International PhD Program in Neuroscience

XXXIV Cycle

Targeting TNFSF10 as a novel immunotherapeutic strategy in Alzheimer's disease

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Ph.D. thesis

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2022

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Acknowledgements

I would like to thank Professor Renato Bernardini and Professor Giuseppina Cantarella who opened my mind to science, believing in my abilities and scientific prospect. I must express my sincere gratitude for their precious guidance and inestimable support throughout this fascinating journey, made up of research, professional and personal growth.

I am grateful to Professor Michal Schwartz who welcomed me in her group at the Weizmann Institute of Science (Rehovot - Israel). It was a great pleasure and honor to work with her in such an inspiring scientific environment. It has been undoubtedly an essential experience for my path, which enriched me both personally and scientifically.

I also want to thank Professor Salvatore Salomone and Professor Claudio Bucolo for their support and for allowing me to discover the fascinating world of neuroscience giving me the opportunity to take part to this prestigious Ph.D. program.

I am also grateful to all friends and colleagues that I met during these years with whom I've shared best and worst moments, for their precious advice in science and life and for enriching my experience.

Last, but not least, special thanks go to my beloved parents, for always supporting me in my choices and for being a safe haven where to dock and stock up on happy moments.

List of abbreviations

AD	Alzheimer's disease
AMD	Age-related macular degeneration
Aβ	Amyloid-beta
APP	Amyloid precursor protein
BBB	Blood brain barrier
CADRO	Common Alzheimer's disease research ontology
CNS	Central nervous system
DD	Death domain
DcRs	Decoy receptors
DR4	Death receptor 4
DR5	Death receptor 5
FDA	Food and Drug Administration
GWAS	Genome-wide association studies
MCI	Mild cognitive impairment
MAPKs	Mitogen-activated protein kinases
NF-κB	Nuclear transcription factor-κB
NFTs	Neurofibrillary tangles
NMDA	N-methyl-D-aspartate
OPG	Osteoprotegerin
TNF	Tumor Necrosis Factor
TRAIL	Tumor Necrosis Factor Apoptosis-Inducing Ligand
TNFSF10	Tumor necrosis factor ligand superfamily member10
p-Tau	Phosphorylated Tau

Abstract

Alzheimer's disease (AD) is the most common cause of dementia worldwide, characterized by extreme unmet needs and a huge disease burden. Cerebral atrophy, amyloid plaques, and neurofibrillary tangles represent the main pathological characteristics of the AD brain.

Despite remarkable research efforts to unravel pathophysiological mechanisms of AD, a disease-modifying therapeutic option is still today far from clinical practice, as numerous phase III clinical trials targeting amyloid-beta (Aβ) and other disease markers have failed to improve clinical outcomes of AD patients enrolled. The identification of reliable biomarkers that reflect disease progression is crucial to design disease-modifying therapies able to act at its earliest preclinical stage for better management of AD patients.

Elevated levels of inflammatory markers in patients with AD and the identification of AD risk genes associated with innate immune functions suggest that inflammation holds a crucial role in AD pathogenesis, implying that immune pathways could represent therapeutic targets.

In this line, inflammatory cytokines are actively involved in AD pathogenesis and may serve as diagnostic or therapeutic targets to keep track of AD-related neurodegeneration. Since a physiologic immune response appears crucial to ensure maintenance of a healthy brain, this research project has focused on Tumor Necrosis Factor Apoptosis-Inducing Ligand (TRAIL), also named as TNFSF10, a cytokine belonging to the TNF superfamily, involved in the pathogenesis of several disorders and, in particular, in the AD-related inflammatory processes.

Taking into account such hypothesized prominent role of immune response in AD, a first objective of the project has been testing whether chronic immunoneutralization of TNFSF10 could imply a re-balance of both central and peripheral immune response and whether this could relate to the decreased Aβ burden in the brain of 3xTg-AD mice, a transgenic strain resembling human AD pathology and already known to display improvement of functional outcome following treatment with an anti-TNFSF10 monoclonal antibody.

Neutralization of TNFSF10 resulted in a significantly reduced expression of an array of immune/inflammatory markers, including those typical of peripheral T cells subgroup referred to as T regulatory cells, and restraint of microglial activity, paralleled by dramatically decreased burden of Aβ and p-Tau. Consistently, overshoot of splenic

inflammatory/immune parameters associated with parenchymal amyloidosis were shut down as a consequence of the treatment.

In the attempt to assess a suitable model to identify AD in its early phases, the second objective of the study was to investigate in 3xTg-AD mice the role of the TNFSF10 system and miRNAs usually associated to neuroinflammatory processes in the retina, a tissue regarded as an extension of the central nervous system. To accomplish this task, the focus was made on a set of miRNAs, linked to both AD and age-related macular degeneration (AMD).

Specific age-related miRNA dysregulation was found in the retina of 3xTg-AD mice in a way related to the TNFSF10 signaling pathway, as assessed by bioinformatic analysis. The whole miRNA/TNFSF10 network was shut down following chronic TNFSF10 immunoneutralizing treatment, paralleled by a dramatic improvement of both tissue and inflammatory parameters in the retina of 3xTg-AD mice.

In conclusion, immunopharmacological modulation of TNFSF10 brings about significant amelioration of the AD pathology, suggesting that therapeutic exploitation of TNFSF10 signaling represents a novel strategy for effective treatment of AD and related phenomena.

General introduction

1. ALZHEIMER'S DISEASE: A GENERAL BACKGROUND

Dementia has become a global challenge for public health. Alzheimer's disease (AD) is the leading cause of dementia in the elderly population worldwide, accounting for an estimated 60% to 80% of cases, characterized by the highest clinical unmet needs and a huge disease burden [1].

AD is a devastating neurodegenerative disorder that inexorably upset memory, cognitive functions, and the ability to carry out common daily activities [2,3].

Aging is the strongest risk factor for the disease, the incidence of which doubles every 5 years after the age of 65. With the increase of life expectancy, the incidence is estimated at 10% for individuals over the age of 65 years and 40% for those over 80 years [4]. Other than aging, which is not sufficient to cause AD, genetics and having a family history of AD can contribute to the probability of developing this disorder [1,5].

The neuropathology of AD manifests in several features.

Although the brain of AD patients does not show any macroscopic alteration that can be considered diagnostic [6], a typical symmetric pattern of cortical thinning and atrophy, predominantly affecting the medial temporal lobes, and subsequent enlargement of the frontal and temporal horns of the lateral ventricles (*ex vacuo hydrocephalus*) [7] are considered highly suggestive of AD (**Figure 1**).

The cardinal microscopic pathological hallmarks of the disease, currently used for diagnostic interpretation, are represented by amyloid plaques that contain extracellularly deposited amyloid β (Aβ) obtained from cleaved amyloid precursor protein (APP), and neurofibrillary tangles (NFTs) generated by intracellular accumulation of hyperphosphorylated and misfolded tau protein. These neuropathological features show a different degree of correlation with the dementia severity or duration in AD [8].

Aβ plaques are detectable in the brain many years or even decades before dementia onset [9] but appear to have only subtle effects on cognition and brain health in humans [10,11]. In contrast, the spreading of tau neurofibrillary tangles, a process that animal models have suggested may be accelerated by the presence of brain Aβ plaques, is strongly correlated with local neurodegeneration and frank cognitive impairment [12].

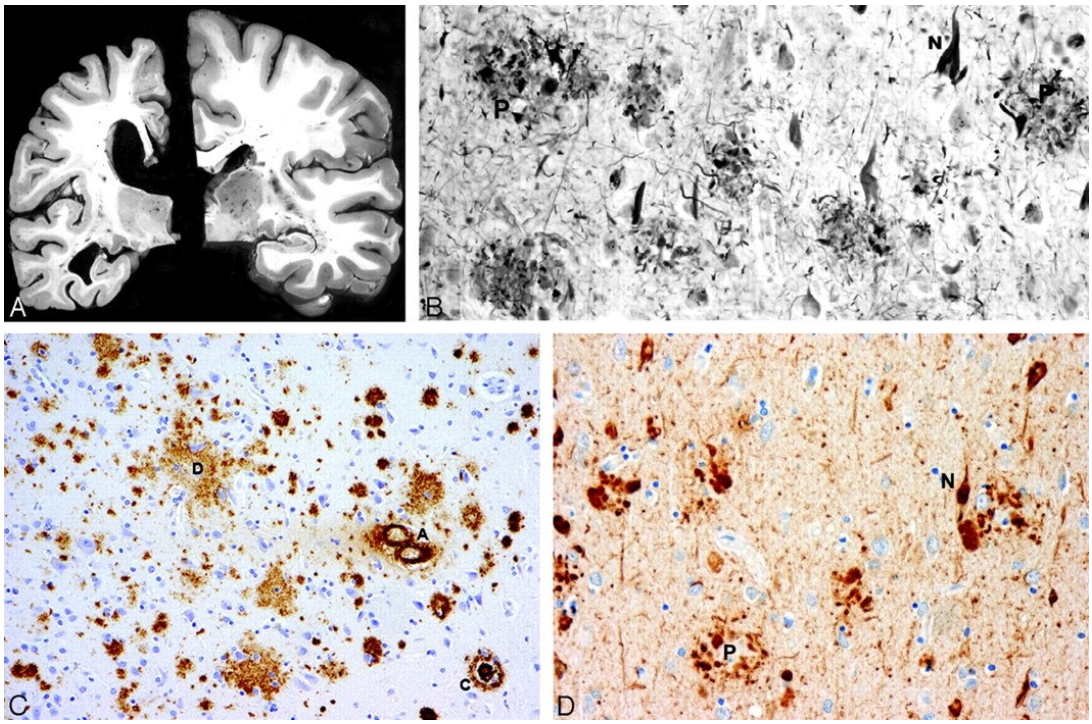

Figure 1. Macroscopic and microscopic features of Alzheimer's brain. A. Brain atrophy. Section of the cerebral hemisphere of a 70-year-old AD patient and, on the right, a healthy aged brain. The AD brain shows marked atrophy, often accompanied by enlargement of the frontal and temporal horns of the lateral ventricles, and a small hippocampus. **B**. Neurofibrillary tangles (N) and Aβ plaques (P) in the hippocampus. Image obtained by silver impregnation. **C**. β-amyloidosis in the frontal lobe: a diffuse plaque (D), a cored plaque (C), and cerebral amyloid angiopathy (A). **D**. Neurofibrillary tangles (N) and Aβ plaques (P) in the frontal lobe. [13]

Despite the efforts made to validate approaches for early diagnosis, the advent of sophisticated neuroimaging techniques [14], and the search for reliable biomarkers [15,16], to date, clinical AD dementia cannot be definitively diagnosed until post-mortem neuropathological examination [4].

Although remarkable research efforts have been made to uncover the pathophysiological mechanisms of AD and to further translate these findings into the clinic, currently available Food and Drug Administration (FDA)-approved pharmacotherapies for AD (listed in **Table 1**) [17] represents only symptomatic treatments with no disease-modifying potential, licensed for the management of cognitive impairment and for the dementia stage of AD [3].

Except for memantine, which blocks excessive stimulation of N-methyl-D-aspartate (NMDA) receptors in the brain preventing nerve cells damage, these drugs include cholinesterase inhibitors actively involved in counteracting the neurotransmitter imbalances typical of the disease [18].

Drug	Time (approved by FDA)	Chemical class	Action	Type of inhibition	Route of administration	Indication	Status
Tacrine	1995	Alkaline	AChE inhibitor	Rapidly reversible	Oral or rectal	_	Withdrawal
Donepezil	1996	Piperidine	AChE inhibitor	Rapidly reversible	Oral	Mild- moderately (mod) AD	Approved
Rivastigmine	1997	Carbamate	AChE and BChE inhibitor	Pseudoreversible	Oral or transdermal patch	Mild-mod AD	Approved
Galantamine	2001	Phenanthrene alkaloid	AChE inhibitor	Rapidly reversible	Oral	Mild-mod AD	Approved
Memantine	2003	Glutamatergic modulator	NMDA antagonist	N/A	Oral	Mod-severe AD	Approved

Table 1. FDA-approved pharmacotherapies for Alzheimer's disease.

Available data from a wide number of clinical trials in which various hypotheses for AD have been tested [2,19], and the limited progress of therapeutics with potentially disease-modifying properties in phase III clinical trials (**Figure 2**), suggest that it is time to adopt alternative strategies for AD treatment [20].

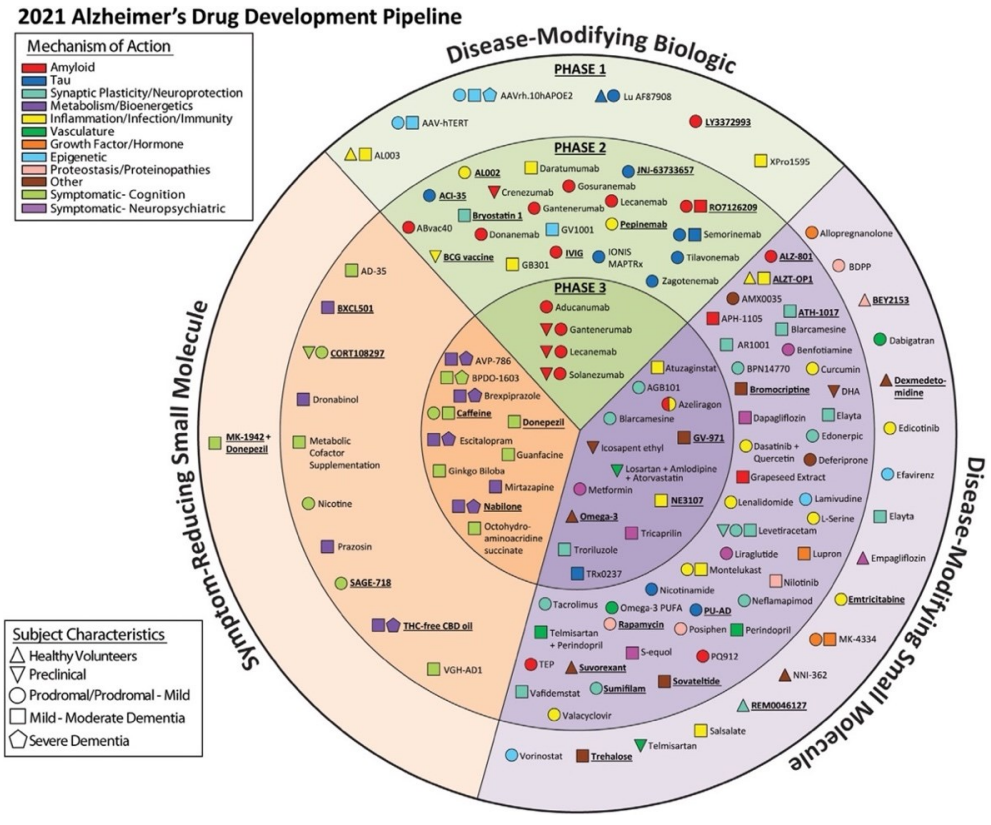

Figure 2. Agents in clinical trials for treatment of Alzheimer's disease in 2021. Phase 1, 2 and 3 agents are respectively shown in the outer, middle, and inner rings. Agents in green area are biologics; agents in purple are disease-modifying small molecules; agents in orange are symptomatic agents addressing cognitive, behavioral and neuropsychiatric symptoms; the shape of the icon shows the population of the trial; the icon color shows the Common Alzheimer's Disease Research Ontology (CADRO)-based class of the agent [19].

Most of the failed phase III trials intervened on patients with mild-to-moderate symptomatic AD, a stage in which significant and irreversible synaptic and neuronal loss has already occurred, and the pathological cascade would likely be very difficult to reverse [17,21].

In this scenario, the identification of reliable biomarkers that reflects disease progression is crucial to design disease-modifying clinical interventions able to act early in the disease continuum, either during the preclinical or mild cognitive impairment (MCI) phases [1]. At these stages, therapies might have a better chance of changing disease trajectory [17,22,23].

In short, at present, all the new strategies in AD drug development seems to shift the focus from treatment to prevention by examining the potential neuroprotective activity of disease-modifying drugs in the pre-symptomatic stages of AD, with the help of biomarkers that predict disease progression before the development of overt dementia.

2. NEUROIMMUNE INTERACTIONS IN ALZHEIMER'S DISEASE

Over the years, several hypotheses have been proposed to unveil the complex pathological mechanisms underlying AD-related neurodegeneration [2,24]. However, the ultimate etiology of AD remains obscure.

Recent genome-wide association studies (GWAS) emphasize the crucial causal role of the immune system, rather than immune response as a consequence of disease pathology, by establishing an enrichment of variants in genes or loci that contribute to AD risk in immune-related tissues (whole blood, spleen, and liver) as well as in the main brain resident immune cells [25].

These observation and the discovery of elevated levels of inflammatory markers associated with cognitive decline in AD patients [26,27] suggest the existence of tight interactions of immunological mechanisms within the central nervous system (CNS) [28].

As it is now well-recognized that AD is a disorder not completely restricted to pathology and biomarkers within the brain, it might become necessary for AD to be studied as a generalized systemic disorder by targeting surrogate tissues [29].

Is it time to shift tactics on AD?

After years of failed therapeutic attempts targeting Aβ and other disease markers, several studies suggest that neuroinflammation holds a crucial role in AD pathogenesis, implying that immune pathways could represent primary therapeutic targets [27].

2.1 Focus on neuroinflammation and mediators of immune/inflammatory response in Alzheimer's Disease

Despite the established perception of the brain as a site of immune privilege had hindered research and therapeutic approaches that involve immune pathways for years [30], only recently neuroinflammation and the immune response in the CNS have been appreciated as major driving forces in AD pathogenesis [31,32].

The immune privilege is not absolute, and it is now accepted that cells of the CNS are sensitive to the inflammatory events occurring both within the brain and in the periphery, as well as to the infiltration of peripheral immune cells [33,34].

The inflammatory response in AD is a double-edged sword. Primarily, it constitutes a self-defense reaction aimed at eliminating harmful stimuli and restoring tissue integrity and becomes detrimental when a chronic response is mounted [35,36].

In particular, brain damage due to Aβ and NFTs in AD triggers a local immune response mediated by astrocytes and microglia that respond to these stimuli producing various mediators of the inflammatory/immune response (i.e., pro-inflammatory cytokines, acute-phase proteins, and complement components) [37,38].

When such a first immune-related process aimed at removing the harmful stimuli is not completely resolutive and the inflammatory stimuli persist, glia-mediated mechanisms remain trapped in a vicious cycle characterized by chronicized release of pro-inflammatory cytokines and chemokines [39,40].

These inflammatory mediators and all the pathological components of the unresolved response promote the recruitment of the peripheral leukocytes, which infiltrate the brain via the compromised blood-brain barrier (BBB). Immune cells infiltrating the brain parenchyma release neurotoxic and proinflammatory factors that act on glial cells, fueling any existing central inflammation [40].

The neuroinflammatory process increases disease severity by exacerbating Aβ and tau pathologies [35,37].

Moreover, the scenario of an inflammatory response not limited only to the CNS but also involving the peripheral compartment suggests that AD could be considered as a generalized systemic disorder [31].

Although the description of pathways for periphery-brain communication is beyond the scope of the present work and have been reviewed in detail elsewhere [41,42], inflammatory reaction in peripheral tissues can induce the production of cytokines which in turn communicate with the brain via blood flow causing damage of BBB integrity. Subsequent cerebral infiltration of peripheral immune cells further contribute to brain pathology and synthesis of inflammatory mediators in the brain parenchyma [27].

In this scenario, the study of pathological changes occurring beyond the brain could offer new opportunities for the early diagnosis of AD and lead to the design of specific therapeutic strategies.

3. FINE-TUNING OF THE IMMUNE RESPONSE BY CYTOKINES: THE CRUCIAL ROLE OF TNFSF10

Convincing evidence supports the pathophysiologic relevance of mediators of inflammatory/immune response in neurodegeneration, as for instance injured neurons and activated glia express and release substantial amounts of cytokines, which amplify and eventually exacerbate the ongoing neurodegenerative process [43].

In this scenario, neuroinflammation acts as an independent factor at a very early stage of AD, where the immune-related genes and cytokines represent the key participants [44]. Cytokines are a heterogeneous and multifunctional group of proteins that provide cells with the ability to communicate, generally acting in a paracrine or autocrine manner, orchestrating complex multicellular behavior [37].

Although a balance between pro- and anti-inflammatory cytokines is important to timely withdraw excessive reaction that leads to neurodegeneration, pro-inflammatory cytokines represent the major payload delivered by the main CNS and peripheral cell mediators of inflammatory response in AD [45,46].

Cytokines belonging to the Tumor Necrosis Factor (TNF) superfamily, which mainly encompass pro-inflammatory cytokines, are the master regulators of the accelerated cell death rate which characterize neurodegenerative processes and play a crucial role in the orchestration of immunity and inflammation [47,48].

Among these, Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), now known as TNFSF10, originally discovered as a tumor cell killer with a vital module in the field of cancer treatment and identified by Wiley and colleagues (1995) [49,50], is a pleiotropic cytokine involved in immune regulation and in a plethora of other biological effects in non-malignant cells [51].

TNFSF10 is expressed in various tissues, including immunogenic organs (i.e., spleen and thymus) and in several immune cells (i.e., monocytes, macrophages, T lymphocytes, neutrophils, NK cells, and dendritic cells) [52].

TNFSF10 represents an interface between immunity and apoptosis, as it regulates either fine co-tuning of the innate and adaptive immune response (as extensively reviewed elsewhere [39,53]) or cell death signaling pathway in different settings [54]. Moreover, this cytokine predominantly regulates various pathophysiological processes involving multiple systems, such as autoimmune and inflammatory diseases [55–57].

Interestingly, TNFSF10 also represents an effector of immune-surveillance in all tissue and organs, including the CNS and the retina, a tissue regarded as an extension of the CNS [39,58,59].

TNFSF10 is not constitutively expressed in the normal brain [60], while in course of inflammation, is abundantly released by a wide range of activated immune-competent cells and injured neurons [61–63] and may act as a potential cell death signal by interacting with TNFSF10 receptor-expressing cells resident in the CNS [64–66].

3.1 The TNFSF10 signaling system

Endogenous TNFSF10, encoded by the *TNFSF10* gene, can be detected as a 281 amino acid (aa), 33 kDa type II transmembrane protein with a small intracellular domain of 17 aa, and as a soluble protein (~20 kDa) due to proteolytic cleavage from the cell surface [52,67].

In humans, TNFSF10 binds to two death domain (DD) containing death-inducing receptors which include TRAIL-R1 (also known as DR4 and TNFRSF10A), TRAIL-R2 (also known as DR5 and TNFRSF10B), leading to receptor trimerization and recruitment of intracellular mediators which transduce a caspase-dependent death signal [57].

In contrast to humans, mice express only one functional TNFSF10 DD-containing receptor (mTRAIL-R), equally homologous to human TRAIL-R1 and TRAIL-R2, which can signal apoptosis upon binding of TNFSF10 [68].

In addition, TNFSF10 can bind non-DD-containing decoy receptors (DcRs), TRAIL-R3 (also known as DcR1 and TNFRSF10C), TRAIL-R4 (also known as DcR2 and TNFRSF10D), and osteoprotegerin (also known as OPG and TNFRSF11B), decreasing the concentration of the TNFSF10 available for binding with death-inducing receptors; thus, leading to negative regulation of apoptosis induction [69–71].

The homotrimeric and biologically active form of TNFSF10 interacts with the complex system of receptors, leading to different signaling outcomes, ranging from pro-apoptotic (extrinsically mediated death pathway in cells generating enough caspase-8 (or -10) activation and apoptotic signaling, and an intrinsic pathway in cells that require additional processes to lead to full apoptosis through cleavage of the pro-apoptotic protein Bid) to prosurvival/proliferative effects (nuclear transcription factor-κB (NF-κB), the

mitogen-activated protein kinases (MAPKs), and PI3K/Akt and ERK pathways), as described in detail elsewhere [51] and represented in **Figure 3**.

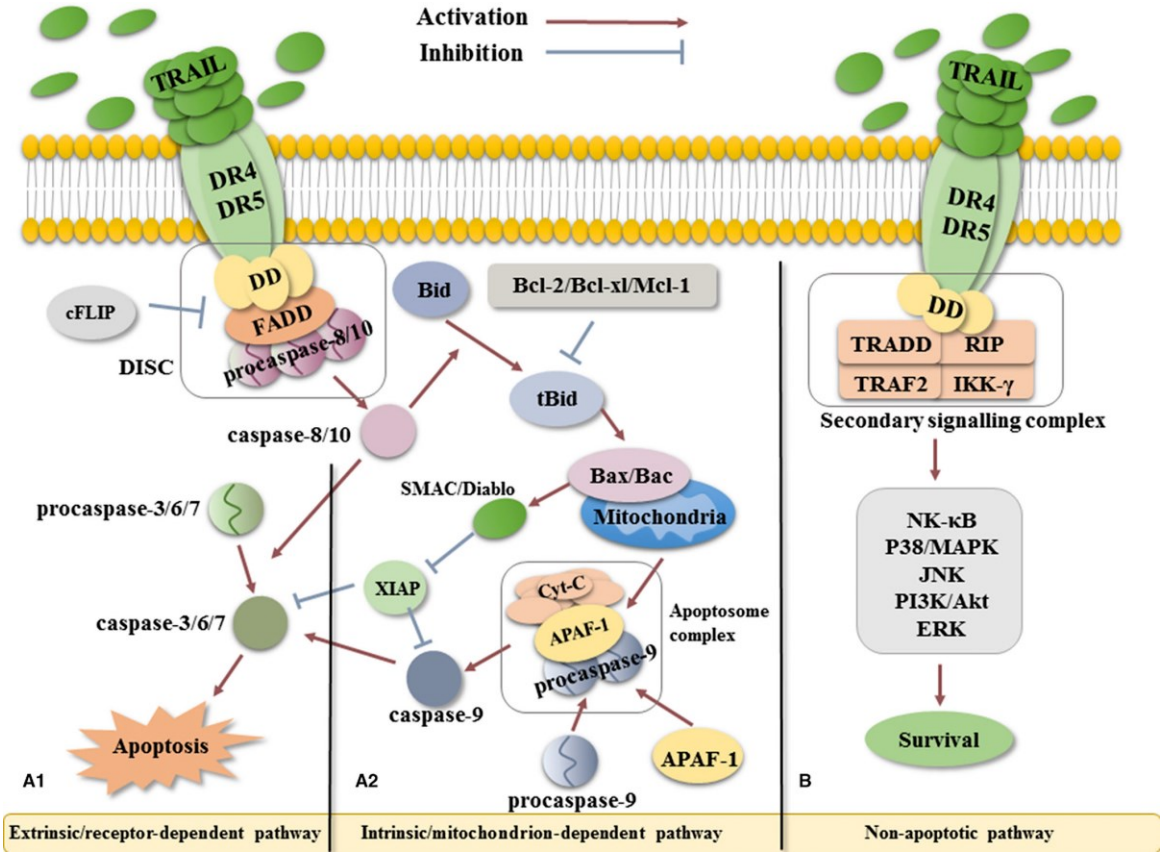

Figure 3. **The TRAIL (TNFSF10) signaling pathway**. **A**: *Apoptotic signaling pathway*. Upon binding of TRAIL, its receptors DR4/5 heterotrimeric receptor-ligand complexes, which subsequently recruit FADD and pro-caspase 8/10 to form the DISC complex, in which pro-caspase 8/10 is cleaved and activated autocatalytically producing caspase 8. Subsequently, in (**A1**) extrinsic pathway, caspase 8 (or -10) activates executioner caspase 3/6/7 directly; in (**A2**) intrinsic pathway, caspase 8 (or -10) cleaves the pro-apoptotic protein Bid into tBid which later interact with Bax/Bac on the mitochondrial membrane leading to lysis of mitochondria and to the release of Cyt-C SMAC/Diablo. Cytochrome c couples with pro-caspase 9 and apoptotic protease-activating factor-1 (APAF-1) to form apoptosome complex, which sequentially activates caspase 9 and executioner caspase 3/6/7. The SMAC/Diablo also promotes apoptosis as it blocks XIAP, which is a direct inhibitor of the caspase 3/9 activation. The effect of SMAC/Diablo might be crucial in case of insufficient caspase 8 activations. **B**, *Non-apoptotic signaling pathway*. Adaptor molecules such as TRADD, TRAF2, RIP1, and IKK-γ are recruited and form the secondary signaling complex that mediates intracellular signal transmission involving several molecular mediators such as NF-κB, P38/MAPK, JNK, PI3K/Akt, and ERK. These pathways induce cell survival, proliferation, and migration [51]. APAF-1, apoptotic protease–activating factor-1; Bcl-2, Bcl-XL, Mcl-1, Bac, Bax all belong to Bcl-2 family, B cell leukaemia 2 family; Bid, BH3 domain–containing protein; c-FLIP, cellular FADD-like IL-1β–converting enzyme inhibitory protein; DISC, death-inducing signaling complex; DR4/5, death receptor 4/5; ERK, extracellular regulated kinase; FADD, Fas-associated death domain; IKK-γ, inhibitor of κB (IκB) kinase-γ; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinases; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; PI3K, phosphatidylinositide 3-kinases; RIP1, receptor-interacting kinase 1; tBid, truncated Bid; TRADD, TNFR1-associated death domain; TRAF2, TNF receptor-associated factor 2; TRAIL, TNF-related apoptosis-inducing ligand; TRAIL-R, TNF-related apoptosis-inducing ligand receptor; XIAP, X-linked inhibitor of apoptosis protein

4. TARGETING THE IMMUNE MOLECULE TNFSF10 TO DAMPEN NEURODEGENERATION AND ITS PHENOMENA

Treatments designed to target specific immune pathways or molecules crucial for neurodegenerative processes represent an attractive therapeutic approach for neurodegenerative disorders, such as AD [72,73].

