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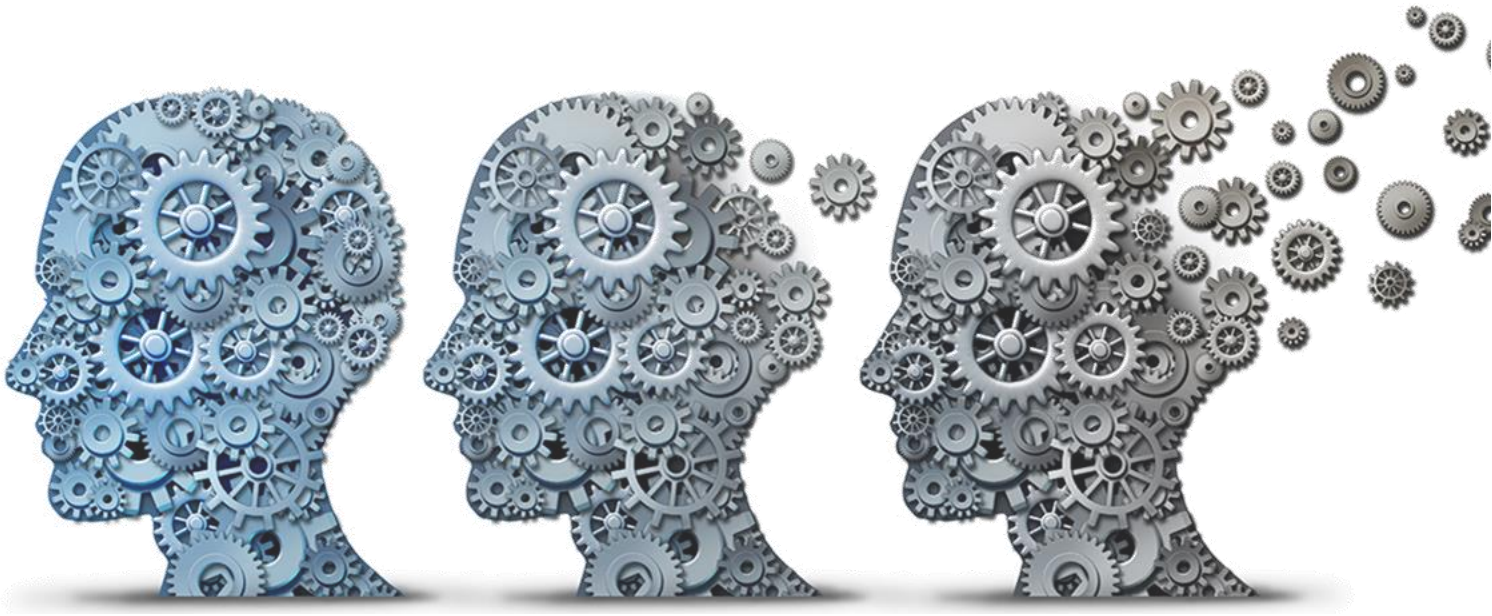

*(Vallet, 2011)*


Author: A.A. Hooijsma
Supervisors: Prof. Dr. U.L.M Eisel, N. Ortí-Casañ

Date: 11/07/2021

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# Abstract

Upon ageing, the risk for developing Alzheimer's Disease (AD) increases. As the population ages, the interest in curing this disease rises. Within AD, inflammation plays an important role during its pathogenesis, whereby tumour necrosis factor α (TNF) is considered a major contributor. When released, TNF can bind to tumour necrosis factor receptor (TNFR)1, inducing inflammation and neurodegeneration and TNFR2 enhancing neuroprotection. Regarding its protective role, this study aims to analyse the effect of TNFR2 stimulation on neuroinflammation in a humanized AD's mouse model. For analysing the effect in human context, a crossbreed between J20 mice and mice with a human knock-in TNFR2 was treated with a selective mouse TNF-based agonist of TNFR2 (STAR2) whereby treatment with PBS was used as a control. The results showed that STAR2 induced a significant decrease in microglia in hippocampal areas cornu ammonus (CA)1, CA3 and the dentate gyrus (DG). A significant decrease in microglial activity was found in the DG upon STAR2 treatment. Concerning astrocytes, no significant differences were found. Regarding these results, it can be concluded that treatment with STAR2 may contribute to reducing inflammation in AD. In addition, STAR2 used for this study had an activity of 15%. Since this study is a follow-up study, and the previous study, where the effect of TNFR2 stimulation in an AD's J20 mouse model was analysed, had a STAR2 with 85% activity, further research must be done using STAR2 with an 85% activity to determine its effect in the humanized AD's mouse model.

# Abbreviations

| | |
|---|---|
| Alzheimer's Disease | AD |
| Amyloid-beta | αβ |
| Amyloid precursor protein | APP |
| Blood brain barrier | BBB |
| Bovine goat serum | BSA |
| Cornu ammonus | CA |
| Cellular inhibitor of apoptosis proteins | cIAPs |
| Central nervous system | CNS |
| c-Jun N-terminal kinase | JNK |
| C-X-C Motif Chemokine Ligand | CXCL |
| Dead domain | DD |
| Dental Gyrus | DG |
| Diominobenzidine | DAB |
| Endoplasmic reticulum | ER |
| Experimental autoimmune encephalomyelitis | EAE |
| Extracellular signal regulated kinase | ERK |
| Granylocte colony stimulating factor | G-CSF |
| Inhibitor of κB | IκB |
| Interferon gamma | IFN-γ |
| Interleukin | IL |
| Intraperitoneal injection | IP |
| IκBα kinase | IKK |
| Leukemia inhibitory factor | LIF |
| Linear ubiquitin chain assembly complex | LUBAC |
| Mitogen-activated protein kinase | MAPK |
| Multiple sclerosis | MS |
| Natural killer cells | NK-cells |
| Neurofibrillary tangles | NFTs |
| NFκB inducing kinase | NIK |
| N-methyl-D-asparaginezuur receptor | NMDR |
| Phosphoinositide 3-kinases | PI3K |
| Promoter driven amyloid precursor protein | PDAPP |
| Protein kinase B/serine-threonine kinase | PKB/Akt |
| Receptor interacting protein kinase 1 | RIP1 |
| Selective mouse TNF-based agonist of TNFR2 | STAR2 |
| Soluble TNF | solTNF |
| TGF-B kinase | TAK |
| TNF converting enzyme | TACE |
| TNF receptor 1 | TNFR1 |
| TNF receptor 2 | TNFR2 |
| TNFR1-associated dead domain protein | TRADD |
| Transforming growth factor-beta | TGF-B |
| Transgene | tg |
| Trans-Golgi network | TGN |
| Transmembrane TNF | tmTNF |
| Triss-buffered saline | TBS |
| Tumor necrosis factor α | TNF |
| Vascular endothelial growth factor 2 | VEGFR2 |

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# 1. Introduction

As the population ages, the risk for developing AD increases (Xia et al., 2018). Despite all the research that has been done, no cure has been found for this disorder.

AD is a neurodegenerative disorder defined by cognitive impairments such as deterioration of visuospatial skills, memory deficits and executive dysfunction as a result of neuronal loss (Mattson, 2004; Ortí-Casañ et al., 2019). Hallmarks of AD's pathology are deposition of amyloid-β (αβ) and neurofibrillary tangles (NFTs) (Ortí-Casañ et al., 2019; Sung et al., 2020). Besides αβ deposition and NFTs, dystrophic neurites, microglial activation, neuropil threads and astrogliosis are also seen in AD (Ortí-Casañ et al., 2019; Serrano-Pozo et al., 2011). Deposition of αβ can be induced by overproduction or reduced degradation of αβ. Overproduction of αβ is induced by a metabolic shift towards amyloidogenic processing of amyloid precursor protein (APP), which can be induced by genetic, ageing or environmental factors (Sung et al., 2020). During amyloidogenic processing, APP is cleaved by β-secretase (BACE) and γ-secretase leading to the production of an Aβ40/42 monomer. Aggregation of these monomers results in production of neurotoxic oligomers and finally formation of Aβ plaques (Kumar, V., Abbas, A. K., Fausto, N., & Aster, 2014; Mattson, 2004). Next, decreased clearance of αβ can be a result of reduced production of degradation enzymes, produced by glial cells, as well as a reduced phagocytotic capacity of microglia and astrocytes (Ries & Sastre, 2016). Focussing on the effect of deposition of αβ as well as NFTs, one of the major consequences is the induction of neuroinflammation, stimulating AD's pathogenesis. Within this process, TNF plays an important role (Ortí-Casañ et al., 2019; Sung et al., 2020).

