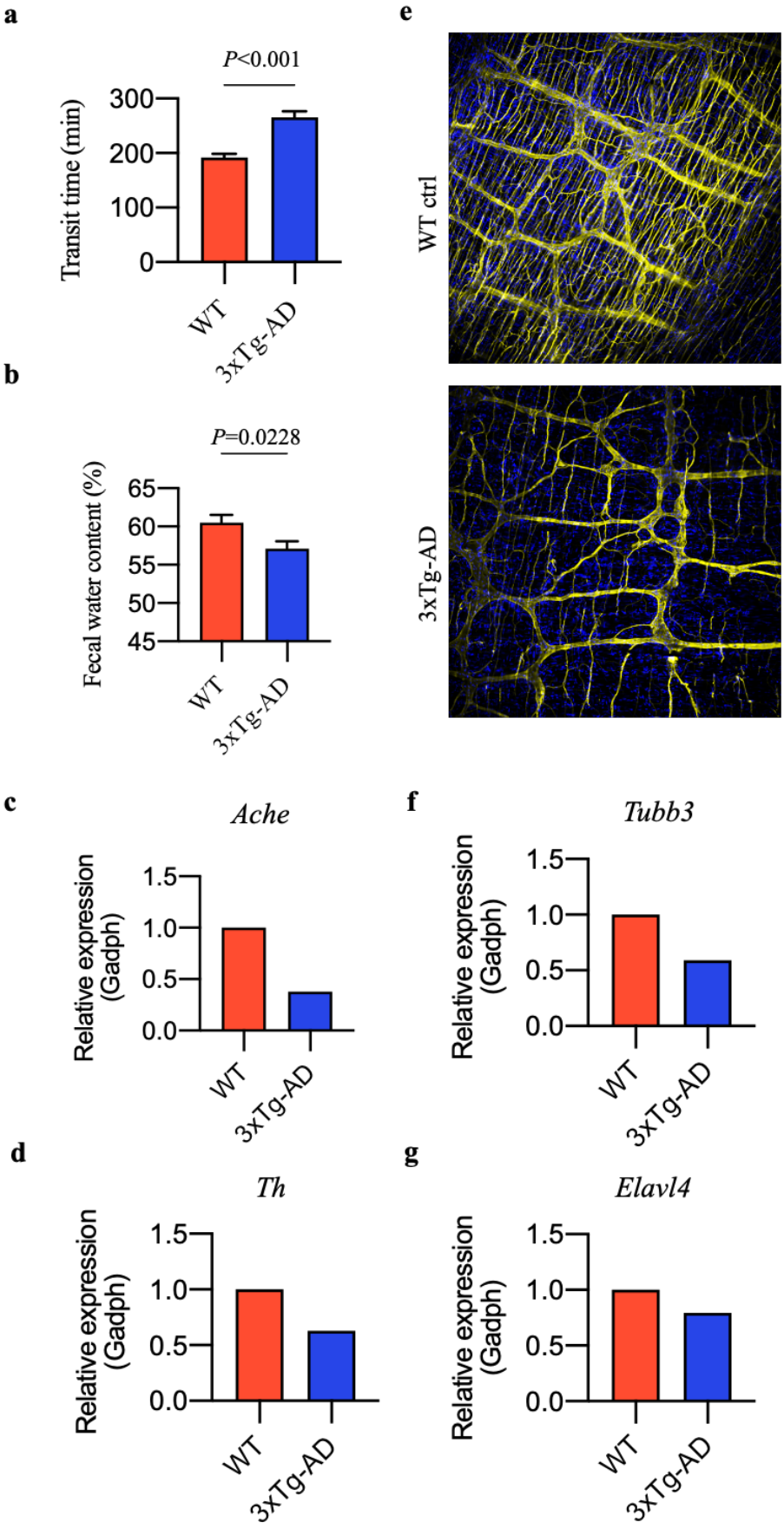
a
P<0.001
Transit time (min)
300
200
100
0
WT
3xTg-AD
b
P=0.0228
Fecal water content (%)
65
60
55
50
45
WT
3xTg-AD
e
WT ctrl
3xTg-AD
c
Ache
Relative expression (Gadph)
1.5
1.0
0.5
0.0
WT
3xTg-AD
f
Tubb3
Relative expression (Gadph)
1.5
1.0
0.5
0.0
WT
3xTg-AD
d
Th
Relative expression (Gadph)
1.5
1.0
0.5
0.0
WT
3xTg-AD
g
Elavl4
Relative expression (Gadph)
1.5
1.0
0.5
0.0
WT
3xTg-AD

Figure 12. 3xTg-AD mice show signs of ENS degeneration*. (a) Intestinal transit times were assessed using carmine red and measuring the time it took for mice to release the first red fecal pellet (n=9-10 animals per group). (b) Fecal water content was assessed by weighting fecal samples shortly after collection and after incubation at 65ºC O/N (n =8-10 animals per group). (a, b) Unpaired t-test was used. (c) Representative images of whole-mount immunofluorescence of muscularis externa of WT and 3xTg-AD mice (n=1 animal per group). Quantitative PCR of intestinal samples from WT and 3xTg-AD mice of Tubb3 (d), Th (e) Ache (f), and Elavl4 (g) (n=2 animals per group).*

4.1.4 Neutrophils infiltrate the myenteric plexus in animal models of Alzheimer's disease

Although the infiltration of neutrophils is beneficial to eliminate foreign particles and kill bacteria, their persistent infiltration and hyperactivation are known to drive pathological conditions and cause tissue damage[210,211]. Using whole-mount immunofluorescence of the muscularis externa, we show a strong recruitment of neutrophils to the myenteric plexus of the distal ileum in the 3xTg-AD mice (Figure 13), a phenomenon not observed in WT mice, supporting a role for neutrophils in intestinal inflammation in these mice and, bringing evidence of a possible systemic neutrophil dysregulation in AD.

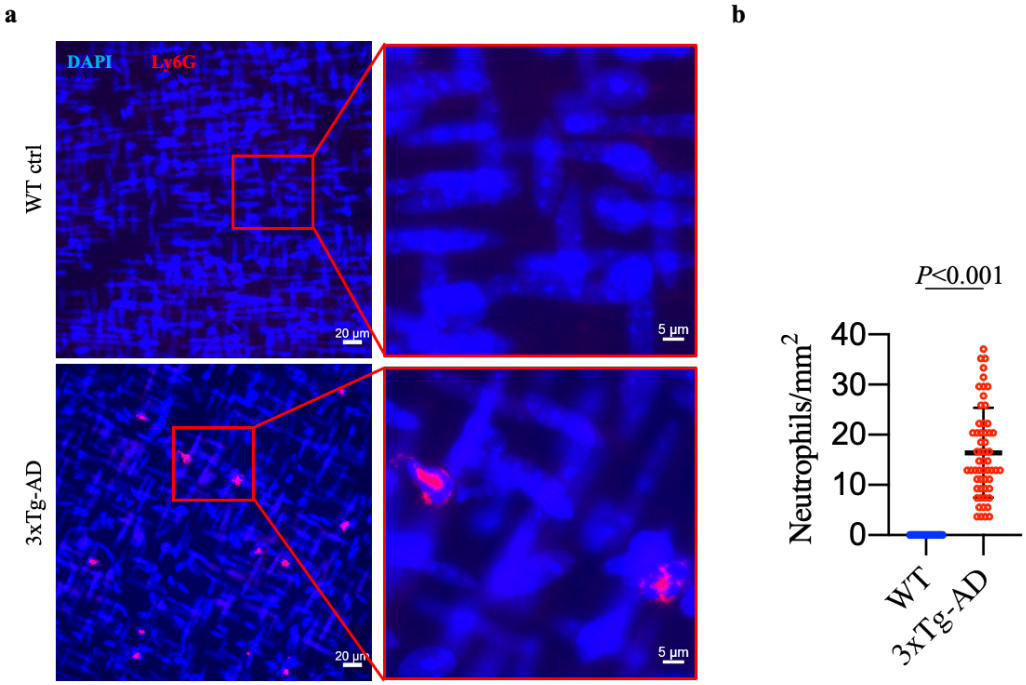

Figure 13. Neutrophils infiltrate the myenteric plexus in 3xTg-AD mice. *(a) Representative images of Ly6G (Red) positive neutrophils and DAPI (blue) positive nuclei in the myenteric plexus of WT and 3xTg-AD mice at 6 months of age. Red squares in the left images are shown at a higher magnification in the images on the right. (b) The plot represents the quantification of neutrophils per mm^2 of tissue in the Ileal portion of the intestine. Unpaired t-test was used.*