Cytokines have attracted much attention towards their exact roles in different stages of neurodegenerative disease and the possibility of being exploited as therapies [73].

Although targeting cytokines represents a relatively new approach, it is noteworthy several cytokine inhibitors have already been used successfully for the treatment of different disease conditions [74,75].

In this line, besides the best characterized anti-cancer activity, mounting evidence suggests that TNFSF10, with its prominent death signaling and immune-modulating properties, and by its involvement in many processes of the innate and adaptive immune response [54], plays a pivotal role in CNS disorders of various nature and in neurodegeneration [51,65].

Indeed, TNFSF10 has been recognized as a biomarker in various CNS non-neoplastic diseases [51].

TNFSF10 represents a potent mediator of prominent neuronal loss induced in both chronic and acute neurodegenerative processes. Such involvement in neurodegeneration as a damaging factor has led to its recognition as a potential target to be neutralized.

TNFSF10 is specifically expressed in the human AD brain but not in the normal brain, and its expression is mainly localized in AD-affected regions, such as the cerebral cortex, often in the proximity of Congo-red-positive amyloid plaques [76].

This cytokine substantially contributes to amyloid-induced neurotoxicity in a human neuronal-like cell line, whereas blockade of its cascade via a TNFSF10-neutralizing monoclonal antibody appears to prevent neurotoxicity *in vitro* [77].

This evidence suggests that neurons represent an independent and potential source of TNFSF10. Thus, the latter acts redundantly with other noxious stimuli in neurodegenerative diseases characterized by amyloidosis and neuroinflammation [76].

Consistently with these findings, immunoneutralization of TNFSF10 by means of a neutralizing monoclonal antibody resulted in restored cognitive behavior, reduced deposition of Aβ, and dramatically decreased expression of immune/inflammatory mediators in the brain of 3xTg-AD mice [43].

In addition, TNFSF10 is upregulated in the apoptotic areas of the post-ischemic brain triggering apoptosis following focal brain ischemia *in vivo* [78] and its blockade reduced the rate of post-ischemic neuronal death [79].

TNFSF10 seems to play a pivotal role in the post-ischemic inflammatory process, as its immunoneutralization restrains post-ischemic tissue damage.

Prolonged ischemic injury induces overexpression of TNFSF10 and its death receptors, whereas dramatically reduce the expression of both DcR1 and DcR2 [79].

Preconditioning-mediated neuroprotection largely occurs also through both upregulation of TRAIL decoy receptors, an event which, thus, guarantees TNFSF10 molecule neutralization, as well as downregulation of TNFSF10 itself and of its death receptors, perhaps in the attempt of the tissue to set into motion a self-protective strategy [80].

TNFSF10 mediates also neuronal damage in HIV encephalopathy, a neurodegenerative disorder characterized by infiltration of HIV-infected monocyte-derived macrophages.

TNFSF10 expressed on macrophages interacts with death receptors on neurons triggering neuronal apoptosis in the brain, and its neutralization results in neuroprotection [81].

Moreover, it has been discovered that TNFSF10 is involved in the inflammatory response and cellular apoptosis after spinal cord injury [82,83].

Immunoneutralization of TNFSF10 resulted in improved functional recovery, reduced apoptotic cell number, modulation of molecules involved in the inflammatory response, and the corresponding signaling [83].

In synthesis, TNFSF10 efficiently sets into motion redundant neurodegeneration-related cell death processes, and its neutralization implies either significant attenuation or abrogation of phenomena typical of neurodegeneration [84].

Nevertheless, due to the crucial role of TNFSF10 in neurodegeneration, we propose the hypothesis of targeting TNFSF10 as a potential immunomodulatory strategy to improve outcomes in AD studying the pathology either in the periphery or in the brain, as well as in the retina, regarded to as a developmental outgrowth of the brain.

Chapter I

Beneficial effects of curtailing immune susceptibility in an Alzheimer's disease model

J Neuroinflammation. 2019;16(1):166. doi: 10.1186/s12974-019-1554-9.

BENEFICIAL EFFECTS OF CURTAILING IMMUNE SUSCEPTIBILITY IN AN ALZHEIMER'S DISEASE MODEL

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Abstract

Background: Currently, there are no effective therapeutic options for Alzheimer's disease, the most common, multifactorial form of dementia, characterized by anomalous amyloid accumulation in the brain. Growing evidence points to neuroinflammation as a major promoter of AD. We have previously shown that the proinflammatory cytokine TNFSF10 fuels AD neuroinflammation, and that its immunoneutralization results in improved cognition in the 3xTg-AD mouse.

Methods: Here, we hypothesize that inflammatory hallmarks of AD might parallel with central and peripheral immune response dysfunction. To verify such hypothesis, we used a triple transgenic mouse model of AD. 3xTg-AD mice were treated for 12 months with an anti-TNFSF10 antibody, and thereafter immune/inflammatory markers including COX2, iNOS, IL-1β and TNF-α, CD3, GITR, and FoxP3 (markers of regulatory T cells) were measured in the spleen as well as in the hippocampus.

Results: Spleens displayed accumulation of amyloid-β_{1-42} ($A\beta_{1-42}$), as well as high expression of Treg cell markers FoxP3 and GITR, in parallel with the increased levels of inflammatory markers COX2, iNOS, IL-1β and TNF-α, and blunted IL-10 expression. Moreover, CD3 expression was increased in the hippocampus, consistently with FoxP3 and GITR. After chronic treatment of 3xTg-AD mice with an anti-TNFSF10 antibody, splenic FoxP3, GITR, and the above-mentioned inflammatory markers expression was restored to basal levels, while expression of IL-10 was increased. A similar picture was observed in the hippocampus. Such improvement of peripheral and CNS inflammatory/immune response was associated with decreased microglial activity in terms of TNFα production, as well as decreased expression of both amyloid and phosphorylated tau protein in the hippocampus of treated 3xTg-AD mice. Interestingly, we also reported an increased expression of both CD3 and FoxP3, in sections from human AD brain.

Conclusions: We suggest that neuroinflammation in the brain of 3xTg-AD mice triggered by TNFSF10 might result in a more general overshooting of the immune response. Treatment with an anti-TNFSF10 antibody blunted inflammatory processes both in the spleen and hippocampus. These data confirm the detrimental role of TNFSF10 in neurodegeneration, and corroborate the hypothesis of the anti-TNFSF10 strategy as a potential treatment to improve outcomes in AD.

Keywords: AD target therapy; Immune response; Inflammation; Neurodegeneration.

Background

Alzheimer's disease (AD) is the most common form of dementia [1] and is characterized, besides a high burden of disease, by substantial unmet need [2]. In fact, despite the considerable number of attempts of introducing innovative neuroprotective treatments, such need remains widely unsatisfied.

Although evidence shows that the anomalous protein amyloid beta (Aβ), which exceedingly accumulates in the hippocampus and other cerebral areas of the Alzheimer's brain, plays a pivotal role in the pathogenesis of the disease [3, 4], outcomes of Aβ-based clinical trials have been, so far, deluding [5].

On the other hand, in the past two decades, laboratory research has shed more light on various AD-related candidate pathogenetic factors, including superoxides [6, 7], excitotoxicity [8, 9], as well as inflammation [10, 11]. In this line, peripheral immune cells have more recently been indicated as factors disrupting the immune equilibrium of the brain, potentially contributing to neurodegeneration [12].

Consistently, TNF-related apoptosis inducing ligand (TNFSF10), a potent pro-apoptotic member of the TNF superfamily also produced by injured neurons [13] and activated glia [14], is known to modulate cell-mediated immune response [15], for example, by inducing an increase of the Treg cell subset of T lymphocytes [16], and it is in fact regarded as a critical regulator of autoimmune T cells [17]. While there is evidence that the blockade of TNFSF10 improves pathology in models of multiple sclerosis, beneficial effects in other neurological pathologies, included AD, remain to be investigated [18].

In the AD brain, TNFSF10 sets into motion the inflammatory machinery, and mediates Aβ-induced neuronal death [19]. In fact, the role of TNFSF10 in Aβ-related neurotoxicity has been demonstrated by different studies showing that neutralization of TNFSF10 death pathway protects human neuronal cell line from beta-amyloid neurotoxicity [13], and, that the blockade of the TNFSF10-death receptor DR5 with a specific antibody completely prevents Aβ-induced neurotoxicity in neuronal cells in vitro [20]. Moreover, TNFSF-10 immunoreactivity has been detected in the vicinity of Aβ plaques in post-mortem human AD brains [21]. Consistently, age-related, Aβ-dependent progression of cognitive decline is efficiently prevented by chronic anti-

TNFSF10 treatment in vivo in the 3xTg-AD, a triple transgenic mouse model of Alzheimer's disease [19].

Such prominent neurotoxic effect of TNFSF10 relies upon its capacity of redundantly recruiting other inflammatory cytokines, so to synergistically contribute to the worsening of neuronal function [22].

Despite poor evidence is currently available regarding the influence of peripheral immune response upon the pathology of the AD brain and the related clinical outcome [23, 24], more recent data indicate in fact that not only peripheral immunocytes can enter the brain in murine models of AD but also their modulation significantly influence progression of brain pathology in the same animals [12].

With such rationale, and with the aim to better highlight a hypothetical prominent role of the peripheral immune system in AD, we here investigate whether immunoneutralization of TNFSF10 is related to a re-balance of both the central and the peripheral immune response, and whether this could correlate with decreased Aβ burden in the CNS, consistently with the previously demonstrated improvement of the cognitive outcome in 3xTg-AD mice [19].

To accomplish this task, we used a triple transgenic mouse model of AD (3xTg-AD), a strain homozygous for the Psen1 mutation and homozygous for the co-injected APPSwe and tauP301L transgenes, which presents an age-dependent increase of Aβ oligomer accumulation, extracellular plaques in the cortex and the hippocampus, and tau pathology paralleled by learning and memory impairment [25]. Mice were treated chronically, twice a month for 12 months with intraperitoneal injection of an anti-TNFSF10 monoclonal antibody as previously described [19], to evaluate the effects of TNFSF10 neutralization, upon parameters of neuroinflammation, as well as upon those related to the systemic immune response.

Methods

Animals

Male 3xTg-AD mice [B6129-Psen1tm1MpmTg (APPSwe, tauP30L) 1Lfa/J] [25] and wild-type mice (B6129SF2/J) were purchased from Jackson Laboratories. The 3xTg-AD, overexpressing mutant APP (APPSwe), PSEN1 (PS1M146V), and hyperphosphorylated tau (tauP301L), were originally generated by co-injecting two independent transgene constructs encoding human APPSwe and tauP301L (4R/0 N) (controlled by murine Thy1.2 regulatory elements) in single-cell embryos harvested from mutant homozygous PS1M146V knock-in mice. Wild-type mice of mixed genetic background 129/C57BL6 were used as controls. These mice have been characterized and described by Oddo et al. [25]. The animals were maintained on a 12-h light/dark cycle in temperature- and humidity-controlled rooms, and food and water were available ad libitum. All experiments were carried out according to the Directive 2010/63/EU and the Italian law (D.Lgs. 26/2014) and were approved by the Italian Ministry of Health.

Drug administration and experimental groups

Twenty 3xTg-AD and 20 wild-type mice were enrolled at 3 months of age and four study groups were used: (i) wild-type plus vehicle (Purified Rat IgG2ακ Isotype Control; BD Biosciences, San Jose, CA, USA); (ii) wild-type plus TNFSF10-neutralizing antibody (Purified Rat Anti-Mouse CD253;BD Biosciences); (iii) 3xTg-AD plus vehicle; and (iv) 3xTg-AD plus TNFSF10-neutralizing antibody. Animals (ten per experimental group) were administered with TNFSF10-neutralizing antibody (concentration: 0.05 mg/ml; 200 μl/ mouse; i.p.) or vehicle (concentration: 0.05 mg/ml; 200 μl/ mouse; i.p.) twice a month (Monday at 12 a.m.) and sacrificed at 15 months of age 2-weeks after the last injection.

Human brain samples

Hippocampus tissue slides were obtained from four healthy donors per group (two males, two females, age 59–85 years; post-mortem delay 5–8 h, hippocampal CA2-CA3 subfields) who had no known history of neurological or psychiatric disease. Human AD samples were obtained from patients (two males, two females; age 65–80 years; post-mortem delay 4–6 h; hippocampal CA2-CA3 subfields). Tissues were purchased from Abcam (Cambridge, UK).

Immunohistochemistry

For immunohistochemical analysis, sections were incubated for 30 min in 0.3% H_2O_2/methanol to quench endogenous peroxidase activity, then rinsed for 20 min with phosphate-buffered saline (PBS; Bio-Optica, Italy). High-temperature antigen unmasking was conducted using a microwave oven.

Then, the sections were incubated with diluted rabbit anti-beta-amyloid 1–42 (Merck Millipore, Darmstadt, Germany). After overnight incubation in a humidified chamber (4 °C), sections were incubated with the secondary antibody (for 30 min at RT); detection was performed with the Streptavidin-biotin method using 3,3′-diaminobenzidine (DAB) as chromogen (LSAB 2 System-HRP, Dako, Denmark). Sections were counterstained with hematoxylin (Histolab Products AB, Goteborg, Sweden) mounted in GVA mount (Zymed, Laboratories Inc., San Francisco, CA, USA) and observed under an Axioplan (Zeiss, Germany) light microscope and photographed with a digital camera (Canon, Japan). The antibodies-staining (beta-amyloid 1–42) status was identified as either negative or positive. Immunohistochemical positive staining was defined by the presence of brown chromogen on the edge of the hematoxylin-stained cell nucleus, distributed within the cytoplasm or in the membrane via evaluation with light microscope. Five fields of each sample, randomly selected from each section, were analyzed for morphometric and densitometric analysis. The percentage areas (morphometric analysis) stained with antibodies (anti-beta-amyloid 1–42), expressed as % positive, dark brown pixels of the analyzed fields, and the level (high/low) of staining intensity of positive areas (densitometric analysis), expressed as densitometric count

(pixel2) of positive, dark brown pixels of the analyzed fields, were calculated using an image acquisition software (AxioVision Release 4.8.2—SP2 Software, Carl Zeiss Microscopy GmbH, Jena, Germany). Digital micrographs were taken using the Zeiss Axioplan light microscope (Carl Zeiss, Oberkochen, Germany) fitted with a digital camera (AxioCam MRc5, Carl Zeiss, Oberkochen, Germany).

Protein extraction

Tissues were lysed in a lysis buffer containing 150 mM NaCl, 50 mM Tris–HCl (pH 7.5), 5 mM EDTA, 1 mM Na_3VO_4, 30 mM sodium pyrophosphate, 50 mM NaF, 1 mM acid phenyl-methyl-sulphonyl-fluoride, 5 μg/ml aprotinin, 2 μg/ml leupeptin, 1 μg/ml pepstatin, 10% glycerol, and 0.2% TritonTM X-100. The homogenates were then centrifuged at 14,000 rpm for 10 min at 4 °C. The protein concentration of the supernatant was determined by the Bradford method [26].

Western blot analysis

Equal amounts of protein (50 μg) were resolved by 8–12% SDS-PAGE gels and transferred onto Hybond-ECL nitrocellulose membranes (GE Healthcare, Little Chalfont, UK). Membranes were blocked at room temperature overnight with a blocking solution composed of 5% nonfat dry milk (Bio-Rad Laboratories, Segrate, Italy) in phosphate-buffered saline plus 0.1% Tween 20 (PBS-T) and were then probed with the following appropriate primary antibodies: rabbit anti-beta-amyloid1–42 antibody (Merck Millipore, Darmstadt, Germany); rabbit anti-TNFRSF10B (Abcam); rabbit anti-TNFSF10 (Abcam); goat anti-GITR (R&D Systems, Inc.); rat anti-FoxP3 (eBioscience, San Diego, CA, USA); rabbit anti-IL10 antibody (Abbiotec, San Diego, CA, USA); mouse anti-COX2 (BD Biosciences); rabbit anti-NOS2 (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA); goat anti-IL-1β (R&D Systems, Inc.); and rabbit anti-TNF-α antibody (Abbiotec). After that, the membranes were washed with PBS-T, and finally were probed with the appropriate horseradish peroxidase-conjugated secondary antibodies (GE Healthcare) for 60 min at room temperature in 5% non-fat dry milk (full details of the antibodies used are reported in Additional file 8: Table S1). For immunodetection, the membranes were exposed to film after enhanced chemiluminescence (ECL) (GE Healthcare). β-actin and β-tubulin (Santa Cruz Biotechnology Inc.) were used as an internal control to validate the right amount of protein loaded on the gels. Densitometric analysis of band intensity was performed with the aid of ImageJ software (https://imagej.nih.gov/ij/).

Immunofluorescence and confocal microscopy analysis

Splenic and hippocampal tissue specimens were fixed overnight in 10% neutral-buffered formalin (Bio-Optica). After overnight washing, they were dehydrated in graded ethanol and paraffin-embedded taking care to preserve their anatomical orientation. Tissue sections of 5 mm were then

cut and mounted on silanized glass slides and air dried. To remove the paraffin, slides were immersed in xylene two times, for 3 min each; rehydrated with graded ethanol, 100%, 95%, 80%, 70%, and 50%, for 3 min each; and transferred to tap water. Antigens were retrieved in sodium citrate buffer (10 mM sodium citrate, 0.05% Tween-20, pH 6.0) by microwave for 15 min, followed by rinsing with tap water. The slides were then washed in PBS containing 0.25% Triton X-100 (PBST) twice for 5 min each, blocked in PBST/1% BSA for 1 h at room temperature, briefly rinsed with PBST, and incubated at 4 °C overnight with the following primary antibodies: a rabbit anti-CD3 antibody (Abcam), or a rat anti-FoxP3 antibody (eBioscience), or a mouse anti-GITR antibody (Santa Cruz Biotechnology Inc.), or a rabbit anti-IL10 antibody (Abbiotec), or a rabbit anti-beta-amyloid1–42 antibody (Merck Millipore), or a goat anti-p-TAU antibody (Santa Cruz Biotechnology Inc.), or a rat anti-CD11b antibody (Serotec, Kidlington, UK), or a rabbit anti-TNF-α antibody (Abbiotec). Antibodies were applied directly onto sections before overnight slide incubation (4 °C) in a humid chamber. For fluorescence visualization, after washing in PBS three times for 5 min each, sections were incubated with the corresponding fluorescent-labeled secondary antibodies at dark for 1 h at room temperature: goat anti-mouse IgG-TR (Santa Cruz Biotechnology Inc.); or goat anti-rat IgG antibody, FITC conjugate (Merck Millipore); or goat anti-rabbit IgG-TR (Santa Cruz Biotechnology Inc.); or Alexa Fluor 488 goat anti-rabbit IgG (Life Technologies, Carlsbad, CA, USA); or Alexa Fluor 488 donkey anti-goat (Life Technologies). See Additional file 8: Table S1 for full details of the antibodies used. Finally, for staining of nuclei and stabilization of fluorescent signals, slides were covered in mounting medium (Fluoroshield with DAPI; Sigma-Aldrich, Milan, Italy) and secured with a coverslip. Images were observed using a laser scanning confocal microscope (Zeiss LSM 700, Germany).

Qualitative and quantitative analysis for CD11b and TNF-α immunoreactivity was performed using a Leica 4D confocal laser scanning microscope, equipped with an argon–krypton laser. Confocal images were generated and processed as described [27]. Volume of co-localized elements was determined by Imaris 7.3 as previously described [27]. Four regions of interest (x = 40 μm; y = 40 μm; z = 10 μm) were randomly chosen within each slide and their respective volumes were calculated, summed, and expressed as volume/μm3 [28].

Statistical analysis

Data were analyzed either by the one-way or the two-way analysis of variance (ANOVA), followed, respectively, by the Duncan's least significant difference test or by Bonferroni post-hoc test. Vertical bars are means ± S.E.M. of at least three different experiments; significance was set at a p value < 0.05 or $p < 0.001$. The graphs were made using Graph Pad Prism (Ver. 7, La Jolla, USA). Statistical evaluation was performed using standard computer software (SPSS software package, ver. 23.0, SPSS Inc., Chicago, IL, USA).

Results

Chronic anti-TNFSF10 treatment beneficially influences body, spleen, and brain weight and decreases the amount of amyloid beta in the spleen of 3xTg-AD mice

In light of the reported differences in the size of 3xTg-AD animals compared to wild-type [29], we measured the weight of the body, brains, and spleens, confirming that while both body and brain weight were significantly lower in 3xTg-AD mice vs. wild-type animals, spleens weight was significantly augmented (Fig. 1a). Treatment with the anti-TNFSF10 antibody resulted in increased body and brain weight in 3xTg-AD animals and, on the other hand, in a significant reduction of the spleen weight (Fig. 1a–d).

Immunohistochemical and Western blot analysis performed on spleen samples revealed specific immunoreactivity for amyloid beta 1–42 in AD vehicle (Fig. 2(a, C, black arrows; b)) and AD anti-TNFSF10 (Fig. 2(a, D, black arrows; b)) spleen sections, while spleens from WT animals (untreated; treated with anti-TNFS10) did not show any specific immunoreactivity for amyloid beta 1–42 (Fig. 2(a, A and B, respectively; b)).

Densitometric analysis showed that the expression of amyloid beta 1–42 was significantly higher in untreated AD animals compared to anti-TNFSF10 treated AD animals (Fig. 2(a', b')).

Effects of chronic anti-TNFSF10 treatment on the expression of either proinflammatory and anti-inflammatory molecules in the spleen of 3xTg-AD mice

To corroborate the hypothesis that such changes in 3xTg-AD mice spleen could be related with impairment of some proinflammatory parameters, Western blot analysis was performed on spleen homogenates for the expression of TNFSF10 and its receptor TNFRSF10B, and that of the inflammatory molecules COX2, iNOS, IL-1β, TNF-α, the anti-inflammatory cytokine IL-10, as well as that of the Treg lymphocyte markers GITR and FoxP3.

The expression of COX2, iNOS, IL-1β, and TNF-α was substantially increased in untreated 3xTg-AD mice while treatment with the anti-TNFSF10 monoclonal antibody determined a significant attenuation of their expression. On the other hand, the expression of IL-10 was significantly increased in anti-TNFSF10-treated animals. Furthermore, the expression of GITR and Foxp3, constitutively highly expressed in the spleen of untreated 3xTg-AD mice, was significantly attenuated in animals undergone the anti-TNFSF10 treatment, as confirmed by densitometric analysis (Fig. 3a, b).

In order to investigate the relationship between the variation in the expression of both GITR and FoxP3, we performed immunofluorescence of splenic sections from the same animals. Indeed, immunofluorescence confirmed that the expression of the two molecules was higher in untreated 3xTg-AD mice and it was decreased after treatment with the anti-TNFSF10 antibody. Interestingly, GITR and FoxP3 co-localized within the same cells (Fig. 3c, white arrows).

The neuroinflammatory hallmarks in the hippocampus of 3xTg-AD mice are consistent with the splenic inflammatory pattern and are modulated by the anti-TNFSF10 treatment

In light of the observed splenic alterations, which reflected objective impairment of the peripheral immune response, we further investigated whether the spleen findings could relate with the neuroinflammatory processes in the hippocampus of the same animals. Thus, we studied Treg cells profile markers by means of Western blot analysis (and respective densitometry) in the hippocampus. Robust expression of GITR and FoxP3 was detectable in the brain of untreated AD mice, while treatment with the anti-TNFSF10 antibody resulted in blunted expression of both GITR and FoxP3 (Fig. 4a).

In contrast, Western blot analysis (and relative densitometry) showed that, while the expression of the anti-inflammatory cytokine IL-10 was absent in the hippocampus of 3xTg-AD mice, it became detectable in animals undergone the anti-TNFSF10 treatment (Fig. 4a).

Consistently, additional immunofluorescence experiments demonstrated that, in the same hippocampal areas, FoxP3 was abundantly expressed and co-localized with GITR immunoreactivity in untreated 3xTg-AD mice (Fig. 4b; white arrows). Treatment with the anti-TNFSF10 antibody significantly reduced the expression of both FoxP3 and GITR in the hippocampi of 3xTg-AD mice (Fig. 4b). See Additional file 1: Figure S1 for negative controls.

Moreover, as the observed immune processes were blunted by the anti TNFSF10 treatment, it was of interest that the expression of IL-10 was increased in 3xTg-AD mice treated with the anti TNFSF10 antibody, suggesting a setting into motion of the anti-inflammatory response in the brain of the 3xTg-AD animals, while FoxP3 expression was downregulated (Fig. 4c; white arrows). See Additional file 2: Figure S2 for negative controls.

To verify whether the expression of both GITR and FoxP3 in the hippocampus of 3xTg-AD mice was associated with lymphocytes infiltration, immunostaining for CD3 was performed in the CA2 and CA3 hippocampal areas of untreated 3xTg-AD mice revealing a substantial immunoreactivity in both areas as compared with wild type mice. On the other hand, CD3 immunoreactivity was reduced in the hippocampus of 3xTg-AD mice treated with the anti TNFSF10 antibody compared to untreated animals (Fig. 5a).

To better understand whether such decrease of CD3 immunoreactivity in the hippocampus of anti TNFSF10-treated animals could be due to a decreased number of Treg cell (as per the blunted expression of FoxP3 in the hippocampus), we performed specific immunofluorescence experiments and observed that FoxP3 expression decreased following anti TNFSF10 treatment, in parallel with decreased CD3 specific immunoreactivity (Fig. 5b). See Additional file 3: Figure S3 for negative controls.

Expression of immune markers in the human AD brain

We asked whether changes in CD3, FoxP3, and GITR immunoreactivity observed in the hippocampus of the 3xTg-AD mice similarly occurred in the brain of AD patients. Interestingly, immunohistochemistry showed that while CD3 and FoxP3 were absent in in the hippocampus from healthy individuals (Fig. 6(a)), both proteins were detected in the hippocampus from AD brain, showing a co-localized immunostaining, consistently with the data obtained in mice (Fig. 6(a; white arrows)). Furthermore, the human AD hippocampus also expressed GITR, which co-localized with FoxP3 (Fig. 6(b; white arrows)). Respective densitometric counts are shown in panels a' and b'. See Additional file 4: Figure S4 and Additional file 5: Figure S5 for negative controls.