## 1.1. TNF and its Receptors

TNF is a 26 kDA monomeric type II transmembrane protein (tmTNF) that can also be transformed into a 17 kDA soluble monomeric protein (solTNF) by cleavage via TNF converting enzyme (TACE/ADAM17) (Dong et al., 2015; Fischer et al., 2015; Ortí-Casañ et al., 2019). TNF is a major regulator of the adaptive and the innate immune system and interacts with its transmembrane receptors; TNFR1 and TNFR2 (Fischer et al., 2015). When comparing both receptors, a 28% homology can be found in the extracellular domain, consisting of a four cysteine-rich region. The intracellular domain, on the contrary, lacks homology, suggesting that both receptors activate various pathways (Idriss & Naismith, 2000; Ortí-Casañ et al., 2019). One of the major differences between both receptors is the presence of an intracellular death domain (DD) (Fischer et al., 2015; Idriss & Naismith, 2000; Ortí-Casañ et al., 2019). TNFR1, containing this domain, can therefore bind TNFR1-associated dead domain protein (TRADD), activating Fas-associated dead domain (FADD) and thereby inducing apoptosis. Contrary to TNFR1, TNFR2, lacking this DD, stimulates cell survival by interaction with TNF-associated factor 2 (TRAF2) (Ortí-Casañ et al., 2019). TNFR1 is expressed by different tissues and cell types and can be stimulated by both tmTNF and solTNF. TNFR2, on the contrary, only interacts with tmTNF and is expressed in low levels by endothelial and immune cells (Dong et al., 2015; Fischer et al., 2015; Ortí-Casañ et al., 2019). Generally, activation of TNFR1 induces a pro-inflammatory response characterized by pro-apoptotic functions, whereby activation of TNFR2 leads to an anti-inflammatory response stimulating regeneration, cellular proliferation, cell survival, and tissue homeostasis with a neuroprotective role in the brain (Dong et al., 2015; Ortí-Casañ et al., 2019).

Regarding AD, increased protein levels of TNF were found in plasma and serum of AD patients (Álvarez et al., 2007; Bruunsgaard, 1999; Tan et al., 2007). *In vitro* studies showed that increased TNF expression is induced by αβ, explaining its presence around αβ plaques in brain tissue post-mortem (Decourt et al., 2016; Dickson, 1997). Concerning both TNFRs, post-mortem studies also showed increased levels of TNFR1 as well as an increased affinity for this receptor, whereas TNFR2 levels were decreased as well as the affinity for this receptor (Cheng et al., 2010; Zhao et al., 2003). Regarding AD's pathology, the effect of TNF depends on the interaction with its receptors.

## 1.2. TNF and its receptors in the brain

Since AD is a neurodegenerative disease of the central nervous system (CNS), understanding the role of TNF in the brain is highly important. In healthy individuals, TNF is expressed in physiological levels by neurons and microglia and plays an important role in, among others, synaptic plasticity, ionic homeostasis and host defence (Decourt et al., 2016). Upon brain injury, as well as neurodegenerative diseases, increased levels of TNF are expressed by oligodendrocytes, reactive astrocytes, activated microglia, neurons, ependymal cells, endothelial cells, and epithelial cells (Decourt et al., 2016).

Focusing on AD, previous studies tried to determine the driving factor behind the increased TNF levels. *In vitro* studies showed that stimulating human and rodent microglial cells with αβ induces a significant release of TNF (Decourt et al., 2016). Moreover, when stimulating neurons with αβ, TNF-induced apoptosis is stimulated (Blasko et al., 1997). Also, specific overexpression of TNF in neurons, of hippocampal region CA1, lead to microglial activation and neuronal death, revealing that TNF induced inflammation may have a damaging effect on neurons (Janelsins et al., 2008). Regarding these data, it is suggested that TNF is chronically released by microglia, and neurons, as a result of elevated αβ levels (Decourt et al., 2016).

### 1.2.1. Microglial secretion of TNF

Concerning microglia, the primary immune cells within the brain, activation occurs upon detection of microenvironmental changes (Lynch, 2014; Nayak et al., 2014; Subramaniam & Federoff, 2017). When becoming activated, different pathways are induced leading to release of cytokines and chemokines. Regarding TNF, upregulation is induced when microglia become activated in response to pathological conditions, such as infections, injury, neurotoxicity, and neurodegenerative diseases like Parkinson's disease, AD, and multiple sclerosis (Veroni et al., 2010).

Focusing on pathological events, CNS injury leads to increased TNF secretion as a result of significant release of ATP by damaged cells and microglia at the site of injury (Raffaele et al., 2020). In AD, this ATP release is induced by its neurodegenerative effect, as well as accumulation of αβ itself (Cieślak & Wojtczak, 2018). Upon ATP release, P2 purinoceptors, expressed by glia and neurons, become activated (Raffaele et al., 2020). Regarding microglia, released ATP activates Purinoceptor P2X7, inducing activation of extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 mitogen-activated protein kinase (MAPK), resulting in secretion of TNF (Holbrook et al., 2019). Transcription of TNF is particularly regulated by ERK and JNK, through activation of transcription factors p65 (RelA) and nuclear factors of activated T cell (NFAT). After transcription of TNF, p38 regulates expression of RNA-binding proteins, necessary for post-transcriptional modifications as well as nucleocytoplasmic transport of TNF (Raffaele et al., 2020).

When synthesized, release of TNF occurs through the conventional or non-conventional pathway (Figure 1). Regarding the conventional pathway, tmTNF is rapidly transcribed and translated containing a signal peptide, capable of targeting the endoplasmic reticulum (ER) for correctly folding the protein. After folding the protein, the Golgi complex carries out the final modifications, followed by coverage of tmTNF by the trans-Golgi network (TGN). Within the TGN, tmTNF is sorted into vesicular or tubular carriers, containing an N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) complex, followed by transport to the plasma membrane and exposure in a phagocytic cup (Kay et al., 2006; Raffaele et al., 2020). When presented in the phagocytic cup, TACE cleaves tmTNF, and solTNF is released (Raffaele et al., 2020). Since tmTNF can regulate its release, release of solTNF depends on signalling with the recycling endosome during transport to the plasma membrane (Raffaele et al., 2020). After release, solTNF can bind TNFR1 present on adjacent cells such as neurons, astrocytes, and oligodendrocytes, inducing inflammation and neurodegeneration.

Concerning the non-conventional pathway, activation of P2X7 induces release of tmTNF. In contrary to the conventional pathway, release of tmTNF does not depend on an ER-Golgi transport mechanism. Here, synthesizing occurs remotely from the phagocytic cup and TACE-enzymes, protecting cleavage of tmTNF. Release of tmTNF occurs by transport via extracellular vesicles (EVs). When released, tmTNF can activate TNFR2 on recipient cells, inducing tissue remodelling and neuroprotection (Raffaele et al., 2020). Interestingly, upon ATP activation, P2X7 can affect its downstream pathways inducing a switch to the non-conventional pathway, stimulating release of tmTNF

and therefore inducing a neuroprotective and anti-inflammatory response (Raffaele et al., 2020). Furthermore, when released, solTNF, as well as tmTNF, also interacts with the corresponding TNFR expressed by the donor microglia, inducing a positive feedback loop, enhancing production of TNF (Raffaele et al., 2020; Veroni et al., 2010). Concerning expression of both TNFRs, it is shown that released TNF increases the expression of both receptors on microglia (Nadeau & Rivest, 1999).

Focussing on the net effect of TNFR activation, interferon-gamma (IFN-γ) is indicated as a major regulator (Raffaele et al., 2020; Veroni et al., 2010, 2020). When produced by NK, Th1, or dendritic cells, IFN-γ induces TNFR1 shedding, and inhibition of TNFR2 expression, resulting in a shift towards TNFR1 signalling leading to inflammation and tissue degeneration (Darwich et al., 2009; Raffaele et al., 2020; Veroni et al., 2010). In addition, some studies also show that IFN-γ may also be produced by microglia, upon stimulation with IL-8 and IL12 (Kawanokuchi et al., 2006; Zhang et al., 2020).

