Alzheimer's
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Novel fully human high-affinity anti-TREM2 antibody shows efficacy in clinically relevant Alzheimer´s mouse model

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Abstract

Background New drugs to treat Alzheimer´s disease (AD) are urgently needed. Human triggering receptor expressed on myeloid cells 2 (hTREM2) is a validated drug target which is genetically associated with AD. Existing anti-hTREM2 antibodies were raised in animal immune systems, and subsequently humanized, which may incur immunological complications upon repeated preventive or therapeutic applications in vivo in AD patients. In addition, anti-hTREM2 antibodies should be optimized for both, efficacy and safety.

Methods A novel fully human monoclonal brain-targeting anti-hTREM2 antibody M07-TFN was created. Binding affinities, cell viabilities, and agonist potencies were investigated on rhTREM2 and in human microglia. Transcytosis assays modeled blood–brain barrier translocation (BBB). Behavior tests were carried out in 5 × familiar AD (5xFAD) mice of both genders, to test for brain function and cognition as well as hippocampus-dependent spatial memory using the Barnes maze. In addition, amyloid plaque formation was determined on brain sections at the end of the study.

Results M07-TFN showed higher binding affinities and stronger activation of hTREM2 signaling than all previously described anti-hTREM2 antibodies. p-Syk activation was increased 30-fold in hTREM2-overexpressing HEK293 cells and fourfold in human microglia cells compared to baseline. Human microglia viability significantly improved after stress testing. M07-TFN showed strong BBB translocation in a human BBB model, and exerted cross-reactivity to the mouse TREM2 stalk region, which allowed us to investigate M07-TFN directly in an AD mouse model. In 5xFAD mice, M07-TFN resulted in improved novel object location and better spatial orientation and memory, and significantly reduced plaque load. Additional safety investigations in mice showed no negative effects on blood cells or major organs.

Conclusion Compared to existing humanized anti-hTREM2 antibodies that have been investigated in clinical trials, M07-TFN showed best-in-class affinities and agonist potencies. Being a fully human anti-hTREM2 antibody, M07-TFN holds the promise of reduced immunogenicity for use in human patients.

Keywords Alzheimer's disease, Microglia, Neurodegeneration, TREM2, Antibody

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Introduction

Neurodegenerative disorders such as Alzheimer´s disease (AD) result in age-associated progressive deterioration of neuronal structures, ultimately leading to cognitive disability and dementia. AD is the most common form of dementia. It affects millions of people worldwide, occurs spontaneously or familial, and is favored by a combination of risk factors such as aging and genetic predisposition [1]. There is currently no cure for AD and current treatments are mainly aimed at relieving symptoms and slowing progression. Therefore, new strategies to interfere with plaque deposition need to be developed.

Triggering receptor expressed on myeloid cells 2 (TREM2) is a transmembrane receptor expressed on myeloid cells and is essential for activation of microglia cells. TREM2 mutations resulting in TREM2 loss of function (LOF) through a variety of mechanisms have been identified in neurodegenerative disorders such as AD [2–5]. Mutations in TREM2 coding region significantly increase AD risk [6, 7], suggesting that TREM2 LOF contributes to AD onset. Therefore, increased TREM2 function may be beneficial in AD.

TREM2-mediated signaling in microglia cells induces a transition of homeostatic microglia into disease-associated microglia (DAM) [8]. This transition is phenotypically characterized by enhanced phagocytosis, migration, and cell survival. Activation of TREM2 signaling is mediated through the adaptor protein DAP12. Upon ligand binding to TREM2, the ITAM motif of DAP12 becomes phosphorylated, which results in the recruitment of Syk kinase and activation of downstream signaling molecules. The signaling is terminated by shedding of the extracellular domain of TREM2 mediated by α-secretases, which results in the release of soluble TREM2 (sTREM2) [9, 10]. Altered levels of sTREM2 have been reported in the cerebrospinal fluid of AD patients and sTREM2 has been suggested as a potential disease biomarker [11]. The cleavage site of TREM2 has been identified at His 157 within the stalk region [5, 12, 13]. Based on published humanized anti-TREM2 antibodies that bind to this epitope [14, 15], we now report fully human anti-hTREM2 antibodies which were identified in a phage display screening using this peptide epitope. In a number of studies, agonistic monoclonal antibodies have been investigated that target mouse or human TREM2. The published antibodies have been shown to activate TREM2 to varying levels determined by Syk phosphorylation (p-Syk increase from twofold up to 18-fold). In addition, the anti-TREM2 antibodies have been tested in myeloid cells employing either bone marrow derived macrophages (BMDMs) [16–19], primary mouse microglia cells [14, 20] or one specific model of serum-exposed human induced pluripotent stem cell (hiPSC)-derived microglia cells [15] in order to determine activating effects in functional assays. Some of the antibodies have been tested for their effects on cognition in the 5xFAD mouse model through behavioral studies [17, 20–22].