Reactive proinflammatory microglia is blunted by the anti-TNFSF10 treatment in 3xTg-AD mice

Chronically activated microglia and the related proinflammatory response play a pivotal role in AD neuropathology [10]. Here, we showed confocal images of the microglial marker CD11b, co-localized with the proinflammatory cytokine TNF-α in the hippocampus of mice treated as detailed above.

Microglia in untreated 3xTg-AD mice showed an activated morphology, as suggested by the cell body enlargement and ticker processes while in wild-type mice microglia displayed a resting morphology. Moreover, untreated 3xTg-AD mice showed a dramatic increase in microglial TNF-α, i.e., TNFα co-localized with CD11b, as compared with WT mice (Fig. 7(a, A–C, G–I)). Of note, the treatment with anti-TNFSF10, significantly reduced TNF-α levels in microglia (Fig. 7(a, L–N)).

Expression of Aβ and phosphorylated-tau protein (p-tau) are significantly attenuated in 3xTg-AD mice treated chronically with anti TNFSF10

Amyloid beta and the phosphorylated tau protein are recognize hallmarks of AD, as it is well established that their amount correlates with the disease staging in the AD brain [30] as well as in the 3xTg-AD model [25]. In the light of our findings on the immune response in 3xTg-AD mice, we attempted to establish whether the expression of both Abeta 1–42 (Fig. 8(a)) and p-tau (Fig. 8(b)) paralleled the immune activity pattern observed in the spleen and in the hippocampus of 3xTg-AD mice. Immunohistochemical analysis showed that both amyloid beta 1–42 and p-tau were abundantly expressed in untreated 3xTg-AD mice, while their expression was greatly attenuated after treatment of the animals with the anti-TNFSF10 antibody (Fig. 8). See Additional file 6: Figure S6 and Additional file 7: Figure S7 for negative controls.

Discussion

Here, we showed that 3xTg-AD mice with age-related cognitive decline manifest also an imbalance of the immune/inflammatory response.

We observed an increased spleen weight, as well as decreased brain and body weight in untreated 3xTg-AD mice in their advanced age, confirming data from other authors [29, 31]. At a first glance, one could argue that such spleen enlargement could be due to the presence of a transgene. However, the risk for genome insertion site-related phenotypes and consequent splenomegaly is highly unlikely, because APPswe and PS1M145V transgenes are expressed but not translated in the spleen. In fact, Oddo et al. [25] reported the absence of protein translates in various organs of the 3xTg-AD mouse, with the exception of the central nervous system. In addition, it has been reported that splenic alterations occur in 3xTg-AD mice fairly ahead of the onset of the amyloid pathology [29]. Accordingly, an altered rate of CD3 lymphocytes in the spleen has been reported, suggesting an autoimmune/inflammatory involvement [29]. In this line, light behavioral dysfunctions occurring in the early life of 3xTg-AD mice have been associated with the human parameters of minimal cognitive impairment (MCI), with episodic memory loss in the absence of major cognitive dysfunction [32].

Interestingly, an accumulation of human beta-amyloid 1–42 originating in the transgenic mouse brain was revealed in the spleens of 3xTg-AD mice by immunohistochemical and Western blot analysis. Although in this mouse model beta-amyloid 1–42 and tau transgenes are limited expressed by brain and spinal cord [25], different reports showed that beta-amyloid 1–42 and tau are also present in the blood and peripheral organs [33,34,35,36]. Therefore, we may speculate that over-accumulated brain Aβ might be transported to peripheral organs [37], thereby functioning as a signal to trigger peripheral immune responses [38]. In this scenario, beta-amyloid 1–42 deposits in the spleen could be responsible for the peripheral inflammation observed in the AD mice, and for changes in immune cell setting, with special regard to the increased CD3 population [31].

Consistently, we found that a set of inflammatory molecules, including TNFSF10, and its death receptor, as well as COX2, iNOS, IL-1β, and TNF-α were significantly increased in the spleen homogenates from 3xTg-AD mice. It is also noteworthy that the splenic expression of the antigen GITR [39], as well as that of the Treg cell-related transcription factor FoxP3 [40], were dramatically increased in the spleen of the same animals, whereas the expression of the anti-inflammatory cytokine IL-10 was negligible.

Now, keeping in mind the concept that molecules from the CNS, such as Aβ, may influence also peripheral immune/inflammatory response [41], the exceeding synthesis of Aβ in the brain could set into motion specific immune response, as suggested by the increased expression of splenic Treg markers GITR and FoxP3. In fact, it is noteworthy that Treg cells may suppress, for example, autoreactive T lymphocytes recruited in course of chronic inflammation [42], which could be envisioned as a sort of feedback mechanism to avoid overshooting of the immune/inflammatory

response and tissue damage, as, for example, activation of glucocorticoid secretion set into motion by cytokines release in the course of an immune/inflammatory response [39].

Although contrasting data are available on the role of the GITR system in Treg setting [43], nevertheless, more recent research has defined its role as a marker for human Treg cells [44], confirmed by data showing that GITR could be regarded as a receptor belonging to the immune checkpoint family [43]. In fact, it is known that when Treg cell function is pharmacologically inhibited by the immune checkpoint inhibitors, the restoration of a balanced immune response is achieved, due to inactivation of the Treg cells enabled by tumors [45].

TNFSF10 produced by macrophages is known to intervene in immune processes, such as graft-versus-host disease (GVHD), protection of privileged barriers, and others [16]. In the same line, our results indicated that high splenic expression of TNFSF10 was associated with an increased GITR and FoxP3 expression in aged 3xTg-AD mice. Consistently, the treatment with the anti-TNFSF10 antibody resulted in a decreased expression of Treg cells markers and in a significant attenuation of inflammatory molecules COX2, iNOS, IL-1β, and TNF-α, with shrunk splenic volume. Similarly, there is evidence that PD-1 checkpoint inhibitors can reduce brain pathology and improve cognition in the AD murine model [46].

Our data indeed confirm the potent inflammatory properties of TNFSF10, mediated by its death receptor TNFRSF10B [19], in peripheral organs, in which a chronic inflammatory/immune response was set into motion by a noxious challenge, such as Aβ, and support the value of TNFSF10 neutralization in shutting the inflammatory process down [19, 47, 48].

Evidence shows that peripheral lymphocytes, including Treg cells, are able to crawl across the BBB in neuropathologic conditions [49]. In this line, we have previously shown that TNFSF10 is substantially expressed in both the human AD brain [50], as well as in the hippocampus of 3xTg-AD mice [19], and that its immunoneutralization by means of a monoclonal antibody against TNFS10, is associated with an almost complete recovery of cognitive capacities, along with blunted expression of inflammatory mediators in the brain [19].

Although poor evidence is currently available on the influence of peripheral immune response upon the AD brain pathology [23, 24], recent data indicate that not only peripheral immunocytes have privileged gates to enter the brain [51] but also they can significantly influence the progression of brain pathology in murine models of AD [12]. More recently, a relationship has been demonstrated between adaptive immune-related impairment and AD neuropathology in the 3xTg-AD mouse model, suggesting a causal role for typical Aβ and tau pathologies [38].

Here, we showed high expression of FoxP3 and GITR and their co-localization in the hippocampus of untreated 3xTg-AD mice while their expression was attenuated by treatment with the anti-TNFSF10 antibody, suggesting that neutralization of TNFSF10, known to increase the number and activity of Treg cells [16], achieves a significant anti-inflammatory effect. In light of the capability of TNFSF10 of recruiting other immune/inflammatory mediators during the

neurodegenerative process [19, 47], it is noteworthy that also proinflammatory mediators COX2, iNOS, IL-1β, and TNF-α were downregulated by anti-TNFSF10 treatment along with the increased levels of the anti-inflammatory cytokine IL-10, [52], which is also produced by activated microglia [53], injured neurons [54], and other cell types in the CNS. In fact, it appears that IL-10 works as a protective tool in course of brain damage, likely to avoid propagation of neuroinflammatory areas and related detrimental effects [55].

We also found that CD3 positive cells were significantly represented in the hippocampus of untreated 3xTg-AD animals, eventually returning to their basal expression after the anti-TNFSF10 treatment. In addition, we also demonstrated that FoxP3-specific immunofluorescence was consistent with that of CD3. As postulated by other authors, FoxP3-positive cells crossing into the brain could create favorable conditions for an overshooting immune/inflammatory response to Aβ [12]. In a similar line, a role for Treg cells has been identified in other tissues chronically inflamed and in models of impaired immune response [16, 56, 57]. This may partly account for the significant attenuation of the inflammatory molecules in the brain obtained by neutralization of TNFSF10, which also recruits Treg cells [16], Interestingly, we showed that the human post-mortem AD brain expressed CD3 (which was not expressed by the healthy human brain), that co-localized with FoxP3 and GITR, corroborating the hypothesis that the AD brain is also characterized by the presence of immunocytes, in analogy with other immune-related CNS disorders [49].

Although there are no clinical data linking AD to splenomegaly, prominent inflammatory and innate immune responses have been observed in both AD and minimal cognitive impairment (MCI) [58,59,60]. Accordingly, there are reports that suggest that circulating plasma levels of cytokines are increased in AD [61, 62]. Moreover, increased plasma levels of TNF-α have been associated with scores in MMS [62]. More recently, an increase in the CD4/CD8 ratio in 3xTg-AD mice was reported [38], suggesting a deficit in the adaptive immune response consistently with data reporting aberrant lymphocyte populations in AD individuals [63,64,65,66,67,68].

Comparing the immunological scenario in the 3xTg-AD animals with the data from human brains, it might be speculated that neutralization of TNFSF10 may produces beneficial effects through two possible mechanisms. The first one relates to the decreased expression of the inflammatory/immune mediators [16], with a second alternative mechanism relating to the removal of the Treg influence on immunocytes, allowing a re-balance of the immune response and reduced brain accumulation of Aβ [12].

The hypothesis of the restoration of a proper immune reactivity in the hippocampus of 3xTg-AD mice by the anti-TNFSF10 treatment was corroborated by the demonstration that proinflammatory microglia in 3xTg-AD mice was blunted after anti-TNFSF10 treatment in the same area. As known, activated microglia produces, in addition to TNFSF10 [69], several inflammatory cytokines included TNF-α [28], contributing to accelerate neuronal death [70].

Interestingly, microglia still displayed an activated morphology after anti-TNFSF10, which likely relates to an increased production of the anti-inflammatory cytokine IL-10 by these cells [27]. There is extensive evidence that microglia is activated by noxious stimuli within the CNS [71], including Aβ [72]. In addition, activated microglia has been show to influence both humoral and cell-mediated peripheral immune response [73], through the release of cytokines including TNF-α that may cross the BBB in the course of neurodegenerative processes [49]. This clearly support the hypothesis that soluble inflammatory molecules may influence tissues distant from the site of production [74]. Overall, this result suggests that the anti-TNFSF10 treatment induced a dampening of proinflammatory detrimental microglia, while boosting the production of anti-inflammatory molecules.

Restraint of the overall inflammatory/immune response achieved by the anti-TNFSF10 treatment came along with significantly decreased levels of both Aβ and p-Tau protein. Immunofluorescence data showed significant decrease of Aβ amounts in the hippocampus of 3xTg-AD mice treated with the anti-TNFSF10 antibody, in parallel with significant decrease of another AD hallmark, the hyperphosphorylated Tau protein, known to substantially contribute to neuronal death [61]. Cognitive decline is associated with a progressively increasing amount of both proteins in the hippocampus of 3xTg-AD animals [19]. Assuming that the Aβ excess induces TNFSF10 expression in neurons [13], we suggest that neutralization of TNFSF10 represents a potential strategy to limit Aβ production, with consistent subsequent improvement of the cognitive outcome in AD [19].

Conclusions

In conclusion, the neurotoxic effects of TNFSF10 may partly ensue from an unbalanced equilibrium of the overall immune response, triggered by Aβ which accumulates in both the CNS and in the spleen. Aβ may be, at any rate, the candidate antigenic challenge setting into motion a systemic immune response associated with neuroinflammation. Re-trafficking of immunocytes between the periphery and the brain [46] of 3xTg-AD mice might contribute to neuroinflammation, with the involvement of Treg cells. Neutralization of TNFSF10 may significantly attenuate its detrimental effects along with those of other inflammatory mediators in the brain; on the other hand, decreased Treg cells in both the periphery and the brain may enhance the activity of other cells such as macrophages/microglia and monocytes [12], to efficiently clear Aβ from the brain tissue [75].

Finally, our data demonstrate that TNFSF10 substantially cooperates with other cytokines in sustaining inflammation in the 3xTg-AD mouse brain. TNFSF10 also inhibits the beneficial activity of the immune response by recruiting Treg cells, resulting in the inhibition of the anti-inflammatory machinery. Blockade of TNFSF10 may thus be envisioned as an innovative treatment of neurodegeneration in AD.

Abbreviations

AD: Alzheimer's disease; APPswe: Swedish mutation of the amyloid precursor protein; Aβ: Amyloid beta; BBB: Blood-brain barrier; CD11b: Cluster of differentiation 11b; CD3: Cluster of differentiation 3; CNS: Central nervous system; COX2: Cyclooxygenase-2; FoxP3: Forkhead box P3; GITR: Glucocorticoid-induced; i.p: Intraperitoneally; IL-10: Interleukin-10;; IL- 1β: Interleukin-1 beta; iNOS: Inducible nitric oxide synthase; PD-1: Programmed cell death protein 1; Psen1: Presenilin-1; p-Tau: Phosphorylated-tau protein; TNF: Receptor-related protein; TNFSF10: Tumor necrosis factor (ligand) superfamily, member 10; TNF- α: Tumor necrosis factor-alpha; Treg: Regulatory T cells; WT: Wild-type

Acknowledgements

The authors wish to thank Chiesi Farmaceutici S.p.A., Parma, Italy for generous, unconditioned support to this research. The authors did not or will not receive any direct or indirect financial benefit by Chiesi Farmaceutici from this work. The data presented in the manuscript is solely owned by the University of Catania. Chiesi Farmaceutici will not apply for intellectual property with the data.

Authors'contributions

RB and GC designed the research; RB, GDB, ARC, CB, CL, GM, and GC performed the research; LL and SS analyzed data; and RB, GDB, and GC wrote the paper. All authors read and approved the final manuscript.

Funding

Not applicable.

Availability of data and materials

The dataset used and analyzed during the current study are included within the article and its additional files. All material used in this manuscript will be made available to researcher subject to confidentiality.

Ethics approval and consent to participate

Animal studies: All experiments were carried out according to the Directive 2010/63/EU and the Italian law (D.Lgs. 26/2014) and were approved by the Italian Ministry of Health on 03-02-2015-authorization n°. 916/2015-PR, exp. date August-2020.

Human tissues: Human tissue collection and the informed consent agreement (complied with all requirements set forth by Title 21 Code of Federal Regulations Chapter I, Part 50.25, Elements of informed consent, Food and Drug Administration Department of Health and Human Services) were in line with the official regulations and the Abcam's internal Ethics Committee approved the products.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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a

Strain (*sample size*)	Age (*months*)	Body weight (g)	Brain (mg)	Spleen (mg)
WT, vehicle (n=10)	15	41.09	604	205
WT, anti-TNFSF10 (n=10)	15	39.33	612	181
AD, vehicle (n=10)	15	33.51	518	1062
AD, anti-TNFSF10 (n=10)	15	37.43	580	335

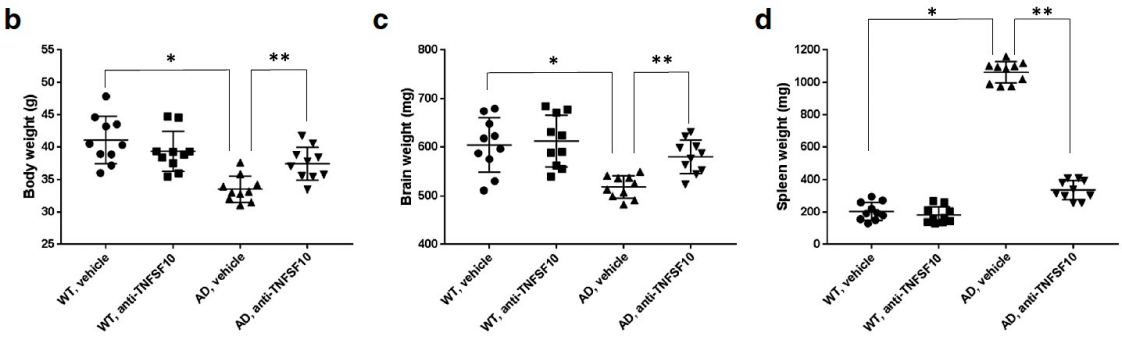


Fig. 1 Panel **a** (table): body, brain, and spleen weight changes in 15-month-old 3xTg-AD mice treated for 12 months with an anti-TNFSF10 monoclonal antibody (10 µg, i.p. twice a month). Panels **b–d**: comparison of body, brain and spleen weights in the same animals. *$p < 0.05$ vs WT treated with vehicle; **$p < 0.05$ vs. untreated 3xTg-AD mice (one-way ANOVA, followed by a Duncan's multiple range test). Vertical bars are means ± S.E.M. WT wild-type (n = 10/group); AD: 3xTg-AD mice (n = 10/group)

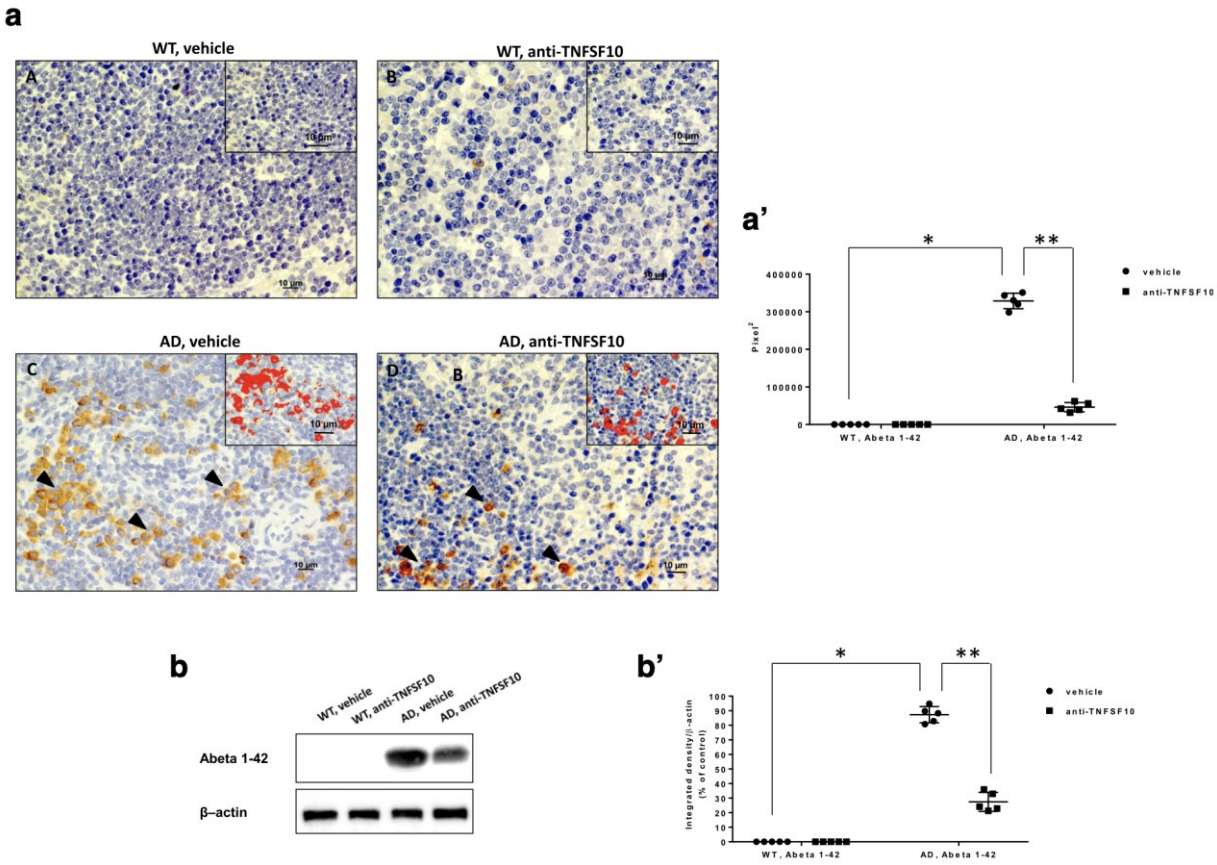
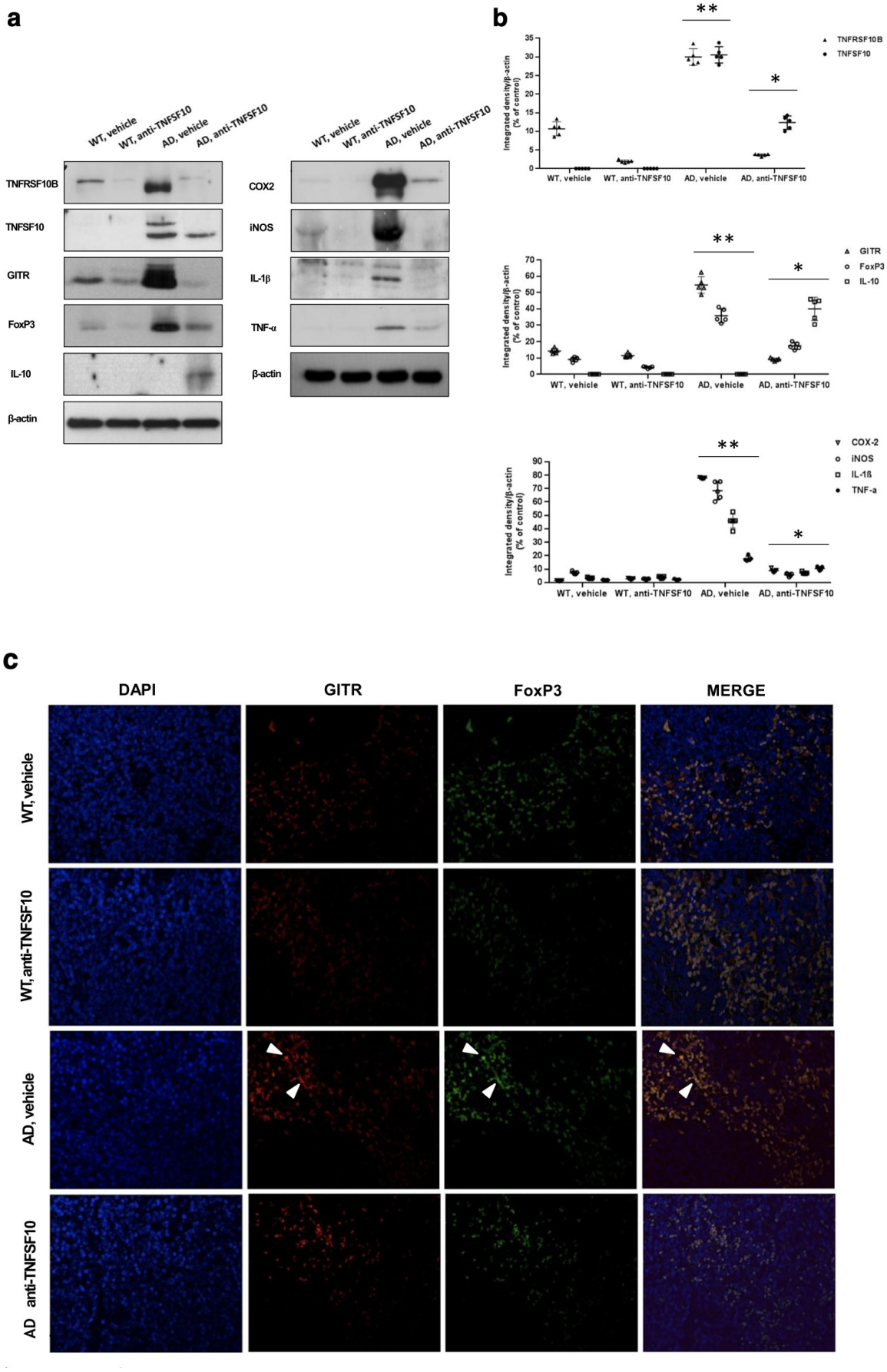
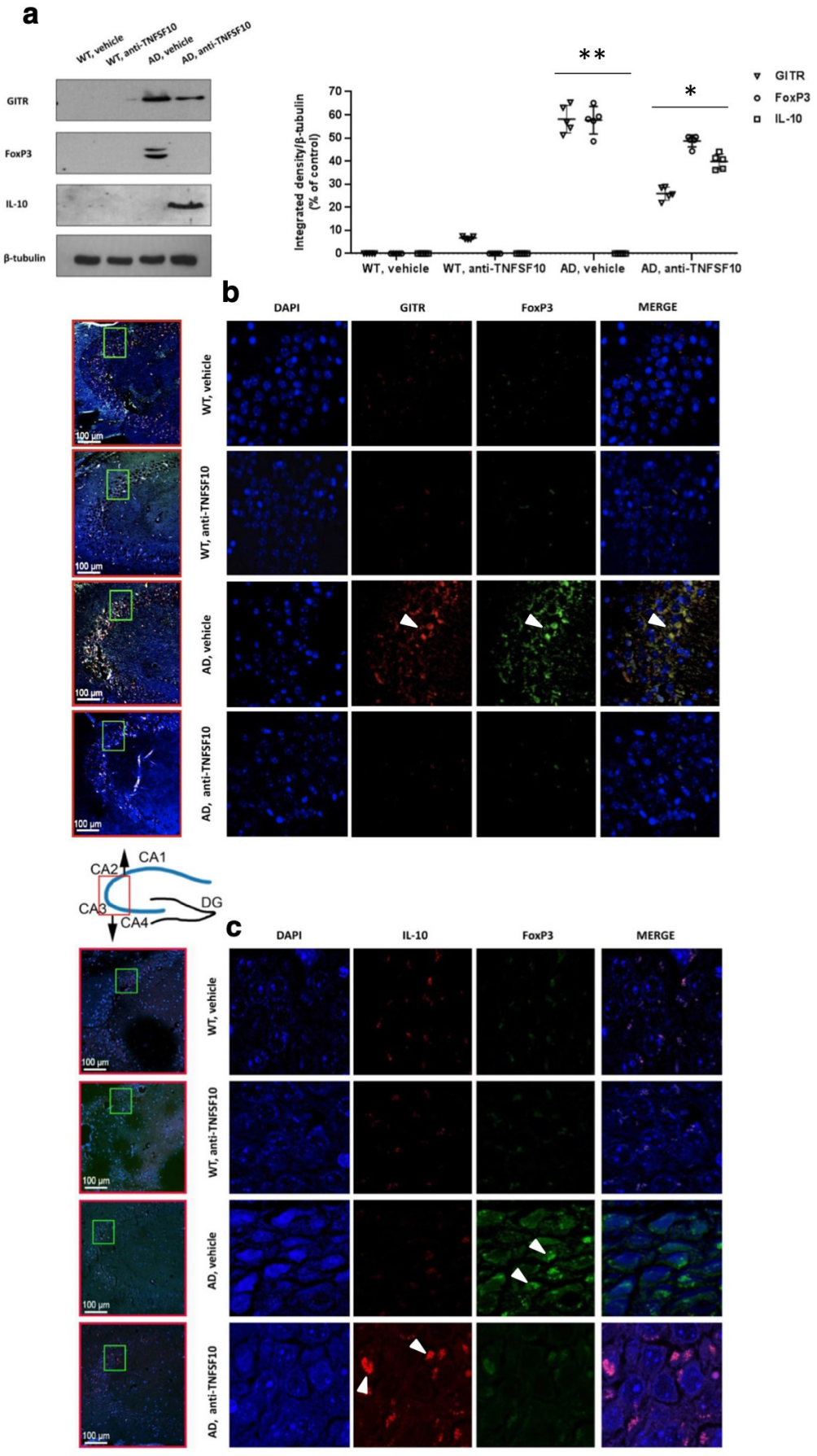