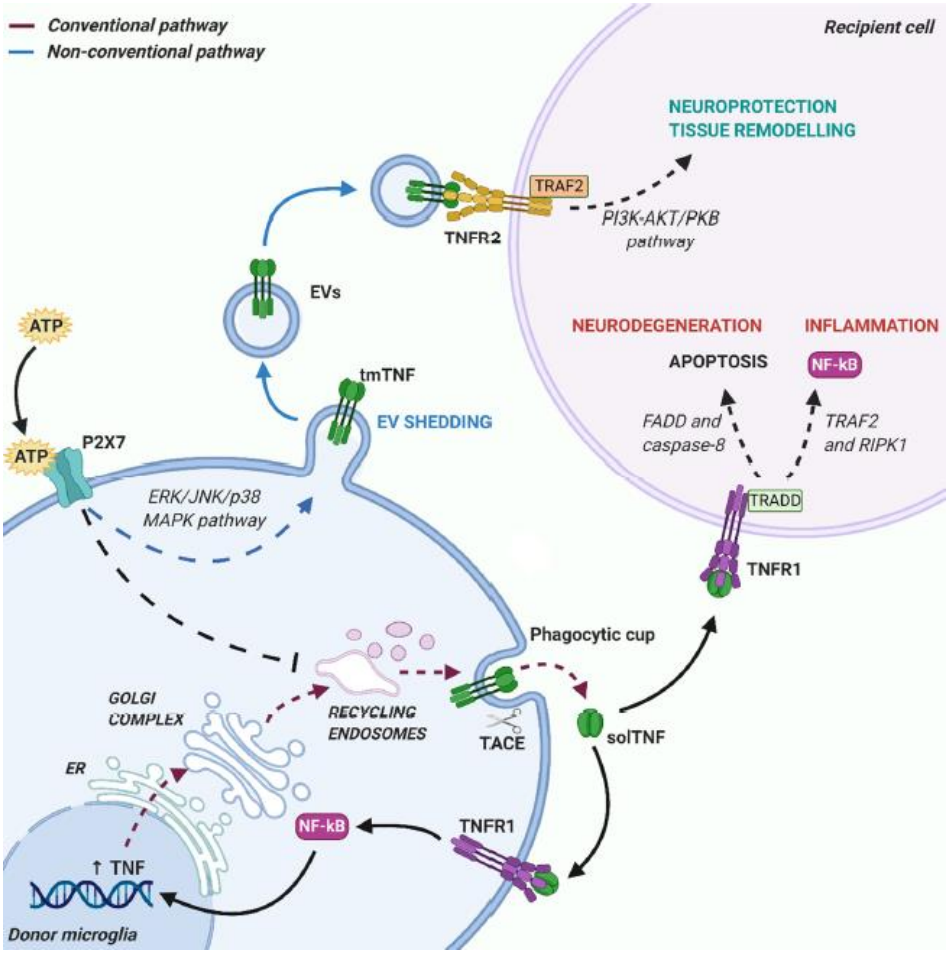


**Figure 2. Microglial TNF release** (Raffaele et al., 2020)
Schematic overview of microglial TNF release via conventional(purple) and non-conventional(blue) pathway. The conventional pathway: synthesised TNF is correctly folded in the ER, followed by post-translational modification in the Golgi complex. tmTNF is then transported to the membrane and exposed in the phagocytic cup where it is cleaved by TACE enzymes and released as solTNF. solTNF can activate TNFR1 on the recipients' cells, inducing neurodegeneration via FADD and caspase 8, and inflammation by activation of NFκB. solTNF can also bind TNFR1 expressed on donor microglia, inducing a positive feedback loop. Non-conventional pathway: release of tmTNF occurs without post-translational modifications by ER and the Golgi complex. tmTNF is not exposed in the phagocytic cup avoiding cleavage by TACE enzyme. After packed into shedding EVs, tmTNF is released in the extracellular space where it interacts with TNFR2, inducing neuroprotection and tissue remodelling by activating the P13K/PKB pathway. ER; endoplasmic reticulum, EV's; extracellular vesicle, TACE; TNF converting enzyme, TNF; tumour necrosis factor, tmTNF; transmembrane TNF, solTNF; soluble TNF (Raffaele et al., 2020).

### 1.2.2. TNFR2 receptor microglia

As mentioned above, when solTNF is released, it can bind TNFR1 present on microglia cells, inducing a positive feedback loop leading to elongate production of TNF, enhancing inflammation and neurodegeneration. Focusing on TNFR2 much remains unclear regarding the effect of TNFR2 stimulation on microglia. A study by Veroni et al. (2010) showed that stimulation of TNFR2 induces an increase in gene expression of the receptor itself. In addition, stimulation of TNFR2 also leads to an increase in gene expression of adrenomedullin, an anti-inflammatory peptide, ZFP36, encoding for tristetraprolin, a negative biosynthesis regulator, and lipase acylxyacyl hydrolase (AOAH), involved in decreasing endotoxin activity (Veroni et al., 2010). When classifying upregulated genes into pathways, a significant increase of genes (IL-10r1, STAT1, heme oxygenase-1, TNF and IL-6) elaborating in the anti-inflammatory IL-10 signalling pathway were found (Veroni et al., 2010). Furthermore, stimulation of TNFR2 also induces upregulation of granulocyte colony-stimulating factor (G-CSF), a neurotrophic factor stimulating survival of neurons, neurogenesis, and inducing a repair response (Roberts, 2005; Song et al., 2016; Veroni et al., 2010). Combined, these data indicate that stimulation of microglial TNFR2 may induce a neuroprotective effect in the brain. Supporting, a study by Gao et al. (2017) showed that ablation of microglial TNFR2 induces early onset of experimental autoimmune encephalomyelitis (EAE), a model for studying multiple sclerosis (MS), accompanied by elevated T cell activation, leukocyte infiltration, and demyelination of neurons in the CNS (Gao et al., 2017). Interestingly, focusing on the effect in the periphery, activation of TNFR2 on monocytes/macrophages induces the opposite effect, whereby the activation induces EAE accompanied by increased peripheral T-cell activation, T-cell infiltration in the CNS and demyelination (Gao et al., 2017).

### 1.2.3. TNF induced stimulation of astrocytes

After TNF is released, it binds to its receptor on recipient cells. One of these cells are the astrocytes. Astrocytes elaborate in, among others, nutritional supplementation of neurons, maintenance of the blood-brain barrier (BBB), clearance of waste and synaptic communication (Osborn et al., 2016; Sung et al., 2020). Upon brain injury or neurodegenerative diseases, astrocytes undergo functional and morphological changes, called astrogliosis. Characteristics for astrogliosis is the increased expression of, among others, glial fibrillary acidic protein (GFAP)(Kamphuis et al., 2014).

In AD, TNF, released by microglia, induces astrogliosis upon binding to its receptors expressed by astrocytes. When binding the receptors, the effect can be neuroprotective as well as neurotoxic dependent on the receptor interaction. (Osborn et al., 2016). When TNF binds astrocyte expressed TNFR1, toxicity towards oligodendrocyte precursors is induced, leading to demyelination (Kim et al., 2011). In addition, binding to TNFR1 also induces release of neurotransmitters glutamate (Habbas et al., 2015; Osso & Chan, 2015). When released, glutamate can bind the presynaptic N-methyl-D-aspartic acid receptor (NMDR), inducing presynaptic release of glutamate able to bind post-synaptic NMDRs, stimulating synaptic activity and survival (Habbas et al., 2015; Wang & Reddy, 2017). However, chronically stimulation of this glutamatergic signalling, as a result of chronically TNFR1 activation on astrocytes, for example, leads to excitotoxicity, with neuronal cell death, induction of neuronal trauma as well as neurodegeneration as a result (Wang & Reddy, 2017). Regarding AD, increased levels of glutamate also enhance increased activation of extrasynaptic NMDRs, triggering pro-apoptosis. Since signalling through the extrasynaptic NMDRs exceed the induced survival by endosynaptic NMDRs, increased activation of these receptors enhances further neuronal damage (Wang & Reddy, 2017).

Concerning TNFR2, activation of this receptor on astrocytes stimulates the PI3K/Akt/PKB pathway leading to increased expression and release of neuroprotective factors. These factors include leukaemia inhibitor factor (LIF) and C-X-C Motif Chemokine Ligand(CXCL) 12 (Raffaele et al., 2020), which both induce proliferation and differentiation of oligodendrocytes and therefore stimulating remyelination of axons (Fischer et al., 2014; Patel et al., 2012). Finally, induced astrogliosis also leads to the release of IL-1 and TNF, stimulating the inflammatory response and enhancing a positive feedback loop of TNF production (Osborn et al., 2016).

## 1.3 Therapeutic approach Alzheimer's Disease

Since it is becoming clearer that TNF and its receptor interaction plays an important role in AD and its pathogenesis, many *in vitro* studies have been done to understand this signalling for determining a therapeutic target. A study by Montgomery et al. (2011) showed that deletion of both TNF receptors leads to a significant aggravation of AD pathology. In addition, knock-down of TNFR1 and/or TNFR2 enhances neuroinflammation (Dong et al., 2015; Montgomery et al., 2011). These data suggest that the focus should lie on modulation of one or both receptors, instead of inhibiting TNF signalling (Dong et al., 2015; Montgomery et al., 2011). Focussing on TNFR1, deletion of this receptor inhibits αβ production and prevents memory and learning deficits, indicating its stimulating role in AD's pathology when activated (Dong et al., 2015; He et al., 2007). On the contrary, inhibition of TNFR2 exacerbates AD pathology, by enhanced TNFR1-induced tau and αβ pathology, while overexpression of TNFR2 reversed these results (Dong et al., 2015; Jiang et al., 2014; Montgomery et al., 2013). Moreover, specific stimulation of TNFR2 protects oligodendrocytes and neurons from oxidative stress, induces myelination and differentiation of oligodendrocytes, and protects neurons from glutamate-induced excitotoxicity (Dong et al., 2016). Because of the protective features of TNFR2, specific activation of TNFR2 is considered a potential therapeutic target against AD (Dong et al., 2016).

## 1.4 The project

As TNFR2 is suggested as a potential target for AD, this study will focus on the effect of TNFR2 stimulation on neuroinflammation in AD (Dong et al., 2016; Ortí-Casañ et al., 2019). Unpublished work of N. Ortí Casañ et al. showed that TNFR2 stimulation, using the TNFR2 agonist STAR2, led to cognitive improvements and reduction of αβ plaques in an AD mouse model. This study will be a follow-up study where the effect of TNFR2 stimulation will be analysed, focussing on neuroinflammation, in a humanized AD mouse model.

The humanized mouse model used for this project consists of a crossbreed between J20 and human TNFR2 knock-in (kiHuTNFR2) mice. Genetically, the crossbred contains mutations for overexpression of mutant human APP protein (J20), inducing ADs' pathology, and a knock-in of human TNFR2 to simulate the human context (Fisher et al., 2018). The STAR2 used in this study consist of three mouse mTNF (D221N-A223R) molecules, interconnected by a linker and fused to the chicken tenascin (TNC) precursor trimerization domain (figure 3a). A study by Chopra et al. (2016) analysed the binding capacity of STAR2 and showed that stimulating Hek293 cells, transfected with mouse TNFR2, with STAR2 almost lead to the same specific binding results, compared to tmTNF itself (Figure 3b). Compared to TNFR1, no specific binding of STAR2 was found (Figure 3c). These results showed that STAR2 can be used as a functional agonist for TNFR2 (Chopra et al., 2016).

