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Targeting Tumor Necrosis Factor Alpha for Alzheimer's Disease

Boris Decourt[1,2,*], **Debomoy K. Lahiri**[3,4], and **Marwan N. Sabbagh**[5,*]

[1]School of Life Sciences, Arizona State University, Tempe, AZ, USA

[2]Banner Sun Health Research Institute, Sun City, AZ, USA

[3]Institute of Psychiatry Research, Department of Psychiatry, School of Medicine, Indiana University-Purdue University, Indianapolis, IN, USA

[4]Department of Medical and Molecular Genetics, School of Medicine, Indiana University-Purdue University, Indianapolis, IN, USA

[5]Barrow Neurological Institute, Phoenix, AZ, USA

Abstract

Alzheimer's disease (AD) affects an estimated 44 million individuals worldwide, yet no therapeutic intervention is available to stop the progression of the dementia. Neuropathological hallmarks of AD are extracellular deposits of amyloid beta (Aβ) peptides into plaques, intraneuronal accumulation of hyperphosphorylated tau protein forming tangles, and chronic inflammation. A pivotal molecule in inflammation is the pro-inflammatory cytokine TNF-α. Several lines of evidence using genetic and pharmacological manipulations indicate that TNF-α signaling exacerbates both Aβ and tau pathologies *in vivo*. Interestingly, preventive and intervention anti-inflammatory strategies demonstrated a reduction in brain pathology and an amelioration of cognitive function in rodent models of AD. Phase I and IIa clinical trials suggest that TNF-α inhibitors might slow down cognitive decline and improve daily activities in AD patients. In the present review, we summarize the evidence pointing towards a beneficial role of anti-TNF-α therapies to prevent or slow the progression of AD. We also present possible physical and pharmacological interventions to modulate TNF-α signaling in AD subjects along with their limitations.

Keywords

Alzheimer's disease; BACE1; Etanercept; Inflammation; Neuroinflammation; thalidomide; TNF-α

[*]Address correspondence to these authors at the Banner Sun Health Research Institute, 10515 W. Santa Fe Dr., Sun City AZ 85351, USA; Tel: +1 623-832-2973; Boris.Decourt@bannerhealth.com; and Director, Alzheimer's and Memory Disorders Division, Barrow Neurological Institute, 240 West Thomas, Ste 301, Phoenix, AZ 85013, USA; Tel: +1-602-406-4784; Marwan.Sabbagh@DignityHealth.org.

CONFLICT OF INTEREST

The author(s) confirm that this article content has no conflict of interest.

1. INTRODUCTION

Alzheimer's disease (AD) is the most prominent form of dementia worldwide, affecting an estimated 44 million people [1]. Patients progressively become debilitated, placing a heavy emotional and financial burden on caregivers and healthcare systems [1]. Neuropathologically, AD is characterized by 1- the extracellular accumulation of amyloid beta (Aβ) oligomers and other materials into dense senile plaques; 2- the intraneuronal hyperphosphorylation of the microtubule-binding protein tau which induces its aggregation into tangles; and 3- chronic inflammation [2]. Together, these events disrupt the homeostatic functioning of neurons by affecting synaptic transmission and intracellular transport, ultimately leading to neuronal death. Amyloid beta is generated via the consecutive proteolysis of the amyloid precursor protein (APP) by β- and γ-secretases. The main β-secretase in AD was identified as beta amyloid converting enzyme 1 (BACE1) [3]. Gamma secretase is a tetramer comprising presenilin 1 or 2 (PSEN1/2), anterior pharynx-defective 1 (APH-1), nicastrin (NCT), and presenilin enhancer 2 (PEN-2) [4].

Following its discovery in 1984 [5], Aβ was found to be a hallmark of AD- and Down syndrome-associated brain plaques [6]. These observations seeded the formulation of the amyloid cascade hypothesis which describes Aβ as the central cause of AD neuropathology [7, 8]. Based on this hypothesis, blocking amyloidogenesis or preventing Aβ aggregation should lower brain Aβ loads and plaque formation, and thus improve cognition in AD subjects. Different strategies have been tested in clinical trials, including 1- BACE1 inhibitors [9]; 2- γ-secretase inhibitors and modulators [10]; and 3- active and passive immunization targeting monomeric, oligomeric, and protofibril Aβ [11]. However, all trials completed to date have failed to meet the clinical end-points of significantly improving the cognition and daily living functions of AD patients. Furthermore, although bio-marker and post-mortem analyses suggest target engagement as identified by lower brain and CSF Aβ loads [12–14], most trials reported substantial adverse events, such as unexpected liver toxicity for the BACE1 inhibitor LY2886721 [15], weight loss and skin cancers for γ-secretase inhibitors [16], and microhemorrhages, vasogenic edema, and meningoencephalitis for Aβ immunization [11, 17], sometimes leading to early termination of the trials. These results, combined with observations of elevated brain Aβ42 in aged, non-demented individuals with cerebral atherosclerosis [18], and the lack of correlation between dementia progression and brain Aβ levels [19] are feeding the debate about the validity of the amyloid cascade hypothesis (for recent discussions see [20–22]), and prompting the investigation of additional targets and methods for therapeutic interventions.

An alternative approach to Aβ modulation is immunization against hyperphosphorylated tau [23]. For example, following an encouraging Phase I study AXON Neuroscience is starting a Phase II trial with its AADVAC1 vaccine [24, 25].

Another possible strategy is the use of anti-inflammatory agents. A growing body of evidence implicates proinflammatory cytokines as enhancers of Aβ and tau pathologies (see section 3 below) [26, 27]. Several epidemiological studies suggest a sparing of AD in individuals taking non-steroidal anti-inflammatory drugs (NSAIDs) for at least two years prior to symptom onset (reviewed in [22]). NSAIDs are non-selective inhibitors of

cyclooxygenase-1 (COX-1) and COX-2. However, clinical trials did not corroborate these epidemiological findings [28]. A possible explanation for the latter result is that the cohorts treated during clinical trials were already suffering AD while epidemiological studies suggest a preventive action of NSAIDs on presymptomatic individuals. Nonetheless, further studies found no AD prevention effect by COX-2 specific inhibitors [22]. To note, NSAIDs are known to induce gastrointestinal and kidney adverse events [29], which limit enthusiasm for their chronic use on AD patients.