To improve upon existing humanized anti-hTREM2 antibodies, we identified a fully human monoclonal anti-hTREM2 antibody. Therapeutic fully human antibodies have shown less immunogenicity and hence increased long-term efficacy in larger human patient populations, as demonstrated e. g. by the recent approval of fully human vs. humanized anti-proprotein convertase subtilisin/kexin type 9 (PCSK9) antibodies [23] to treat hypercholesterinemia, which are also being developed for Alzheimer´s disease.

In addition, we aimed to reduce antibody doses by maximizing binding affinities, p-Syk activation, viability of target cells (as determined in hiPSC-derived microglia cells), and blood–brain barrier (BBB) translocation. The best-performing candidate was further investigated in vitro in an iPSC-based model of human microglial stress, and in a relevant Alzheimer´s disease model for brain alterations and in extensive cognition tests in 5xFAD mice.

Results

M07 directed against hTREM2 stalk region is specific for human and mouse TREM2

We generated a fully human antibody binding to human TREM2 (hTREM2) termed M07. We introduced an IgG1-LALA modification in order to reduce effector function of the antibody (M07-WT) [24]. In addition, we generated a modified M07-WT version termed M07-TFN, which binds to the human transferrin receptor 1 (TfR1) to allow for efficient uptake via the human BBB [25]. The full antibodies M07-WT and M07-TFN were engineered in symmetric bispecific designs, as outlined in Fig. 1A. In order to determine the apparent binding affinities of both antibodies to hTREM2, we performed ELISA binding assays using full-length hTREM2. We observed a strong binding of both antibodies, M07-WT and M07-TFN to hTREM2, as compared to the Isotype control antibody (Fig. 1B). M07-WT bound with an apparent EC50 value of 0,103 nM and M07–TFN with an apparent EC50 of 0,093 nM, corresponding to approximate K_D values of 0,051 nM (M07-WT) and 0,046 nM (M07-TFN). We ruled out any unspecific binding of the antibodies to hTREM1.

Agonistic anti-hTREM2 antibodies have previously been designed to bind to the stalk region of TREM2 in order to prohibit TREM2 cleavage and thereby increase the amount of TREM2 receptor on the cell surface [14]. This enhanced both binding to the TREM2 receptor and subsequent signal transmission. To identify precise