4.1.5 Immature neutrophils are increased in the blood of mice with Alzheimer-like disease and correlate with intestinal inflammation

While advances in the understanding of peripheral inflammation in AD are still being made, hyperactivation of peripheral neutrophils and increased neutrophil to lymphocyte ratio are established features of AD[170,173]. Given that the numbers of circulating and bone marrow neutrophils are affected by the microbiota[212] and considering that intestinal microbiota augments the host response against pathogens, such as *Streptococcus pneumoniae* and *Staphylococcus aureus*, through constitutive stimulation of neutrophils derived from the bone marrow[213] we wondered if that would also be the case in our mouse model of AD. We showed above that 3xTg-AD mice present intestinal dysbiosis and inflammation and further

identified neutrophil infiltration in the myenteric plexus of these mice, suggesting a tight correlation between the microbiota and neutrophil dysregulation in our mice with AD-like disease. Indeed, flow cytometry analysis of the peripheral blood of 3xTg-AD at early disease stages showed higher numbers of neutrophils in the circulation, regardless of sex (Figure 14a and b). Interestingly, we identified a subset of immature neutrophils that is increased in both males and females, but was more predominant in male 3xTg-AD mice. The contribution of the immature neutrophil fraction to the circulating neutrophil pool in female 3xTg-AD mice was higher in comparison to the WT counterpart (especially at 6 months), but lower than that observed in male 3xTg-mice, suggesting a sex-dependent diversity in AD-like disease mice (Figure 14c and d). Moreover, it is worth noting an increase in the immature neutrophils with time (from 4 to 6 months old) in both males and females, suggesting not only an early activation of circulating neutrophils but also a continuous increase in their number with disease progression.

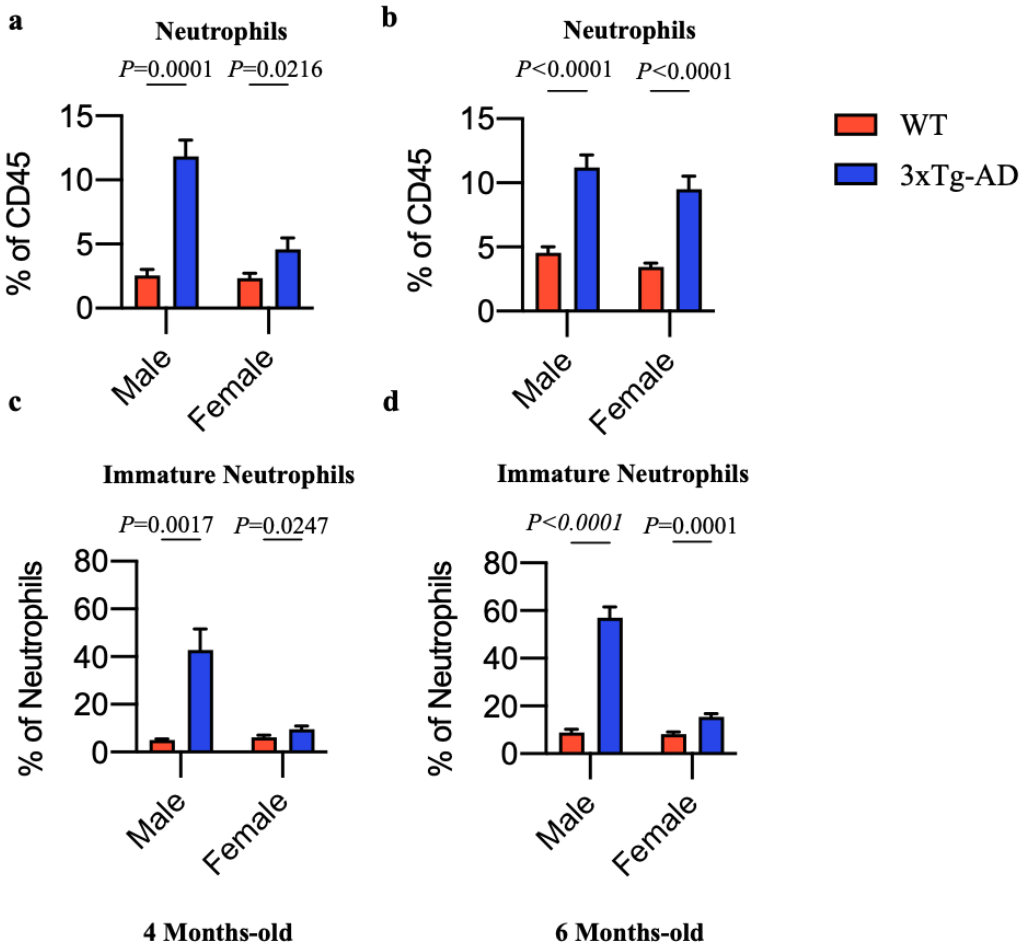

Figure 14. Neutrophils increase in the blood of 3xTg-AD mice*. Flow cytometry analysis of peripheral blood showing: (a and b) the percentage of neutrophils within peripheral leukocytes (CD45$^+$ cells) or (c and d) the percentage of immature neutrophils (CD101$^-$ /CXCR2$^-$ cells) within neutrophils of 3xTg-AD and WT mice at 4 (Left graphs) and 6 months of age (Right graphs). P values were determined using one-way ANOVA, followed by Dunnett's multiple comparisons test.*

Next, we further studied the intriguing immature level of circulating neutrophils in our 3xTg-AD mouse model. Giemsa staining of sorted neutrophils revealed a ring-shaped nucleus in the CD101$^-$ neutrophils, supporting an immature phenotype of these cells (Figure 15a). To further understand the phenotype of circulating neutrophils, we isolated CD101$^-$ and CD101$^+$ neutrophils and performed low-input RNAseq analysis of these populations. Accordingly, RNAseq analysis revealed that CD101- neutrophils in the circulation present classical immature mRNA signatures

as observed by the high expression of LCN-2, CD177, CEBPB, (Figure 15b), as it has been described by others[214]. Altogether, our data suggest a condition of emergency granulopoiesis that releases immature myeloid cells into the circulation, suggesting an ongoing chronic inflammatory condition that might be favored by gut dysbiosis taking place before the onset of cognitive deficits and brain pathology in the transgenic mice with AD-like disease. Considering the increased permeability of the intestinal tract, we correlated the gut leakage with the percentage of blood immature neutrophils. Importantly, we found a positive and statistically significant correlation between these two processes thus supporting the **interplay between gut dysbiosis and increased granulopoiesis** in the 3xTg-AD mice (Figure 15c), bringing further evidence on the role of **peripheral neutrophil dysregulation** in disease development.