Aged-related changes in the immune system, known as immunosenescence, and increased secretion of cytokines by adipose tissue represent the major causes of chronic inflammation during aging. This phenomenon is known as “in-flamm-aging” [30]. A major pro-inflammatory molecule involved in neurological disorders, including AD, is tumor necrosis factor alpha (TNF-α) [30–33]. Anti-TNF-α interventions have been proposed to modulate AD neuropathology, and current data suggests it may also improve cognitive function (see section 4 below). In the present article we review the reasons behind targeting TNF-α for AD treatment and list several options to lower TNF-α, along with their potential challenges.

2. THE BIOLOGY OF TNF-α IN THE CENTRAL NERVOUS SYSTEM

TNF-α and its receptors regulate a plethora of physiological functions in the body, including immune surveillance, immune reactions to fight microbial infections, induction of cell death, for example to eliminate cancer cells or in pathological conditions like allergies [34–37]. In this section we provide a summary of TNF-α expression, signaling pathways, and physiological roles in the central nervous system (CNS).

2.1. TNF-α Expression

TNF-α controls numerous physiological processes in the CNS [34]. Therefore, it is not surprising that its expression is tightly regulated at the transcriptional, post-transcriptional, and translational levels to maintain homeostasis. The *TNF*-α gene is located within the Major Histocompatibility Complex (MHC) II region on the short arm of chromosome 6, and comprises four exons and three introns. A variety of transcription factors can bind and activate the TNF-α promoter, including NF-κβ, CCAAT/enhancer binding protein β (C/EBPβ), SP-1, Erg-1, and c-Jun (summarized in [32]). TNF-α protein is synthesized as a type II transmembrane protein (tmTNF-α) of 26 kDa [38]. The metalloprotease TNF-α converting enzyme (TACE/ADAM17) can shed tmTNF-α from the plasma membrane and release a soluble form (sTNF-α) of 17 kDa into the extracellular milieu [39]. Interestingly, both tmTNF-α and sTNF-α assemble in homotrimers that are biologically active when engaging TNF-α receptors [32].

2.2. TNF-α Signaling

TNF-α exerts its actions by binding two distinct type I, high-affinity receptors located at the cell surface. TNF-RI (p55 or p60) is ubiquitously and constitutively expressed, except on erythrocytes, while TNF-RII (p75 or p80) expression is restricted to myeloid cells, endothelial cells, myocytes, thymocytes, oligodendrocytes, microglia, astrocytes, and sub-populations of neurons (summarized in [40, 41]). TNF-RI binds equally well with the sTNF-

α and the tmTNF-α, however TNF-RII preferentially binds to the tmTNF-α [42]. Historically, the two receptors were ascribed antagonistic effects with TNF-RI being pro-apoptotic and TNF-RII playing a protective role. However, recent studies have shown that both receptors are capable of driving cell survival and cytotoxicity depending on age, extracellular TNF-α levels, the cell type expressing the receptors, and intracellular signaling pathways activated by other trophic factors (reviewed in [32,33]). TNF receptors mediate signals via the recruitment and inhibition of adapter proteins (for details see [32, 33]). Briefly, both receptors share similar signaling cascades that activate the mitogen-activated protein kinase (p38 MPAK), c-jun N-terminal kinase (JNK), extracellular-signal-regulated kinases (ERKs), acid sphingomyelinase (A-SMase), neutral sphingomyelinase (N-SMase) pathways, and the transcription factors AP-1 and NF-κB, which then induce the expression of molecules participating in inflammation and amyloidogenesis [32, 42, 43]. Interestingly, tmTNF-α is capable of reverse signaling, i.e. when tmTNF-α (or an agonist/antagonist) binds to its receptor it can increase intracellular calcium levels (in the cell bearing tmTNF-α), which activates p38 MAPK, upregulates NF-κB signaling, and induces cell survival or death [44].

2.3. Sources of TNF-α and Its Roles in the Central Nervous System

In order to target TNF-α for AD treatment it is important to identify the cells expressing this pro-inflammatory cytokine in the CNS. In the past three decades, an increasing number of physiological and deleterious functions have been credited to TNF-α signaling in the CNS [32, 45, 46]. A non-exhaustive list of TNF-α roles in the CNS is provided in Table 1. Since TNF-α signaling stimulates NF-κB, many (though not all) of the functions attributed to TNF-α in the CNS are also controlled by NF-κB signaling (see recent reviews [47, 48] for details).

Numerous studies have shown that TNF-α is expressed at physiological levels by microglia and neurons; but its expression increases in activated microglia, neurons, oligodendrocytes, reactive astrocytes, epithelial cells, endothelial cells, and ependymal cells upon brain and peripheral injuries, and in chronic disorders [49–51]. Moreover, like other cytokines [52, 53], TNF-α can cross the intact blood brain barrier (BBB) via transcytosis involving both TNF-RI and TNF-RII [54], and affect cognitive functions [55]. In addition, peripheral immunocompetent cells that produce TNF-α may migrate across the BBB to enter the brain parenchyma and cerebrospinal fluid (CSF) [56]. In aggregate, these findings suggest that several central and peripheral sources of TNF-α may contribute to AD neuropathology (see section 3 below) [57], although it is still unclear which source of TNF-α seeds chronic neuroinflammatory reactions.

3.TNF-α IN ALZHEIMER'S DISEASE

3.1. TNF-α Levels in Alzheimer's Disease

Numerous studies have described elevated TNF-α levels in biological fluids in aging, mild cognitive impairment (MCI) and in AD patients (meta-analysis conducted in [77]). Early reports indicated that TNF-α levels are elevated in the CSF of AD patients compared to cognitively normal controls (e.g. [78]). However, a recent meta-analysis did not find a

significant difference between the two populations of patients [79]. From recent discussions [80] and our own experience, we hypothesize that part of the controversy might originate from the lack of standardization for sample collection, and the very low concentration of TNF-α in biological fluids (e.g. serum values ranging 0–4 pg/mL in healthy subjects), which likely impedes reproducible measurements across study centers. Nevertheless, it is widely accepted that TNF-α plasma levels are higher in MCI and AD vs. cognitively normal individuals [79, 81]. Importantly, both TNF-α and TNF-RI levels were shown to be increased in the postmortem brain of early-stage AD patients [82]. Furthermore, challenging primary cultures of rodent and human microglial cells with Aβ induces the release of high levels of TNF-α [83–85]. In addition, in a recent study human TNF-α cDNA flanked with a human cytomegalovirus promoter was delivered to the hippocampal CA1 region via an adeno-associated virus in 2 month-old 3xTg-AD mice (pre-pathological stage) for specific overexpression of TNF-α in neurons [86]. This manipulation induced the activation of microglia and neuronal death, revealing that TNF-α-driven inflammation may have a deleterious effect on neurons [86]. Combined with studies reporting activated microglia surrounding amyloid oligomers and senile plaques in acute and genetic rodent AD models [87], as well as in human brains [2], the data strongly suggests that TNF-α is chronically released during the course of AD, likely by activated microglia, neurons, and astrocytes stimulated by increased levels of extracellular Aβ [88].