Fig. 2 Panel a: representative pictures of the immunohistochemical detection of Aβ1–42 expression (black arrows), in the spleen of 3xTg-AD treated for 12 months with an anti-TNFSF10 monoclonal antibody (10 µg, i.p. twice a month). Photos A and B: respectively, wild-type mice untreated, or treated with the anti-TNFSF10 antibody. Photos C and D: respectively 3xTg-AD mice untreated or treated with the anti-TNFSF10 antibody. The inserts in photos represent the respective areas magnified. Scale bar = 10 µM. Panel (a'): densitometric count of Aβ1–42 immunopositive cells. *p < 0.05 3xTg-AD mice untreated vs WT treated with vehicle; **p < 0.05 3xTg-AD mice treated with anti-TNFSF10 vs. untreated 3xTg-AD mice (one-way ANOVA, followed by a Duncan's multiple range test). Vertical bars are means ± S.E.M. WT wild-type (n = 5/group); AD: 3xTg-AD mice (n = 5/group). Panel (b): Western blot analysis of Aβ1–42 in splenic homogenates. Panel (b'): Densitometric analysis of the representative Western blot *p < 0.05 3xTg-AD mice untreated vs WT treated with vehicle; **p < 0.05 3xTg-AD mice treated with anti-TNFSF10 vs. untreated 3xTg-AD mice (one-way ANOVA, followed by a Duncan's multiple range test). Vertical bars are means ± S.E.M. WT wild-type (n = 5/group); AD: 3xTg-AD mice (n = 5/group)


a
WT, vehicle
WT, anti-TNFSF10
AD, vehicle
AD, anti-TNFSF10
TNFRSF10B
TNFSF10
GITR
FoxP3
IL-10
β-actin
COX2
iNOS
IL-1β
TNF-α
β-actin
b
Integrated density/β-actin (% of control)
TNFRSF10B
TNFSF10
GITR
FoxP3
IL-10
COX-2
iNOS
IL-1ß
TNF-a
c
DAPI
GITR
FoxP3
MERGE
WT, vehicle
WT, anti-TNFSF10
AD, vehicle
AD anti-TNFSF10

(See figure on previous page)

Fig. 3 Panel a: effects of the treatment with an anti-TNFSF10 monoclonal antibody on the expression of either TNFSF10, TNFRSF10B receptor, proinflammatory, and anti-inflammatory molecules in the spleen of 3xTg-AD mice. Left blots: changes in the expression of TNFSF10 and its TNFRSF10B receptor, as well as in GITR and Foxp3 and the anti-inflammatory cytokine IL-10 expression in 3xTg-AD mice, following chronic treatment (12 months) with an anti-TNFSF10 monoclonal antibody (10 μg/animal twice a month, i.p.) or vehicle (10 μg/animal twice a month, i.p.). Right blots: changes in the expression of the proinflammatory mediators COX2, iNOS, IL-1β, TNF-α in 3xTg-AD mice, following chronic treatment (12 months) with an anti-TNFSF10 monoclonal antibody or vehicle (10 μg/animal twice a month, i.p.). Panel b: densitometric analysis of respective western blots. *$p < 0.05$ vs untreated 3xTg-AD mice; **$p < 0.05$ vs. all other matching groups (one-way ANOVA, followed by a Duncan's multiple range test). Vertical bars are means ± S.E.M. Panel c: representative immunofluorescence photographs of mice spleens for GITR (red) and FoxP3 (green) expression and co-localization of the two molecules (white arrows; merge column; DAPI = nuclear staining) in WT and 3xTg-AD animals receiving either vehicle or an anti-TNFSF10 monoclonal antibody (10 μg/animal twice a month, i.p.) for 12 months. WT wild-type mice (n = 5/group); AD: 3xTg-AD mice (n = 5/group)


a
WT, vehicle
WT, anti-TNFSF10
AD, vehicle
AD, anti-TNFSF10
GITR
FoxP3
IL-10
β-tubulin
Integrated density/β-tubulin (% of control)
**
*
GITR
FoxP3
IL-10
WT, vehicle
WT, anti-TNFSF10
AD, vehicle
AD, anti-TNFSF10
b
DAPI
GITR
FoxP3
MERGE
100 μm
CA1
CA2
CA3
CA4
DG
c
DAPI
IL-10
FoxP3
MERGE

(See figure on previous page.)

Fig. 4 Effects of the treatment with an anti-TNFSF10 monoclonal antibody on the expression of GITR and FoxP3, as well as of the anti-inflammatory protein IL-10 in the hippocampus of 3xTg-AD mice Panel a: Left: Western blot analysis of GITR, FoxP3, and IL-10 protein expression in the mice following chronic treatment (12 months) with an anti-TNFSF10 monoclonal antibody (10 μg/animal twice a month, i.p.) or vehicle (10 μg/animal twice a month, i.p.). Right: Densitometric analysis of Western blots. *$p < 0.05$ vs untreated 3xTg-AD mice; **$p < 0.05$ vs. all other matching groups (one-way ANOVA, followed by a Duncan's multiple range test). Vertical bars are means ± S.E.M. Panel b: representative immunofluorescence images of hippocampi for GITR (red) and FoxP3 (green) expression and co-localization from the same animal groups as above (white arrows; merge column; DAPI = nuclear staining). Panel c: immunofluorescence by confocal microscopy of hippocampi for IL-10 (red; white arrows) and FoxP3 (green; white arrows) expression and co-localization from the same animal groups as above (merge column; DAPI = nuclear staining). WT wild-type animals (n = 5/group); AD: 3xTg-AD animals (n = 5/group); the respective side columns are the lower magnification samples where the areas to analyze (CA1, CA2, CA3, and CA4) were magnified (framed in a green box)

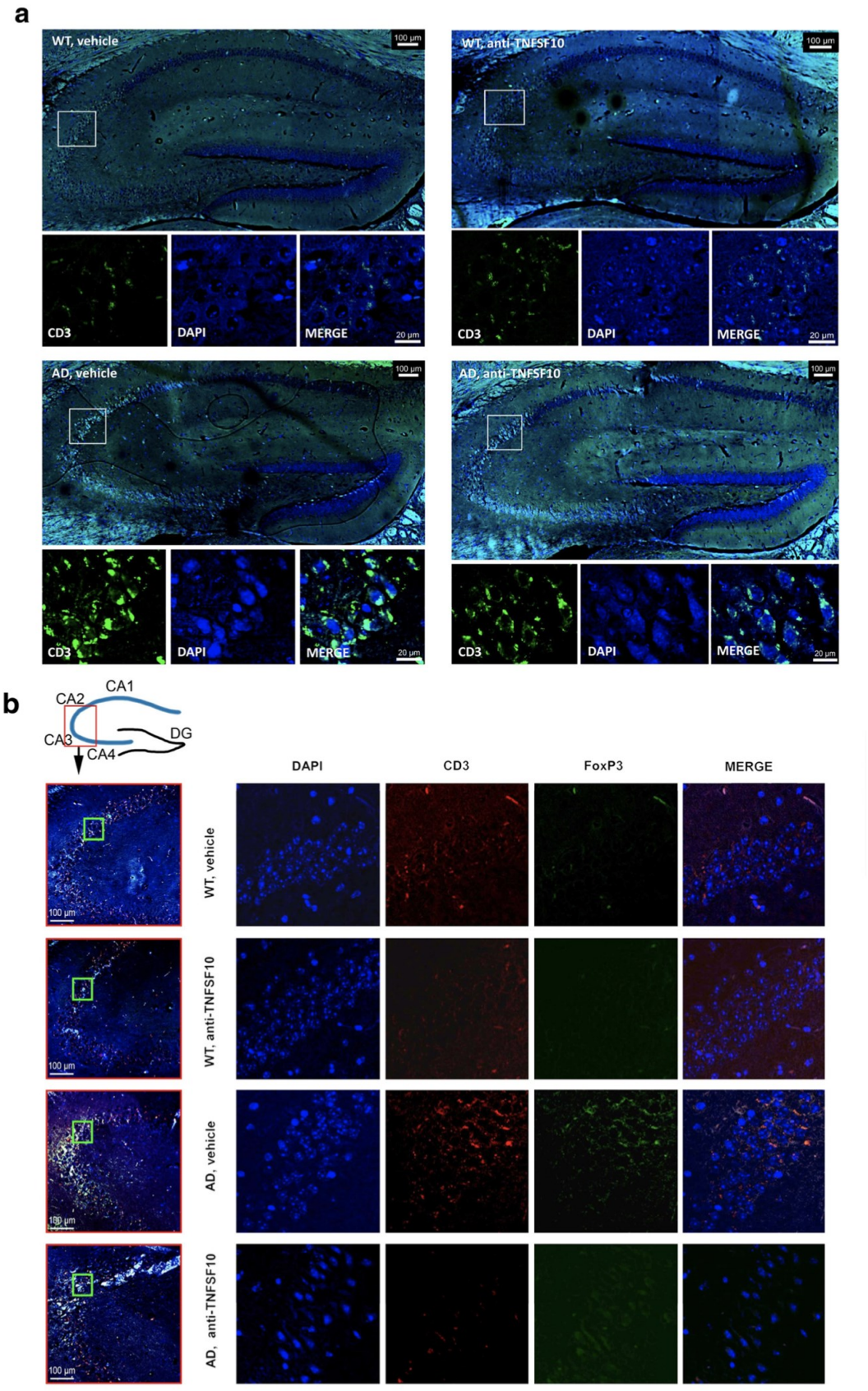


Fig. 5 Confocal microscopy for detection of CD3 and FoxP3 positive cells in the hippocampus of 3xTg AD mice, following chronic treatment (12 months) with an anti-TNFSF10 monoclonal antibody (10 µg/animal twice a month, i.p.) or vehicle (10 µg/animal twice a month, i.p.). Panel a: each picture represents a single group of treatment and illustrates either the whole hippocampus

(sagittal section) or, below, magnification of CA2-CA3 areas displaying specific CD3 (green) immunofluorescence (magnifications of respective white frames) in the mice following chronic treatment (12 months) with an anti-TNFSF10 monoclonal antibody (10 µg/animal twice a month, i.p.) or vehicle (10 µg/animal twice a month, i.p.). Panel b: immunofluorescence of hippocampi for CD3 (red) and FoxP3 (green) expression and co-localization from the same animal groups as above (merge column; DAPI = nuclear staining). The respective side columns are the lower magnification samples where the areas to analyze (CA1, CA2, CA3, and CA4) were magnified (framed in a green box). WT wild-type animals (n = 5/group).; AD: 3xTg-AD animals (n = 5/group)

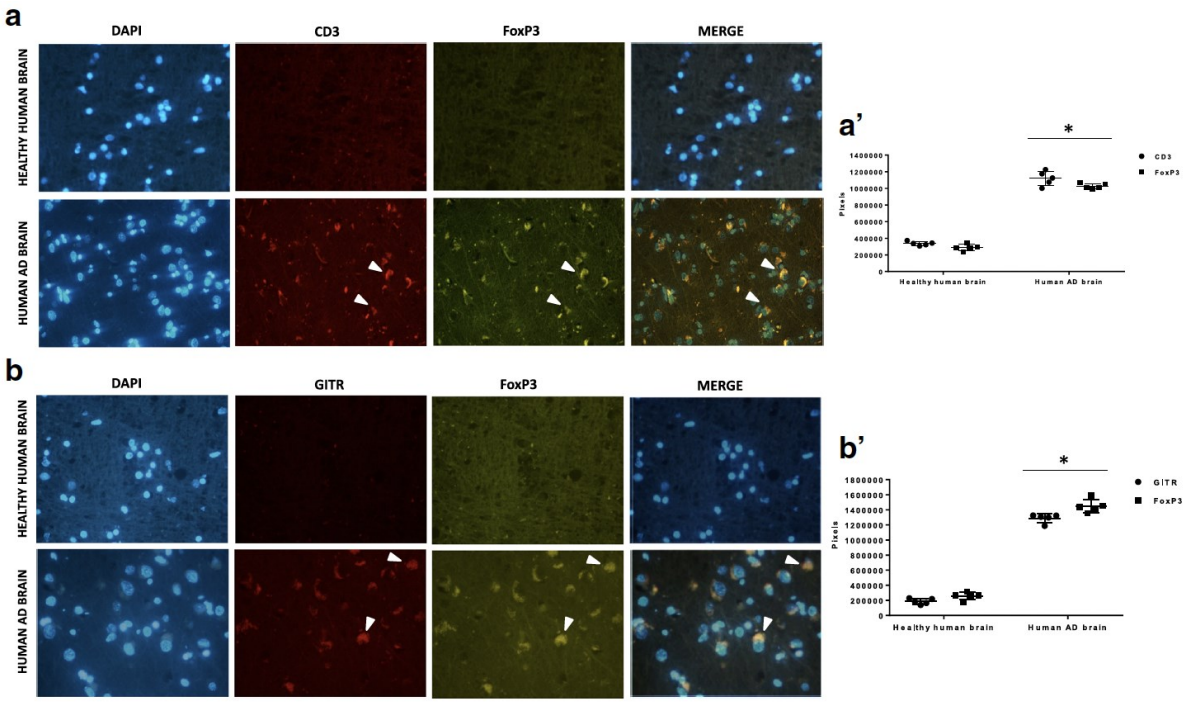


Fig. 6 Confocal microscopy for detection of CD3, FoxP3, and GITR positive cells in the human AD brain (n = 4). Panel (a): CD3 (red) and FoxP3 (green) expression in the human AD brain and respective co-localization (white arrows; merge column; DAPI = nuclear staining). Panel (b): GITR (red) and Foxp3 (green) expression in the human AD brain and respective co-localization (white arrows; merge column; DAPI = nuclear staining). Panels (a', b'): densitometric counts of immunopositive cells. *p < 0.001 vs. healthy human brain; (one-way ANOVA, followed by a Duncan's multiple range test). Vertical bars are means ± S.E.M.

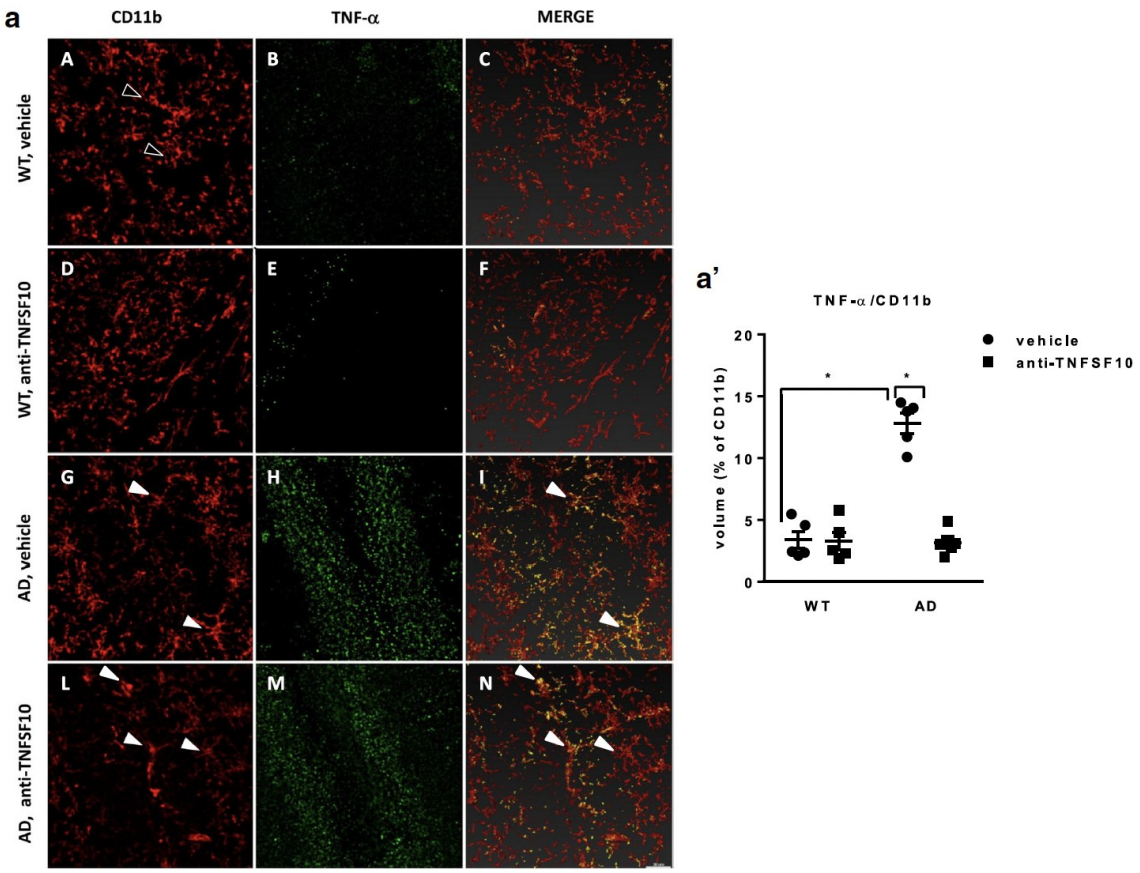


Fig. 7 Confocal microscopy for detection of TNF-α and CD11b expression in the microglia of the CA2-CA3 regions of the hippocampus of 3xTg AD mice, following chronic treatment (12 months) with an anti-TNFSF10 monoclonal antibody (10 μg/animal twice a month, i.p.; (n = 10/group) or vehicle (10 μg/animal twice a month, i.p.). Representative images showing respectively CD11b (red, panels A, D, G, L), TNF-α (green, panels B, E, H, M), and TNF-α co-localized with CD11b (panels C, F, I, N). Side graphic represents the densitometric count of positive cells; *$p \leq 0.05$ vs. all other groups; (two-way ANOVA, followed by Bonferroni post-hoc test). Vertical bars are means ± S.E.M. WT wild-type animals (n = 5/group); AD: 3xTg-AD animals (n = 5/group). Black arrows point resting microglia; white arrows point activated microglia (panels G and I), or co-localization TNF-α/CD11b (panels I and N)

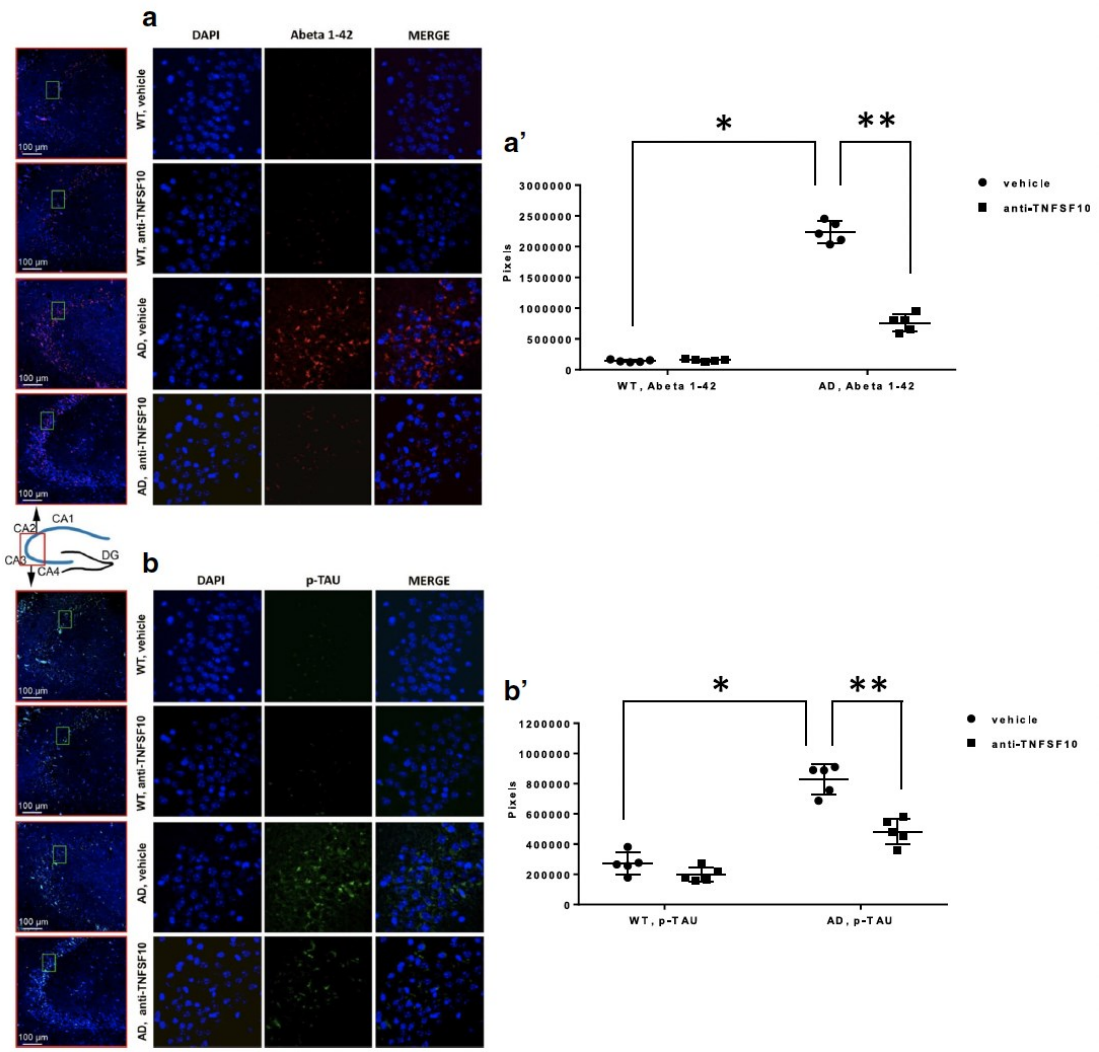


Fig. 8 Effects of the treatment with an anti-TNFSF10 monoclonal antibody on the expression of either Aβ 1–42 (panel a) and phosphorylated Tau protein (p-TAU) (panel b) in the hippocampus of 3xTg-AD mice: representative immunofluorescence images of hippocampi from each animal group (merge column; DAPI = nuclear staining). The respective left-hand side columns are the samples where the areas to be magnified for analysis were picked (green frame boxes; CA1, CA2, CA3, and CA4). Panels (a', b'): densitometric count of immune-positive cells, for, respectively, Aβ 1–42 and p-TAU. *p < 0.001 3xTg-AD mice untreated vs WT-vehicle; **p < 0.001 3xTg-AD mice treated with anti-TNFSF10 vs. untreated 3xTg-AD mice (one-way ANOVA, followed by a Duncan's multiple range test). Vertical bars are means ± S.E.M. WT wild-type animals (n = 5/group); AD: 3xTg-AD animals (n = 5/group)

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Chapter II

The immune system on the TRAIL of Alzheimer's disease

J Neuroinflammation. 2020; 17: 298.. doi: 10.1186/s12974-020-01968-1

THE IMMUNE SYSTEM ON THE TRAIL OF ALZHEIMER'S DISEASE

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Abstract

Alzheimer's disease (AD) is the most common form of dementia, characterized by progressive degeneration and loss of neurons in specific regions of the central nervous system. Chronic activation of the immune cells resident in the brain, peripheral immune cell trafficking across the blood-brain barrier, and release of inflammatory and neurotoxic factors, appear critical contributors of the neuroinflammatory response that drives the progression of neurodegenerative processes in AD. As the neuro-immune network is impaired in course of AD, this review is aimed to point out the essential supportive role of innate and adaptive immune response either in normal brain as well as in brain recovery from injury. Since a fine-tuning of the immune response appears crucial to ensure proper nervous system functioning, we focused on the role of the TNF superfamily member, TNF-related apoptosis- inducing ligand (TRAIL), which modulates both the innate and adaptive immune response in the pathogenesis of several immunological disorders and, in particular, in AD-related neuroinflammation. We here summarized mounting evidence of potential involvement of TRAIL signaling in AD pathogenesis, with the aim to provide clearer insights about potential novel therapeutic approaches in AD.

Keywords: Immune response, Neuroinflammation, Proinflammatory cytokines, Regulatory T cells

Background

Alzheimer's disease (AD) is an age-related neurodegenerative disorder with an insidious onset characterized by cerebral atrophy and progressive cognitive decline [1]. The acknowledged neuropathological hallmarks of AD are represented by extracellular senile plaques, composed of amyloid-β (Aβ) peptide and intracellular neurofibrillary tangles (NFTs) generated by hyperphosphorylated protein tau [2].

Growing evidence suggests that the multifactorial pathophysiological mechanisms of AD is not restricted to the neuronal compartment, as relevant role has been attributed to the tight interactions of immunological mechanisms within the brain [3].

Since decades, active research has investigated network connections between the immune system and the nervous system. In fact, it has been described a reciprocal functional control between the immune system and the central nervous system (CNS) [4], a mechanism essential to tissue repair and regeneration as well as removal of damaged tissues and cells [5]. A low-grade peripheral immune/inflammatory response and the basal release of cytokines are needed to maintain brain homeostasis and functional plasticity, including hippocampal-dependent cognitive functions and neurogenesis, suggesting that the systemic immune response exerts a healing role in the CNS [6,7].

Now, it is a common notion that systemic inflammatory disorders may be associated with cognitive decline [8], and, in fact, chronic inflammation is known to inhibit neuronal functions and contribute to onset and progression of AD [9]. In this line, robust data support the crucial relevance of mediators of the inflammatory/immune response in neurodegeneration, as, for instance, injured neurons release arrays of these molecules, which redundantly sustain neuronal damage and death [10].

Cytokines belonging to the tumor necrosis factor (TNF) superfamily are considered substantial contributors of the accelerated cell death rate which characterizes neurodegenerative processes. Among these, the proapoptotic/proinflammatory cytokine Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), first discovered as a tumor cell killer, is expressed in macrophages, T lymphocytes, neutrophils, and dendritic cells [11, 12].

TRAIL, which acts through two death receptors referred to as DR4 and DR5, is a potent mediator of prominent neuronal loss induced in both chronic and acute neurodegenerative processes, including those related to Aβ accumulation [13], trauma [14], and brain ischemia [15, 16], consistent with boosted peri-damage neuroinflammation.

Furthermore, sustained TRAIL expression appears related to functional decline in animal models of AD [17]. In fact, its immunoneutralization by means of a monoclonal antibody is associated with a significant rescue of neurons from death [13], reduced accumulation of Aβ and attenuated expression of inflammatory/immune mediators [17], paralleled by a re-balance of both central, and peripheral immune response [18].

In synthesis, TRAIL efficiently sets into motion and sustain neurodegeneration-related neuroinflammation, as its neutralization implies significant attenuation of inflammatory processes [19], corroborating the hypothesis that is represents an important molecular clue to Aβ-dependent neurodegenerative processes, and may thus well be envisioned as a potential candidate target for innovative immunotherapeutic strategies in AD.

The impact of central and peripheral inflammatory/immune response in Alzheimer's disease

Neurodegenerative disorders share selective neuronal vulnerability in specific brain regions, which is related to the neuronal responses to detrimental stimuli, such as, for instance, disease-related misfolding proteins, that finally become unsupportive to neurons [20].

In addition to the pathogenetic role of Aβ and tau proteins in AD, recent evidence favors the hypothesis that the immune system plays a pivotal role in the onset and progression of this disease [21].

In fact, neuronal damage in AD is associated with chronic activation of the CNS-resident innate immune cells and increased peripheral leukocyte access across the blood-brain barrier (BBB) [22], consistent with the demonstration of a functional meningeal lymphatic system [23], as well as of a substantial peripheral immunocyte trafficking through the choroid plexus (CP) [24], supporting the notion of a cross-talk system between peripheral and CNS immunocytes.

Moreover, the innate immune system indeed represents the first line of defense against pathogens serving as a link to adaptive T and B cells, by means of antigen presentation processes and transfer of information [25, 26], and in this line, both branches of immune response, adaptive, and innate, may affect the neuroinflammatory process and related progression of neurodegeneration in AD and other CNS disorders [27].