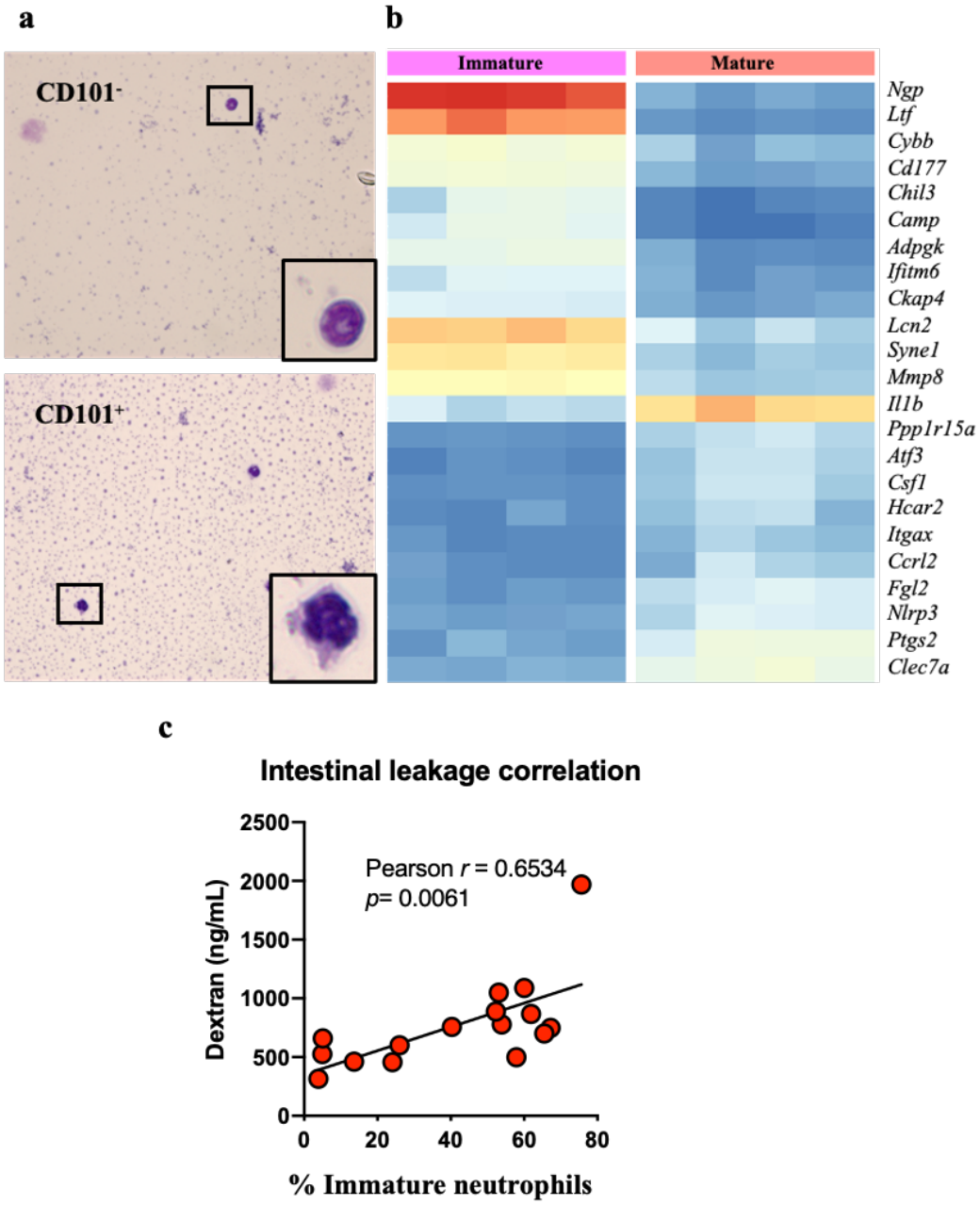

Figure 15. CD101- neutrophils present characteristic features of immature neutrophils. *Peripheral blood leukocytes were isolated from 3xTg-AD and WT mice at 6 months of age and CD101+ and CD101- neutrophils were sorted. Cells were either (a) spotted on a slide using a cytospin and stained with Giemsa or (b) processed for RNA extraction and low-input RNA sequencing. (c) Graphical plot of the Pearson correlation between circulating immature neutrophils and serum dextran.*

4.2 NEUTROPHIL-MEDIATED NEUROTOXICITY IN AN ANIMAL MODEL OF ALZHEIMER'S DISEASE

4.2.1 scRNAseq of brain-infiltrating neutrophils shows activation and degranulation phenotypes in 3xTg-AD mice

We have previously shown that neutrophils infiltrate the brain of 3xTg-AD mice at early time points and promote memory decline and the neuropathological characteristics of AD in 3xTg-AD mice[75]. To gain insight into the immune signature of neutrophils in AD pathogenesis, we isolated brain-infiltrating CD45high cells from 6-month-old 3xTg-AD and WT mice and studied their gene expression profiles by scRNA-seq. We analyzed 2435 individual cells from 3xTg-AD mice and 3264 cells from WT mice. Transcriptionally distinct leukocyte subpopulations were identified by unsupervised clustering and dimensionality reduction using a smart local moving clustering algorithm and *t*-SNE. Using an automatic annotation method to screen the ImmGen database, we identified three main neutrophil populations: Clusters 3, 5 and 9 (Figure 16a). The analysis of DEGs showed an activated phenotype of neutrophils from 3xTg-AD mice (e.g., *Plaur, Pglyrp1, Rab6a, Ctsd, Adam8, Man2B1*). Interestingly, scRNA-seq analysis also showed an upregulation of genes involved in neutrophil granules and degranulation processes (e.g., *Ctse, Plaur, Lyst, Pglyrp1, Ctsd, Adam8, Man2b1*)[215–219] (Figure 16b), as well as genes involved in cell migration and cell adhesion (e.g., *Cebpb, Adam8, Selplg)*[220–222] (Figure 16c), suggesting these processes as putative neurotoxic mechanisms driving neuronal damage in AD.

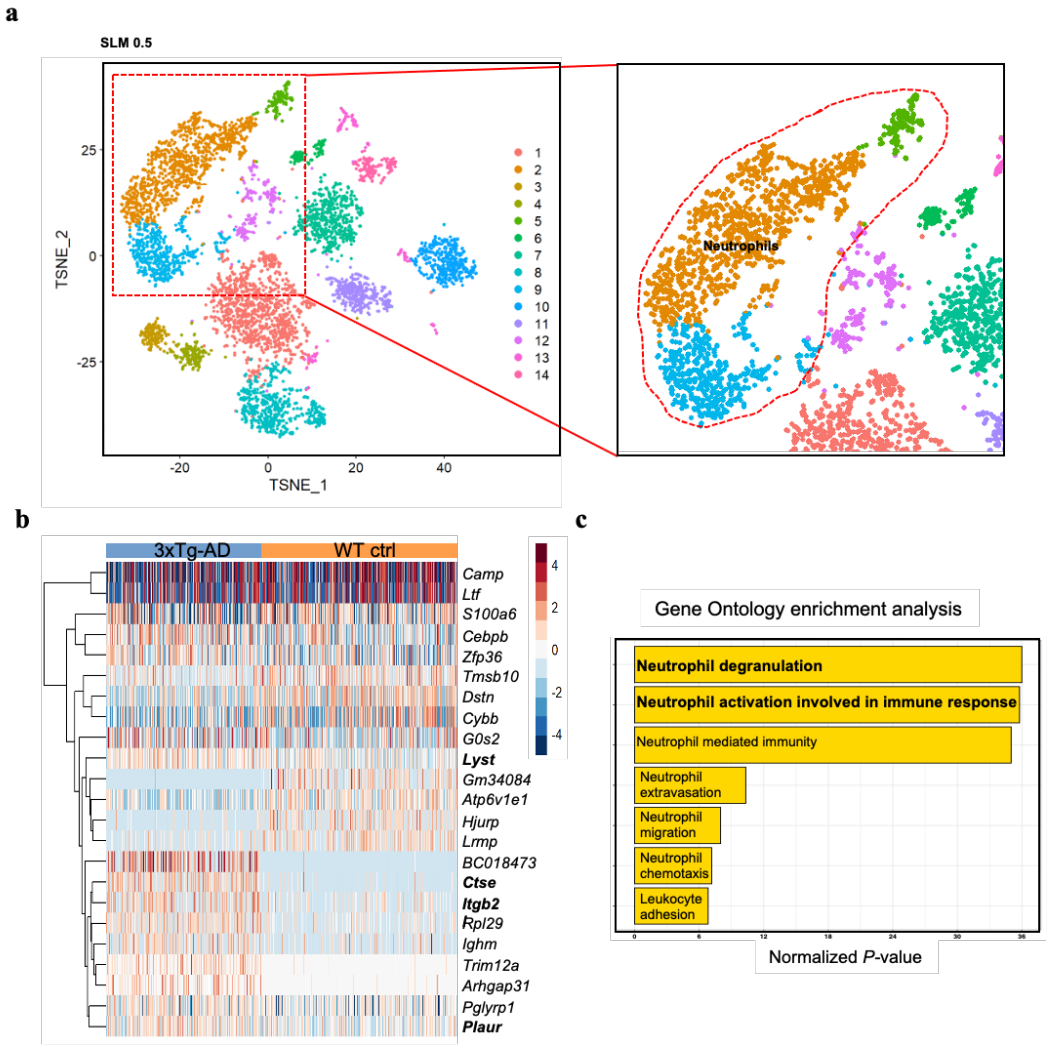

Figure 16. Brain neutrophils show a gene signature associated with activation and degranulation in 3xTg-AD mice. *Single-cell RNA sequencing of CD45high cells derived from brain tissue was used for leukocyte profiling and expression analysis. (a), The t-SNE projection of 5699 individual leukocytes from 20 mice resolved to 14 major clusters, including three for neutrophils. Each dot corresponds to a single cell, colored according to the cell cluster. The red-boxed region in the left panel is magnified in the right panel. (b), The heat map represents normalized, centered and rescaled log-count values for each differentially expressed gene (DEG) in the neutrophils of 3xTg-AD mice and WT. Each column represents a single cell. (c), Gene Ontology (GO) analysis indicates enrichment for biological processes involved in neutrophil degranulation and activation.*