Neurodegenerative disorders are associated with chronic central inflammation [73]. TNF-α increases the production of other pro-inflammatory cytokines, such as IL-1, IL-6, and IL-8 [89], that can participate in the development of chronic inflammation when not counter-balanced by anti-inflammatory cytokines (e.g. IL-10). Furthermore, TNF-α was shown to stimulate the expression of APP and BACE1 in primary cultures of mouse astrocytes, as well as stimulate γ-secretase activity in HEK cells, which results in the release of Aβ peptides in large amounts [70–72, 90]. Interestingly, a recent study indicated that rat neurons are differentially affected by TNF-α and Aβ42 during aging, with older neurons showing a decreased capacity to express TNF-α receptors than middle-aged neurons [91]. Collectively, the data suggests that, once chronic brain inflammation is engaged, a detrimental, auto-amplified upward spiral maintains excessive levels of TNF-α, which could stimulate Aβ synthesis and neuronal loss, as well as inhibit microglia phagocytosis of Aβ [92]. Much less is known about the role of TNF-α in tau hyperphosphorylation and the molecular pathway(s) involved, though recent *in vivo* data suggests there may be a connection [93, 94].

3.2. TNF-α Single Nucleotide Polymorphism and Alzheimer's Disease

The promoter region of the *TNF*-α gene bears several single nucleotide polymorphisms (SNPs). Of particular interest for AD, and other chronic disorders, is the mutation G308A (i.e. 308 bp prior to the start codon), which increases the expression of TNF-α mRNA and protein [95]. Careful experimentation revealed that the control of transcription does not occur via the regulation of transcription factors; instead it involves the generation of a high-order of chromatin structure when G is at position −308, versus a low-order of chromatin structure that makes the region more accessible to transcription factors when the A mutation is present [96].

Disease and meta-analyses studies reported that the frequency of the three possible −308 mutation alleles in the general population are 70–80% (GG), 20–25% (GA), and 1–4% (AA) [97, 98]. Because of increased TNF-α expression with the A mutation, some authors suggested that this SNP could be linked with AD [99]. A recent meta-analysis did find, on one hand, a possible correlation between TNF-α G308A and the risk of AD in Chinese cohorts, but on the other hand reported a lower risk of suffering AD in northern European populations bearing this specific mutation [98]. Therefore, whether TNF-α G308A correlates with a higher probability of developing AD requires further investigation.

4. EVIDENCE THAT TNF-α MODULATION MAY SLOW OR PREVENT ALZHEIMER'S DISEASE

4.1. Evidence From Genetic Manipulations in AD Rodent Models

A large number of transgenic mouse and rat models have been generated to study AD. Those interested in such models are referred to recent reviews [100–102]. With regards to TNF-α signaling, there are a few lines of evidence from work on rodents support the idea of modulating TNF-α to manage AD progression. Genetic manipulations showed that altering TNF-α signaling reduces AD-like brain pathology in mice. For example, the deletion of TNF-RI gene in both APP23 (mimics Aβ pathology only; knock in of human APP bearing the Swedish mutation [sw; K595N/M596L] driven by the mouse Thy1.2 promoter [87]) and 3xTg-AD (mimics both Aβ and tau pathologies; triple knock in of human presenilin-1 mutated M146V driven by the presenilin 1 mouse promoter, and human APPsw and tau bearing the mutation P301L driven by the mouse Thy1.2 promoter [103]) mice resulted in decreased brain inflammation and Aβ burden [104, 105], while tau pathology was also reduced in 3xTg-AD mice which had their TNF-RI knocked out (TNF-RI$^{-/-}$) [105]. Partial analysis of the mechanisms revealed a reduction in BACE1 expression in APP23 mice [104], confirming that TNF-α likely regulates brain BACE1 expression. In addition, McAlpine and collaborators [105] used the intra-ventricular delivery of a lentivirus to overexpress a dominant negative (DN) soluble isoform of TNF-α in 3xTg-AD mice. The competition of DN against endogenous TNF-α for receptor binding resulted in decreased brain amyloid burden [105], similar to the TNF-RI manipulations indicated above.

4.2. Pre-Clinical Pharmacological Evidence

4.2.1. Anti-TNF-α Biologics—Several anti-TNF-α antibodies and recombinant fusion proteins, often developed for rheumatoid arthritis or cancer treatment, have been tested on AD rodent models using both central and peripheral routes of administration. For instance, the acute intracerebral delivery of the anti-TNF-α antibody infliximab (150 μg) to 12 month-old APP/PS1 mice (knock in of both human APPsw driven by the hamster prion protein promoter [mouse model Tg2576] and human presenilin-1 mutated M146V driven by the PDGF-β promoter [106]) induced a rapid and transient reduction in Aβ loads and tau phosphorylation [107]. The authors described the activation of brain immune cells in the process, though it is unclear whether these cells play a role in Aβ and tau clearance. In addition, the bio-engineered, anti-rheumatoid, anti-TNF-α fusion protein etanercept (TNF-RII extracellular domain fused to IgG1 Fc) was administered subcutaneously (range 3–30

mg/kg) in an acute $A\beta_{25–35}$-infused mouse model of AD. The peripheral administration of 30 mg/kg etanercept resulted in improved cognitive outcome measures accompanied by reduced TNF-α levels in the hippocampus [108]. Whether etanercept is able to alter AD-like neuropathology in AD chronic models remains to be explored.

4.2.2. Anti-TNF-α Pharmacological Compounds—Anti-inflammatory drugs capable of altering TNF-α levels and NF-κB signaling after peripheral administration showed promise in treating brain pathology in AD-like mice.