After treatment of the mice with STAR2, the effect of TNFR2 stimulation on neuroinflammation will be analysed, focussing on microglia and astrocytes. Since AD is accompanied by cognitive impairments, the area of interest will be the hippocampus, focussing on the regions CA1, CA3 and the DG (Ortí-Casañ et al., 2019). Concerning microglia, microglial markers ionized calcium-binding adapter molecule (IBA-1) will be analyzed for determining the coverage and activation status (Walker et al., 2020). Regarding astrocytes, glial fibrillary acidic protein (GFAP) will be used to determine the effect of TNFR2 stimulation on astrogliosis.

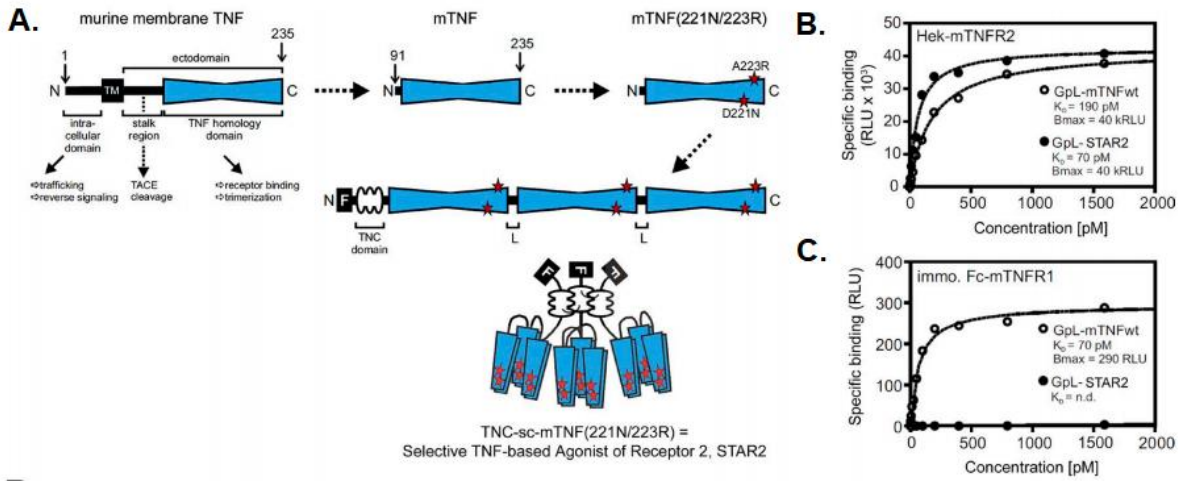


**Figure 3. TNFR2 agonist STAR2**

Schematic representation of STAR2, consisting of 3 linked mTNF (221N/223R) promoters interconnected by a linker and fused to the trimerization domain of the chicken tenascin (TNC) precursor (a). STAR2 stimulation of Hek293 cells, transfected with mouse TNFR2, showed specific binding for TNFR2 (b) with no binding for TNFR1 (c).

When treated with STAR2 it is expected that the induced TNFR2 stimulation dampens the inflammation by release of anti-inflammatory cytokines (Raffaele et al., 2020; Veroni et al., 2010, 2020). Regarding microglia it is expected that the reduced neuroinflammation results in lower coverage and activation of $IBA^+$ microglia. Concerning astrocytes, it is expected that the reduced inflammation dampens astrogliosis leading to lower levels of $GFAP^+$ astrocytes. In addition, it is expected that the reduced inflammation may be accompanied by increased phagocytotic capacity of microglia and astrocytes (Koenigsknecht-Talboo & Landreth, 2005)

# 2. Material and methods

**kiHuTNFR2xJ20 mouse model**

The kihuTNFR2xJ20 mouse model was generated by a crossbred between J20 and kiHuTNFR2 mice, bred at the University of Groningen. The J20 mice used for this crossbreed included C57BL/6J mice with transgenic (tg) APPswind containing the Indiana and Swedish familial AD (FAD) mutations, inducing overexpression of mutant human APP protein (Bearer et al., 2018; Fisher et al., 2018)). For the expression of human TNFR2, to simulate the human context, the J20 mice were crossbred with C57BL/6J mice with a knock-in of human TNFR2. Offspring hemizygous for the tg APPswind mutation and homozygous/heterozygous for kihuTNFR2 were included in this study. Genotyping, using PCR, was performed for controlling the genetic features of the mice. All mice were group-housed followed by single housing starting one week before the onset of the treatment. They were exposed to a day/night cycle of 12/12 h with an environmental temperature of around 21°C, and ad libitum access to water and food. Concerning the animals' health, a daily check was performed combined with monitoring weight every week.

**STAR2 treatment**

For stimulation of the TNFR2 receptor, mice were treated with 375µg/ml STAR2 (University of Würzburg; (Chopra et al., 2016)), twice a week, with an interval of 2 or 3 days, for 6 weeks starting at the age of 6 months. Treatment with PBS was used as a control. Administration of STAR2 was performed using Intraperitoneal (IP) injections.

**Perfusion of the mice and fixation of the brains**

After finishing the treatment, behavioural tests were performed analysing anxiety behaviour (elevated-plus maze), short term memory (Y-maze) and memory and spatial learning (Morris water maze). After performance of the behavioural tests, the mice were sacrificed via perfusion. Brains were post fixated in 0.01M PBS containing 4% PFA for 24 h at RT. The brains were then washed intensively with 0.01M PB, followed by overnight incubation at RT in a 30% sucrose solution to prevent crystal forming. Next, the brains were frozen and stored at -80°C.

**Diaminobenzidine immunohistochemistry – GFAP**

The effect of the TNFR2 activation on $GFAP^+$ astrocytes was determined by the performance of diaminobenzidine (DAB) immunohistochemistry. 20 µM free-floating brain sections were washed 3x 5 min in 0.01 M TBS (pH 7.4) followed by blocking endogenous peroxidase activity using 0.01 M TBS containing 3% $H_2O_2$ for 30 min. at RT. Next, sections were treated with 0.01 M TBS containing 0.1% Triton X-100 (TBST) and 3% BSA (blocking solution) for 30 min. at RT, to prevent non-specific antibody binding. Sections were then treated with primary mouse anti-GFAP (1:10000; 0000078061, Sigma) in blocking solution for 3 nights at 4°C. After another washing step, the sections were incubated with secondary antibody goat anti-mouse (1:500; 147137, Jackson ImmunoResearch) in blocking solution for 2hr at RT. Subsequently, the sections were treated with horseradish peroxidase-labelled avidin-biotin complex (ABC complex 1:500; Vector Laboratories), followed by another washing step. The immunoreactivity was visualised using DAB according to manufactures protocol (SLBR2966V; SigmaFast 3,3'-Diaminobenzidine). All different steps described above were performed on an orbital shaker (100 RPM). After mounting and overnight drying of the sections, a dehydration step was performed. To prevent signal loss, sections were dehydrated in a series of ethanol shifting in Xylene, with a duration of 5 min. for each solution, according to the following order: 70% EtOH, 100% EtOH (2x), 70%/30% EtOH/Xylene, 30%/70% EtOH, and 100% Xylol (3x). Sections were then coverslipped using DPX. Hippocampus regions were imaged with a 10-time magnification using a Leica microscope with Las-X software.

**Diaminobenzidine immunohistochemistry – IBA1**

For analysing the effect of TNFR2 activation on IBA+ microglia, a similar protocol described for detection of $GFAP^+$ astrocytes was used. Adjustments to this protocol are the use of a solution containing 0.01 M TBS containing 3% $H_2O_2$ for blocking the endogenous peroxidase activity. Moreover, no blocking step was used because of the antibody specificity. For detection of IBA-1, primary antibody rabbit anti-IBA (1:2500; CEA1308, LER0547, Wako) and secondary antibody goat anti-rabbit (1:500; 139009, Jackson ImmunoResearch) were used in a blocking solution of 0.01 M TBS containing 0.1% Triton X-100 and 1% BSA. For imaging, the sections a magnification of 20-times was used.

**Data analysis**

The coverage of the antibody complex in hippocampal regions CA1, CA3 and DG were analysed using ImageJ version 1.50i. For statistical analysis, the normal distribution was determined using the Anderson-Darling test, the D'Agostino & Pearson test, the Shapiro-Wilk test, and the Kolmogorov test. Next, the unpaired T-test with Welch's correction was used for normally distributed data, whereby the Mann Withney test was used for data without normal distribution. A P-value $\leq 0.05$ was considered significant. For performing the statistical test Graphpad Prism version 9.0 (Graphpas Software, USA) was used.