4.2.2 Brain-infiltrating neutrophils from 3xTg-AD mice show higher degranulation phenotypes

While increased expression of CD11b in neutrophils has been described in AD[169] patients, this is often associated with neutrophil activation and no study regarding neutrophil degranulation in AD has been published. Accordingly, flow cytometry analysis on brain-infiltrating neutrophils confirmed the scRNA-seq data on neutrophil activation. At 6 months of age, we observed an increased surface expression of CD11b and CD177 as well as CD63 (Figure 17). CD11b is a cell surface receptor important for neutrophil and monocyte migration to sites of inflammation. It is also involved in the degranulation of secretory vesicles and its expression in the cell surface has been shown to correlate with neutrophil activation and degranulation[223]. Similar roles in neutrophil activation, degranulation and migration were shown also for CD177. While CD11b, together with CD18, is able to mediate adhesion processes by interactions with ICAM-1, CD177 has been described to bind to PECAM-1 and is believed to be involved in neutrophil motility. Interestingly, CD177 is expressed both at the cell surface and in specific granules and its expression is almost exclusively restricted to neutrophils[224,225]. On the other hand, CD63is associated with azurophilic granules and has been reported to increase in the cell surface upon neutrophil degranulation[226]. This suggests a massive involvement of neutrophils in AD, emphasizing the importance of further understanding the dysregulation of this population in these settings.

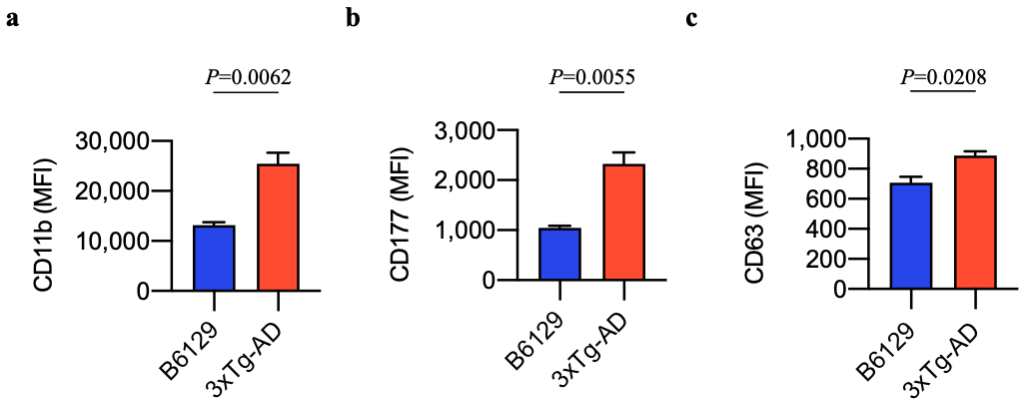

Figure 17. Brain infiltrating neutrophils express classical markers of neutrophil degranulation in 3xTg-AD mice*. FACS analysis on brain infiltrating neutrophils of 3xTg-AD and WT control mice at 6 months of age. The expression of a) CD11b, b) CD177 and c) CD63 is presented as the mean fluorescence intensity (MFI). Unpaired t-test was used.*

4.2.3 Amyloid-β acts as a trigger of neutrophil degranulation

Given the increased degranulation observed in brain-infiltrating neutrophils in the 3xTg-AD mouse model, we wondered whether Aβ could play a role in the induction of degranulation. Aβ is known to activate neutrophils and induce the production of ROS[75], however, its effect on neutrophil degranulation has not been addressed yet. We isolated human peripheral blood neutrophils from buffy coats by means of Ficoll gradient, and mouse bone marrow neutrophils from 2 months-old WT mice by percoll gradient and studied the effect of oligomeric $A\beta_{1-42}$ in the induction of neutrophil degranulation. Oligomerization of Aβ is believed to be a crucial step for the pathology of Alzheimer's disease. Interestingly, oligomeric forms of Aβ are known to induce proinflammatory responses in innate immune cells and this responses seems to differ from those induced by amyloid fibrils[227]. Mouse and human neutrophils were stimulated with Aβ for 30 minutes after 10 minute priming with cytochalasin B to facilitate the degranulation of neutrophils, and the surface expression of granule markers was analyzed by flow cytometry. Interestingly, our preliminary data show a clear upregulation of CD11b and CD66b in human neutrophils (Figure 18a and c) and the upregulation of CD11b and CD63 (Figure 18b and d) in mouse neutrophils when stimulated with Aβ, confirming its capacity to induce neutrophil degranulation.

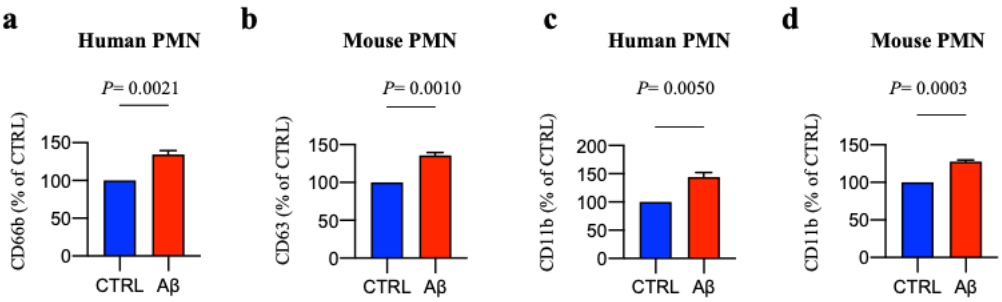

***Figure 18**. Aβ is a potent inducer of neutrophil degranulation. Neutrophils were isolated and treated with 5 μM of oligomeric $A\beta_{1-42}$. Degranulation was assessed by evaluating the expression of CD66b (a) and CD11b (c) in human neutrophils and the expression of CD63 (b) and CD11b (d) in mouse neutrophils by FACS. Unpaired t-test was used for the statistical analysis.*

4.2.4 Cathepsin E mediates neutrophil-dependent cytotoxicity on neurons from 3xTg-AD mice

scRNA-seq data from brain-infiltrating neutrophils revealed *Ctse* as one of the most upregulated genes in AD mice (Figure 19a). To validate these data, we performed flow cytometry analysis on brain infiltrating neutrophils and we found an upregulation of CTSE also at the protein level (Figure 19b) in brain-infiltrating neutrophils from 3xTg-AD mice at 6 months of age. Interestingly, it was recently described in an EAE (experimental autoimmune encephalomyelitis), the animal model of MS, that CTSE plays a key role in the induction of neuropathic pain, thus suggesting its role in another neurodegenerative disorder. Indeed, the authors reported an increase in CTSE in CNS-infiltrating neutrophils and further suggested that this increase is mediated by activation of neutrophils by MOG (myelin oligodendrocyte glycoprotein), the peptide used to induce the disease[216]. Hence, we further addressed its role also in Aβ-mediated neutrophil activation. Interestingly, our preliminary data show an increase in CTSE in both human and mouse neutrophils upon Aβ stimulation (Figure 19c), supporting CTSE involvement in neutrophil activation.