4.2.2.1. Rapamycin: A low dose of the immunosuppressant rapamycin (2.24 mg/kg in food pellets) improved cognitive measures while reducing both amyloid and tau pathologies in 3xTg-AD mice after 10 weeks of treatment [109]. This may be mediated by modulating the expression of pro-inflammatory cytokines during the blockage of T and B cell activation via the inhibition of mammalian target of rapamycin (mTOR) kinase (see section 5.9. below).

4.2.2.2. Minocycline: The antibiotic minocycline (~55 mg/kg/day from food pellets for 3 months) lowered Aβ burden and improved cognitive performance in middle-aged J20 mice (knock in of human APP bearing both the Swedish and Indiana mutations [ind; V717F] driven by the PDGF-β promoter) [110]. Although the exact molecular mechanisms regulated by minocycline in J20 mice were not reported, it was observed that minocycline is a neuroprotectant and might possess anti-inflammatory properties [111]. Similar positive effects on cognition were obtained when minocycline was administered intraperitoneally (i.p.; 45 mg/kg/day for three weeks) to an $A\beta_{1\text{-}42}$-infused rat model and Tg2576 mice (knock in of human APPsw driven by the hamster prion protein promoter; 10 mg/kg/day minocycline, 5 days a week for 9 months) [112]. Importantly, minocycline (50 mg/kg/day) lowered brain inflammatory markers (COX-2, iNOS, and IL-1β), APP expression, and BACE1 activity when administered intraperitoneally for one month to young, pre-plaque McGill-Thy1-APP mice (knock in of human APPsw/ind driven by the murine Thy1.2 promoter) [113]. However, in the latter study the authors reported liver and peritoneal toxicity, which “precluded the completion of behavioral testing for learning and memory” [113].

4.2.2.3. Thalidomide and Analogs: Compounds of the thalidomide family are referred to as immunomodulators and are known to reduce the half-life of TNF-α mRNA [114], thereby lowering TNF-α protein levels. Interestingly, thalidomide administered *per orens* to an $A\beta_{1–40}$-infused mouse model (20 mg/kg/day thalidomide for two hours to three days after $A\beta_{1–40}$ infusion) lowered hippocampal TNF-α mRNA levels, which translated to improved cognitive performance in an object recognition task carried out seven days after $A\beta_{1–40}$ infusion [115]. Test experiments conducted in our laboratory showed a 25% reduction in brain TNF-α protein levels in 12 month-old APP23 mice receiving thalidomide 100 mg/kg/day via i.p. injections for three months (unpublished data). In addition, a recently developed analog, 3,6’-dithiothalidomide, at the dose of 50 mg/kg/day i.p. significantly reduced brain TNF-α mRNA and protein levels in 6 month-old 3xTg-AD mice (pre-plaque and tau pathologies), and this was accompanied by improved cognitive measures [116]. These studies showed the increased potency of 3,6’-dithiothalidomide compared to

thalidomide [116]. Furthermore, 3,6'-dithiothalidomide (42 mg/kg/daily. i.p. for 6 weeks) reduced Aβ loads and tau hyperphosphorylation, while improving cognitive performance in 17 monthold 3xTg-AD mice [117]. In the latter study there had no demonstration that brain TNF-α levels were lowered in old 3xTg-AD mice, although complementary experiments suggest this. Further, the authors reported a significant down-regulation of the human APPsw transgene expression in old 3xTg-AD mice treated with the drug [117], concluding that "the possibility that some actions of 3,6′-dithiothalidomide may be mediated via suppression of this unnatural transgene promoter cannot be ruled out". Complementary experiments using different AD mouse models could help solving this issue and ascertain that the compound reduces Aβ loads when a different promoter than Thy1.2 drives the expression of an hAPPsw transgene.

4.2.2.4. Celastrol: Celastrol is known for its antioxidant and anti-inflammatory properties, such as lowering of TNF-α and IL-1β expression in human monocytes and macrophages [118]. Recently, celastrol (1 mg/kg/day i.p. for four days) was reported to alter Aβ loads in PSAPP mice (knock in of both human APPsw driven by the hamster prion protein promoter [model Tg2576] and human presenilin-1 mutated M146V driven by the HMG-CoA reductase promoter [119]) [120].

4.2.2.5. NF-κB Modulation: As indicated in section 2.2 above, the binding of TNF-α to TNF-RI and TNF-RII triggers NF-κB signaling [42, 43, 48]. Consequently, most anti-TNF-α drugs (including etanercept [121], infliximab [122], minocyclin [113], thalidomide [123], and celastrol [120]) were shown to reduce NF-κB phosphorylation (active state [48]) in various research models of inflammation. Interestingly, all these drugs reduced AD-like neuropathological features and, when assessed, improved cognitive measures (see references in sections 4.2.2.1–4 above).

4.2.2.6. Translation to Human Studies: Extrapolating pre-clinical pharmacological data to Phase I clinical trials involves deciding the starting drug dose to administer to human volunteers while ensuring their safety. The method recommended by the U.S. Food and Drug Administration (FDA) is the body surface area (BSA) normalization method [124]. This method calculates the quantity of drug per body surface area (e.g. mg/m^2) rather than weight (e.g. mg/kg). While helpful, the BSA method does not necessarily correlate with the pharmacologically active dose. Thus, the FDA allows scaling to demonstrate therapeutic effect in human subjects using physiologic, pharmacokinetic, and toxicology data instead of the BSA method if scientifically supported (for recent discussion, see [125]).

4.3. Clinical Evidence

In addition to pre-clinical studies, two bodies of work in humans suggest that medications altering TNF-α signaling might prevent or lower AD neuropathology.

4.3.1. Non-Steroidal Anti-Inflammatory Drugs—The first agents are non-steroidal anti-inflammatory drugs (NSAIDs). Epidemiological studies indicate that some of these compounds, like ibuprofen, might help prevent AD-related cognitive decline if taken chronically by prodromal subjects [126]. However, clinical trials failed to detect cognitive

improvements in AD patients; thus it appears unlikely that NSAIDs alone are sufficient to treat AD after cognitive deficiencies develop [28].