# 3. Results

## 3.1 STAR2 reduces coverage microglia in hippocampal regions CA1, CA3 and DG.

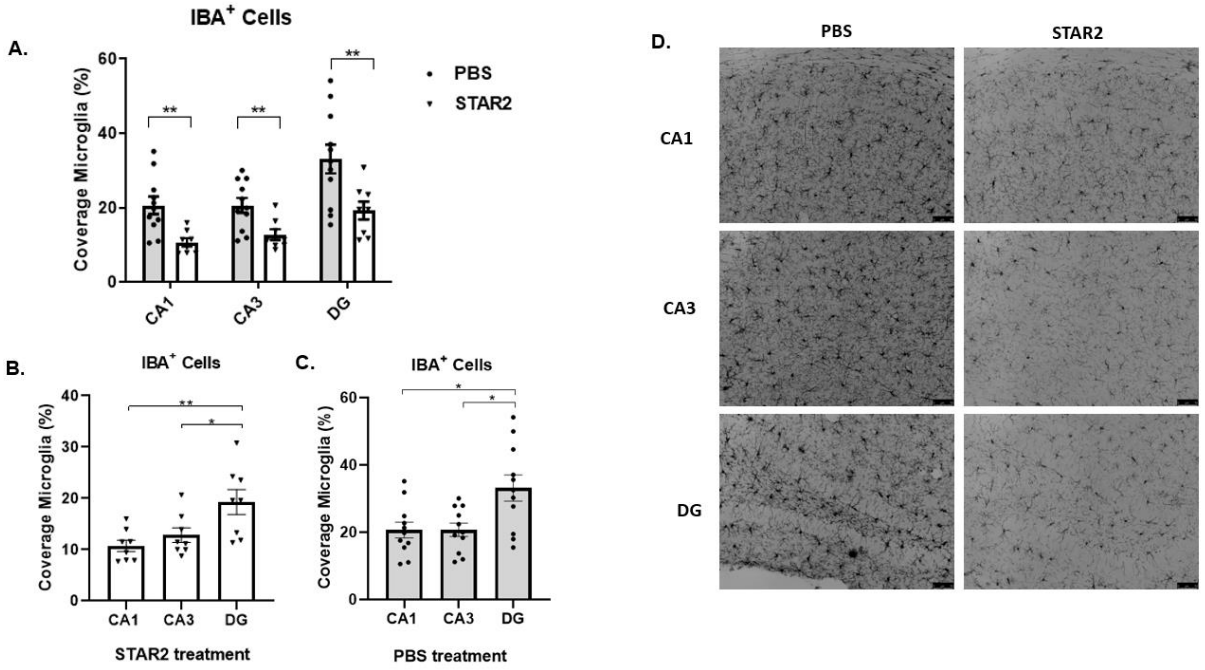


**Figure 4. Effect of STAR2 on microglia**

Effect of STAR2 treatment on the coverage (A-C) of $IBA^+$ microglia in hippocampal regions CA1, CA3 and DG, with visualisation of the distribution of $IBA^+$ microglia in the CA1, CA3 and DG upon treatment with STAR2 and PBS (C). Samples of STAR2 treatment (N=8) were compared with PBS treatment as a control (N=11) (A), as well as the comparison of $IBA^+$ coverage in the CA1, CA3 and DG per treatment (B-C). Error bars represent the standard error of the mean (SEM). Unpaired t-test with Welch correction was performed whereby significant P-values are noted as followed: $P \leq 0.05$(*), $P \leq 0.01$ (**).

To analyse the influence of TNFR2 stimulation on neuroinflammation in AD, the effect of STAR2 on the coverage of $IBA^+$ microglia was analysed. The results show a significant decrease in coverage when treated with STAR2 (Figure 4a). Focussing on the different areas the microglial coverage decreased from 20.66% (+/- 2.34) to 10.76% (+/- 1.087) in the CA1, from 20.70% (+/- 1.99) to 12.83% (+/- 2.43) in the CA3, and from 33.17 % (+/-3.87) to 19.30% (+/- 2.43) in the DG. Concerning the coverage of microglia, a significantly higher percentage of $IBA^+$ microglia were found in the DG, compared to the CA1 and CA3 regions in both the STAR2 treatment group (B) and the PBS treatment group (C). To illustrate, microscopic visualisation of the sections also shows a decrease in coverage in the CA1, CA3, and DG upon STAR2 treatment.

## 3.2 STAR2 reduces activation microglia in the DG.

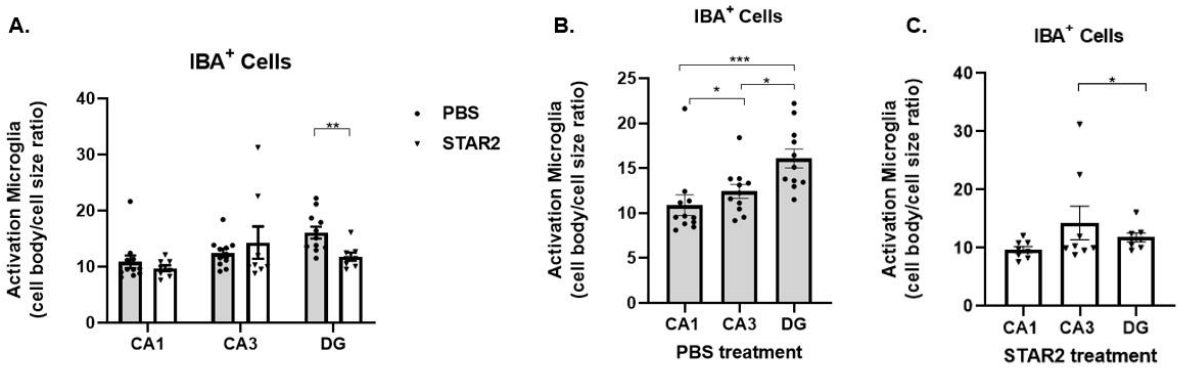


**Figure 5. Effect STAR2 on microglial activation**
Influence STAR2 on the activation of $IBA^+$ microglia in hippocampal regions CA1, CA3, and DG. Samples of STAR2 treatment (N=8) were compared with PBS treatment as a control (N=11) (A), as well as the comparison of microglial activation in the CA1, CA3 and DG per treatment (B, C). Error bars represent SEM. Unpaired t-test with Welch correction and the Mann-Withney test were performed whereby the significant P-values are noted as followed: P≤0.05(*), P≤0.01 (**), P≤0.001 (***).

Since active microglia induce the upregulation of TNF and therefore contribute to the immune response in AD (Veroni et al., 2010), the influence of STAR2 on the activation of microglia was analysed. When treated with STAR2, a significant decrease of active microglia was found in the DG (Figure 5a). Here, the cell body/cell size ratio decreased from 16.08 (+/- 1.061) to 11.81 (+/- 0.73). No significant differences were found in the CA1 and CA3. Focussing on the distribution of active microglia per treatment, a significantly higher ratio of activated microglia was found in the CA3(12.43 +/- 0.77) compared to the CA1 (10.88 +/- 1.15), whereby the DG (16.08 +/- 1.06) contained the highest ratio of activated microglia when treated with PBS. Focussing on the STAR2 treatment, a significantly higher ratio of activated microglia was found in the CA3 (14.31 +/- 2.89) compared to the DG (11.81 +/- 0.73).

## 3.3 STAR2 did not influence the coverage of astrocytes

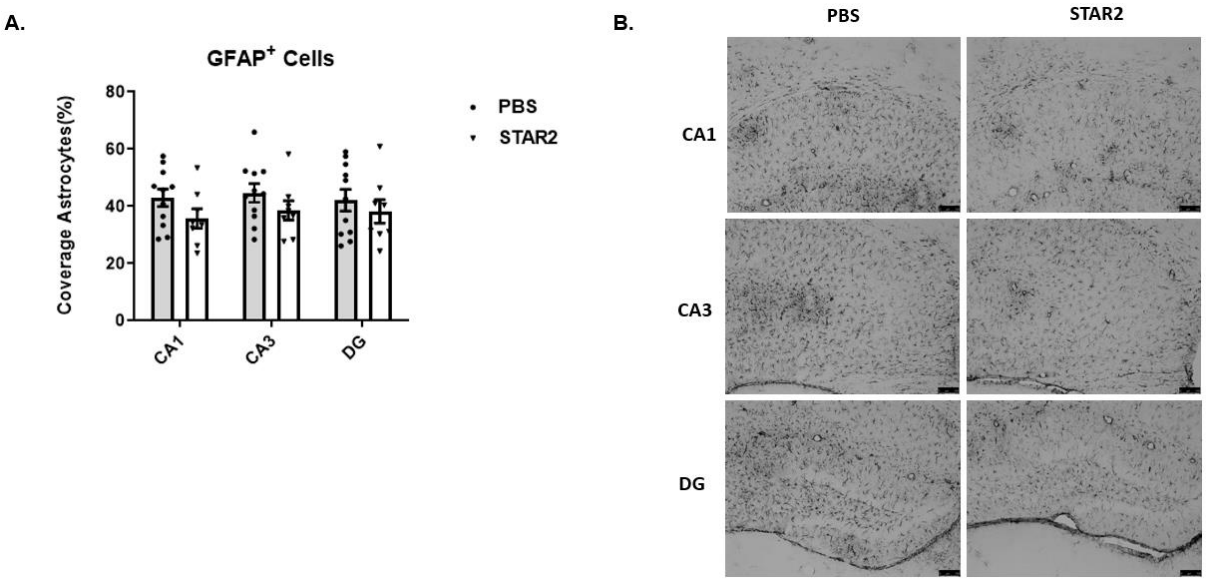


**Figure 6. Effect of STAR2 on astrocytes**
Effect of STAR2 treatment on the coverage (A) of GFAP$^+$ astrocytes in hippocampal regions CA1, CA3 and DG, with visualisation of the distribution of GFAP$^+$ astrocytes in hippocampal regions CA1, CA3 and DG upon treatment with STAR2 and PBS (B). Samples treated with STAR2 (N=8) were compared with PBS treatment as a control (N=11). Bars represent the mean of each group, with error bars representing the SEM. Unpaired t-test with Welch correction was performed whereby the significant P-values are noted as followed: P≤0.05(*), P≤0.01 (**), P≤0.001 (***).