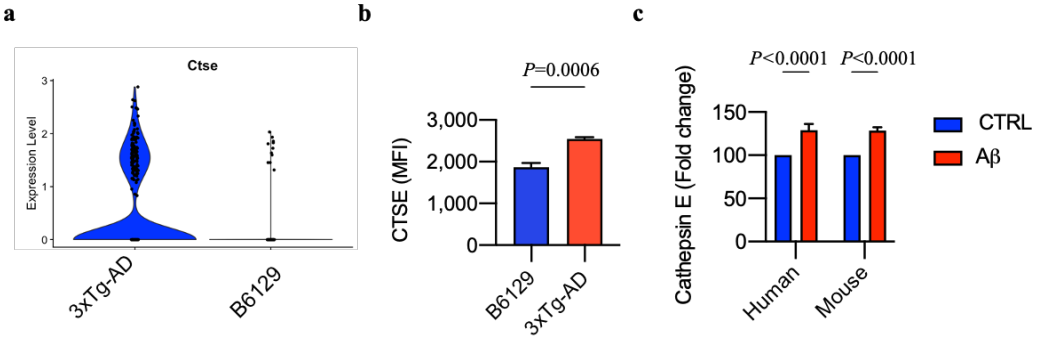

Figure 19. Aβ induces the upregulation of CTSE in vitro. (a) Violin plots showing average log-normalized counts for Ctse in 3xTg-AD and WT brain-infiltrating neutrophils *(b) Brain-infiltrating neutrophils were isolated and stained for intracellular CTSE and analyzed by FACS. (c) Human and mouse neutrophils were isolated from buffy coats or bone marrow, respectively, and incubated with oligomeric Aβ (5 μM) and assessed for CTSE Unpaired t-test was used.*

As in the EAE model, the authors also showed that the increase in neutrophil CTSE is associated with the regulation of elastase release[216], a protein contained in their granules, we next evaluated the role of CTSE in the degranulation process mediated by Aβ. Hence, we took advantage of ritonavir, a food and drug administration-approved drug for the treatment of human immunodeficiency virus patients described to inhibit CTSE activity[228]. Here, we show the complete abrogation of CD63 and CD11b upregulation on mouse neutrophils (Figure 20), supporting its involvement in the degranulation process. Altogether, these data point to CTSE as a key player in Aβ-mediated neutrophil degranulation, suggesting its inhibition as a possible pharmacological approach for neutrophil-mediated neurotoxicity in AD.

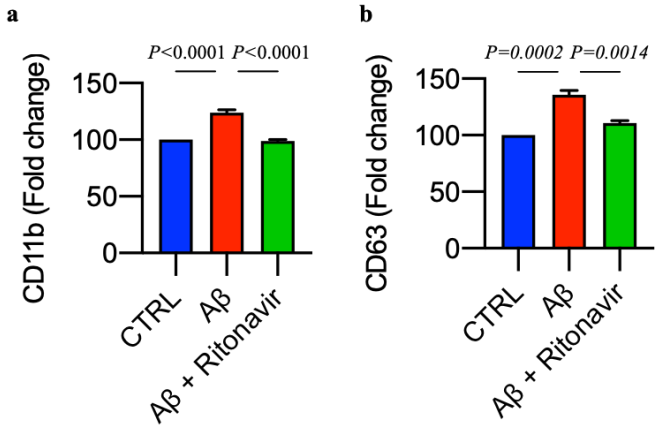

Figure 20. CTSE is involved in Aβ-induced neutrophil degranulation. *Bone marrow neutrophils were isolated and pre-treated with Ritonavir 1 μM for 30 min. Afterward, neutrophils were treated with 5 μM of oligomeric $A\beta_{1-42}$. Degranulation was assessed by evaluating the expression of CD11b (a) and CD63 (b) by FACS. One-way ANOVA, followed by Dunnett's multiple comparisons test was used for the statistical analysis.*

4.2.5 CTSE as a central player in neutrophil-mediated neurotoxicity

To get further insights on neutrophil-dependent neurotoxicity, we co-cultured neurons and neutrophils isolated from 3xTg-AD mice. Recent data obtained in our laboratory indicates strong cell-cell contacts between neutrophils and neurons and revealed the induction of neuronal cell death after direct contact with neutrophils. Given the increased expression of CTSE in neutrophils of 3xTg-AD mice, we asked if the inhibition of this molecule could prevent neutrophil-dependent neuronal death. Notably, neuronal death caused by direct contact with neutrophils was significantly decreased when neutrophils were pretreated with two CTSE inhibitors (Ritonavir and Pepstatin A) (Figure 21a and b). Furthermore, the pre-treatment of neutrophils with the two CTSE inhibitors at several concentrations did not result in increased neutrophil apoptosis (Figure 21c), indicating that the reduction of the neurotoxicity was due to CTSE inhibition but not neutrophil death. Altogether, these data point to CTSE as a crucial mediator of neutrophil-dependent neuronal damage, and its therapeutic targeting may yield significant advances in AD treatment.

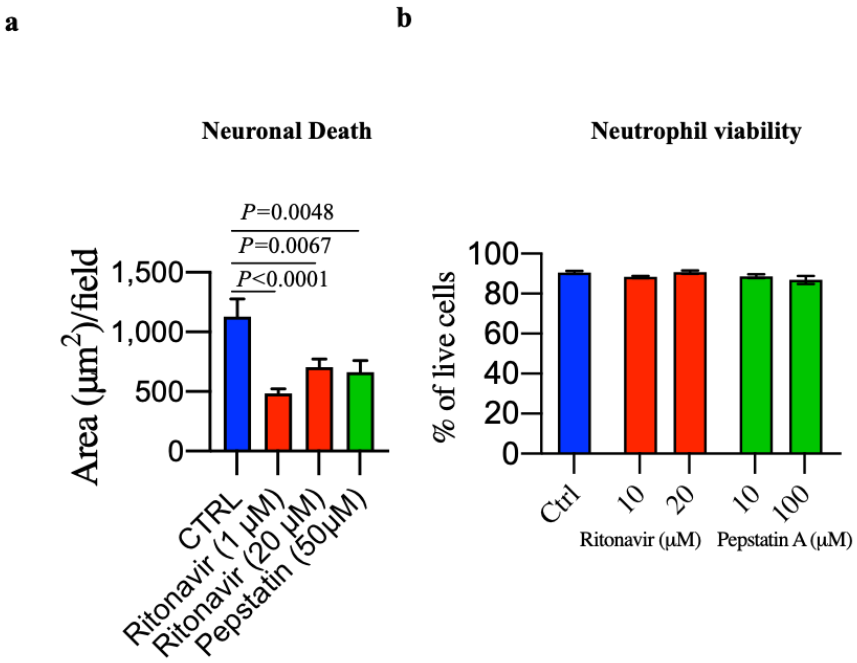

Figure 21. CTSE inhibition blocks neutrophil-mediated neurotoxicity. *Neurons from P7 WT pups were obtained using the adult dissociation kit from Miltenyi biotec and cultured for 5 days. Neutrophils isolated from the 3xTg-AD mice were incubated with ritonavir and pepstatin A for 1 hour at 37 ºC, washed and laid over neuronal cells. After 4 hours, neutrophils were washed off the neuronal culture and neurons were stained for PI and imaged at an inverted fluorescence microscope (Zeiss) (a) The area of PI-stained neurons was quantified using the Zeiss Software. (b) Neutrophils after drug treatment were stained using vybrant/sytox apoptosis kit and analyzed by flow cytometry. One-way ANOVA, followed by Dunnett's multiple comparisons test was used for the statistical analysis.*

CHAPTER V

DISCUSSION

We have previously reported that neutrophils migrate near Aβ deposits, adhere and spread in brain vessels and release NETs in the human AD brain[75]. Additionally, we showed that the number of neutrophils in the brain of two transgenic mouse models of AD and human patients is higher compared to the control groups[75]. However, how these cells contribute to AD pathogenesis and the mechanism behind neutrophil activation remain to be elucidated. Our study first aimed at understanding the mechanisms leading to **peripheral neutrophil dysregulation in AD** by studying a potential role for dysbiosis in altering circulating neutrophils. Gut dysbiosis and the involvement of the gastrointestinal tract in CNS disorders were first described for Parkinson's disease, and soon after, similar data were found in other diseases. Indeed, the reports on altered microbiota and its modulation of disease course have been described for stroke, MS, and AD[22,175]. Still, most of the studies in AD models showed only a characterization of gut dysbiosis and its effect on disease hallmarks such as Aβ accumulation in the brain[229]. Understanding the role of microbiota on immune peripheral changes during disease development may provide clues on how to modulate these effects and slow down disease progression. Our results confirmed the gut dysbiosis already described in AD patients and other animal models of AD and our data showed a significant alteration of microbiota composition in 3xTg-AD mice. Interestingly, we have also observed a slight difference between male and female AD mice, that was not observed in the wild type counterpart, and these data are in line with several reports that have already addressed sex-related differences in AD with the incidence of the disease higher in females than in males[3]. Our data showed a decrease in species belonging to the *Clostridia* class, among which *Lachnospiraceae* and *Ruminococcaceae* families accounted for the main players behind the decrease in *Clostridia*. Interestingly, species belonging to these two families have been shown to play an important role in the metabolization of starch to produce butyrate and other SCFAs[230]. SCFAs have been shown to play important roles in the communication between the gut and the immune system. *Arpaia et a*l.[231] demonstrated that butyrate induces extra-thymic peripheral polarization of naïve T cells to Foxp3$^+$ Tregs both *in vivo* and *in vitro*. These authors showed a significant increase in the number of peripheral Tregs in antibiotic-treated mice that received

butyrate. Interestingly, these authors also showed that this effect was not seen in the thymus, suggesting a broad regulation of Tregs in the peripheral blood and no effect on their initial thymic development.