4.3.2. Etanercept—Etanercept was reported to dramatically improve the condition and cognition of an AD patient after a single dose (25 mg) via perispinal administration [127]. Perispinal extrathecal administration is defined as the injection of molecules into the anatomic area within 10 cm of the spine (for etanercept, between the spinous processes of the C6 and C7 vertebrae) which contains numerous veins proposed to allow some transport into the brain via retrograde venous flow [128]. The chosen route of administration is due to etanercept's large size (150 kDa) which prevents it crossing the BBB by passive diffusion [129]. Thus, this chimeric molecule must be delivered into the CNS for central inhibition of TNF-α. Further testing in 12 mild to moderate AD patients confirmed the potential of etanercept (25–50 mg weekly for 6 months) to slow AD-associated cognitive decline [130]. While encouraging, it is to note that results about etanercept on AD patients were collected during open-label trials without placebo controls, and the long-term effects on the sample population after drug wash out has not been reported. Moreover, a recent study using I-125-labeled etanercept in rats did not demonstrate any penetration of the compound into the CNS after perispinal delivery [131], which contrasts with a previous study using the same paradigm [132]. Therefore, it is currently unclear whether the fast improvement of cognitive abilities recorded for etanercept in AD patients is due to a central or peripheral action of the drug, or possibly the placebo effect.

In summary, both animal and human studies suggest that modulating TNF-α synthesis and/or signaling may be viable therapeutic interventions to prevent or slow the progression of AD. Recent discussions argue that mouse models of AD recapitulate asymptomatic phases of the disease [133]. In addition, an increasing body of evidence suggest that neuroinflammation occurs at early stages of AD neuropathology (summarized in [134]). Taken together, pre-clinical and clinical data support the idea that anti-inflammatory strategies, including anti-TNF-α, might be more efficient when administered at early, or even at prodromal stages of AD, rather than at later stages of the disease. TNF-α inhibition strategies now need to be explored in clinical trials to confirm this hypothesis and assess tolerability and efficacy.

5. STRATEGIES AND CHALLENGES INVOLVING CENTRAL TNF-α SIGNALING MODULATION

Using the knowledge acquired in the past decades about the synthesis and physiopathological roles of TNF-α (see sections 2 and 3 above), physical and pharmacological interventions can be tested in clinical trials to modulate TNF-α levels or signaling, and to lower inflammation-driven AD neuropathology. In this section we present a non-exhaustive list of options and their limitations, which are summarized in Table 2.

5.1. Physical Exercise

In the past decade, a very interesting line of research has shown that physical exercise is able to reduce age-related low-grade inflammation (reviewed in [135, 136]). Several laboratories

reported that muscle contraction induces the synthesis and release of IL-6 from muscle cells during acute (single 2–4h long) exercises, as well as other cytokines which are referred to as myokines [137–140]. In addition, long-term (several weeks long) physical exercise lowered peripheral markers of inflammation in a small group of MCI patients (n=20) above 60 years of age and age-matched cognitively normal (CN) subjects (n=15) [141]. At baseline, both plasma IL-6 and TNF-α were elevated in MCI vs. control individuals (IL-6: 1.53 vs. 1.17 pg/mL; TNF: 1.74 vs 1.48 pg/ml, respectively; $p<0.05$). After a 16 week period of multimodal exercise adapted to a population of this age range (see [142] for details), at the frequency of three weekly-one-hour-sessions, both plasma IL-6 and TNF-α levels dropped compared to baseline in the two cohorts (MCI IL-6: 1.36 vs 1.53 pg/L; MCI TNF: 1.49 vs. 1.74; CN IL-6: 1.02 vs 1.17 pg/L; and CN TNF: 1.29 vs. 1.48; all values post- vs. pre-exercise respectively; $p<0.05$) [141]. Furthermore, this chronic regimen of physical exercise improved cognition in active MCI patients vs. inactive, control MCI individuals [141], although it is not clear whether the benefit is solely the product of exercising or is combined with increased social interactions during exercise. While IL-6 is generally considered a proinflammatory cytokine, it may also act as an anti-inflammatory cytokine in human monocytic cells [143], likely via a post-transcriptional regulation mechanism [144], though the exact molecular mechanisms are unclear. Nonetheless, if these findings are confirmed, such therapeutic intervention might be safer to administer to large scale populations than some of the pharmacological agents described below. Therefore, the potential benefits might warrant broader experimentation, although attrition is often recorded in trials involving long-term physical exercise (e.g. [145]) and such trials are open-label rather than double-blind.

5.2. IL-6 Supplementation

Since physical exercise may induce the release of IL-6 from muscle cells into the blood, one could ask whether the administration of IL-6 alone is sufficient to reduce peripheral TNF-α levels. Starkie and collaborators infused physiological levels (dose not described) of recombinant human IL-6 (rhIL-6) for 3h to a small group of healthy subjects [139]. The authors recorded a drop in plasma TNF-α levels in a manner similar to physical exercise [139]. In addition, they observed that pre-infusion of rhIL-6 blocks the induction of TNF-α production following acute administration of endotoxin, again very similar to the effect of physical exercise. The authors concluded that administering rhIL-6 or physical exercise produce an anti-inflammatory effect that has the potential to reduce low-grade-inflammation [139].

Interestingly, another group showed that both IL-6 and IL-10 reduce TNF-α expression in primary rat astrocytes via regulation of translation [146]. Furthermore experimentation in healthy humans revealed that an acute arterial infusion of rhIL-6 (30 μg/h for 3h) induces a transient increase in plasma anti-inflammatory cytokines IL-1 receptor antagonist (IL-1ra) and IL-10 [147], paralleling results obtained in subjects who completed a marathon race [140]. Surprisingly, plasma TNF-α levels remained unchanged during and after rhIL-6 infusion. Importantly, however, both blood cortisol levels and the number of circulating neutrophils increased during rhIL-6 infusion [147]. This is of great importance for TNF-α modulation because 1- corticoids are inhibitors of TNF-α synthesis (see section 5.7 below); and 2- activated neutrophils release pro-inflammatory cyctokines, incuding TNF-α, which

facilitate extravasation through the endothelium (including in the BBB) and increase the levels of cytokines in tissues [148], but cortisol alters the binding of neutrophils to the endothelial membrane and prevents infiltration and release of cytokines into tissues [149]. Thus, treatment of patients with rhIL-6 appears to be a possible therapeutic intervention, for example for subjects with limited physical abilities such as after amputation or when suffering joint diseases. However, we did not find any literature about the long-term effect of IL-6 supplementation on inflammatory markers. Consequently, we believe that caution should be exerted before treating large AD cohorts since high levels of IL-6 were reported to correlate with increased risks of metabolic and immune diseases, such as coronary heart diseases [150, 151], and diabetes [152], though it is unclear whether IL-6 causes these diseases or is released by the body to prevent them.