As mentioned before, after the release of TNF by microglia, TNF can activate astrocytes, inducing astrogliosis, whereby the downstream signalling pathways depend on the interaction with its receptors (Osborn et al., 2016). Similarly, as seen by microglia, STAR2 may also interact with the TNFR2 receptor located on astrocytes. Therefore, the coverage of GFAP$^+$ astrocytes was analysed. The results showed no significant differences in the CA1, CA3 nor DG upon treatment with STAR2 (Figure 6a). Also, no significant differences were found between the different areas upon STAR2 or PBS treatment. However, a non-significant decrease is found in all the hippocampal areas. To illustrate, microscopic visualisation of the sections also shows a slight decrease in GFAP$^+$ astrocytes upon STAR2 treatment(Figure 6b).

# 4. Conclusion and discussion

This study aims to determine the effect of TNFR2 stimulation on neuroinflammation in an AD humanized mouse model, whereby STAR2 was used to stimulate TNFR2. Concerning inflammation, this study focused on the coverage and activation of $IBA^+$ microglia and the coverage of $GFAP^+$ astrocytes. The results of this study showed that STAR2 led to a significant decrease in the coverage of $IBA^+$ microglia in hippocampal regions CA1, CA3 and the DG, as well as a significant decrease of activated microglia in the DG. Regarding astrocytes, a non-significant decrease was found in the CA1, CA3 and DG upon STAR2 treatment. These data suggest that STAR2 treatment may reduce inflammation in an AD mouse model.

Concerning the coverage of $IBA^+$ microglia, the significant decrease in $IBA^+$ microglia can be a result of decreased neuroinflammation within the brain. Stimulation of TNFR2 with STAR2 may overrule the maintaining effect of TNFR1 stimulation, which remains since TNFR1 is not inhibited. This can be explained by the expectation that TNFR2 stimulation dampens inflammation by the release of anti-inflammatory cytokines, leading to decreased release of, among others, IFN-γ. Since IFN-γ stimulates a shift towards TNFR1 signalling, decreased inflammation may restore this shift, leading to less TNFR1 activation with reduced inflammation and neurodegeneration as a result (Raffaele et al., 2020; Veroni et al., 2010, 2020). Furthermore, decreased coverage of $IBA^+$ microglia can also be a consequence of reduced αβ plaques. In AD, deposition of αβ may be a result of decreased phagocytotic capacity of microglia and astrocytes (Koenigsknecht-Talboo & Landreth, 2005; Ries & Sastre, 2016). A study by Koeningsknecht-Talboo et al. (2015) showed that pro-inflammatory cytokines inhibit phagocytosis of fibrillar αβ, which can be resolved by anti-inflammatory cytokines, such as ibuprofen, a cyclooxygenase inhibitor, or an E-prostanoid receptor antagonist. They suggest that pro-inflammatory cytokines stimulate formation of prostaglandins, inducing inhibition of phagocytosis (Koenigsknecht-Talboo & Landreth, 2005). Since TNFR2 activation may induce release of anti-inflammatory cytokines, treatment with STAR2 may restore the phagocytotic capacity resulting in increased clearance of αβ plaques. Since αβ plaques induce neuroinflammation on its term, by activating microglia (Cieślak & Wojtczak, 2018), increased clearance may explain the decrease in coverage of $IBA^+$ microglia as well as the decreased activation seen in the DG. Regarding literature findings, Ugoli et al. (2018) showed no differences between CA1 and CA3 in untreated AD mice at the age of 3 and 6 months (Ugolini et al., 2018). These data correspond to the findings within the PBS treatment group. Since mice, included in the STAR2 study were already 6 months old before the onset of the treatment, the data cannot be supported by Ugolini's findings. However, this data may indicate that the coverage between the CA1 and CA3 in AD may be similar.

Concerning microglial activity, a decreased activation can also be explained by the features of TNFR2 downstream signalling. Stimulation of TNFR2 generally induces neuroprotection, tissue remodelling as well as remyelination of axons. This may lead to less neuronal damage accompanied by decreased ATP release. Since ATP activates microglia, lower levels of ATP may explain a decrease in activated microglia (Raffaele et al., 2020). In addition, ATP-induced activation of microglia also stimulates release of TNF, inducing further activation of microglia (Raffaele et al., 2020). Lower levels of ATP may therefore lead to decreased TNF release, resulting in reduced microglial activation. Interestingly, only a significant decreased activation was found in the DG, suggesting that the interaction between STAR2 and TNFR2 may differ in the CA1 and CA3. Since TNFR2 is essential for the effect of STAR2, and previous studies showed a decrease in TNFR2 receptors in AD (Cheng et al., 2010; Zhao et al., 2003), lower amounts of TNFR2 may be expressed in the CA1 and CA3. Therefore, it would be interesting to analyse the TNFR1/TNFR2 ratio within the different hippocampal regions of the brain. Substantiating, when focusing on both treatments individually, the results showed that the DG significantly contains the highest coverage of $IBA^+$ microglia upon STAR2 and PBS treatment. Regarding treatment with PBS, the DG contains the highest ratio of activated microglia, followed by the CA1 and CA3. These data suggest that in untreated AD, the coverage of microglia may correlate with the activation of these microglia. Meaning the higher the coverage, the higher the activation ratio which can be explained by the increased activation of microglia in general upon simulation by, among others αβ plaques, ATP and inflammatory cytokines such as TNF. Concerning the STAR2 treatment, the DG

contained significantly lower activated microglia compared to the CA3, suggesting that the effect of STAR2 treatment may depend on the number of microglia present. This can be explained by the increased anti-inflammatory response upon TNFR2 activation in the DG compared to the CA3, resulting in decreased microglial activation. However, when comparing the DG and CA3 with CA1, no significant differences were found in activated microglia, despite the significant higher coverage of microglia in the DG.

When comparing these data with literature findings, no other studies could be found analysing $IBA1^+$ microglia in the CA1, CA3 and DG upon STAR2 and PBS treatment to compare the studies data with. However, a study by Pomilo et al (2016) analysed the amount of activated $IBA^+$ microglia in the hilus and stratum radiatum at the age of 3, 9 and 15 months in promoter-driven amyloid precursor protein (PDAPP) J20 mice (Pomilio et al., 2016). They demonstrated that activation of IBA+ microglia starts in the hilus, followed by activation in the stratum radiatum in PDAPP-J20 mice (Pomilio et al., 2016). Since the hilus is represented by the DG and the stratum radiatum covered by the CA1 and DG, these data suggest that the onset of the activation occurs in the DG and followed by the CA1 and CA3. This data may explain the significantly increased microglia activation ratio in the DG compared to the CA1 and CA3 upon PBS treatment. Concerning the STAR2 treatment, it might be that the influence of TNFR2 stimulation is most effective in the DG because of the increased microglia immunoreactivity in this region in AD.

Furthermore, since STAR2 induces a significant decrease in coverage of $IBA^+$ microglia and a decreased activity in the DG, it would be expected that this decrease would also be seen in coverage of $GFAP^+$ astrocytes. Besides the induced astrogliosis upon TNF, released by microglia, it is expected that STAR2 may also interact with the TNFR2 receptor on astrocytes. This would, among others, stimulate release of neuroprotective factors CXCL12 and LIF (Raffaele et al., 2020) leading to neuroprotection, dampening of neuroinflammation and an increase of αβ clearance as mentioned before, which ultimately leads to reduced $GFAP^+$ astrocytes levels. Since the results show a non-significant decrease in the coverage of $GFAP^+$ astrocytes, the induced effect by STAR2 may not be strong enough to also modulate $GFAP^+$ astrocytes directly or via microglia. Focusing on AD, no significant differences were found within the PBS treatment. A study by Ugolini et al. (2018) showed a significantly increased density in the CA1 compared to the CA3 in AD mice at the age of 3 and 6 months (Ugolini et al., 2018). They suggest that the difference in areas may depend on the variety in involvement of these cells in AD and its pathogenesis (Ugolini et al., 2018).

Next, as mentioned in the introduction, this study is a follow-up study, analysing the effect of TNFR2 stimulation in a human context. Importantly, this study used STAR2 with an activity of 15%, compared to an 85% activity used in the unpublished previous study of N. Ortí Casañ et al. The 15% activity was a result of an incorrect dilution of the ordered product. However, this different activity provides the opportunity to analyse whether there might be a different response upon different STAR2 concentrations (Appendix A – B).