While some effects of intestinal dysbiosis on neutrophils have been previously described, consistent evidence has yet to be published. Reports have shown both a decrease in the production of pro-inflammatory cytokines such as TNF-α, and an increase in the production of CXCL8 when neutrophils are stimulated with TLR agonists and treated with SCFAs[232,233]. An increase in the *Akkermansiacaeae* family was also observed in the 3xTg-AD mice and it has been reported that *Akkermansia muciniphila* is also increased in MS patients and can induce pro-inflammatory responses[234]. Moreover, mucin, the component of the protective gastrointestinal mucus preventing the interaction between microbes and the epithelial layer, is known to be degraded by this family of bacteria[235]. In our mouse model of AD, we observed a decrease in the overall mucin quantity, and an increase in the length of the villi in the ileum, both characteristic of inflammatory reactions as described for inflammatory conditions such as colitis ([236]). Interestingly, in addition to the mucin decrease in the ileum, we also have preliminary data (Figure A2) indicating a substantial decrease of the mucus barrier in the colon of these mice. The mucus layer is known to be thicker in the colon, reaching 800 μM in humans[237]. This decrease in the mucus layer could be indeed a result of the increased abundance of the *Akkermansiaceae* family. This increase in mucus degradation, and therefore a decrease in the mucus protective layer may lead to increased contact between microbes and epithelial cells, a pathological change known to induce pro-inflammatory reactions[238]. To corroborate the findings indicating a dysfunctional intestine, we have shown that 3xTg-AD mice present increased intestinal permeability as shown by increased translocation of dextran into the circulation, a feature usually described as “leaky gut”. Along with the increase in intestinal permeability, the translocation of microbial-derived products such as LPS has been described to contribute to systemic inflammation[239]. Similarly, in our model we also have strong indications that LPS levels in peripheral blood are increased in AD, as we observed increased levels of LBP in the feces of 3xTg-AD mice. Interestingly, this finding has already been confirmed by other groups[191], supporting a

dysregulated intestinal homeostasis in AD. While reports on intestinal inflammation across several disorders have been published, only a small portion has focused on the dysregulation of the ENS. The ENS has been shown to play an important role in intestinal function, by regulating functions such as muscle activity, transmucosal fluid fluxes, local blood flow[203], but still its role in AD is unclear. Previous reports have pointed to an accumulation of Aβ in intestine sections of AD patients[240], but the correct characterization of its deposition in the ENS is still lacking. We have shown that 3xTg-AD mice present increased intestinal transit time and lower fecal water content, suggesting constipation. While reports on gastrointestinal symptoms in AD are still rare, a few publications have suggested the same effect in AD patients[209]. We speculate that this is associated with a dysfunction of the ENS, given its role in the regulation of gastrointestinal motility. In fact, we show a clear reduction in our preliminary β-tubulin isoform III whole-mount staining (a general neuronal marker) and a reduction of several RNA transcripts associated with neuronal subtypes. Given our previously published data on neutrophil accumulation inside the CNS and its role in AD pathogenesis, we wondered whether these cells could also influence the ENS. Upon the initiation of an inflammatory response, circulating neutrophils transmigrate into the intestinal mucosa as an essential process of the immune defense. However, neutrophil infiltration near myenteric ganglion cells was not reported so far in AD. Surprisingly, we have striking evidence of neutrophil infiltration inside the myenteric plexus of 3xTg-AD mice, suggesting neutrophils as pivotal players also in ENS degeneration. Postoperative ileus, a condition resulting from the manipulation of the intestine during laparotomy, shows intense inflammatory reactions and alterations of gastrointestinal functions such as motility[241,242]. Interestingly, in line with our findings, previous studies have shown that in mice with this condition, neutrophils migrate in the myenteric plexus in large numbers whereas control animals do not present any neutrophil infiltration to the myenteric plexus[243], further suggesting neutrophil presence in the myenteric plexus as factor in the induction of intestinal dysfunction. Importantly, the recruitment of neutrophils to the myenteric plexus was limited through the stimulation of the serotonin-4 receptor 5-HT_4R,[243] expressed by cholinergic motor neurons in the

myenteric plexus of the gastrointestinal tract, suggesting a neural-immune interplay possibly mediated by acetylcholine release and neutrophils.

Data obtained by others in AD patients and mouse models have shown a clear hyperactivation of circulating neutrophils and have suggested this peripheral activation may promote neutrophil infiltration into the CNS[173]. In addition, our results have further elucidated the mechanisms behind neutrophil activation in the peripheral circulation. We show that, in our mouse model, peripheral blood is characterized by increased neutrophil levels, a finding that has also been suggested by other authors[170]. More importantly, we also find a substantial increase in immature neutrophil forms in the circulation of these mice by flow cytometry. While the maturation step of these neutrophils is yet to be determined, we provide supporting evidence of their immature phenotype by both nucleus segmentation and characteristic RNAseq transcriptomics. The regulation of neutrophil development and release is known to be dependent on several environmental cues. Indeed, activation of TLRs has been shown to modulate the shift between homeostatic and emergency granulopoiesis[119]. In accordance, our results have shown a clear correlation between the levels of immature neutrophils and the grade of intestinal permeability, indicating the role of the intestinal inflammatory state in increasing granulopoiesis in AD. Interestingly, a recent study on experimental spondyloarthritis has shown that systemic and intestinal inflammation drive GM-CSF–dependent dysregulated hematopoiesis and pathogenic extramedullary myelopoiesis[244]. While we are still working on deciphering the alterations of medullary and extramedullary hematopoietic events in AD, our results point to a strong interaction between gut dysbiosis and neutrophil homeostasis in 3xTg-AD mice.

Neutrophils have been described to play a central role in the pathogenesis of AD, however, the exact mechanisms leading to neuronal damage remain elusive. We previously have shown that neutrophils infiltrate the AD brain and produce NETs, however this process seems to be present only in a small percentage of infiltrating neutrophils[190]. Neuronal damage constitutes one of the prominent hallmarks in AD pathology and most of the studies regarding this matter have focused on the role of Aβ and tau as neurotoxic factors in AD. Still, therapies targeting these molecules

have produced controversial results, without any substantial therapeutic benefit[19]. The role of inflammation in AD has acquired an important place in AD pathogenesis in the last decade, not only due to the description of reactive microglia in AD but also by the discovery of a tight connection between blood-borne leukocytes and CNS homeostasis[166]. To further understand a more central role of neutrophils in AD, we performed scRNA-seq of brain infiltrating neutrophils at disease onset, and identified a pathogenic signature of neutrophils characterized by genes involved in cell activation and neutrophil degranulation. This signature was further verified at the protein level by flow cytometry, confirming an activated and degranulating neutrophil phenotype. Interestingly, the involvement of neutrophil granule proteins in AD pathogenesis has been recently suggested and a systematic review of several proteomics studies in both blood and CSF has reported neutrophil granule molecules such as LCN-2 and MPO to be elevated in AD[245]. Moreover, a study on 5xFAD, a mouse model of AD, has further confirmed the important role of MPO in AD pathogenesis by showing that 5xFAD carrying hematologic MPO deficiency show superior cognitive functions in comparison to control 5xFAD. Interestingly, the authors of this study also showed that these MPO deficient mice do not show changes in Aβ accumulation[246], suggesting that cognitive amelioration is associated with MPO-dependent mechanisms rather than Aβ-mediated toxicity.