5.3. Acetylcholinesterase Inhibitors and Nicotine

Acetylcholine (ACh) is one of the major neurotransmitters affected by AD. But, beside its role at the synaptic level in the brain, in recent years it was shown that ACh also modulates inflammation via what is referred to as the “immune cholinergic system” [153]. The system works thanks to lymphocytes and monocytes possessing the enzymatic machinery to synthesize (Choline acetyltransferase; ChAT) and degrade (Acetylcholinesterase; AChE) ACh. Activation of these immune cells leads to increased expression of ChAT and synthesis of ACh. After release, ACh can bind muscarinic and nicotinic receptors located on effector cells, which lowers the production of pro-inflammatory cytokines. The effects of ACh were mimicked by infusion of macrophages with nicotine (1–100 μM range), but not muscarine (1–100 μM range) [154]. Interestingly, both macrophages and microglia were shown to express the α7 subunit of the nicotinic receptor (α7 nAChR) [155]. This particular subunit was demonstrated to downregulate the NF-κB-mediated transcription of pro-inflammatory cytokines (summarized in [156]).

Of particular interest for AD, Aβ was shown to increase the activity of AChE in a neuroblastoma cell line, likely by slowing down the degradation of this enzyme rather than increasing its synthesis [157]. Similarly, challenging human THP-1 and peripheral blood mononuclear cells with Aβ induced the synthesis of pro-inflammatory cytokines, including TNF-α [158]. This increase was mitigated by infusing the cells with AChE inhibitors [158]. Furthermore, it was recently shown that anti-ACh drugs exacerbate systemic inflammation in a mouse model of tauopathy [93], though the exact molecular mechanisms remain to investigate. In addition, the acute administration of the AChE inhibitor galantamine (1–4 mg/kg i.p.) 1h prior to endotoxin (6 mg i.p.) in mice significantly reduced TNF-α serum levels, and this process required normal signaling by the vagus nerve [159]. Taken together, the data suggests that a systemic reduction in ACh may drive chronic inflammation by stimulating the production of pro-inflammatory cytokines, which could be modulated by AChE inhibitors. AChE inhibitors are one of the few FDA-approved drugs for AD treatment to date. Given their potential on the immune cholinergic system, it is possible that their transient slowing down of AD symptoms is mediated not only by central neurotransmission regulation, but also by affecting peripheral and central inflammation. However, it is well known that the effect of AChE inhibitors is short in duration (several months only), suggesting that this approach alone is not sufficient to counteract AD-related inflammation

after symptoms appear, and additional therapeutic interventions are likely required to produce lasting effects.

5.4. Etanercept

Following the positive outcome of etanercept perispinal administrations on cognitive performance in AD subjects in small scale studies (see section 4.3 above), this biologic could be tested on a larger sample population, in a randomized, double-blind, placebo-controlled study, to confirm the original findings. Since it is not clear whether perispinal administration carries etanercept into the CNS [131], while TNF-α is transported across the BBB (as explained in section 2 above), it is possible that altering peripheral TNF-α might lower its brain levels since less TNF-α would cross the BBB.

Interestingly, the subcutaneous administration of etanercept in an acute murine model of AD (30 mg/kg) improved cognitive measures and lowered TNF-α levels in the hippocampus [108] (section 4.2 above). This approach was then translated into a double-blind, randomized clinical trial on 41 mild to moderate AD patients receiving etanercept (n=20) and placebo control (n=21). The study reported that weekly subcutaneous injections of etanercept (50 mg for 24 weeks) was well tolerated in AD patients whose main adverse events were increased, but manageable infections [160]. However, compared to placebo controls, no significant amelioration in cognitive outcomes and daily activities were demonstrated in patients treated with etanercept [160]. The authors concluded that increasing the number of patients might be needed to obtain significant improvement of cognitive performance.

Since inflammation exacerbates AD neuropathological features, which build up during the pre-symptomatic phase of the disease [134, 161], we suggest that clinical trials targeting inflammatory molecules and pathways should recruit patients suffering mild cognitive impairment (MCI) to early AD to try preventing further neuronal death rather than treating patients at mild to moderate stages who already suffer significant neuronal loss. But caution is required for long term administration of etanercept because psychiatric adverse events, such as schizophrenia-like disorders [162], have been reported, indicating that some patients may require close monitoring. Nonetheless, if the peripheral administration of etanercept proves effective to slow AD progression, then peripheral administration of other biologics, such as anti-TNF-α and anti-TNFRI antibodies, could also be tested in the future.

5.5. Anti-TNF-α Antibodies

Another option to capture soluble TNF-α and prevent its binding and activation of cellular receptors is to use highly specific, bioengineered anti-TNF-α antibodies such as infliximab and adalimumab [163]. Infliximab was administered intracerebroventricularly (150 μg daily injection for three days) to the AD mouse model APP/PS1 at 12 months of age, i.e. when brain pathology is established. The authors reported a rapid reduction in brain TNF-α, Aβ, and tau phosphorylation levels, which were accompanied by an activation of monocytic CD11c-positive cells [107]. However, no cognitive data were provided. On a cautious note, adverse events have been reported in patients treated with anti-TNF-α antibodies for rheumatoid arthritis, including lymphoma, neuropathies, and infections [164]. In addition,

like etanercept [129] most antibodies show very limited crossing of the BBB via passive diffusion [165], thus they must be administered either centrally to efficiently inhibit CNS TNF-α, or in the periphery to lower circulating TNF-α and its crossing of the BBB. In addition, the ability of anti-TNF-α antibodies, administered peripherally, to reduce central TNF-α and AD-like neuropathology in rodent models has not been reported yet.