It is well-established that microglia and astrocytes, the predominant innate immune cells in the CNS, are strongly implicated in aberrant molecular pathways that underlie AD pathogenetic alterations [10].

Microglial cells represent the major immunological effector of the innate immune system in the brain and mediate functions such as tissue surveillance, removal of pathogens, and response to injury [28, 29], also contributing to neuronal survival and synaptogenesis [30].

Under resting condition, microglia are characterized by a ramified morphology and a weak antigen-presenting activity, partly due to low level of expression of the major histocompatibility complex (MHC) on its surface [31]. Activated microglia eventually convert to an amoeboid-like morphology which displays upregulated expression of both MHC and co-stimulatory molecules involved in antigen presentation, leading to interactions with peripheral immune cells [32].

Upon injury, disease, or inflammation, healthy neurons may get damage, which in turn causes release of self-antigens or aberrant proteins that activate resting microglia (Fig. 1). In fact, pathogenic stimuli break the delicate balance between neurotoxic and neuroprotective mechanisms, inducing microglial activation, triggering for example, the Toll-like receptors 4 (TLR4) signaling pathway and conversion to the pro-inflammatory phenotype [33,34,35]. The latter microglial state is characterized, not only by a morphological changes, but also by release of pro-inflammatory molecules, such as interleukin-1 beta (IL-1β), interleukin-6 (IL-6), tumor necrosis factor alpha (TNFα), interferon gamma (IFN-γ), chemokines, as well as reactive oxygen

and nitrogen species (ROS/RNS), which promote diapedesis of peripheral leukocytes through the BBB, further contributing to fuel local detrimental inflammatory response [28, 36].

In addition to its pro-inflammatory pattern, microglia can also adopt an alternative activation pathway [37], associated with increased production of anti-inflammatory cytokines and neurotrophic factors to facilitate phagocytosis of cell debris and promote neuronal repair and survival [38, 39].

In the occurrence that innate immune-related processes are not completely resolutive and the inflammatory stimuli persist, microglia-mediated mechanisms are trapped in a vicious cycle, characterized by chronicized release of pro-inflammatory cytokine initiating a cascade of toxic events leading to neuronal death [40].

Although microglia represent the main mediators of brain immune surveillance, under pathological conditions the infiltrating monocytes transiently supplement the brain mononuclear phagocyte compartment (microglia itself) playing a major role in controlling neuropathological events in the CNS [41, 42]. Notably, infiltrated monocytes contribute to tissue repair, inflammation resolution, and production of neurotrophic factors [41].

Astrocytes, the other major innate effector cells in the CNS, contribute to maintainance of CNS homeostasis and sustain neuronal survival through the release of metabolites and neurotrophic factors essential for normal brain functions and organized cognitive activity [43], and they also safeguard BBB structural integrity and permeability, eventually exerting gate-controlled recruitment of peripheral immune cells into the brain parenchyma [44].

Recent work has highlighted the pathophysiological relevance of the microglia-astrocyte crosstalk [45]. In particular, activated microglia releases specific astrocyte-activating signal molecules, such as interleukin-1 alpha (IL-1α), TNFα, and complement component 1q (C1q), all inductors of a neuroinflammatory reactive astrocyte phenotype, which, similarly to activated microglia, highly express many complement components, including MHC class II molecules [46, 47], as well as an array of cytokines and chemokines that act as chemoattractants, crucial for the recruitment of T cells into the CNS [48,49,50].

In addition, also adaptive immune cells infiltrating the brain parenchyma seem able to support the neuroinflammatory process [51], as demonstrated for B and T lymphocytes, which are endowed with protective functions from pathogens and trigger a fast specific immune response in case of repeated infections due to the same agent [52].

Under conditions of neurodegeneration, high frequencies of T lymphocytes have been found to infiltrate the brain parenchyma, suggesting a critical pathophysiological role [53]. In fact, the chronic neuroinflammatory status associated with neurodegenerative disorders and driven by the main reactive components of the CNS affects the structural integrity and the permeability of BBB, enhancing transmigration of peripheral immune cells into the CNS and diffusion of inflammatory molecules across the BBB [54,55,56], thus contributing to the development and progression of

lesions [57, 58]. Nevertheless, it has been shown that the CP of the blood-cerebrospinal fluid barrier (BCSFB) works mainly as a selective gateway for leukocyte entry, rather than a firm barrier (BBB) for immune surveillance. Schwartz and colleagues proposed CP as a selective and "educative" gate for recruitment of leukocytes to the inflamed CNS parenchyma [40]. This hypothesis is supported by the findings that neutrophils, monocytes, and T cells enter the injured CNS through the BCSFB in response to brain parenchyma damage [59].

T cells can be classified into CD4+T cells, main regulators of the immune response, and CD8+T cells, designated as cytotoxic T cells for their ability to remove damaged and infected cells [60]. Depending either upon specific stimuli, tissue environment and antigen-presenting cell signaling, naïve CD4+ T cells (Th0) differentiate into antigen-specific T effector including T-helper1 (Th1), T-helper2 (Th2) and T-helper17 (Th17) cells, as well as cytotoxic T lymphocytes (CTLs), or regulatory T cells (Tregs). While Th1 and Th17 cells, which are overactivated in neurodegenerative disorders [61], directly contribute to neuroinflammation through the release of pro-inflammatory cytokines (IFNγ, TNFα, and IL-17) and other inflammatory mediators, Th2 cells, produce anti-inflammatory cytokines (e.g., IL-4), and, for this reason, they have been considered for development of potential intervention strategies [62]. Both Th1 and Th2 cells, are essential for the maintenance of a healthy CNS environment, as an altered Th1/Th2 ratio has been regarded as a causative event in neurodegeneration [63].

Moreover, also antigen-specific CD8+ CTLs have been shown to participate to the pathophysiology of chronic inflammatory disorders includeed those related to neurodegeneration, through production of cytolysis mediators such as perforins and granzymes [64, 65].

Another cell subset, Treg cells have been shown to dampen down neuroinflammation by inhibiting antigen presentation, and upregulating glial neurotrophic factors [66]. As immunoregulatory cells, Tregs release anti-inflammatory factors, such as interleukin-10 (IL-10), and transforming growth factor beta (TGFβ), that suppress activation of effector T lymphocytes, assuming a key role in the development and maintenance of immune tolerance [67].

Because of their immunosuppressive properties, Treg cells, extensively studied in autoimmune disorders [68], actually represent potential elements for improvement of the outcome in neurodegenerative disorders [69, 70].

Recently, the possibility of a dual role of Tregs in the progression of AD has been object of debate. In this condition, Tregs may have a beneficial role at early disease stages, restraining detrimental gliosis, promoting beneficial activation of microglia, and allowing leukocyte re-trafficking through CP [71].

A deficit of TGF-β1, the main cytokine produced by Tregs, can critically contribute to neuroinflammation in AD brain [72, 73]. Additional preclinical studies in experimental models of AD are needed to understand whether Treg cells might exert neuroprotective effects in an early phase of the amyloid-related neurodegeneration by rescue of TGF-β1.

On the other hand, at later disease stages, Tregs appear to take over a detrimental function, by altering CP function and reducing the recruitment of inflammation-resolving leucocytes to CNS [74].

TRAIL: a potent, pleiotropic fine-tuning effector of the immune response

TRAIL, also known as TNFSF10, is a pleiotropic cytokine belonging to the TNF superfamily, involved in many peripheral and CNS functions, including cell death signaling pathway, immune response, and inflammation [75].

TRAIL can be detected as a soluble and type II transmembrane protein [76, 77]. The homotrimeric and biologically active form is able to interact with a complex system of receptors with different signaling outcomes, from pro-apoptotic to prosurvival/proliferative effects [78, 79].

In humans, TRAIL binds two death-inducing receptors, DR4/TRAIL-R1 and DR5/TRAIL-R2, which contain a functional intracellular death domain, and two transmembrane decoy receptors (DcRs), DcR1/TRAIL-R3 and DcR2/TRAIL-R4, which downregulate the activity of the former receptors by sequestration of the bioactive ligand [80]. Finally, TRAIL has also been shown to bind with very low affinity to osteoprotegerin (OPG), a secreted member of the TNF receptor family involved with the regulation of bone turnover, which acts as a soluble neutralizing receptor [81, 82]. Unlike humans, mice express only three TRAIL receptors: DR5, DcR1, and DcR2 [83, 84].

Two TRAIL-activated death pathway have been identified: an extrinsic pathway, linked to caspase-8 activation, through the recruitment of the adaptor molecule Fas-associated death domain protein (FADD), and an intrinsic mitochondrial pathway in which effector caspases are activated after a BH3 interacting-domain (Bid)-mediated signaling cascade causing mitochondrial outer membrane permeabilization, and the release of cytochrome c which promotes the formation of multimeric complex called “apoptosome” [85].

Several studies suggest the existence of a crosstalk between the two pathways, as demonstrated by the evidence that Bid is cleaved by active caspase-8 [86].

Since its discovery, TRAIL has been extensively studied in the cancer area because of its ability to induce selective apoptosis in a wide variety of tumor cell lines [87]. While the latter has long represented the best characterized function of TRAIL, increasing evidence suggest that TRAIL mediates several alternative functions in normal cells [88]. In fact, TRAIL can stimulate also prosurvival pathways, through factors such as nuclear factor κB (NF-κB) and Akt [89]. In addition, TRAIL promotes proliferation and migration of endothelial cells, suggesting its role in endothelial cell physiology and in the pathophysiology of the vascular system [90, 91].

Among others, a major role of TRAIL appears related to the fine-co-tuning of the immune response in the CNS (Fig. 2).

TRAIL is not costitutively expressed in the normal brain, which, instead, expresses its receptors [92], while under inflammatory brain conditions, TRAIL is abundantly released by activated glia

[93], CNS-infiltrating macrophages [94], and damaged neurons [13] acting as a potential cell death signal after interacting with TRAIL receptor-expressing cells resident in the CNS [92, 95,96,97].

An interesting aspect of TRAIL is its involvement in the homeostatic regulation of the immune system, as in fact, it is expressed on various innate and adaptive immune cell types [98], including monocytes, macrophages, natural killer cells, natural killer T cells, dendritic cells, and neutrophils after stimulation with lipopolysaccharide and pro-inflammatory cytokines such as IFNα, IFNβ, and IFNγ, as well as on T lymphocytes following T cell receptor (TCR)-mediated activation [99,100,101,102,103,104]. In contrast to the ligand, TRAIL-receptors are ubiquitously expressed also outside the immune system [105], and, for this reason, TRAIL seems to substantially modulate both the immune responses and their cellular components via the apoptotic cell-death pathway [106], and to participate to the immune response in different tissues and conditions [107,108,109,110].

Nevertheless, the immune system employs apoptosis not only as a self-restricting regulatory mechanism but also as an effector mechanism of immune-competent cells which can selectively eliminate virus-infected, transformed cells [111], and also normal cells in case of autoimmune inflammation [97], or in post-transplantion disorders [112]. TRAIL also represents an effector of immune-surveillance function and contribute to apoptosis of tumor and virus-infected cells [84]. TRAIL-induced apoptosis is involved in various processes, such as removal of lymphocytes with dangerous self-reactive specificities (autoreactive T and B cells) [113] and infiltrating immune cells [114, 115]. Data from studies carried out on TRAIL-deficient animal model suggest that TRAIL is essential for the maintenance of central immune tolerance by an indirect negative selection of autoreactive thymocytes [106, 116]. Additionally, TRAIL is involved in the regulation of peripheral tolerance by apoptosis-induction in mature lymphocytes after sensitization with IL-2, as well as by promoting the proliferation of Treg cells, elements with an essential role in maintaining immune tolerance [117, 118]. TRAIL increases anti-inflammatory Treg cell population as demonstrated by recent in vivo studies with systemically administered long-acting PEGylated TRAIL [119].

Moreover, TRAIL affects immune cells not only by inducing apoptotic death, but also by inhibiting their activation and expansion [120] as it directly inhibits T cell activation, suppresses T cell proliferation, and production of T cell-derived proinflammatory cytokines [108].

Finally, TRAIL system regulates innate and adaptive immune responses playing a role of crucial relevance in autoimmune and inflammatory diseases [11, 121, 122], as well as in immune surveillance in virtually all tissue and organs, including the CNS [123].

TRAIL: a conductor of the inflammatory/immune orchestra in Alzheimer's disease?

AD represents one of the greatest future global healthcare challenges. Owing to the increasing life expectancy in the general population and the consequent rising AD prevalence, this widely diffused disorder has become a major concern [124].

Neuropathologically, AD is characterized by the presence of amyloid plaques in the brain, as well as intracellular NFTs generated by hyperphosphorylated forms of the protein tau [2, 125], and in addition, by an inflammatory/immune response susceptibility, which plays a major role in various phases of the disease from its onset to later, progressive stages [21, 126].

It is noteworthy that neuroinflammatory foci in the AD brain localize in close vicinity of Aβ plaques, and they are associated with glia activation [127] and release of mediators of the inflammatory/immune response [128], including, among others, pro-inflammatory cytokines [129].

In this regard, TRAIL with its prominent death signaling and potent immune modulating properties [11] assumes an orchestrating role in the complex scenario of the AD brain.

TRAIL, specifically expressed in the human AD brain [130], is abundantly released by human neural cells challenged with Aβ in vitro [13] and activated glia [131], and is also associated with reduced expression of the Na+-Ca2+ exchanger neuroprotective isoform NCX3, with a subsequent reduction of the energetic supply to neurons, in such manner providing redundant contribution to its potent proapoptotic effect in course of neurodegenerative process [132].

Growing evidence suggest that TRAIL has a relevant coordinating function in the inflammatory roundabouts of AD, while it also directly mediates Aβ-related neurotoxicity (Table 1) [13, 14]. In fact, it has been demonstrated that immunoneutralization of TRAIL is associated with rescue from death of human neuronal cells challenged in vitro with Aβ [13], and that blockade of the DR5 TRAIL-death receptor signaling with specific antibodies completely abrogates Aβ-induced neurotoxicity in both human neuronal cell lines and primary cortical neurons [133], suggesting a direct, Aβ-additive neurotoxic effect of TRAIL in the AD brain. Based on these findings, it has been demonstrated that TRAIL immunoneutralization resulted in functional improvement, reduced deposition of Aβ and dramatically decreased expression of immune/inflammatory mediators in a transgenic mouse model of AD which develops progressive, age-related, cognitive decline [17].

We have previously mentioned how misfolded proteins, such as Aβ, when not adequately removed, may drain into peripheral lymphoid organs, setting into motion and chronically maintaining an immune response [134], which, in turn, can result unbalanced in its outcome. In light of the fact that increased exchange of immunocytes may occur between peripheral lymphoid organs and the brain [135], it is noteworthy that the integrity of the BBB may not necessarily subsists in course of neurodegenerative disorders [136]. In fact, peripheral immunocytes have been indicated as factors that, when the inflammatory/immune equilibrium within the CNS is disrupted, are able to significantly influence progression of the AD brain pathology [74, 137].

Now, considering the pleiotropic role of TRAIL in orchestrating key events of the inflammatory/immune response, it appears of interest how its immunoneutralization also leads to a rebalance of immunocytes ratios, with special regard to the Treg cell population either in the spleen and in the brain [18].

Treg cells, besides their role as "controllers" of the overshooting inflammatory/immune response [138], when not represented in an adequate number, may be hired as causative elements of either hyperinflammatory [139] or proliferative [140] disorders. Thus, it is plausible to hypothesize that Treg cells represent, in a first phase of the response, a key factor in preventing fast progression of overshooting brain inflammation and consequent accelerated neurodegeneration, as a fruit of the adjustment of Treg (and, perhaps, of other immunocytes) flow to the brain, paralleled by decreased amount of Aβ and blunted immune reactivity.

Apparently, after a first attempt of the immune response to restain AD-related brain inflammation by means of TRAIL-driven increase of Treg cells, the latter may assume an overwhelming attitude, thus, limiting the beneficial effects of the immune response against accumulating Aβ [74], allowing the inflammatory response to overshoot, and resulting in noxious effects.

Consistently, central and peripheral immune/inflammatory markers, including specific Treg cells markers FoxP3 and GITR, as well as cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS), IL-1β and TNFα are restored to basal levels, while, on the other hand, expression of anti-inflammatory cytokines such as IL-10 is significantly upregulated after chronic treatment of transgenic AD mice with anti-TRAIL antibody [18]. Moreover, such TRAIL-related restrain of peripheral and CNS inflammatory/immune response in murine model of AD occurs along with decreased both microglial TNFα production, along with reduced accumulation of both Aβ and p-Tau protein in the hippocampus of 3xTg-AD mice treated with an anti-TRAIL antibody [18].

Conclusions

Redundant, persistent, and self-activating inflammatory processes in the brain undoubtedly represent one main factor fueling the progression of AD.

The concept of a dynamic, balanced modulation of the inflammatory/immune response has a relevant strength that should be exploited for discovery of innovative therapeutic strategies.

The pleiotropic effects of TRAIL appear evident within different outcomes of the inflammatory immune/response, consistently, either in the peripheral lymphoid organs and in the brain. The TRAIL system greatly influences neuronal death rate during neurodegeneration. Secondly, TRAIL also appears to be a connector of peripheral immune response with the degenerating inflamed brain, leading to activation of Treg cells and probably driving them to over-respond with detrimental consequences for the AD brain.

In conclusion, it is plausible to hypothesize that clinically meaningful treatment options for AD could be achieved through pharmacological modulation of the TRAIL system.

Abbreviations

Aβ: Amyloid-beta; AD: Alzheimer's disease; APCs: Antigen presenting cells; BBB: Blood-brain barrier; BCSFB: Blood-cerebrospinal fluid barrier; C1q: Complement component 1q; CNS: Central nervous system; COX-2: Cyclooxygenase-2; CP: Choroid plexus; CTLs: Cytotoxic T lymphocytes; DcRs: Decoy receptors; DR4: Death receptor 4; DR5: Death receptor 5; IFN- γ: Interferon gamma; IL-10: Interlekin-10; IL-1α: Interleukin-1alpha; IL-1β: Interleukin-1beta; IL-6: Interleukin-6; iNOS: Inducible nitric oxide synthase; MHC: Major histocompatibility complex; NFT: Neurofibrillary tangles; OPG: Osteoprotegerin; RNS: Reactive nitrogen species; ROS: Reactive oxygen species; TGFβ: Transforming growth factor beta; Th1: T-helper1 cells; Th17: T- helper17 cells; Th2: T-helper2 cells; TLR4: Toll-like receptors 4; TNF: Tumor necrosis factor; TNFSF10: TNF Superfamily Member 10; TNFα: Tumor necrosis factor alpha; TRAIL: TNF-related apoptosis-inducing ligand; Treg: Regulatory T cells; rTRAIL: Recombinant TRAIL

Acknowledgements

Not applicable.

Authors' contributions

ChB drafted and edited the manuscript and prepared figures. AM contributed the part of CNS diseases. GDB contributed the part on TRAIL and Alzheimer's disease. CDF and RDM performed literature searching and drafted the manuscript. FC, ClB, and GC critically reviewed the manuscript. RB conceived the idea of this review. All authors read and approved the final manuscript.

Funding

The work has been funded by the PRIN grant n. 2017YH3SXK from the Italian Ministry of Research.

Availability of data and materials

Not applicable.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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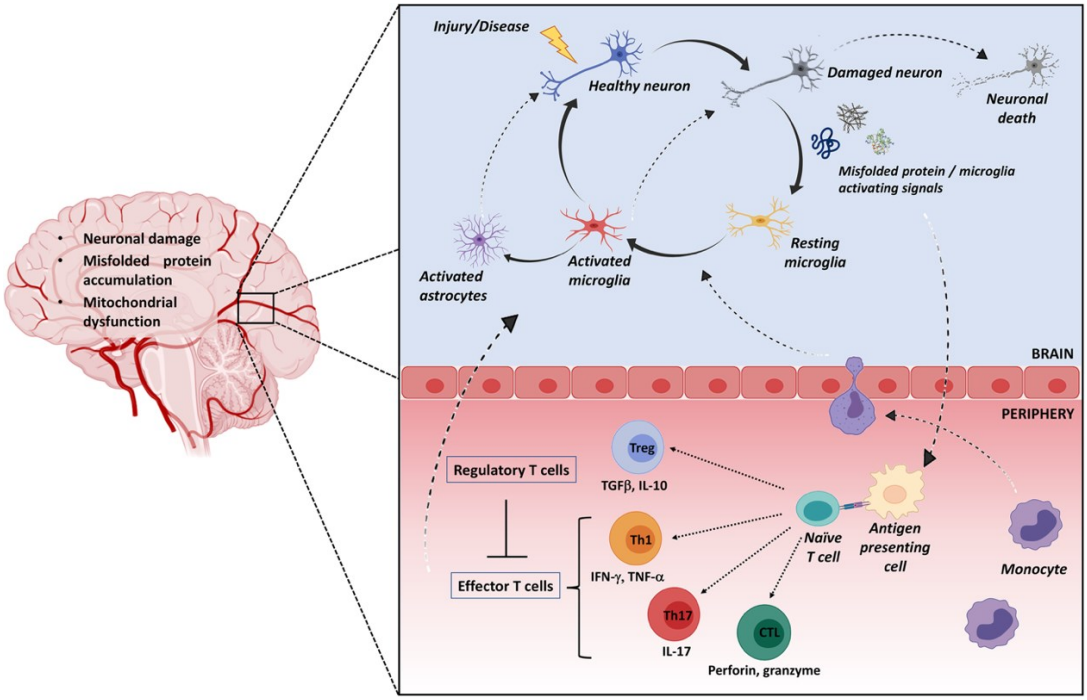

Figure 1. Central and peripheral inflammatory/immune response in neurodegeneration. Upon injury, disease, or inflammation, damaged neurons could release self-antigens or modified proteins that activate resting microglia. Activated microglia responds to these stimuli, by production of proinflammatory cytokines and chemokines, reactive oxygen, and nitrogen species. When such first innate immune-related process is not completely resolutive and the inflammatory stimuli persist, the microglia-mediated mechanisms remain trapped in a vicious cycle, characterized by chronic pro-inflammatory cytokine production linked to a cascade of neurotoxic events leading to neuronal death. Substantial recruitment of monocytes into the AD brain begins when Aβ deposition and associated neuronal damage triggers a local immune response, activating astrocytes and microglia. Activated pro-inflammatory microglia also release astrocyte-activating signals which induce neuroinflammatory astrocytes that, in turn, amplify the neurodegenerative cycle. In addition, misfolded proteins not adequately removed may drain into peripheral lymphoid tissues, wherein they are presented by antigen presenting cells to naïve T cells, thereafter mounting an adaptive immune response against these antigens. Depending upon antigen-presenting cell signals, naïve T cells differentiate into antigen-specific T effector cells (Th1, Th2, Th17, and cytotoxic T lymphocytes (CTL) or regulatory T (Treg) cells). Specifically, Th1 and Th17 cells cross the blood-brain barrier and directly contribute to neuroinflammation through the production of neurotoxic and proinflammatory factors that act on glial cells. Consequently, activated microglia and astrocytes respond by releasing high amounts of chemokines that assist the infiltration of a second wave of effector T cells into the brain. CD8+ CTLs recognize antigen presented by MHC class I on neurons to induce perforin- and/or granzyme-mediated cytolysis. In response to inflammatory events, Treg cells dampen down neuroinflammation and neurodegeneration

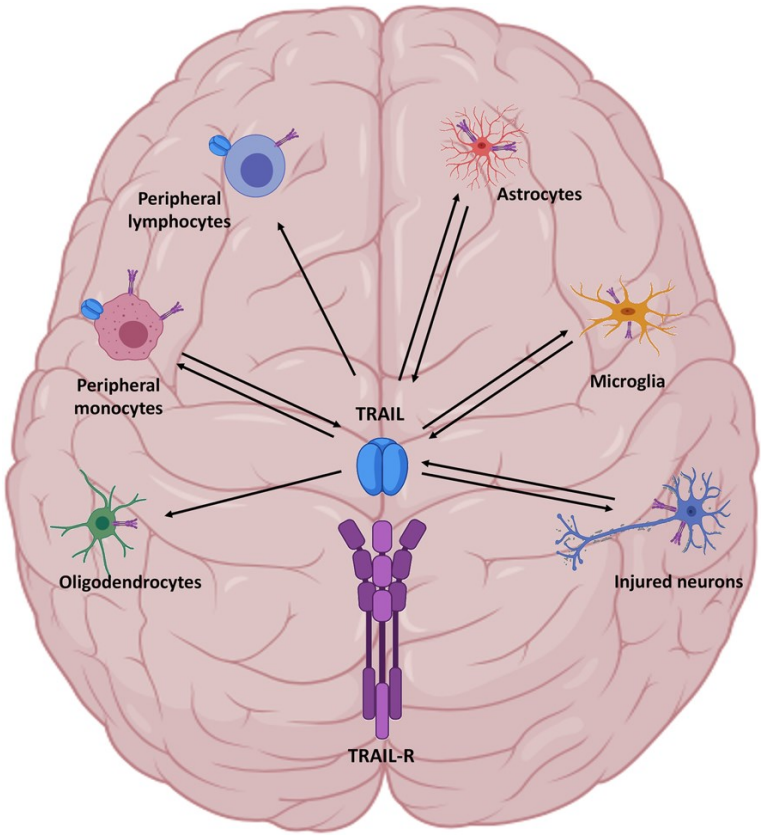

Figure 2. Fine-tuning of immune response by TRAIL in the brain. Under brain inflammatory conditions, TRAIL is abundantly released by activated glia, infiltrated peripheral monocytes and injured neurons. TRAIL acts as a potential death signal by interacting with its receptors expressed in neurons, microglia, monocytes, lymphocytes, astrocytes, and oligodendrocytes

Alzheimer's disease model	TRAIL-based treatment	Main findings	Reference
SH-SY5Y neuronal-like cells	rTRAIL TRAIL-neutralizing monoclonal antibody	TRAIL mediates Aβ-neurotoxicity in vitro	[13]
Human AD brain	/	TRAIL is specifically expressed in Alzheimer's disease brain	[130]
SH-SY5Y neuronal-like cells Primary mouse cortical neurons	anti-TRAIL-R/DR5 antibody	Blockade of TRAIL-death receptor DR5 signaling prevents Aβ-neurotoxicity in vitro	[133]
3xTgAD	TRAIL-neutralizing monoclonal antibody	Neutralization of TRAIL is associated with functional recovery, decreased Aβ burden and rebalance of both central and peripheral immune response in vivo.	[17]
3xTgAD	TRAIL-neutralizing monoclonal antibody	Neutralization of TRAIL restrain peripheral and CNS inflammatory/immune response along with decreased microglial TNFα production, reduced accumulation of both Aβ and p-Tau protein in the hippocampus of 3xTg-AD mice.	[18]

Figure 3. Summary of the most interesting evidences of the involvement of TRAIL in the pathophysiological events related to neuroinflammatory conditions such as Alzheimer's disease, in view of a potential future clinical development of TRAIL-based therapeutic strategies

Chapter III

Targeting the miRNA-155/TNFSF10 network restrains inflammatory response in the retina in a mouse model of Alzheimer's disease

Cell Death Dis 2021;12(10):905. doi: 10.1038/s41419-021-04165-x.