To further dissect the role of degranulation in AD, we stimulated neutrophils with oligomeric Aβ and evaluated the degranulation levels in freshly isolated neutrophils. Aβ has been shown to induce a pro-inflammatory reaction in myeloid cells. Della Bianca *et al.*, showed already in 1994 that oligomeric forms of Aβ induce the activation of the respiratory burst in microglia and neutrophils[247], a study that was later confirmed by others[75]. Furthermore, the chemotactic capacity of Aβ has also been described in microglia and neutrophils, supporting the fact that Aβ constitutes a strong mediator of cell activation[75,248]. In accordance with this, our data showed that oligomeric Aβ is also able to induce the degranulation of both mouse and human neutrophils, identifying Aβ as a potential trigger of neutrophil-mediated neurotoxicity. Additionally, our scRNA-seq data identified *Ctse* as one of the most up-regulated genes in infiltrating neutrophils. CTSE is an aspartyl protease, but little is known regarding its functions. Some reports have associated

CTSE with antigen presentation[249] and a recent study has also reported a pathogenic role for this molecule in regulating neutrophil elastase release in an animal model of MS[216]. The authors showed that CTSE is upregulated in neutrophils and contributes to the generation of mechanical allodynia by mediating elastase production/release by neutrophils. In a similar fashion, we show that brain-infiltrating neutrophils in 3xTg-AD mice present higher levels of CTSE in comparison to WT mice, confirming the scRNA-seq data. Moreover, we also show that Aβ is able to induce an increase of CTSE in neutrophils, suggesting its use as therapeutic target for AD. To further understand CTSE involvement in neutrophil degranulation, we took advantage of ritonavir, a food and drug administration-approved drug for the treatment of human immunodeficiency virus. This drug has been described to inhibit the activity of CTSE[228], and due to its use in clinical settings, the transfer of ritonavir to AD therapies would be rather facilitated. While easily translationable to the clinic due to being already approved, we do have to acknowledge that studies involving inhibitors are usually a limitation when understanding a molecular mechanism. Using a KO model or using silencing techniques would most likely counteract the problem of off-target or unspecificic effects of drug treatments, an issue we plan to tackle in the future by taking advantage of commercially available CTSE-KO mice. Interestingly, inhibition of CTSE with ritonavir prior to the stimulation of neutrophils with Aβ led to a complete abrogation of neutrophil degranulation, without any effect on neutrophil viability, suggesting its role in the degranulation process. To date, evidence for neutrophil degranulation in mediating tissue damage in disease settings is scarce. Most studies focus on the characterization of degranulation phenotypes or stimuli able to trigger degranulation, due to the difficulty of studying this phenomenon *in vivo*. Still, a study let by Allen *et al.* showed an important role for transmigrated neutrophils in mediating neuronal damage[250]. The authors took advantage of *in vitro* time lapse microscopy and have suggested the role of degranulation and NETs release in this neurotoxic mechanism. In support of these data, in this thesis we show a beneficial role for CTSE inhibition in reducing neuronal damage. Particularly, we showed that ritonavir-treated neutrophils induce less neuronal damage than neutrophils treated with vehicle control. Altogether, we speculate that

Aβ and possibly other AD-related triggers such as hyperphosphorylated tau, stimulate neutrophil degranulation through a CTSE-dependent mechanism that can be targeted to decrease neuronal toxicity in AD.

Collectively, the work performed during this thesis was mainly carried out taking advantage of mouse models. Understanding disease mechanisms and providing valuable input for possible new therapies is specially important for conditions where no available cure or modifying therapies are available such as AD. Still, the use of mouse models is often a limitation to fully uncover the specifics of disease pathologenesis. Our mouse models, while recapitulating rather well the main hallmarks of the disease, is considered a familiar AD model, an issue that may provide limitations to the understanding of sporadic AD cases, wich account for 95% of the cases. This however is a limitation we cannot fully overcome given the lack of rodent mouse models presenting sporadic AD. In light of these limitations, we will seek to provide confirmation of the main findings using human samples to bring our data closer to the clinic settings.

In conclusion, in this thesis we demonstrate a role for **peripheral and central neutrophil alterations in 3xTg-AD mice** and suggest that targeting mechanisms leading to neutrophils dysfunction, including dysbiosis, or blocking neutrophil specific molecules leading to neurotoxicity may interfere with AD development and possibly slow down disease progression at early disease stages.

CHAPTER VI

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CHAPTER VII

APPENDIX

Table A1. **List of antibodies used for flow cytometry and immunofluorescence stainings**.

Antibody	Fluorochrome	Concentration (µg/mL)	Clone
Rat anti-mouse CD45	BV480	1.0	30-F11
Rat anti-mouse Ly6G	FITC	1.0	1A8
Rat anti-CD11b	APC-R700	1.0	M1/70
Rat anti-mouse CD182 (CXCR2)	BV650	1.5	V48-2310
Rat anti-mouse Siglec-F	BV711	1.5	E50-2440
Rat anti-mouse CD101	Alexa Fluor 647	1.5	307707
Rat Anti-Mouse CD16/CD32	-	1	2.4G2
Rat anti-mouse CD177	Alexa Fluor 647	1.5	Y127
Rat anti-mouse CD63	PE	1.5	NVG-2
Mouse anti-human CD11b	APC-Cy7	1.0	557754
Mouse anti-human CD66b	APC	1.0	G10F5
Rabbit anti-mouse CTSE	-	10	Polyclonal
Goat anti-rabbit IgG	Alexa Fluor 488	2	Polyclonal
Rabbit anti-mouse beta-3 tubulin	-	0.1	EP1569Y
Rat anti-mouse Ly6G	Alexa Fluor 594	2	1A8
Goat anti-rabbit IgG	Alexa Fluor 647	2	Polyclonal