5.6. Anti-TNFRI Antibodies

As an alternative to capturing sTNF-α, TNF-α signaling could be reduced by antagonizing its receptors. As indicated in section 3.4. above, TNF-RI is mostly pro-apoptotic. The genetic deletion of this receptor showed improvement in AD-like pathology in rodent models (see section 4.1 above). For clinical applications, an anti-TNF-RI antibody has been bioengineered with a modified Fc region and named ATRO-SAB. Although no data are currently available on its effects on AD pathology, its *in vitro* bioactivity showed blockage of TNF-α-induced expression of IL-6 and IL-8 in HeLa cells via reduction of NF-κB activation [166]. In addition, the authors showed that the classical pathway of activation of the complement system was not activated by this antibody, indicating that once it binds to the cell surface it does not stimulate opsonization and cell lysis. Like for etanercept and anti-TNF-α antibodies, inhibition of CNS-located TNF-RI would likely require central administration given the low rate at which antibodies penetrate the brain parenchyma by passive diffusion through the BBB [165]. Furthermore, additional testing is required to determine whether peripheral administration of ATROSAB could reduce AD-like neuropathology and cognitive impairment in rodent models. Finally, the toxicity profile of this antibody will need to be assessed in Phase I studies before planning Phase II clinical trials on AD cohorts.

5.7. Corticoids

Corticoids are well known general inhibitors of inflammation used in many pathological conditions such as asthma and skin rashes. Mechanistically, corticoids are plasma membrane-soluble molecules that bind cytoplasmic glucocorticoid receptors (GR), inducing their nuclear translocation (summarized in [167]). Once in the nucleus, the corticoid-GR complex can either bind DNA glucorticoid response elements (GRE) to increase the transcription of anti-inflammatory cytokines (e.g. IL-10), or regulate other transcription factors, including depressing the NF-κB-driven transcription of pro-inflammatory cytokines. It is also proposed that corticoids may destabilize some pro-inflammatory mRNAs, though the exact mechanisms are unclear [167]. At the cellular level, corticoids prevent the attachment of neutrophils to endothelial cells, which reduces the extravasation of these cells and build up of cytokines in tissues [148]. Pilot experiments were conducted with dexamethasone (10 μM) on LPS-challenged whole blood samples from AD patients and healthy subjects. The authors reported that dexamethasone was more efficient on AD samples vs. controls [168]. Interestingly, dexamethasone is commonly co-administered with thalidomide analogs in cancer treatment (see section 5.8 below) because a cumulative effect on inflammation reduction was noted during clinical trials. However, long term administration of corticoids often results in adverse events such as increased risks of infection, osteoporosis, and mental depression via alteration of the hypothalamic-

pituitaryadrenal (HPA) axis [168, 169], limiting their potential to treat chronic disorders like AD.

5.8. Thalidomide and Analogs

Because AD is a complex disease [161], the use of pleiotropic anti-inflammatory agents may be required to not only reduce pro-inflammatory, but also increase anti-inflammatory cytokines. Examples of this pharmacological class include immunomodulators of the thalidomide family which are very potent TNF-α inhibitors, and which were proposed as therapeutic agents for neurodegenerative disorders [31, 170]. Their mechanism of action is via destabilization of TNF-α mRNA, which reduces protein synthesis [114] , but they also modulate other cytokines [171]. In a separate paper in this special edition of CAR, we report the testing of thalidomide in mild-to-moderate AD patients (see Thalidomide paper in this special edition). Our main conclusion is that the toxicity of thalidomide unfortunately induced too many adverse events (including somnolence, constipation, and peripheral neuropathy), which resulted in participants prematurely withdrawing from the study. In addition, and as indicated above, inflammation exacerbates AD neuropathological features and is detected at early stages of AD [172]. Since our sample population consisted of mild to moderate AD patients, our study design was likely not optimal to detect the preventive effects of thalidomide.

Interestingly, several novel thalidomide analogs generated recently have been shown to be potent TNF-α synthesis inhibitors *in vitro* [173, 174]. For example, the thio-modified compound 3,6'-dithiothalidomide lowered the synthesis of inflammatory markers in the mouse macrophagic cell line RAW 264.7, and in rat blood after LPS stimulation [117]. Of interest for AD research, the drug was also shown to improve cognitive measures and reduced Aβ loads in 3xTg-AD mice [117]. Thus, we suggest that future trials testing the potential of less toxic thalidomide analogs should focus on MCI to early AD cohorts in the hope of preventing further accumulation of neuropathological features and cognitive decline, rather than testing such compounds as disease-modifying therapies in mild- to late-AD patients who suffer advanced cognitive deficits.

5.9. Immunosuppressants

In this section we refer to immunosuppressive drugs known as anti-rejection medications, which inhibit B and T cells activation. The major compounds of this class are the cyclic undecapeptide cyclosporine, the macrolide antibiotic tacrolimus, and the antifungal rapamycin [175]. They are used mostly to 1- prevent the rejection of transplanted organs and tissues; 2- treat autoimmune diseases (e.g., rheumatoid arthritis, multiple sclerosis, myasthenia gravis, systemic lupus erythematosus); and 3- treat some non-autoimmune inflammatory diseases (e.g. long-term allergic asthma).

Cyclosporine and tacrolimus (also named FK-506) are both inhibitors of the calcium/calmodulin dependent serine/threonine protein phosphatase calcineurin. Cyclosporine A binds to proteins located in the cytoplasm of immunocompetent lymphocytes called cyclophilins. Tacrolimus binds to FK-506 binding proteins (FKBP) [176]. The drug-protein complexes then bind calcineurin to inhibit the dephosphorylation (activation) of the

transcription factor, nuclear factor of activated T cell, cytoplasmic (NFATc). This results in the blockage of T cell activation, and altered expression of the pro-inflammatory cytokine IL-2, which prevents further activation of cell-activated immunity that is characterized by the release of TNF-α [177]. Both cyclosporine (50 μM) and tacrolimus (50 μM) were shown to downregulate the expression of APP mRNA and protein in primary cultures of neonatal rat astrocytes [178]. However, we did not find any report on clinical trials for AD. To note, while cyclosporine and tacrolimus are very potent anti-inflammatory drugs, cyclosporin does not cross the BBB because it is endocytosed and then trapped in the cerebral endothelial cells [179], and long term treatment with high doses often results in nephrotoxicity, and occasionally in neurotoxicity [180, 181].

Rapamycin (also named sirolimus) binds to FKBP, and then inhibits the serine/threonine kinase mTOR Complex 1. Contrary to cyclosporine A and tacrolimus which affects the first phase of T lymphocyte activation, rapamycin affects the second phase, i.e. signal transduction and lymphocyte clonal proliferation, preventing their transition from G1 to S phase of the cell cycle [182]. In addition, rapamycin prevents B cell differentiation to plasma cells, reducing the production of IgM, IgG, and IgA antibodies [183]. Oddo and collaborators showed that rapamycin reduces both Aβ and tau pathologies while improving cognitive measures in 3xTg-AD mice [109]. Whether rapamycin could be effective on AD patients remains to be tested in clinical trials, though chronic administration may generate adverse events such as diabetes, thrombocytopenia, anemia and leucopenia [184].