TARGETING THE MIRNA-155/TNFSF10 NETWORK RESTRAINS INFLAMMATORY RESPONSE IN THE RETINA IN A MOUSE MODEL OF ALZHEIMER'S DISEASE

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Abstract

Age-related disorders, such as Alzheimer's disease (AD) and age-related macular degeneration (AMD) share common features such as amyloid-β (Aβ) protein accumulation. Retinal deposition of Aβ aggregates in AMD patients has suggested a potential link between AMD and AD. In the present study, we analyzed the expression pattern of a focused set of miRNAs, previously found to be involved in both AD and AMD, in the retina of a triple transgenic mouse model of AD (3xTg-AD) at different time-points. Several miRNAs were differentially expressed in the retina of 3xTg-AD mice, compared to the retina of age-matched wild-type (WT) mice. In particular, bioinformatic analysis revealed that miR-155 had a central role in miRNA-gene network stability, regulating several pathways, including apoptotic and inflammatory signaling pathways modulated by TNF-related apoptosis-inducing ligand (TNFSF10). We showed that chronic treatment of 3xTg-AD mice with an anti-TNFSF10 monoclonal antibody was able to inhibit the retinal expression of miR-155, which inversely correlated with the expression of its molecular target SOCS-1. Moreover, the fine-tuned mechanism related to TNFSF10 immunoneutralization was tightly linked to modulation of TNFSF10 itself and its death receptor TNFRSF10B, along with cytokine production by microglia, reactive gliosis, and specific AD-related neuropathological hallmarks (i.e., Aβ deposition and Tau phosphorylation) in the retina of 3xTg-AD mice. In conclusion, immunoneutralization of TNFSF10 significantly preserved the retinal tissue in 3xTg-AD mice, suggesting its potential therapeutic application in retinal degenerative disorders.

Subject terms: Alzheimer's disease, Neurodegeneration

Introduction

Alzheimer's Disease (AD) is an age-related neurodegenerative disorder, whose onset precedes the disease's symptoms and diagnosis. Since it was first described, big efforts have been made to validate approaches for early diagnosis, along with effective treatments for AD [1].

Despite the advent of sophisticated neuroimaging techniques and the search for reliable biomarkers, to date, the definitive diagnosis of AD can only be made after the post-mortem identification of amyloid-β (Aβ) plaques and neurofibrillary tangles in the brain of AD patients [2].

Research studies also focused on AD diagnosis through ophthalmic diagnostic procedures because the eye is considered an easily reachable "window to the brain". Furthermore, the retina, a central nervous system tissue formed as a developmental outgrowth of the brain, is profoundly affected by AD [3].

Aβ deposition is considered a hallmark of AD pathology, and retinal Aβ deposits reported in AD patients and early-stage cases matched with brain amyloid pathology [4–6].

Furthermore, visual deficits and retinal ultrastructural modifications, such as ganglion cell degeneration, nerve fiber layer (NFL) thinning and optic nerve degeneration, were experienced by AD patients [6–8], strengthen the hypothesis that the retina represents a valuable site of pre-symptomatic AD stage imaging, and at the preclinical level can be considered as a surrogate tissue to be analyzed for mechanistic studies [9].

Noteworthy, studies on several retinal degenerative diseases such as glaucoma and age-related macular degeneration (AMD), which share some features with AD [10–13], may provide some clues to understand the pathological process underlying such disorder.

Despite major advances in understanding mechanisms of AD, to date, there are no disease-modifying options available to slow down or halt the progression of the neurodegenerative process. Current pharmacological treatments only transiently mitigate the severity of symptoms, with generally unsatisfactory clinical outcomes [14].

In such scenario, it would be helpful to identify reliable targets for AD therapeutic intervention [15].

Besides a leading role as feasible disease biomarkers, microRNAs (miRNAs) expression profiles could provide an overview of the complex network of molecular pathways in AD and AMD, which is defined as the "dementia of the eye" [12].

In this light, our research group has previously identified an overlap in the expression patterns of specific miRNAs (miR-155, miR-126a, miR-23a, miR-34a, miR-9, miR-27a, miR-146a), between the retina of a rat model of AMD (Aβ intravitreal injection) and serum of AMD patients, which were also recognized as potentially useful biomarkers of AD pathology [11, 16].

It is well known that chronic neuroinflammation is one of the prominent hypotheses put forward to describe the pathogenesis of AD [17]. Several miRNA networks, including miRNAs related with innate immunity and neuroinflammation, have been found to be dysregulated in AD [18, 19]. In fact, as previously reported, miR-155 upregulation contributes to neuroinflammation in AD [20], where it plays a central role in the regulation of the innate immune response through the modulation of cytokines and chemokines production [21, 22]. In this regard, cytokine Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), a cytokine formerly known as TNFSF10 and a member of the TNF superfamily, produced by injured neurons [23] and by activated glia [24], with its potent immune-modulatory properties represents a pleiotropic fine-tuning effector of the inflammatory/immune response with an orchestrating role in the complex scenario of AD etiopathogenesis [25]. TNFSF10 mediates death signaling through interaction with its death receptors TNFRSF10B (DR5) and TNFRSF10A (DR4), and interferes with several pathways, including the Wnt pathway concurring to neuronal damage [26].

The prominent role of TNFSF10 in Aβ-related neurodegeneration has been already demonstrated in different studies showing that neutralization of TNFSF10 pathway in an in vitro model of AD protects human neuronal cell line from Aβ−neurotoxicity [23]; as well as in the triple transgenic mouse model of AD (3xTg-AD) where it exerts a beneficial effect on central and peripheral AD-related inflammatory/immune response and disease outcome [27, 28].

Given the emerging role of miRNAs and TNFSF10 system in AD-associated neuroinflammation and considering that the retina is an integral part of the central nervous system originating from the neural tube, in the present study, we investigated the expression of a focused set of miRNAs, linked to AD and AMD, in the retina of 3xTg-AD mice. Furthermore, we investigated the combinatorial effect of miRNAs through bioinformatic approaches. We highlighted a direct link between these miRNAs and the TNFSF10 signaling pathway in AD-related inflammation, and to support our experimental hypothesis we used the retina as a surrogate tissue for mechanistic and pharmacological studies in AD pathology. Furthermore, our pre-clinical data evidenced that anti-TNFSF10 antibody treatment would be of value for the management of sight-threatening retinal degenerative diseases.

Results

3xTg-AD retinal miRNA expression and bioinformatic analysis of related biochemical pathways

In light of the reported link between AD and retinal degeneration associated with AMD [10] and to identify useful biomarkers of disease progression, we analyzed the expression of a specific set of miRNAs, involved in both disease conditions, in retina extracts from 3xTg-AD mice at different time-points (3, 9 and 15 months of age), resembling the evolution of AD-like pathology.

This focused set of miRNAs was chosen with the rationale that it was altered in sera of AMD patients as well as in the retina of rats subjected to intravitreal injection of Aβ oligomers [11], thus resembling a model of early AMD.

Real-time PCR analyses highlighted five miRNAs (miR-155, miR-126a, miR-23a, miR-34a, miR-27a) significantly dysregulated in the retina of 3xTg-AD mice compared to age-matched wild-type (WT) mice, while the miR-9 expression levels were not significantly modulated.

Noteworthy, we found that miR-155 was significantly up-regulated in the retina of the AD mice at all ages (Fig. (Fig.1).1). Retinal miR-126a was significantly up-regulated in the retina of 3- and 9-month-old 3xTg-AD mice, while miR-126a expression level decreased, though not significantly, in 15-month-old 3xTg-AD mice compared to age-matched WT mice. A similar trend was observed for miR-23a and miR-27a expression levels, that after upregulation, significantly decreased in the retinas of 15-month-old 3xTg-AD compared to WT mice. The miR-34a retinal level was significantly up-regulated only in 3-month-old AD mice, compared to WT.

To shed light on the biological effects of miRNAs expression patterns, several bioinformatic approaches were carried out. We hereby predicted the pathways dysregulated by the analyzed miRNAs accessing the miRNet webserver, which generated a complex network of about 17,000 interactions (edge). Degree centrality analysis with Cytoscape has shown that miR-155 is the node with the highest node degree value (Fig.2), demonstrating that within about 2,000 nodes, the miR-155 displays the highest number of incident links with other nodes, which represent target genes. Moreover, miR-155 has shown the highest betweenness centrality, along with miR-34a and miR-27a than other miRNAs.

To carry out a straightforward analysis of this complex miRNA-gene interaction network, we have done a KEGG (Kyoto Encyclopedia of Genes and Genomes) enrichment analysis (Supplementary Table 1) [29]. Top predicted pathways were: “Apoptosis”, “T cell receptor signaling pathway”, “p53 signaling pathway”, “Neurotrophin signaling pathway”, “Alzheimer’s disease”, “Natural killer cell-mediated cytotoxicity”, “Cytokine-cytokine receptor interaction”. Interestingly, the miRNet enriched analysis, for the given miRNA-gene network, has yielded two diseases: “Inflammation” and “Alzheimer’s Disease”.

Computational analysis of the combinatorial effects among this group of miRNAs, specifically miR-155-5p, miR-126-3p and miR-23a-3p revealed that these miRNAs target also genes belonging to the TNF-related apoptosis-inducing ligand (TNFSF10)-mediated apoptotic signaling pathway (Supplementary Fig. 1), including the TNFSF10 death receptors TNFRSF10B and TNFRSF10A. Additionally, dysregulated miRNAs were predicted to modulate other pathways through targeting TNFSF10 related genes: “p53 signaling pathway”, “Cytokine-Cytokine receptor interaction” and “Natural killer cell-mediated cytotoxicity”. In Table 1 we evidenced the combinatorial effect of miRNAs on TNFSF10 related genes. Specifically, Table 1 shows experimentally validated miRNA:mRNA interactions, as regards as Tarbase algorithm output.

Noteworthy, both human TNFRSF10B and TNFRSF10A are experimentally validated targets of miR-155. Looking at the time-dependent pattern of expression of miRNAs obtained in 3xTg-AD mice, we observed that in young mice (3- and 9-month-old mice), most of the miRNAs up-regulated in the retina of AD mice could negatively regulate the expression of the TNFSF10 pathway target genes. On the contrary, in the late phase (15-month-old mice), significantly downregulated miRNAs such as miR-23a and miR-27a could act as positive regulators of the TNFRSF10B receptor and FADD, likely promoting their detrimental effects on the retina.

Neutralization of TNFSF10 modulates the expression of miR-155 and SOCS-1 in the retinas of 3xTg-AD mice

Bioinformatic analysis relied on already validated in-vitro functional assays and correlates dysregulated miRNAs in AD retina with the TNFSF10-signaling pathway. To confirm bioinformatic data in in-vivo studies, we focused our analysis on 15-month-old 3xTg-AD mice which exhibit most of the neuropathological features of the disease. Interestingly, we observed that chronic treatment with an anti-TNFSF10 monoclonal antibody significantly inhibited only the expression of miR-155 (Fig.3A). No significant effect on miR-155 expression levels was detectable in the retina of WT mice. Other miRNAs from the analyzed set were not significantly differentially expressed when comparing treated with untreated animals.

It has been reported that the suppressor of cytokine signaling 1 (SOCS-1) is a validated (Tarbase v8 algorithm) [30] and predicted target (microTG algorithm) [31] of miRNA-155 (Fig.3B). Therefore, given that anti-TNFSF10 treatment modulated miR-155 (Fig.3A), we also investigated the effect of the TNFSF10 immunoneutralization on the expression of its molecular target SOCS-1 in the retinas of the same AD animals. Consistently, western blot analysis revealed that the increased expression of miR-155 in the retina of 15-month-old 3xTg-AD mice was paralleled by a significant decrease of SOCS-1 expression, whereas treatment with anti-TNFSF10 antibody restored SOCS-1 to basal levels (Fig. 3C, D). These data provided the in-vivo functional validation of the tight link between miR-155 and TNFSF10 signaling pathway, along with the in-silico analysis carried out on the basis of experimental validated miRNA:mRNA interactions.

Histological evidence of the efficacy of the anti-TNFSF10 treatment upon the retinal tissue alteration in 3xTg-AD mice

With the aim to verify the role of TNFSF10 immunoneutralization on morphological changes in the retinas of AD mice and to confirm bioinformatic predictions and biomolecular findings, hematoxylin-eosin staining was performed upon retinal sections of 3xTg-AD and WT mice.

While no significant changes throughout the retinal layers were observed in specimens from both treated or untreated WT animals, on the other hand, vacuolization and cell disorganization, as well decreased tissue cellularity were observed in the retinal ganglion cell layer (GCL), along

with a reduced thickness of the NFL in untreated 3xTg-AD mice. Both tissue parameters appeared improved in the retinas of 3xTg-AD mice treated for twelve months with anti-TNFSF10 treatment, suggesting its neuroprotective effect (Fig. 4).

TNFSF10 immunoneutralization brings about downregulation of expression of TNFSF10 and its receptor TNFRSF10B in the retina of 3xTg-AD mice

Since it is known that TNFSF10 and its death receptor TNFRSF10B were specifically upregulated in the brain of 3xTg-AD mice [27], and given that the retina is regarded as a developmental outgrowth of the brain, we explored the role of both mediators in the retinas of 3xTg-AD mice treated chronically with an anti-TNFSF10 antibody.

Western blot analysis revealed that while both TNFSF10 and its death receptor TNFRSF10B were highly expressed in the retinas of untreated 3xTg-AD mice, their expression was significantly attenuated following treatment with an anti-TNFSF10 antibody (Fig. 5A, B).

Biochemical data were confirmed by confocal microscopy experiments, showing that both TNFSF10 and its death receptor TNFRSF10B were highly represented throughout the retina of 3xTg-AD mice, and particularly in the retinal pigmented epithelium (RPE) and the outer plexiform (OPL) layers. While the expression of TNFRSF10B receptor was significantly reduced in the retinal RPE and OPL layers the expression of TNFSF10 was significantly blunted only in the retinal RPE layer of anti-TNFSF10 treated 3xTg-AD mice (Fig. 5C, D, Supplementary Fig. 2). These proteins colocalized in both the retinal RPE and OPL layers of 3xTg-AD mice (Supplementary Fig. 3).

Inhibition of the TNFSF10 signaling pathway protects the retina of 3xTg-AD mice from neuroinflammatory damage

Neurodegeneration-related breaking of the balance between neurotoxic and neuroprotective mechanisms can induce activation of microglia, which can polarize assuming a classical proinflammatory phenotype, or the alternative anti-inflammatory phenotype via cytokine production [32].

A skewed M1 activation over M2 markedly promotes both AD progression and retinal degeneration, and modulation of microglia polarization has been regarded to as a potential therapeutic target for neuroprotection [25, 33, 34].

Concerning the activation status of microglia and related proinflammatory molecules, western blot analysis revealed that, while the expression of microglial marker Iba-1 and of TNF-α was substantially present in the retina of 3xTg-AD mice, anti-TNFSF10 treatment significantly blunted their expression (Fig. 6A, B). Consistently, confocal microscopy analysis showed an increased expression of both TNF-α and Iba-1 (Fig. 6C, E), which, colocalized in both the RPE

and OPL layers (Supplementary Fig. 4) of untreated 3xTg-AD mice. Treatment with anti-TNFSF10 restored TNF-α and Iba-1 to basal levels (Fig. 6C, E, Supplementary Fig. 5A).

On the other hand, although Iba-1 is expressed in all retinal layers of both treated and untreated AD mice, the anti-inflammatory cytokine IL-10 is strongly expressed and colocalized with Iba-1 in the RPE and OPL retinal layers of mice treated with anti-TNFSF10 (Fig. 6D, F, Supplementary Fig. 5B, Supplementary Fig. 6). Therefore, anti-TNFSF10 treatment promoted an anti-inflammatory phenotype in microglial cells confirming the western blot data (Fig. 6A, B). These data suggest that TNFSF10 neutralization boosts anti-inflammatory microglia as a consequence of inflammatory microglia inhibition, resulting in retinal protection.

Moreover, increased glial fibrillary protein (GFAP) and COX2 immunostaining, hallmarks of reactive gliosis, another typical feature appearing during neurodegenerative processes, was observed in retinal RPE and OPL layers of 3xTg-AD mice. Significantly, reduction of both GFAP and COX2 expression occurred in 3xTg-AD mice treated with the TNFSF10 antibody (Fig. 7A, B, Supplementary Fig. 7). These proteins colocalized in both the retinal RPE and OPL layers of 3xTg-AD mice (Supplementary Fig. 8). A similar trend of expression of GFAP and COX2 was observed in western blot analysis (Fig. 7C, D). In addition, as inflammation emerges as crucial common point in AMD and AD pathogenesis, we also evaluated the expression of other inflammatory markers such as Interleukin 6 (IL-6) and Interferon-γ (IFN-γ) in the retina of 3xTg-AD mice [35]. Robust expression of both IL-6 (Supplementary Fig. 9A, B) and IFN-γ (Supplementary Fig. 9C, D) was detectable in the retina of untreated 3xTg-AD mice, while treatment with the anti-TNFSF10 antibody resulted in blunted expression of both inflammatory markers.

Accumulation of both Aβ deposits and phosphorylated Tau (p-Tau) in the retina of 3xTg-AD mice is attenuated by anti-TNFSF10 treatment

In consideration of the well-known contribution of both Aβ1-42 and p-Tau [36] in 3xTg-AD mice, we investigated their expression in the retina of these mice with and without chronic anti-TNFSF10 antibody.

Indeed, p-Tau was detected in retinas of 3xTg-AD mice and eventually colocalized with Aβ deposits in the OPL layer but especially in the RPE cell layer (Supplementary Fig. 10). A remarkable reduction of Aβ and p-Tau immunostaining was observed after treatment with a TNFSF10-neutralizing antibody (Fig. 8A, B, Supplementary Fig. 11). Western blot analysis confirmed these findings (Fig. 8C, D).

Discussion

Circulating serum miRNAs or tissue-specific miRNAs, have been largely considered as feasible disease biomarkers in the oncology field, but also in ocular diseases [11, 37]. The expression pattern of miRNAs has been also analyzed in AD, either in pre-clinical or clinical studies [38,

39]. Several studies have investigated the role of a single miRNA (i.e., miR-181 [40, 41], miR-369 [42], miR-31 [43], miR-342 [44], miR-132/212 [45], miR-34a [46, 47], miR-155 [20], miR-146a [48]) in 3xTg-AD mice, but only a few of these studies were focused on differential expression of more than one noncoding RNA [49, 50].

Here, we evaluated the expression of a focused set of miRNAs previously validated in a rat model of AMD and in serum of AMD patients [11], in the retina of 3xTg-AD mice at different ages, instead of using high-throughput analysis. Considering that, many other retinal miRNAs could be dysregulated in this strain but also that high-throughput analyses are quite expensive and need a mandatory validation step (qPCR) [51], our focused strategy (i.e., literature search and bioinformatic validation) was aimed to increase the success rate of miRNAs and gene target analysis.

Indeed, results obtained hereby can be inferred for mechanistic and pharmacological studies in age-related ocular degenerative diseases, such as glaucoma and AMD, that share common pathogenetic mechanisms with AD [16].

We found that five miRNAs were dysregulated in the retina of 3xTg-AD mice (miR-155-5p, miR-126-3p, miR-34a-5p, miR-27a-3p, miR-23a-3p). According to previous data [46, 47], we found significant up-regulation of miR-34a only in the retinas of 3-month-old 3xTg-AD mice. With regard to miR-155, Guedes et al. in 2014 showed that this miRNA was up-regulated in the brain of 3xTg-AD mice, and it was tightly linked to astrocyte and microglia activation [20]. Consistent with this evidence, we found an age-dependent retinal up-regulation of miR-155 in 3xTg-AD mice and the highest node degree distribution with susceptibility genes in the predicted miRNA-gene network, confirming that miR-155 plays a crucial role in the regulation of several pathways of AD.

Thus, we investigated the role of the dysregulated set of miRNAs through bioinformatic approaches, unraveling a tight link with the TNFSF10 signaling pathway. Furthermore, validated interactions (Tarbase algorithm) [30] were found between miR-155 and TNFSF10 death receptor TNFRSF10B mRNA, along with SOCS-1, which is a protein involved in a negative feedback loop necessary to control the proinflammatory cytokines release [20]. Now, it is well known that cytokine signaling is overactivated in AD [17]. Low expression of SOCS-1 observed in AD, depending upon mir-155 overexpression, was associated with the sustained inflammatory process that characterizes the disease [20].

Moreover, the miR-155 upregulation in 3xTg-AD mice would represent a mechanism aimed to modulate the TNFSF10 system, in response to activation of other detrimental biological pathways. Consistently, we found that retinal miR-155 expression was significantly down-regulated in anti-TNFSF10-treated 3xTg-AD mice. These results are in line with previous studies, showing the tight relationship between the TNFSF10 pathway and miR-155 [52]. Furthermore, we found that SOCS-1 was significantly down-regulated in the retina of 3xTg-AD mice, whereas

it was stabilized at basal level in animals treated with anti-TNFSF10. Overall, our results point out to relevant consequences of TNFSF10 immunoneutralization in counteracting the inflammatory/immune-response sustained by miR-155 upregulation and consequent SOCS-1 downregulation in the AD retina.

The anti-TNFSF10 treatment restored a normal morphology of retinal GCL and NFL. These results are aligned with previous studies showing changes in retinal morphology of AD patients [53] and other types of retinal degeneration [54]. In this scenario, our data are consistent with data demonstrating that immunoneutralization of TNFSF10 is correlated with neuroprotection [27, 55].

The expression of TNFSF10 and its TNFRSF10B receptor was significantly higher in the retina of untreated 3xTg-AD mice, while anti-TNFSF10 treatment resulted in significantly decreased expression of both proteins. This appears in line with other data, showing an increase of TNFSF10 and its death receptor in different neurodegenerative processes, occurring, for example, after spinal cord injury [55], and in the post-ischemic stroke [56].

As the TNFSF10 system has an orchestrating role in immune/inflammatory response during neuroinflammatory processes related to neurodegeneration [25, 28], we found a constitutively increased expression of the microglia marker Iba-1, as well as of the microglia-released cytokine TNF-α in the retina of 3xTg-AD mice. Both proteins colocalized in the RPE and the OPL layers and their colocalization was attenuated following anti-TNFSF10 treatment. This is in line with the decreased proinflammatory microglia activity shown in the brain of 3xTg-AD mice after anti-TNFSF10 treatment [28], indicating that the immunomodulating effect of TNFSF10 is extended to different areas of the central nervous system.

When an inflammatory response is triggered and sustained by arrays of proinflammatory cytokines [17], a counterbalancing anti-inflammatory response is promptly set into motion through the release of inhibitory molecules with the aim to restrain the overshoot of the inflammatory response and consequent tissue damage [25]. In a similar fashion, our results showed that the levels of the anti-inflammatory cytokine IL-10 substantially increase after the anti-TNFSF10 treatment. In this line, decreased IL-10 expression has been founded in neuroinflammatory conditions during neurodegenerative processes caused by trauma [55] or stroke [56], encompassed in its pleiotropic anti-inflammatory role in peripheral inflammatory diseases, such as rheumatoid arthritis [57], and inflammatory bowel disease [58].

Thus, it is plausible to hypothesize that the prominence of neuroinflammatory features in the retina of 3xTg-AD mice is the result of unbalanced occurrences, where the proinflammatory component gains an advantage over the anti-inflammatory one.

Consistent with the above reports, the anti-TNFSF10 treatment resulted in an increased expression of IL-10 associated with increased colocalization within Iba-1-positive cells.

A relevant contribution to neuroinflammation is given by gliosis, which corresponds to activation of repair processes associated with brain inflammation [59]. Gliosis-related overexpression of inflammatory molecules is a typical feature shared by neurodegenerative processes [60]. Gliosis implies an increased expression of its specific marker, GFAP, in the brain [61]. The anti-gliosis effect of the anti-TNFSF10 treatment observed in our experiments demonstrated that the anti-inflammatory effects of the treatment also encompassed a weaker glial response, likely responsible for the rescue of retinal cells, paralleled by the positive effects occurring in the damaged brain areas [27]. Consistently to the decreased number of activated glial cells in the retina of anti-TNFSF10 treated 3xTg-AD mice, we also observed a decreased expression of the inflammatory marker COX2, highly induced in glial cells during neurodegeneration [62].

Moreover, we observed an upregulation of both IL-6 and IFN-γ in retinal lysates from untreated 3xTg-AD mice, as expression of Aβ-induced gliosis. Both IL-6 and IFN-γ expression was significantly attenuated in 3xTg-AD mice following the anti-TNFSF10 treatment. Considering that IL-6 and IFN-γ play a significant role in the pathogenesis of AMD, and that both AD and AMD share a number of striking similarities [35], such results corroborate our hypothesis that the anti-TNFSF10 antibody treatment represents a valuable strategy for the management of sight-threatening retinal degenerative diseases.

Given the tight correlation between neuroinflammatory processes in AD and the accumulation of Aβ, as well as the presence of neurofibrillary tangles [63], we observed that the remarkable amount of retinal Aβ and p-Tau proteins in the retina of 3xTg-AD mice was significantly reduced after anti-TNFSF10 treatment. This is in line with other studies, showing that the functional outcome improvement is related to the amount of Aβ and p-Tau in the brain of 3xTg-AD [27], and that the curtailment of both the central and the peripheral immune response is followed by improvement of brain tissue parameters, along with decreased inflammatory markers and reduced amounts of anomalous proteins in discrete brain areas [28]. These findings are consistent with the hypothesis that TNFSF10 is a driver of the inflammatory/immune response in different conditions of neuronal damage [23, 27, 28, 55, 56]. Immunohistochemical analysis highlighted that the neuroinflammatory hallmarks were expressed in the OPL and in the RPE layer, while retinal histochemical analyses evidenced that anti-TNFSF10 treatment preserved other retinal layers of AD mice, such as GCL and NFL. Indeed, RPE and OPL layers are involved in AMD, and, specifically, RPE and OPL layers were thinner in subjects with early AMD and neurodegeneration [64]. Therefore, it is plausible to hypothesize that the anti-TNFSF10 treatment could exert retrograde neuroprotection and anti-inflammatory action from outer (RPE and OPL) layers to the inner retina (retinal ganglion cells), probably preventing trans-neuronal degeneration [65], and photoreceptor degeneration induced by amyloid aggregation [1, 66].

In conclusion, we demonstrated that five miRNAs were constitutively dysregulated in the retina of 3xTg-AD mice, showing an age-related expression pattern. Furthermore, we observed that

miR-155 expression was significantly modulated by the anti-TNFSF10 treatment, finally resulting in reduced inflammation and neuroprotective effects on the retina of 3xTg-AD mice.
We also showed that the Aβ eye-related pathology observed in the 3xTg-AD mouse model is sustained, to a large extent, by the proapoptotic cytokine TNFSF10, in redundancy with an array of inflammatory molecules. Systemic treatment with a TNFSF10 neutralizing antibody implies a dramatic improvement in either tissue or inflammatory parameters in competent retinal cells.
Finally, our results show that neutralization of TNFSF10 brings about significant amelioration of the Aβ-related eye pathology, suggesting potential therapeutic target for AD-related and other degenerative retinal disorders. Altogether, our findings suggest that TNFSF10 could be a useful tool for immunopharmacological management of age-related ocular diseases.

Materials and methods

Animals

Experiments were performed in 3xTg-AD mice harboring three human mutated genes (B6129-Psen1tm1MpmTg (APPSwe, tauP30L)1Lfa/J) and age-matched wild type (WT) mice (B6129SF2/J), purchased from Jackson Laboratories (Bar Habor, ME, USA).
The 3xTg-AD mice, overexpressing mutant amyloid precursor protein (APP (APPSwe)), presenilin 1 (PSEN1 (PS1M146V)), and tau (tauP301L), were originally generated by co-injecting two independent transgene constructs encoding human APPSwe and tauP301L (4 R/0 N) (controlled by murine Thy1.2 regulatory elements) into single-cell embryos harvested from mutant homozygous PS1M146V knock-in mice, which were reimplanted into foster mothers. Wild-type mice of mixed genetic background 129/C57BL6 were used as controls. The original 3xTg-AD mice strain was generated and described by Oddo et al. [36].
Wild-type mice of mixed genetic background 129/C57BL6 were used as controls.
All animals were housed under controlled light (12 h light/night cycle), in temperature- and humidity-controlled rooms, with access to food and water ad libitum. All experiments using animals were approved by the Italian Ministry of Health and conducted in accordance to the European Community directive guidelines for the use of animals in laboratory (2010/63/EU) and the Italian law (D.Lgs. 26/2014). All procedures minimized the number of animals used and their suffering.