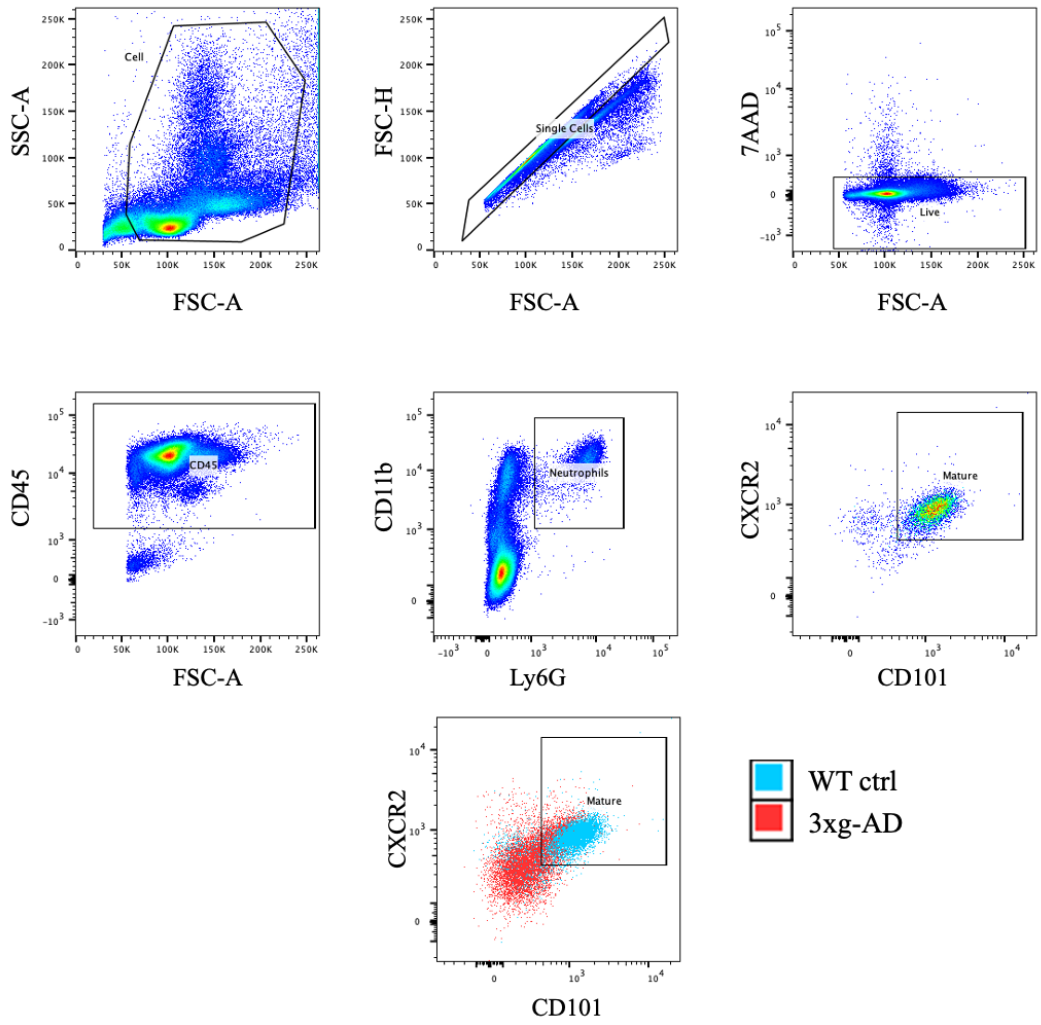

Figure A1. ***Gating strategy applied to circulating neutrophil analysis.*** *Cells were gated based on size and granularity, followed by doublet exclusion. Any anomalies indicative of unstable signal acquisition were excluded using the time parameter. Events were then plotted against the channel containing the viability (7AAD). Leukocytes were then gated using CD45 and neutrophils were identified by plotting leukocytes on a CD11b versus Ly6g plot. To identify the mature and immature neutrophil subpopulation, gated neutrophils were plotted on a CXCR2 against CD101 graph.*

.

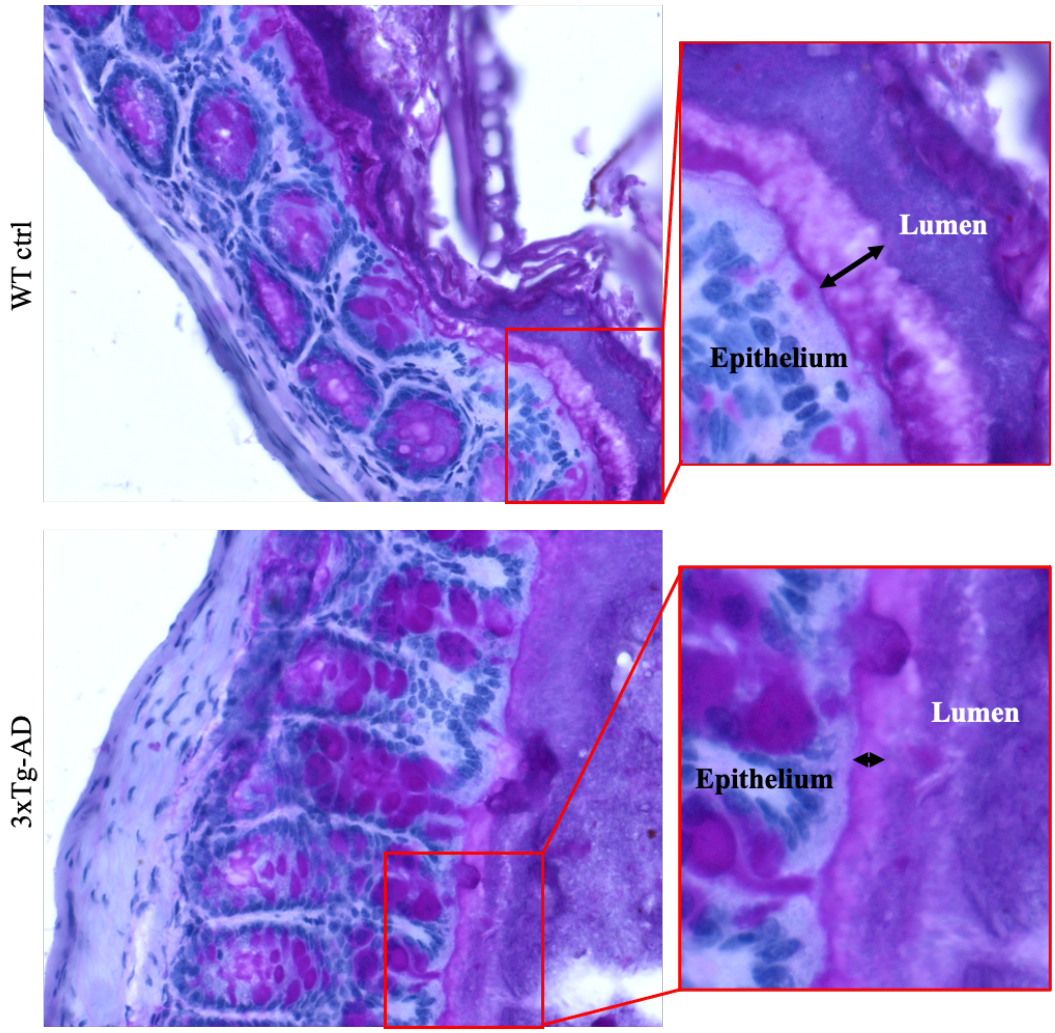

Figure A2. ***Histopathologic changes in the colon of 3xTg-AD mice****. Segments of the colon from male mice were emersed in OCT and flash frozen. 10 μm sections were used for PAS staining. Representative images of WT and 3xTg-AD mice are shown. The right panel images represent zoomed-in versions of the mucosal barrier. Black double-headed arrows indicate mucus barrier thickness.*

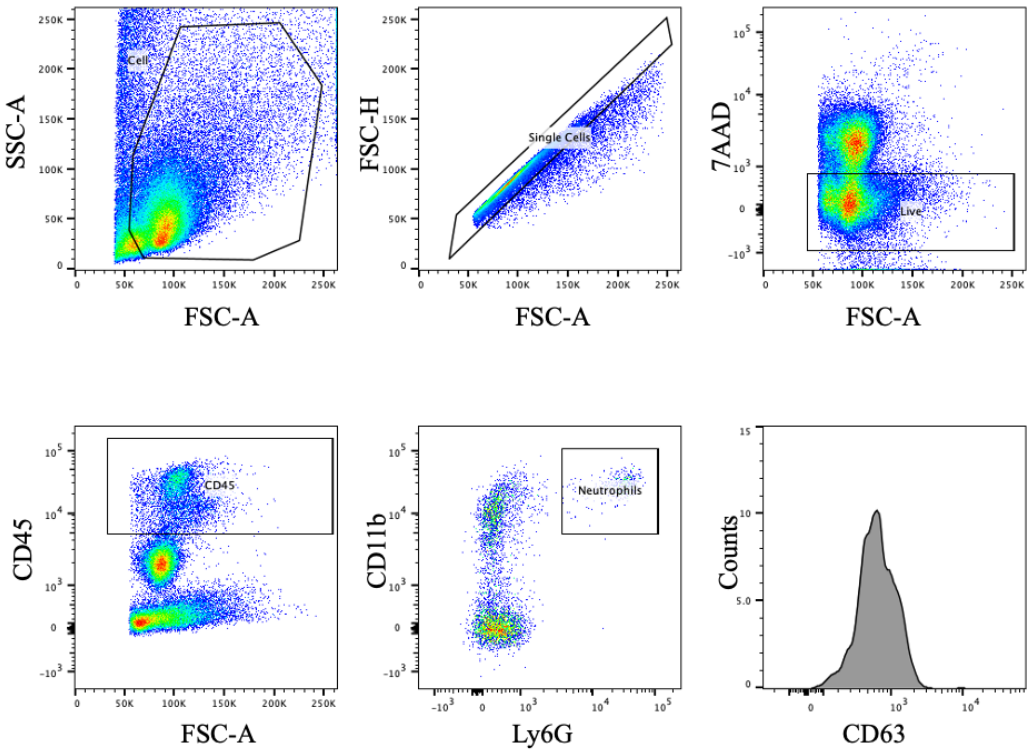

Figure A3. ***Gating strategy applied to CNS-infiltrating neutrophil analysis****. Cells were gated based on size and granularity, followed by doublet exclusion.. Any anomalies indicative of unstable signal acquisition were excluded using the time parameter. Events were then plotted against the channel containing the viability (7AAD). Leukocytes were then gated using CD45. Most of the microglial cells were excluded based on their lower CD45 expression. Neutrophils were identified by plotting leukocytes on a CD11b versus Ly6g plot.*