5.10. Stimulation of Repressors of Transcription

Recently, novel endogenous proteins have been identified which inhibit the transcription of TNF-α. One of these molecules is the Leucine-rich repeat in Flightless-1 interaction protein 1 (GCF2/LRRFIP1). Initial investigations have shown that GCF2/LRRFIP1 occupies the region −308 of the *TNF*-α gene promoter [185]. Very interestingly, GCF2/LRRFIP1 seems to act as a transcription inhibitor that completely represses TNF-α transcription in cells that do not express this cytokine physiologically [185]. A very recent report showed that GCF2/LRRFIP1 regulates pro-survival proteins and pathways in rat astrocytes, including β-catenin, Akt, and mTOR [186]. While still at early stages of investigation, if the effects of GCF2/LRRFIP1 are confirmed, it could become a new therapeutic target to modulate TNF-α expression, although its negative effects must also be studied as another report suggests it may stimulate metastasis in some forms of cancers [187].

5.11. Potential Health Hazards

Finally, as reported in the etanercept clinical trial [188], inhibiting TNF-α in a chronic manner might reduce the potential of AD patients to fight infections and cancers. Furthermore, the complete deletion of TNF-α receptors (TNF-RI and II) in 3xTg-AD mice resulted in exacerbated AD-like neuropathology, suggesting that pan-TNF-α suppressive therapeutic strategies might be detrimental rather than beneficial for AD treatment [189]. Moreover, a few neurological adverse events associated with anti-TNF-α agents were reported, which include central and peripheral demyelination, CNS lupus, encephalitis, and polyneuropathies [181, 190–192]. Therefore, we advocate that all clinical trials using TNF-α

inhibitors on AD patients include a pharmacovigilance section to monitor possible neurological adverse events.

CONCLUSION

Chronic central inflammation plays a major role in the development of AD neuropathology and associated dementia. Therefore, developing therapeutic interventions to modulate inflammation represents a valid option to treat AD. Because of its pivotal role in inflammation, TNF-α is a very attractive pharmacological target. In addition, pre-clinical data indicate that increased TNF-α levels exacerbates amyloidogenesis, and diverse paradigms used to reduce TNF-α signaling in rodent models of AD showed significant reduction in AD-like brain pathology accompanied by an amelioration of cognitive function. Therefore, TNF-α inhibition to prevent or slow AD should be explored in more depth in clinical trials. However, we recommend that the inhibition of TNF-α be kept to mild to moderate levels in order not to induce adverse events resulting from blocking the physiological roles of TNF-α in the CNS (Table 1).

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Table 1

Physiological and deleterious effect of TNF-α signaling in the CNS.

TNF-α Function	References
Neurophysiology (basal expression and acute disease state)	
- Development: act as a neurotrophic factor (neuronal apoptosis)	[32]
- Development: facilitate cell migration and proliferation	[58]
- Cognition	[59, 60]
- Synaptic plasticity	[58, 61]
- Astrocytic gliotransmission	[58]
- Ionic homeostasis	[62]
- Protects from excitotoxicity	[63]
- Facilitator of remyelination by promoting oligodendrocyte survival	[64]
- Sleep	[65]
- Food and water intake	[66]
- Anti-neurogenic effect during adult neurogenesis (cultured ippocampal progenitor cells and SVZ progenitor cells)	[67, 68]
- Host defense	[69]
- Restore brain homeostasis and functions during acute inflammation	[34, 45]
Neuropathology (chronic expression in moderate to high amounts)	
- Promote excitotoxicity (in association with glutamate)	[63]
- Cause synaptic loss	[58, 61,63]
- Stimulate astrogliosis and microgliosis	[57]
- Exacerbate amyloidogenesis in Alzheimer’s disease	[70–72]
- Participate in multiple sclerosis, amyotrophic lateral sclerosis, Parkinson’s disease, ischemia, and other neurological disorders	[32, 34, 73–75]
- Drive HIV-associated dementia	[76]

Table 2

Possible therapeutic interventions to decrease TNF-α signaling in AD, their targeted mechanism of action, and potential health hazards. See text in Section 5. for details and references.

Possible Therapeutic Intervention	Mechanism of action	Potential Limitations
Physical exercise	Muscle contraction induces the release of IL-6 that seems to act as TNF-α inhibitor	None
IL-6 supplementation	Acute IL-6 supplement alone seems to reduce peripheral TNF-αlevels, and increase IL-1ra, IL-10 and cortisol	IL-6 is an ambivalent cytokine that could induce other severe conditions upon chronic administration
Acetylcholinesterase inhibitors and nicotine	Activation of α7 nicotinic acetyl choline receptors which regulate cytokine transcription via NF-κB modulation	AChE inhibitors used currently to treat AD have limited time effects, showing this option alone is not viable for long term treatment
Etanercept	Capture of soluble TNF-α blocking it from binding cellular receptors	Do not cross the BBB; central administration is high risk
Anti-TNF-α antibodies (e.g. Infliximab)	Capture of soluble TNF-α blocking it from binding cellular receptors	Do not cross the BBB; central administration is high risk
Anti-TNF-RI antibodies	Competes against TNF-α to bind TNF-RI, but does not activate signal transduction	Do not cross the BBB; central administration is high risk
Corticoids (e.g. dexamethasone)	General inhibitors of inflammation via regulation of NF-κB by glucocorticoid receptors; may also destabilize mRNAs	Chronic administration may induce endocrine-related pathologies via disregulation of the hypothalamic-pituitary-adrenal axis
Thalidomide and analogs	Destabilize TNF-α mRNA; modulate cytokines, chemokines, and NF-κB	Commercially available thalidomide analogs induce neutropenia, thrombocytopenia, and various adverse events
Immunosuppressants (e.g. cyclosporine, rapamycin)	General inhibitors of the immune response by inhibiting T and B cells activation	Chronic immunosuppression increases the risk of infection, neutropenia, and thrombocytopenia
Stimulation of repressors of transcription (e.g. GCF2/LRRFIP1)	Binding of TNF-α promoter region to repress transcription	May repress other genes than TNF-α leading to adverse events and/or other pathologies