Experimental groups, drug administration, and sample collection

For a first validation experiment, a panel of miRNAs was analyzed in 3xTg-AD at different time-points resembling the evolution of an AD-like pathology (3, 9, and 15 months of age) and in age-matched wild-type mice, 6 mice per group. For this experiment, two retinas, from different animals of the same group were pooled.

For drug administration study, twenty 3xTg-AD and twenty wild-type mice were enrolled at 3 months of age and four study groups were used: (1) ten wild-type mice plus vehicle (Purified Rat IgG2ακ Isotype Control; BD Biosciences, San Jose, CA, USA); ten wild-type mice plus TNFSF10-neutralizing antibody (Purified Rat Anti-Mouse CD253; BD Biosciences); (iii) ten 3xTg-AD mice plus vehicle; and (iv) ten 3xTg-AD mice plus TNFSF10-neutralizing antibody. Animals (n = 10 per each experimental group) were treated with TNFSF10-neutralizing antibody (concentration: 0.05 mg/ml; 200 µl/ mouse; i.p.) or vehicle (concentration: 0.05 mg/ml; 200 µl/ mouse; i.p.) twice a month and sacrificed at 15 months of age, 2-weeks after the last injection.
Given 10 mice per experimental group, 20 eyes per experimental group were isolated. Specifically for western blot analysis, 10 retinas were randomly collected from 5 different mice of the same experimental group, 2 retinas per group were pooled in a vial, then given a total of N = 5 independent retinal samples (biological replicates) per group. Five eye globes from 5 mice per group were used for qPCR analysis, carried out for miRNA expression analysis on anti-TNFSF10 treated and untreated mice. The contralateral remaining 5 eye globes from different 5 animals per group were fixed, then retinas were isolated to carry out hematoxylin and eosin (H&E) and immunofluorescence staining experiments.

microRNA extraction, cDNA synthesis, and qPCR

The retina from the ocular globe was isolated and placed in RNAlater solution (Ambion Biosystems, Austin, TX, USA), stored at 4 °C overnight then transferred to −80 °C. The extraction of total RNA from mice retina samples was carried out with TRIzol Reagent (Invitrogen, Life Technologies, Carlsbad, CA), according to the manufacturer's protocol. The A260/A280 ratio of the optical density of RNA samples (measured with Multimode Reader Flash di Varioskan™) was within 1.95–2.01. cDNA was synthesized from 10 ng of RNA with TaqMan® Advanced miRNA cDNA Synthesis Kit (ThermoFisher Scientific, Cat. No. A28007). According to the manufacturer's instructions, the poly(A) tailing reaction has been performed, followed by the adaptor ligation reaction and by the reverse transcription (RT) reaction. Subsequently, the miR-Amp reaction was carried out to obtain the undiluted miR-Amp reaction product. The miR-Amp reaction product was diluted 1:10, and the amplification was carried out by using Taqman® Advanced MicroRNA Assays (ThermoFisher Scientific) and Taqman® Fast Advanced Master Mix (ThermoFisher Scientific, Cat. No 4444557). The miR-155-5p (mmu480953_mir), miR-126a-3p (mmu482681_mir), miR-23a-3p (mmu478532_mir), miR-34a-5p (mmu481304_mir), miR-9-5p (mmu481285_mir) and miR-27a-3p (mmu478384_mir), miR-146a-5p (mmu478399_mir) has been analyzed. The miR-16-5p (mmu482960_mir) has been used for the normalization. Real-time PCR was carried out on a 7900 HT Fast Real Time PCR System (Applied Biosystems, Monza, Italy). MicroRNA expression was quantified as -ΔCt, where Ct is the threshold cycle, and -ΔCt is the negative of Ct target miRNA minus Ct miR-16.

Bioinformatics analysis

An integrated bioinformatic approach was carried out to predict the biological effect of the differential expression of a specific set of miRNAs, in the retina of 3xTg-AD mice compared to control wild-type mice. Specifically, the input of miRNet analysis [67] were the miRNAs significantly differentially expressed in the retina of 3xTg-AD mice, compared to the retina of age-matched WT mice (miR-155-5p, miR-126-3p, miR-34a-5p, miR-23a-3p, miR-27a-3p). Since human and murine miRNAs share high sequence homology and identity, we selected in miRNet analysis the "human" option as setting for "organism", to characterize our analysis with a translational approach. The miRNA-target genes network was built applying the Tarbase v.8, an algorithm for the prediction of experimentally validated miRNA-mRNA target gene interactions [30]. The miRNet analysis also provided the prediction of diseases, characterized by dysregulation of the input set of miRNAs. The output of miRNet, a miRNA-target genes network, was analyzed as an undirected graph with Cytoscape, through analysis degree metrics, because of its large dimensions (more than 2000 nodes, more than 17000 undirected node-node interactions). Network analysis, i.e. centrality metrics, has been carried out accordingly to principles or network stability parameters, as previously reported [68]. Within the miRNet analysis, we then carried out an enriched analysis of mRNA-target genes network, through the "function explorer" module, setting the hypergeometric test as algorithm. Other specific analyses were carried out with DIANA tools (http://diana.imis.athena-innovation.gr/DianaTools/index.php), such as Kegg pathways enrichment [69] (https://www.genome.jp/kegg/pathway.html).

Tissue homogenization and protein extraction

The retina samples of 3xTg-AD and age-matched wild-type mice were dissected in ice-cold Hank's balanced salt solution (HBSS: 137 mM NaCl, 5.4 mM KCl, 0.45 mM KH2PO4, 0.34 mM Na2HPO4, 4 mM, NaHCO3, 5 mM glucose; pH 7.4), the two retinas per group were pooled and then frozen in liquid nitrogen and stored at −80 °C, until use. For protein extraction, retinal tissues were lysed in a lysis buffer containing 150 mM NaCl, 50 mM Tris–HCl (pH 7.5), 5 mM EDTA, 1 mM Na3VO4, 30 mM sodium pyrophosphate, 50 mM NaF, 1 mM acid phenyl-methyl-sulphonyl-fluoride, 5 µg/ml aprotinin, 2 µg/ml leupeptin, 1 µg/ml pepstatin, 10% glycerol, and 0.2% TritonTM X-100 and sonicated with 3 pulses of 2 s each. The homogenates were then centrifuged at 14,000 rpm for 10 min at 4 °C and the supernatant was collected. The protein concentration of the supernatant was determined by the Bradford method [70].

Western blot analysis

Equal amounts of protein (40 µg) were resolved by 8–12% SDS-PAGE gels and transferred onto Hybond ECL nitrocellulose membranes (GE Healthcare, Little Chalfont, UK). Membranes were blocked for 1 h at RT with 5% nonfat dry milk or 5% BSA in phosphate-buffered saline plus 0.1%

Tween 20 (PBS-T). For primary antibody reactions, a rabbit anti-SOCS1 (Cell Signaling Technology Inc., Danvers, MA, USA), or a rabbit anti-TNFRSF10B (Abcam, Cambridge, UK), or a rabbit anti-TNFSF10 (Abcam), or a mouse anti-Iba1 (Abcam), or a rabbit anti-TNF-α antibody (Novus Biologicals), or a rabbit anti-IL10 antibody (Abbiotec, San Diego, CA, USA), or a mouse GFAP (Cell Signaling Technology Inc.), or a mouse anti-COX-2 (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), or a mouse anti-p-Tau antibody (Santa Cruz Biotechnology Inc.), or a rabbit anti-Tau antibody (Santa Cruz Biotechnology Inc.), or a mouse IFN-γ (Santa Cruz Biotechnology Inc.), or anti-rabbit IL-6 (Cell Signaling Technology Inc.) were added to membranes and stayed overnight at 4 °C on an orbital shaker. Then, the membranes were washed with PBS-T and were probed with the appropriate horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG antibody (Amersham Life Science, Buckinghamshire, UK) for 1 h at RT. Beta-Tubulin or β-actin (Santa Cruz Biotechnology Inc.) or GAPDH (Cell Signaling Technology Inc.) were used as control to validate the amount of protein loaded in the gels. After washing with PBS-T, protein bands were visualized by enhanced chemiluminescence (Thermo Fisher Scientific) and scanned with the iBright FL1500 Imaging System (Thermo Fisher Scientific). Densitometric analysis of band intensity was done on immunoblots by using IMAGE J software (https://imagej.nih.gov/ij/). Full details of the antibodies used are reported in Supplementary Table 2.

Hematoxylin and eosin (H&E) staining

Retinal tissue samples were fixed in 10% neutral-buffered formalin (Bio-Optica) for 24 h. After overnight washing, tissue samples were dehydrated in graded ethanol and paraffin-embedded. Sections of 4–6 μm in thickness were cut and mounted on silanized glass slides and air-dried. To remove the paraffin, slides were immersed in xylene two times, for 3 min each; rehydrated with graded ethanol, 100%, 95%, 80%, 70%, and 50%, for 3 min each; and transferred to tap water. After that, tissues were stained with (H&E) and morphological examination of the samples was performed using an Axioplan Zeiss light microscope (Germany).

Immunofluorescence

After collection, eye globes were fixed in 4% w/v paraformaldehyde in phosphate buffer saline 0.1 M pH 7.4 (PBS) for 2 h at room temperature. Retinal tissues paraffin-embedded were cut in 5 μm sections and placed on glass slides. After deparaffinization and rehydration, tissue specimens were processed as previously described [28] with a few modifications. Briefly, after antigen retrieval in sodium citrate buffer (10 mM sodium citrate, 0.05% Tween-20, pH 6.0)) by microwave for 15 min, slides were washed in PBS containing 0.25% Triton X-100 (PBST) twice for 5 min each, blocked in 1% BSA in PBST for 1 h at RT, briefly rinsed with PBST and incubated for 1 h at RT with the following primary antibodies: a goat anti-TNFRSF10B (Abcam), or a rabbit anti-TNFSF10 (Abcam), or a mouse anti-Iba1 (Abcam), or a rabbit anti-TNF-α antibody (Novus

Biologicals), or a rabbit anti-IL10 antibody (Abbiotec), or a rabbit GFAP (Abcam), or a mouse COX-2 (Santa Cruz Biotechnology Inc.), or a mouse anti-p-Tau antibody (Santa Cruz Biotechnology Inc.). For immunopositive reactions and fluorescence detection, after washing in PBS three times for 5 min each, sections were incubated using the appropriate fluorescent-labeled secondary antibodies (Invitrogen; Thermo Fisher Scientific, Inc, MA, USA) at dark for 1 h at RT. See Supplementary Table 2 for full details of the antibodies used. Finally, for nuclear staining, slides were washed and mounted with DAPI-containing mounting solution (Fluoroshield with DAPI; Sigma-Aldrich, Milan, Italy) and secured with a coverslip. Images were observed using a laser scanning confocal microscope (Zeiss LSM 700, Germany) and ZEN2010 software was used for image acquisition and colocalization analysis. Intensity level of the fluorescent signals was evaluated using the ImageJ software (NIH, Bethesda, MD; available at http://rsb.info.nih.gov/ij/index.html). Mean data from 14 optical fields (4 × 4 µm of 5-µm-thick sections) were analyzed with one-way ANOVA followed by Tukey's test post hoc analysis. Differences between groups were considered significant at $*p < 0.05$.

Statistical evaluation

Investigators that carried out treatment and analyses were blinded to group labels. Group labels were unveiled after draft graph design and statistical analyses. Sample size was chosen considering the calculation provided by power analysis and the possibility that mice would die or be excluded within 15 months-long experimental protocol. For animals and relative samples, the exclusion criteria from experimental protocol were: sudden death, loss of weight >20%, sign of distress (eyes squinted, contraction of the skin around the nose, ears pulled back, and lethargy or non-responsiveness). Within the monitoring of animal health during the experiment, no animals or samples were excluded from the study. Specifically, given the lowest expected difference between the means of two groups and homogeneous variance within the groups, the calculated sample size was $n = 4$, for $1-\beta$ set to 0.80 and α set to 0.05 (G*power software) [71]. The number of animals and independent retinal samples (biological replicates) used was $n = 6$ for miRNAs expression analyses and $n = 5$ for the other analyses, see the "Experimental groups, and drug administration and sample collection" paragraph in the methods section. Data were analyzed to test normality distribution. Data were represented as mean±standard deviation (SD), from at least three independent samples, and three technical replicates. Data were analyzed by the one-way analysis of variance (ANOVA) test, followed by the Tukey post-hoc test for multiple comparisons. Post-hoc tests were carried out only if F had a $p < 0.05$, and no significant variance in homogeneity was found within the analyzed groups. Significance was set at a $p < 0.05$. Graph design and statistical analyses were carried out with SPSS (https://www.ibm.com/analytics/spss-statistics-software) and GraphPad Prism (https://www.graphpad.com/scientific-software/prism/).

Author contributions

ChB designed experiments, performed protein analysis, and drafted and revised the manuscript; CBMP performed formal analysis, the bioinformatic part of the work and drafted relative parts in the manuscript; GDB performed animal experiments; GDB and AM performed immunofluorescence and updated literature; GG and FC performed miRNAs experiments. RC performed histological analysis; CF, SS acquired confocal microscopy images analyzed and interpreted data from immunofluorescence experiments; GC, RB and CB conceived and supervised the research and contributed to manuscript editing and review. All authors read and approved the final manuscript.

Funding

This study was supported by Grant PIano inCEntivi RIcerca Ateneo 2020/2022 - Linea Intervento 2.

Data availability

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics statement

We did not use human samples in this study. The animal experiments were approved by the Italian Ministry of Health.

Competing interests

The authors declare no competing interests.

Supplementary information

The online version contains supplementary material available at 10.1038/s41419-021-04165-x.

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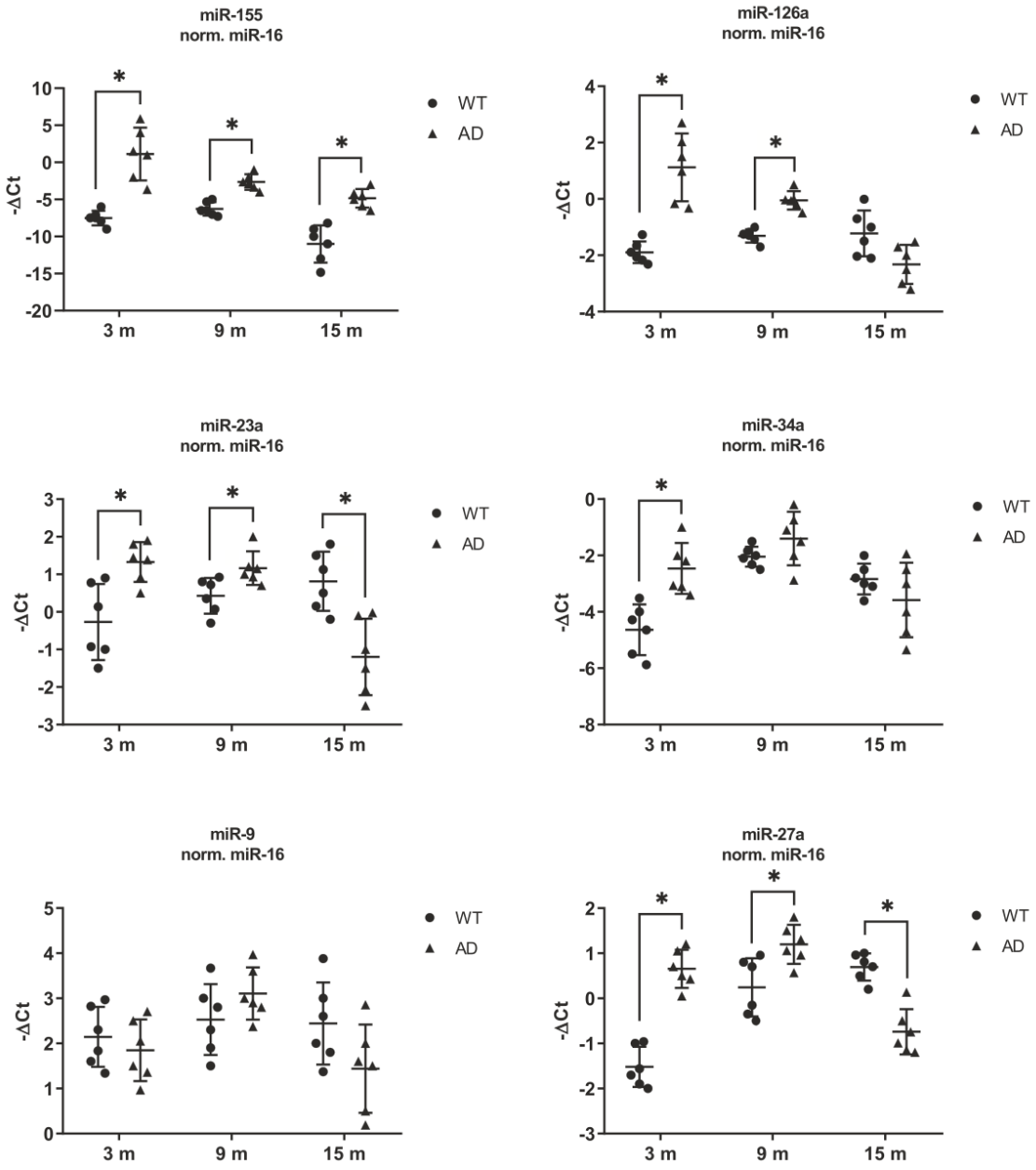

Fig. 1 Differential expression analysis of miRNAs in the retina of 3xTg-AD mice. RT-qPCR was performed to determine the expression of miR-155, miR-126a, miR-23a, miR-34a, miR-9, miR-27a in the retinas from 3xTg-AD mice at three different (3-, 9-, and 15-month-old) age periods. Data are expressed as mean ± standard deviation. One-way ANOVA and Tukey's multiple comparisons test were used to determine statistical significance. *$p < 0.05$ vs. WT age-matched mice. N = 6 animals; 6 independent retinal samples, 2 pooled retinas per sample in each group.

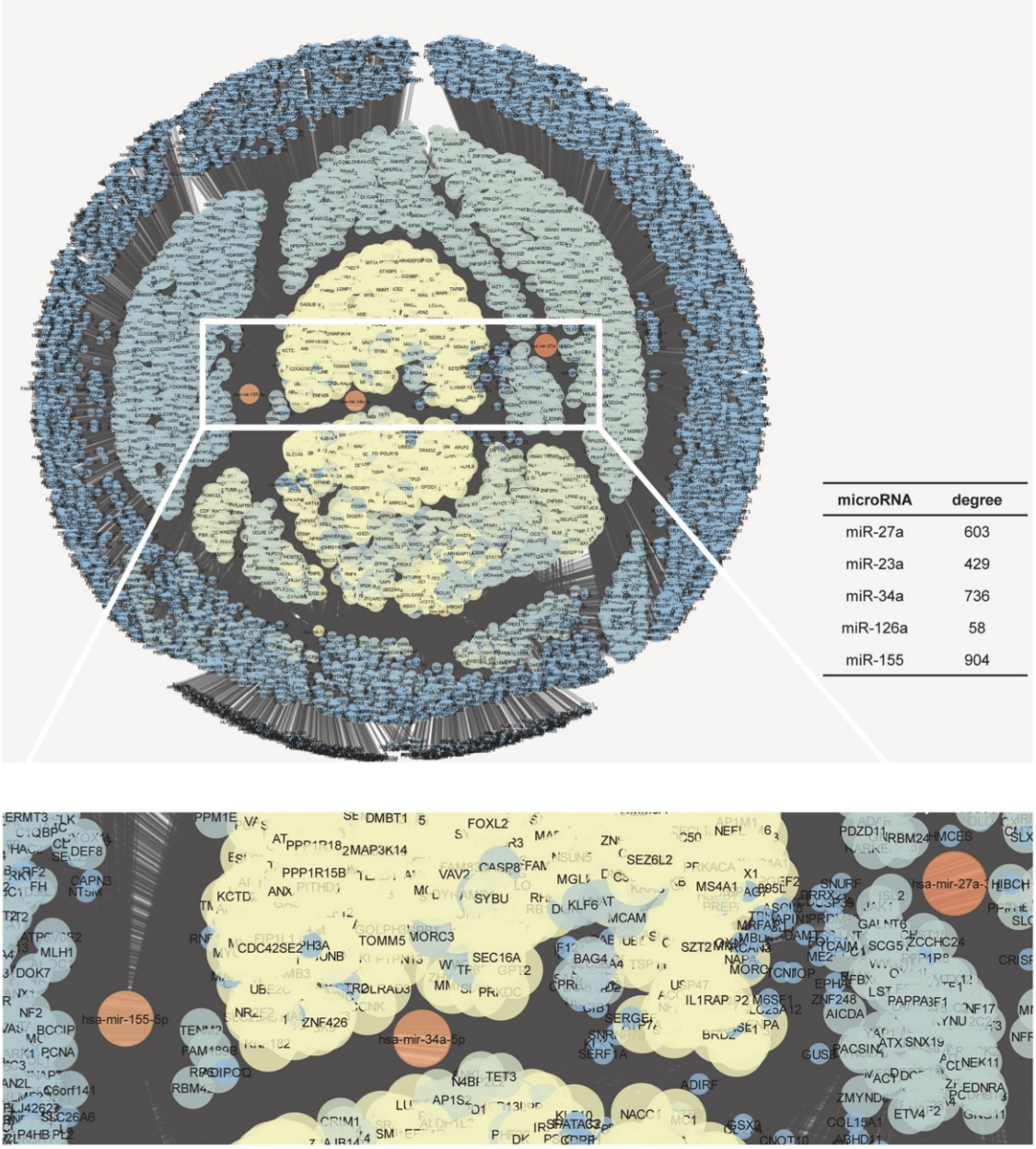

Fig. 2 The miRNA-gene network predicted by miRNet analysis. The predicted network (Prefuse force directed layout based on edge betweenness) included about 20000 edges (connections) and 10000 nodes (miRNAs or genes). Only three nodes showed the highest degree (included table) and particularly the highest betweenness centrality (red color). From left to right, the red nodes represent miR-155, miR-34a, and miR-27a, bearing also the highest degree values. These mentioned node parameters strictly influence the stability of the network. Centrality metric analyses were carried out with Cytoscape and network parameters were plotted in the graphic representation: closeness centrality (proportional to node dimension), betweenness centrality (temperature color scale, blue < red), edge betweenness (proportional to edge thickness).

Table 1. miRNAs-TNFSF10 signaling pathways interactions in retina of 3xTg-AD mice.

	3-month-old 3xTg-AD mice	9-months-old 3xTg-AD mice	15-month-old 3xTg-AD mice
miR-155-5p ***TNFRSF10B*** ***TNFRSF10A***	↑* miRNA fold regulation ↓ predicted target regulation	↑* miRNA fold regulation ↓ predicted target regulation	↑* miRNA fold regulation ↓ predicted target regulation
miR-126-3p ***TNFRSF10B***	↑* miRNA fold regulation ↓ predicted target regulation	↑* miRNA fold regulation ↓ predicted target regulation	↓ miRNA fold regulation ↑ **predicted target regulation**
miR-23a-3p ***TNFRSF10B***	↑* miRNA fold regulation ↓ predicted target regulation	↑ * miRNA fold regulation ↓ predicted target regulation	↓* miRNA fold regulation ↑ **predicted target regulation**
miR-34a-5p ***TNFRSF10D***	↑* miRNA fold regulation ↓ predicted target regulation	↑ miRNA fold regulation ↓ predicted target regulation	↓ miRNA fold regulation ↑ **predicted target regulation**
miR-27a-3p **FADD**	↑* miRNA fold regulation ↓ predicted target regulation	↑* miRNA fold regulation ↓ predicted target regulation	↓* miRNA fold regulation ↑ **predicted target regulation**

Note: * $p < 0.05$ *vs.* WT age-matched mice. Bold characters highlight that most of dysregulated miRNAs in the retina of 15-month-old 3xTg-AD mice, are down-regulated, leading to putative up-regulation of experimentally validated targets of TNFSF10 pathway. In humans, TNFRSF10A is a target of miR-155.

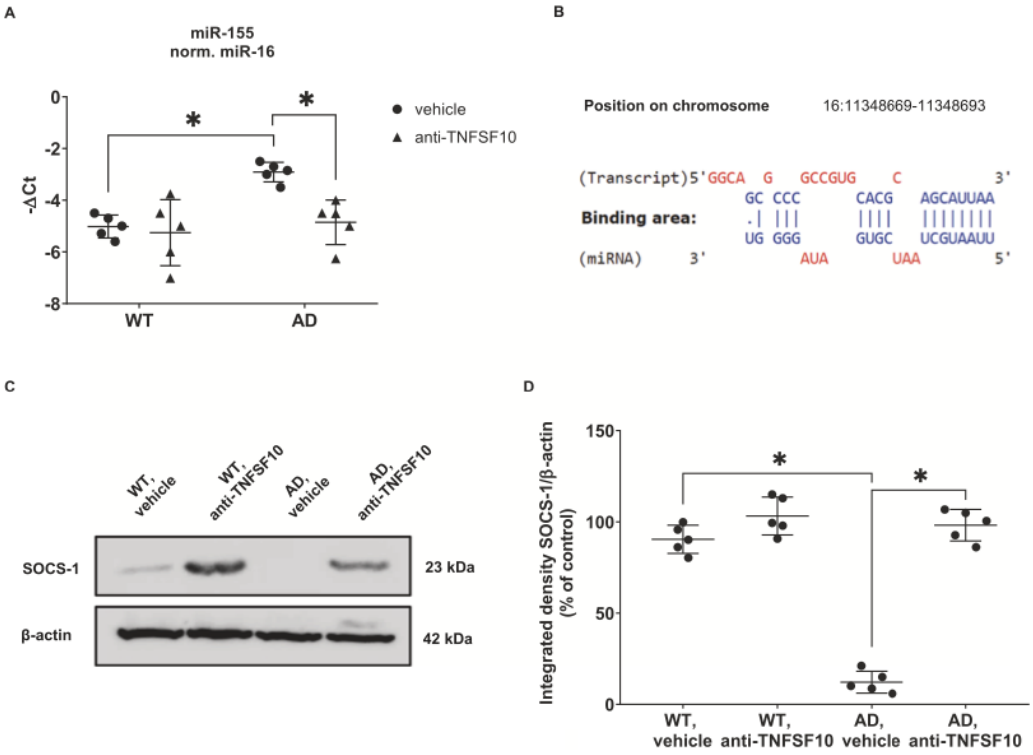

Fig. 3 Anti-TNFSF10 treatment decreased miR-155 retinal levels in 15-month-old 3xTg-AD mice. A RT-qPCR was performed to determine the retinal expression of miR-155-5p in 15-month-old 3xTg-AD mice treated with anti-TNFSF10. One-way ANOVA and post-hoc Tukey's multiple comparisons test were used. N = 5 animals; 5 independent retinal samples, 2 pooled retinas per sample in each group. **B** Bioinformatic prediction of SOCS-1 mRNA binding with miR-155-5p. **C** Western blot analysis was performed to evaluate the expression of the miR-155-5p molecular target SOCS-1 in the retinas of 3xTg-AD mice. **D** Densitometric analysis of western blots. Data are expressed as mean ± standard deviation. One-way ANOVA and post-hoc Tukey's multiple comparisons test were used to determine statistical significance. *$p < 0.05$. N = 5 animals; 5 independent retinal samples, 2 pooled retinas per sample in each group.