When comparing the coverage and activation of $IBA^+$ microglia and the coverage of $GFAP^+$ astrocytes, the results of STAR2 treatment with 85% activity show a significant increase in the coverage (Appendix A: Figure 1b) and activation (Appendix A: Figure 1d) of $IBA^+$ microglia in the DG. Regarding the astrocytes, significant increases in the CA1, CA3 and DG were found upon STAR2 treatment (Appendix B: 1b). These data suggest that increased activity of STAR2 stimulates microglia and astrocytes. These results could be explained by increased phagocytotic capacity of microglia and astrocytes. As mentioned before, release of anti-inflammatory cytokines upon TNFR2 stimulation may restore the phagocytotic capacity, inhibited by the inflammation in AD. The use of STAR2 with an 85% activity may dampen the neuroinflammation even more, compared to STAR2 with an activity of 15%, leading to a higher increase of phagocytotic capacity.

The AD mouse models used for this and the previous study both contained mutations inducing overexpression of mutant human APP(Fisher et al., 2018). Since this mutation is genetically induced, the overexpression of mutant human APP will remain, even after removal of αβ plaques. As a result of decreased inflammation, the restored phagocytotic capacity may lead to increased activation of

microglia and astrocytes stimulating in clearance of αβ plaques. Concerning STAR2 treatment stimulation, lower level of neuroinflammation as a result of STAR2 with an 85% activity, compared to STAR2 with 15% activity, would explain the increased activity of microglia and astrocytes. Therefore, it would be interesting to analyse the coverage of αβ plaques as well as the phagocytic capacity of microglia related to the presence of these plaques. A pilot study using phagocytotic marker CD68 combined with 6e10, a marker for αβ plaques, already showed its potential in analysing the interaction between $CD68^+$ microglia and αβ (Appendix D). Moreover, since IBA1 is used to analyse the activity based on cell body/cell ratio, it would also be interesting to also analyse the coverage of resting/homeostatic microglia, by using P2RY12 as a marker (Walker et al., 2020) to support the activation status of these microglia. A pilot study already showed the presence of $P2RY12^+$ microglia in hippocampal regions in the AD J20 mouse model (Appendix E).

Moreover, when binding TNFR2, STAR2 may also compete with released tmTNF, which may lead to increased levels of tmTNF in the extracellular space. Since tmTNFR can also interact with TNFR1 (Dong et al., 2016), the induced neuroinflammation would then explain the increase in $IBA^+$ microglia when using an increased activity of STAR2. However, this would not explain the reduction in αβ plaques, because the induced inflammation would increase the number of these plaques (Appendix C) (Koenigsknecht-Talboo & Landreth, 2005; Ries & Sastre, 2016). Therefore, it would be interesting to analyse levels of hippocampal tmTNF and solTNF. In addition, downstream products, such as IL-6, IL-8, IL10, and G-CSF released by activation of both receptors should also be analysed to provide knowledge about the inflammation status (Fischer et al., 2015; Holbrook et al., 2019; Veroni et al., 2010). Next, analysis of IFN-γ would also be interesting, since this pro-inflammatory cytokine stimulates a shift towards the TNFR1 signalling (Raffaele et al., 2020; Veroni et al., 2010, 2020).

Besides all the different analysis which should be incorporated in analysing the effect of STAR2 on neuroinflammation, this study must be repeated using the 85% activity of STAR2. Only when using STAR2 with the same activity, it is possible to compare the effect in the kihuTNFR2xJ20 crossbreed with the effect in the J20 mouse model. After completion of this analysis, different percentages of STAR2 could be used to determine whether there are different effects or not. The effect of a higher activity may, for example, induce a more prolonged effect, compared with lower STAR2 activity. Also, when analysing STAR2 with different activities, it is necessary to perform this study in the same mouse model to prevent model-specific interaction.

Finally, future research should also focus on investigating the passage of STAR2 across the blood-brain-barrier (BBB), to analyse whether the effect is in the brain or originates from the periphery. Moreover, since STAR2 is distributed via IP injections, the effect of STAR2 on other cells, containing the TNFR2 receptor, should also be analysed. A study by Gao et al. (2017), for example, showed that ablation of microglial TNFR2 induces the early onset of EAE, suggesting the protective prospects of microglial TNFR2 stimulation. However, when stimulating monocytes and macrophages, located in the periphery, the early onset of EAE was induced (Gao et al., 2017). This study implicates that the effect of TNFR2 stimulation can be contradictive depending on the cell type. To analyse the effectiveness of the treatment, the influence of TNFR2 stimulation on other TNFR2 containing cells should also be analysed preventing adverse effects on the disease's pathogenesis.

In conclusion, stimulation of TNFR2 might be a promising treatment regarding modulation of the immune response seen in AD focusing on microglia and astrocytes. However, further research is necessary to determine the influence of STAR2 on the inflammation status in AD as well as its effect in a humanized AD mouse model.

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# Appendix

## A. High activity STAR2 induces an increase in IBA$^{+}$ microglia.

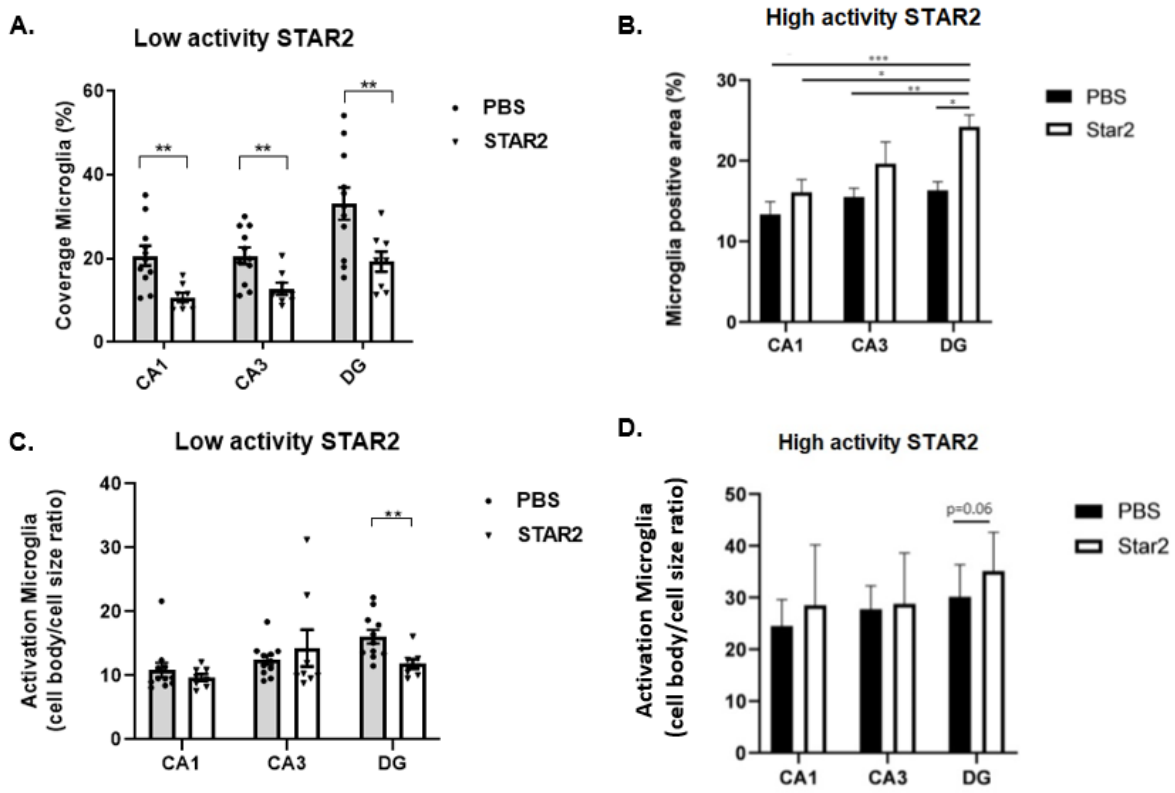


**Figure 1. The effect of STAR2 activity on microglia.**
Influence of low activity of STAR2 (15%) and high activity of STAR2 (85%) on the coverage (A-B) and activation (C-D) of microglia in hippocampal regions CA1, CA3 and DG. Samples of STAR2 treatment were compared with PBS treatment as a control. Low activity STAR2: STAR2 treated group N=8, PBS treated group N = 11. High activity STAR2: STAR2 treated group N= 11. PBS treated group N=13. Error bars representing the SEM. Unpaired t-test and the Mann-Withney test were performed whereby the significant P-values are noted as followed: P<0.05(*), P<0.01 (**), and P<0.001 (***).