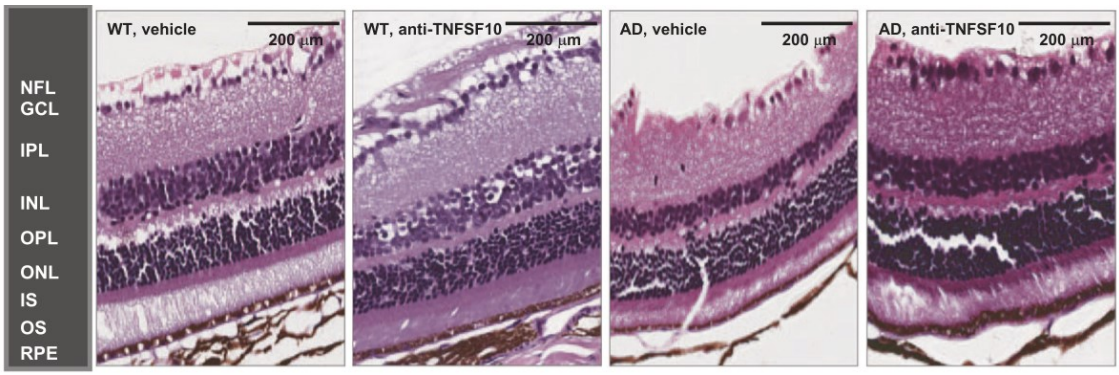

Fig. 4 TNFSF10-neutralizing antibody treatment preserved retinal structure in 15-month-old 3xTg-AD mice. Hematoxylin and eosin staining of retinal tissue of WT and 3xTg-AD mice were performed to analyze retina morphological changes following chronic treatment with vehicle or TNFSF10-neutralizing antibody. Original magnification, x200. Scale bar = 200 μm. N = 5 animals; 5 independent retinal samples per group. NFL nerve fiber layer, GCL ganglion cell layer, IPL inner plexiform layer, INL inner nuclear layer, OPL outer plexiform layer, ONL outer nuclear layer, IS inner segment; OS outer segment, RPE retinal pigment epithelial.

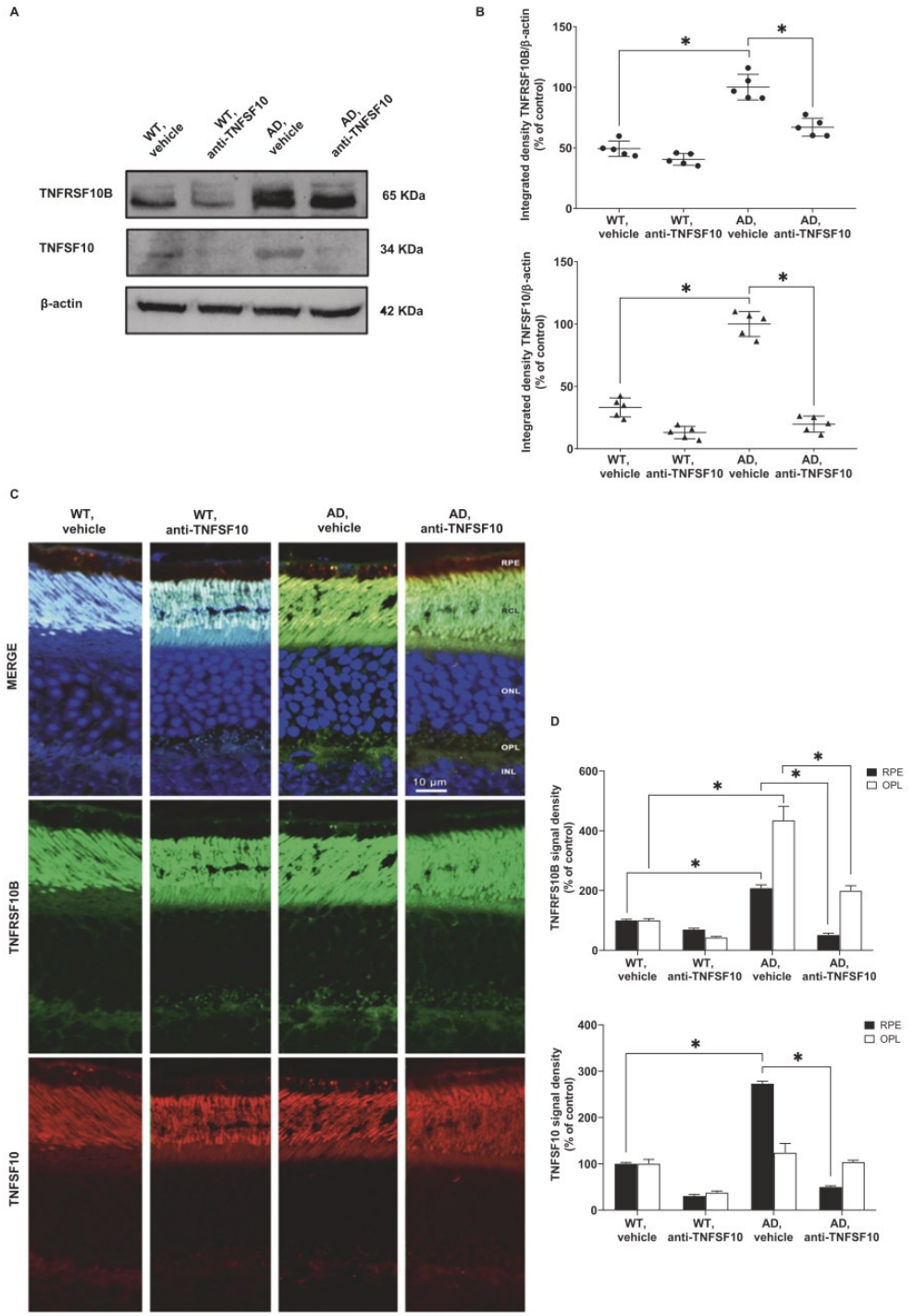

Fig. 5 Anti-TNFSF10 treatment modulated retinal expression of TNFSF10 and its TNFRSF10B receptor in 3xTg-AD mice. A Immunoblots of retinal lysates for the expression of TNFRSF10B and TNFSF10 proteins. **B** Densitometric analysis of western blots. Data are expressed as mean ± standard deviation. One-way ANOVA and post-hoc Tukey's multiple comparisons test were used for statistical analysis. *$p < 0.05$. N = 5 animals; 5 independent retinal samples, 2 pooled retinas per sample in each group. **C** Immunohistochemical staining for TNFSF10 and its receptor TNFRSF10B in the retina of WT and 3xTg-AD mice, treated either with vehicle or anti-TNFSF10 antibody. Original magnification, x63. Scale bar = 10 μm. D Densitometric analysis of the TNFRSF10B and TNFSF10 immunofluorescence signal in the RPE and OPL retinal layers. Data are expressed as mean ± standard deviation. One-way ANOVA and post-hoc Tukey's multiple comparisons test were used for statistical analysis. *$p < 0.05$. N = 5 animals; 5 independent retinal samples per group. For each retinal section, 14 optical fields were analyzed.

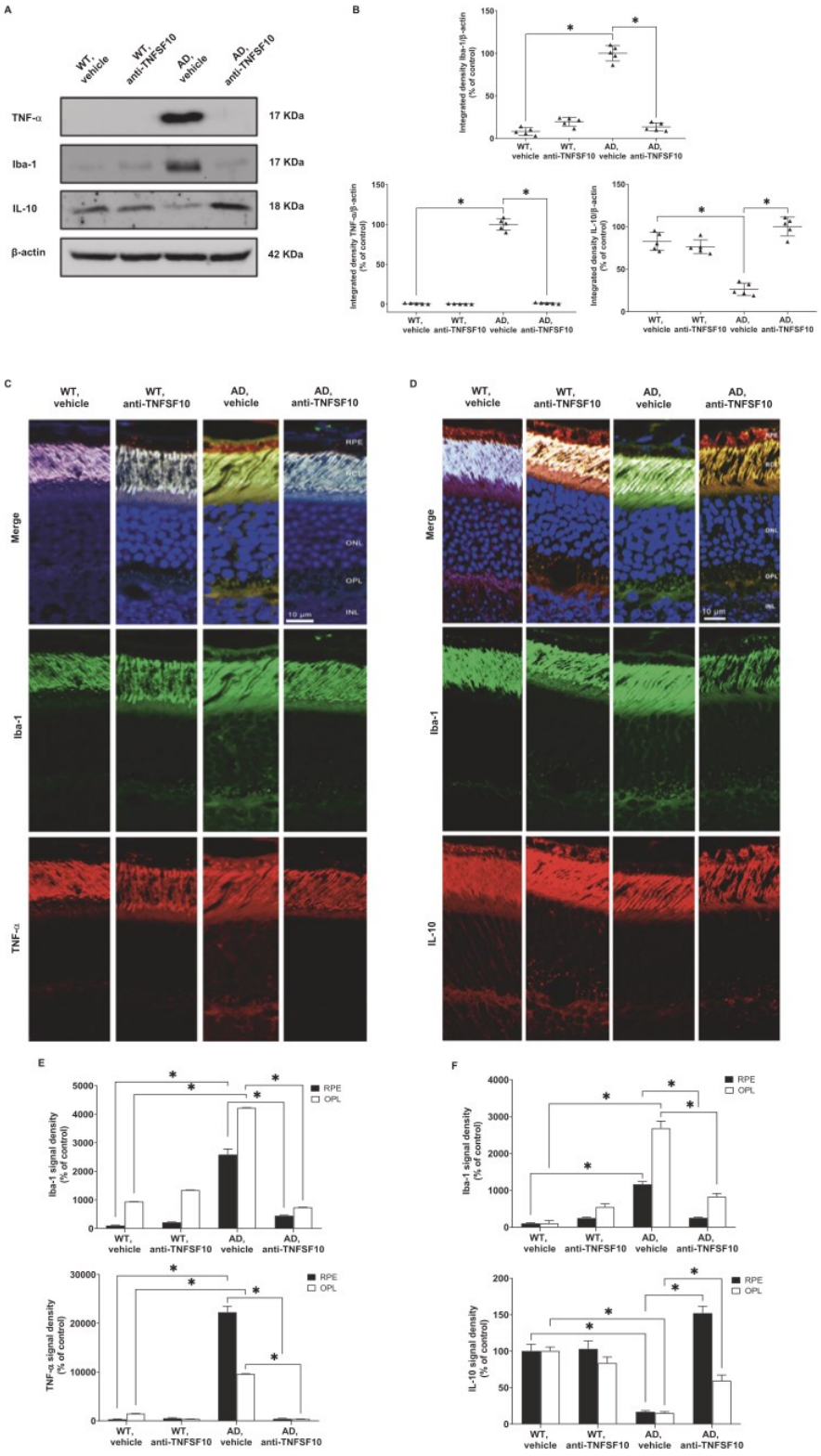

Fig. 6 Anti-TNFSF10 treatment inhibited pro-inflammatory microglia activation in the outer-plexiform and in the RPE layers of 3xTg-AD mouse retina. A Western blots for TNF-α, Iba-1 and IL-10 protein expression in the retinas of 3xTg-AD mice, following chronic treatment with an anti-TNFSF10 monoclonal antibody or vehicle. **B** Densitometric analysis of western blots. Data are expressed as mean ± standard deviation. One-way ANOVA and post-hoc Tukey's multiple comparisons test were used for statistical analysis. *$p < 0.05$. N = 5 animals; 5 independent retinal samples, 2 pooled retinas per sample in each group. **C** Immunohistochemical staining for TNF-α,

Iba-1 in the retina of 3xTg-AD mice, treated with either vehicle or anti-TNFSF10 antibody. Original magnification, x63. Scale bar = 10 μm. **D** Immunohistochemical staining for Iba- 1, IL-10 in the retina of WT and 3xTg-AD mice, treated with either vehicle or anti-TNFSF10 antibody. Original magnification, x63. Scale bar = 10 μm. **E** Densitometric analysis of the Iba-1, and TNF-α immunofluorescence signal in the RPE and OPL retinal layers. **F** Densitometric analysis of the Iba-1, and IL-10 immunofluorescence in the RPE and OPL retinal layers. Data are expressed as mean ± standard deviation. One-way ANOVA and post-hoc Tukey's multiple comparisons test were used for statistical analysis. *$p < 0.05$. N = 5 animals; 5 independent retinal samples per group. For each retinal section, 14 optical fields were analyzed.

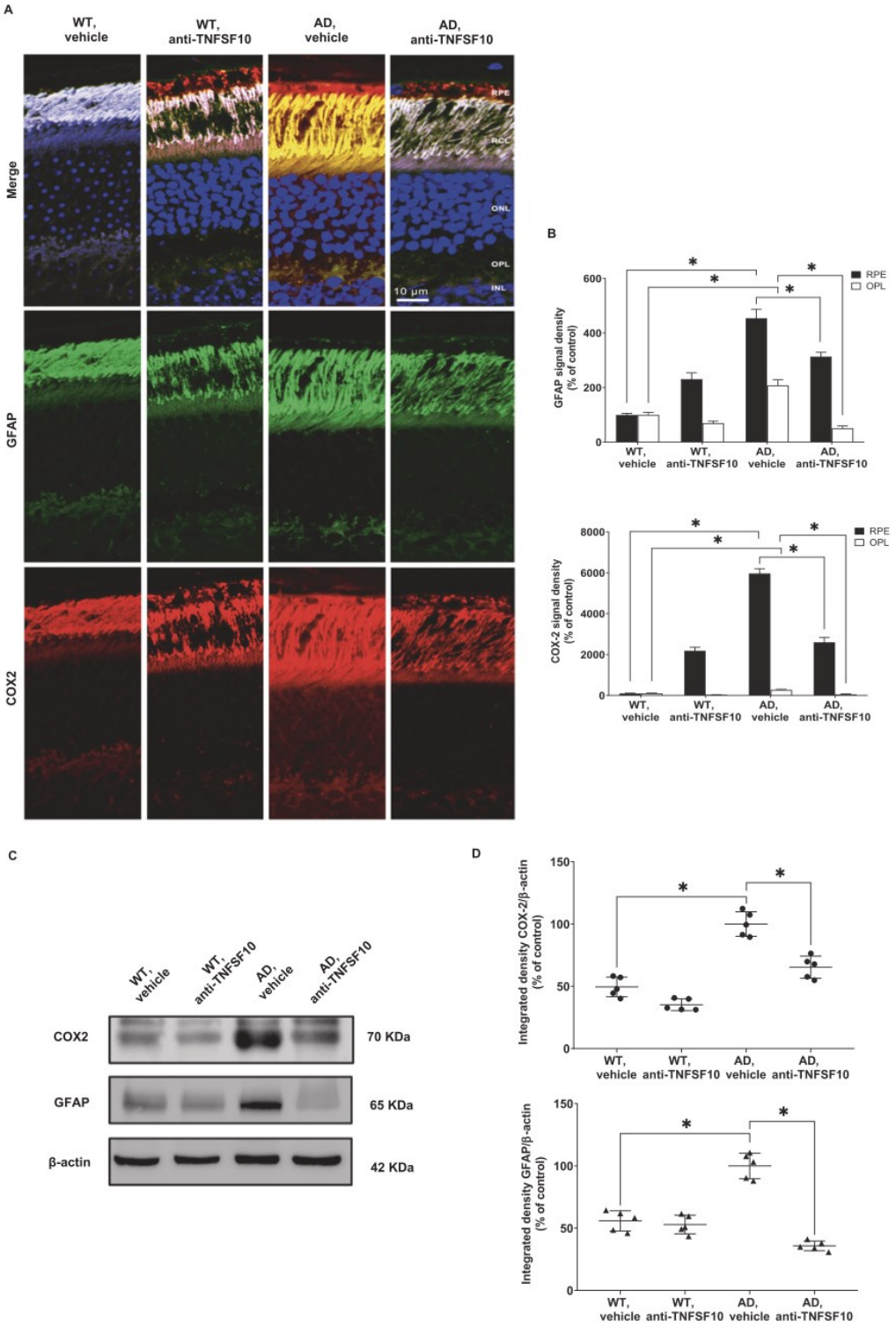

Fig. 7 Anti-TNFSF10 treatment inhibited astrogliosis in the outer-plexiform and in the RPE layers of the 3xTg-AD mouse retina. A Immunohistochemical staining for GFAP, COX2 in the retina of WT and 3xTg-AD mice treated with anti-TNFSF10 or vehicle. Original magnification, x63. Scale bar = 10 µm. **B** Densitometric analysis of the GFAP and COX2 immunofluorescence signal in the RPE and OPL retinal layers. One-way ANOVA and post-hoc Tukey's multiple comparisons test were used for statistical analysis. * $p<0.05$. N=5 animals; 5 independent retinal samples per group. For each retinal section, 14 optical fields were analyzed. **C** Western blot images for GFAP, COX-2 protein expression in the retina of mice following chronic treatment with an anti-TNFSF10 monoclonal antibody or vehicle**. D** Densitometric analysis of western blots. Data are expressed as mean ± standard deviation. One-way ANOVA and post-hoc Tukey's multiple comparisons test were

used to determine statistical significance. *p<0.05. N=5 animals; 5 independent retinal samples, 2 pooled retinas per sample in each group.

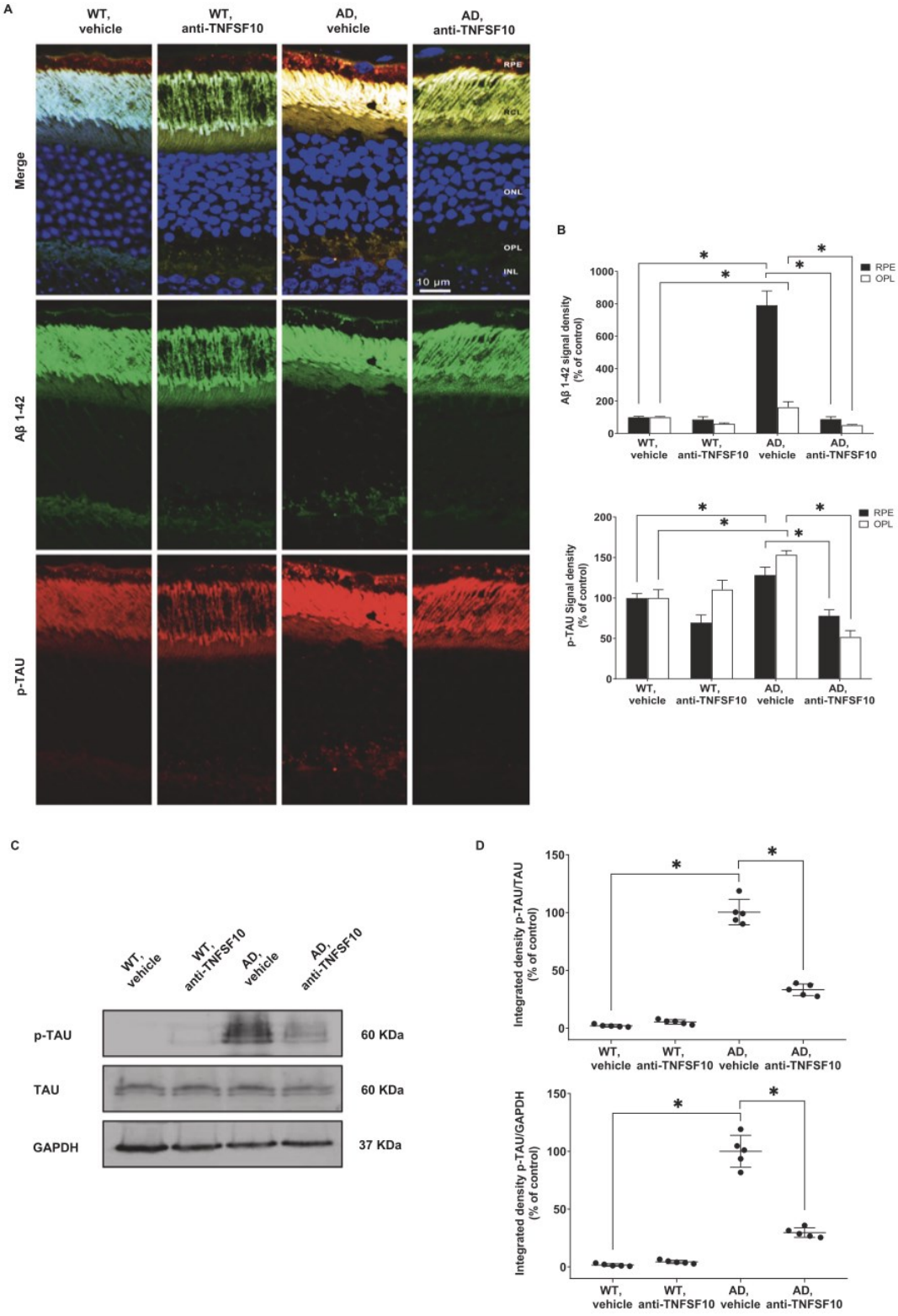

Fig. 8 Anti-TNFSF10 treatment inhibited Aβ and p-TAU deposition in the outer-plexiform and in the RPE layers of the 3xTg-AD mouse retina. A Immunohistochemical staining for p-TAU in the retina of WT and 3xTg-AD mice treated with anti-TNFSF10 or vehicle. Original magnification, x63. Scale bar = 10 µm. **B** Densitometric analysis of the p-TAU immunofluorescence signal in the RPE and OPL retinal layers. One-way ANOVA and post-hoc Tukey's multiple

comparisons test were used for statistical analysis. *p < 0.05. N = 5 animals; 5 independent retinal samples. For each retinal section, 14 optical fields were analyzed. **C** Western blot representative images for p-TAU protein expression in the retina of mice following chronic treatment with an anti-TNFSF10 monoclonal antibody or vehicle. **D** Densitometric analysis of western blots. Data are expressed as mean ± standard deviation. One-way ANOVA and post-hoc Tukey's multiple comparisons test were used for statistical analysis. *p < 0.05. N = 5 animals; 5 independent retinal samples, 2 pooled retinas per sample in each group.

General discussion and conclusions

Although it has been more than 100 years since Alois Alzheimer first described the pathological signs associated with AD, still important gaps remain in our understanding of the condition and the nature of the pathological processes that underlie the disease [6]. Due to the multifaceted nature of the disease, lack of reliable biomarkers and stage-specific molecular targets, and the technical difficulties for effective CNS drug delivery, the task to develop effective treatments for AD is extremely discouraging [72].

Clinical studies suggest that neuroinflammation is an early event in AD pathology and could have a crucial role in disease pathogenesis and progression [36].

The hypothesized role of inflammation as the major driving force in disease pathogenesis has gained strong support from genome-wide association studies which suggest that several genes associated with increased risk for sporadic AD encode for factors that regulate the inflammatory reaction [25,85,86].

In this scenario, interventions to target crucial immune pathways in the pre-disease period and to modulate the immune response along the disease process could bring about promising outcomes as part of a disease-modifying strategy.

Cytokines have drawn much attention to their roles in the different stages of AD and the possibility of being used as targets for treatment [44].

Consistent with the previous observation, the proinflammatory cytokine TNFSF10 plays a critical role, not only in neurodegenerative processes but also in inflammatory disorders of the CNS, likely as the result of different effects of the TNFSF10 pathway in local and peripheral inflammatory processes.

In this line, the approach proposed in this work has been the targeting of cytokine TNFSF10, using a TNFSF10 neutralizing monoclonal antibody, with the aim to investigate its role in the management of AD-related phenomena.

As previously demonstrated by our group, the anti-TNFS10 antibody has the ability to penetrate the brain and to directly neutralize the TNFSF10 pathway [43].

Since immunotherapeutic approaches that use antibodies to target disease hallmarks have not shown convincing results [87], probably due to antibodies administration in the late stages of the disease, our approach was based upon the concept that early intervention might improve functional and tissue outcomes.

Based on these premises, 3xTg-AD mice enrolled at 3 months of age, prior to the appearance of their age-related pathology, and were chronically treated for 12 months with anti-TNFSF10 monoclonal antibody.

As reported in **Chapter I**, the first goal of the project has been testing whether chronic immunoneutralization of cytokine TNFSF10 could imply a rebalance of both central and peripheral immune/inflammatory response in a 3xTg-AD mouse model and whether this could relate to decreased Aβ burden in the brain, beside the already demonstrated amelioration of cognitive performances.

Transgenic AD mice (3xTg-AD) displayed a general immune/inflammatory susceptibility manifested in increased spleen weight, as well as in decreased brain and body weight, confirming data from other authors [88,89]. Moreover, splenic beta-amyloid deposits were paralleled by increased expression of inflammatory/immune markers, including those typical of regulatory T cells (GITR and FoxP3), in 3xTg-AD mice.

Consistently with an already reported altered rate of CD3 lymphocytes in the spleen, which suggest an immune/inflammatory involvement in AD [88], 3xTg-AD showed an increased CD3 expression in the hippocampus, along with increased expression of Treg markers FoxP3 and GITR.

Neutralization of TNFSF10 resulted in a significant reduction of immune/inflammatory markers, including those related to Treg cells. Such restraint of inflammatory/immune response correlated centrally with decreased microglial TNFα production and with a dramatically reduced burden of amyloid and phosphorylated tau proteins.

Therefore, our results suggest that TNFSF10-mediated neuroinflammation merges with a generalized overshooting of the immune response, as reported in **Chapter I** and reviewed in **Chapter II**, and support the rationale for the development of a novel immunotherapeutic strategy for AD, based on the neutralization of the TNFSF10 signaling pathway.

Moreover, the proven existence of a strong link between the retina and the brain is underlined by the presence of retinal manifestations accompanying certain neurodegenerative diseases, such as AD [90,91]. These observations confirm the concept of the retina as an integral part of the CNS, which displays striking similarities to the other CNS structures, in terms of anatomy, response to injury, and immunology [92].

Based on these premises, and in the attempt to assess a suitable model to identify AD in its early phases, the second part of the study reported in **Chapter III** was aimed to

investigate the role of the TNFSF10 system and of miRNAs associated with inflammatory processes in the retina of 3xTg-AD mice.

The involvement of a specific set of miRNAs, already reported both in AD and in age-related macular degeneration (AMD), were evaluated in the retina of 3xTg-AD mice at different ages.

Five miRNAs were found to be dysregulated in the retina 3xTg-AD mice in an age-related manner (miR-155-5p, miR-126-3p, miR-34a-5p, miR-27a-3p, miR-23a-3p). Noteworthy, an age-dependent retinal up-regulation of miR-155 was found, consistent with the evidence of an upregulation of this miR also in the brain of 3xTg-AD mice [93]. These miRNAs, with special regard to miR-155, were found to target genes related to the TNFSF10 pathway signaling pathway, as assessed by means of bioinformatic approaches.

Interestingly, chronic treatment of 3xTg-AD mice with anti-TNFSF10 monoclonal antibody was able to counteract the inflammatory/immune response sustained by miR-155 upregulation and consequent SOCS-1 downregulation, finally resulting in a dramatic improvement in either tissue or inflammatory parameters in the retina of these animals.

In conclusion, neutralization of TNFSF10 brings about significant amelioration of the AD pathology, suggesting that therapeutic exploitation of TNFSF10 signaling potentially represents a novel and efficacious strategy for the immunopharmacological management of AD-related phenomena.

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Oral communications

1. **Burgaletto C,** Di Benedetto G, Bernardini R, Cantarella G**.** Genetic evidence for the detrimental role of the TRAIL receptor DR5 in a murine model of amyloid-beta toxicity. *"New Perspectives in Neuroscience: Research Results of Young Italian Neuroscientists". National Meeting of PhD Student in Neuroscience", Napoli, March 1st*, 2019.
2. **Burgaletto C**. Curtailing inflammatory/immune susceptibility results in beneficial outcome in a murine model of Alzheimer's Disease. *"Retreat 2018", Castiglione di Sicilia (CT); 24-25 novembre 2018.*
3. Di Benedetto G, **Burgaletto C**, Bernardini R, Cantarella G. Restoration of the peripheral immune/inflammatory response correlates with brain injury recovery in a murine model of Alzheimer's disease. *"La Farmacologia Siciliana, tra Scienza e Politica del Farmaco". Prima riunione del Coordinamento dei Farmacologi Siciliani (CoFaS), Roccalumera (ME); 11-12 maggio 2018.*
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Poster presentations

1. Burgaletto C, Di Benedetto G, Bernardini R, Cantarella G**.** The TRAIL death receptor DR5 mediates amyloid-beta toxicity in a mouse model of Alzheimer's disease. *18th SINS NATIONAL CONGRESS, Perugia, September 26th-29th, 2019.*