## B. High activity STAR2 has an increased effect on GFAP$^+$ coverage.

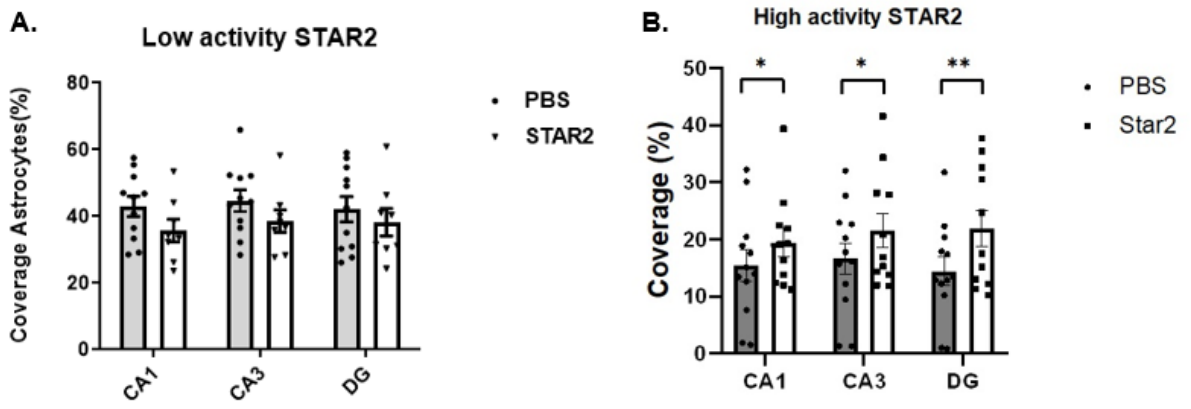


**Figure 2. The effect of STAR2 activity on astrocytes.**
The effect of low activity of STAR2 (15%) and high activity of STAR2 (85%) on the coverage (A-B) of GFAP$^+$ astrocytes in hippocampal regions CA1, CA3 and DG. Samples of STAR2 treatment were compared with PBS treatment as a control. Low activity STAR2: STAR2 treated group N=8, PBS treated group N = 11. High activity STAR2: STAR2 treated group N= 11. PBS treated group N=13. Bars represent the mean of each group, with error bars representing the SEM. An unpaired T-test was performed whereby the significant P-values are noted as followed: P<0.05(*) and P<0.01 (**).

## C. High activity STAR2 induces a decrease in αβ plaque coverage.

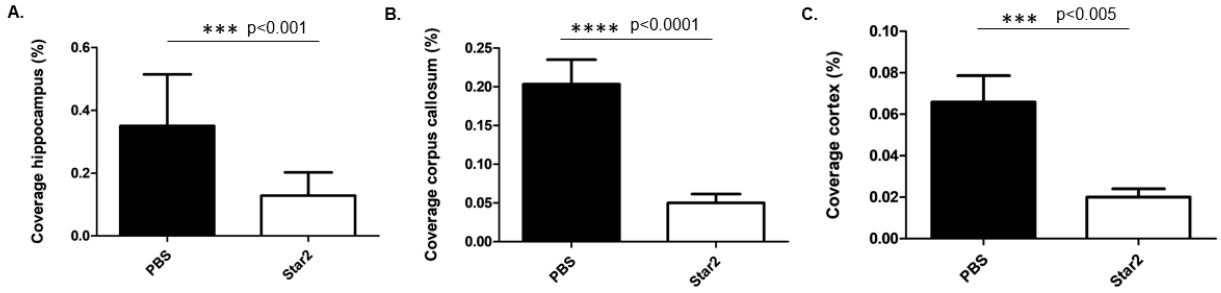


**Figure 3 The effect of 85% activity STAR2 on αβ plaques in different brain regions**
Effect of 85% STAR2 activity on coverage of αβ plaques in the hippocampus (A), corpus callosum (B) and cortex (C). Samples STAR2 treatment (N=11) were compared with treatment using PBS (N=13) as a control. Bars represent the mean of each group, with error bars representing the SEM.

## D. CD68 +6e10 pilot study

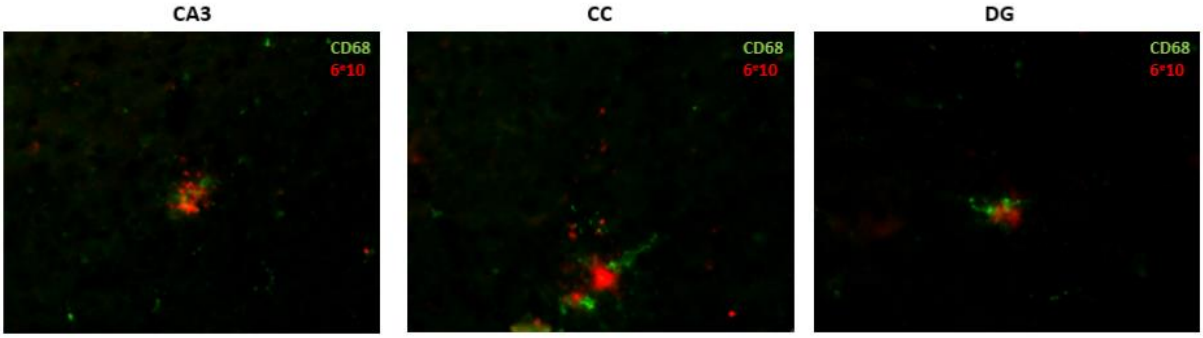


**Figure 4. Pilot staining CD68 + 6e10**
Immunofluorescent pilot staining of microglial marker CD68 (green) and αβ plaque marker 6e10 (red) in hippocampal areas CA3, corpus callosum (CC) and the DG of J20 mice.

**Methods fluorescent confocal immunohistochemistry – CD68 + 6e10**
Two-colour fluorescent confocal immunohistochemistry was performed, to analyse the interaction and deposition of microglia and αβ-plaques. 20 µM Free-floating brain sections were washed 6 times 5 min. with 0.01 M TBS (pH 7.45), followed by blocking endogenous peroxidase activity using TBS containing 1% $H_2O_2$ for 30 min. at RT. Sections were then blocked, after another washing step, with 0.01M TBS containing 0.3% Triton X-100 (TBST) and 3% BSA for 1 h at RT. Incubation with primary antibodies rat anti-CD68 (1:1000; 152755, Bio-rad) and mouse anti-6e10 (1:2000; B287037, Biolegend) in TBST containing 3% BSA was carried out overnight at 4°C. Next, the sections were incubated, after a washing step, with secondary antibodies Alexa Fluor 488 donkey anti-rat IgG (1:500: 2180272, Invitrogen) and Alexa Fluor 555 donkey anti-mouse IgG (1:500; Ti271028, Invitrogen) for 2 h at RT, followed by another washing step. Sections were then mounted and coverslipped using MOWIOL mounting medium. Hippocampus regions were imaged with a 40-time magnification using a Leica microscope with Las-X software. All different steps are performed on an orbital shaker (100 RPM).

## E. P2RY12 pilot study

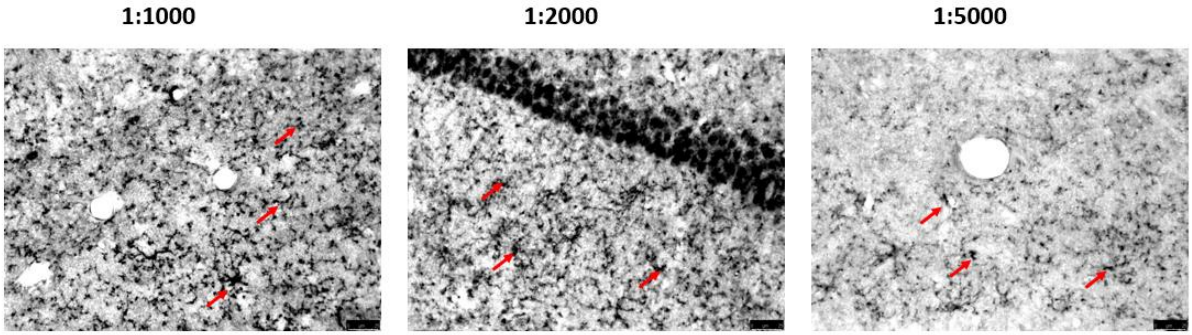


**Figure 3. Pilot staining P2RY12 hippocampal region CA1**
Immunohistochemical pilot staining of P2RY12 in hippocampal region CA1 of J20 mice. Antibody dilutions 1:1000, 1:2000, 1:5000 were used to target the receptor. P2RY12+ microglia are designate by the red arrows.

**Methods diaminobenzidine immunohistochemistry P2RY12**

The effect of the TNFR2 activation on P2Y12+ microglia was determined by the performance of diaminobenzidine (DAB) immunohistochemistry. 20 µM free-floating brain sections were washed 6x 5 min in 0.01 M TBS (pH 7.4) followed by blocking endogenous peroxidase activity using 0.01 M TBS containing 1% $H_2O_2$ for 30 min. at RT. Next, sections were treated with 0.01 M TBS containing 0.3% Triton X-100 (TBST) and 3% BSA (blocking solution) for 1 h at RT, to prevent non-specific antibody binding. Sections were then treated with primary rabbit anti-P2Y12 (1:2000; SCLB3272, Sigma) in blocking solution for 3 nights at 4°C. After another washing step, the sections were incubated with secondary antibody goat anti-rabbit (1:500; 139009, Jackson ImmunoResearch) in blocking solution for 2hr at RT. Subsequently, the sections were treated with horseradish peroxidase-labelled avidin-biotin complex (ABC complex 1:500; Vector Laboratories), followed by another washing step. The immunoreactivity was visualised using DAB according to manufactures protocol (SLBR2966V; SigmaFast 3,3'-Diaminobenzidine). All different steps described above were performed on an orbital shaker (100 RPM). After mounting and overnight drying of the sections, a dehydration step was performed. To prevent signal loss, sections were dehydrated in a series of ethanol shifting in Xylene, with a duration of 5 min. for each solution, according to the following order: 70% EtOH, 100% EtOH (2x), 70%/30% EtOH/Xylene, 30%/70% EtOH, and 100% Xylol (3x). Sections were then coverslipped using DPX. Hippocampus regions were imaged with a 20 or 40-time magnification using a Leica microscope with Las-X software.

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