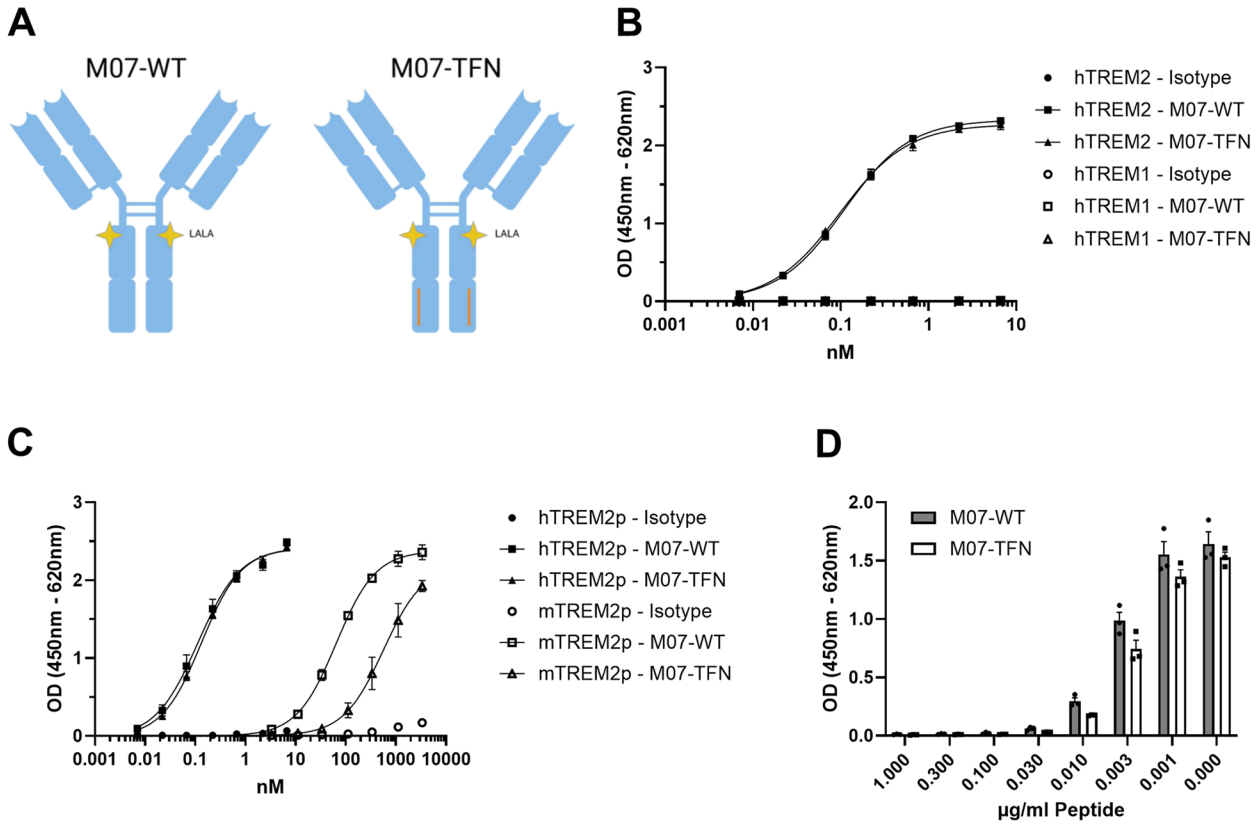

Fig. 1 M07 binds human and mouse TREM2 efficiently **A** Schematic representation of M07-WT and M07-TFN. Both antibodies contain the LALA modification (yellow stars). M07-TFN was modified with a blood–brain-barrier shuttle in both CH3 domains of the Fc region within the heavy chain (orange bars) **B** Binding of M07 to human full-length TREM2 or TREM1. Data represent the mean ± SEM ($n = 3$) **C** Binding of M07 to human or mouse TREM2 peptide fragment of the stalk region. Data represent the mean ± SEM ($n = 4$). **D** Competition ELISA with hTREM2 peptide to determine specific binding of M07 to hTREM2 peptide. Each dot represents an independent experiment. Data represent the mean ± SEM ($n = 3$)

antibody binding epitopes of TREM2, a peptide fragment (DAGDLWFPG) representing a specific epitope from the human TREM2 stalk region (aa 137–162) was investigated by an ELISA binding assay (Fig. 1C). M07-WT showed a high affinity for this specific hTREM2 epitope peptide with an apparent EC50 of 0,107 nM. The apparent EC50 value of M07-TFN was comparable to M07-WT with 0,126 nM, and K_D values were estimated at 0,053 nM (M07-WT) and 0,063 nM (M07-TFN). In addition, M07-WT showed cross-reactivity with mouse TREM2 (mTREM2), and relevant binding, albeit with lower affinity, as shown in Fig. 1C. M07-WT bound mouse TREM2 peptide with an apparent EC50 of 64 nM and M07-TFN with an apparent EC50 of 561 nM. K_D values of this interaction are typically estimated to be less than half of these values.

In order to determine specificity of antibody binding to hTREM2, we performed a competition ELISA. To this aim, we used the peptide fragment from the hTREM2 stalk region (DAGDLWFPG) to block binding of M07 to hTREM2 peptide (Fig. 1D). Binding of M07-WT and M07-TFN to hTREM2 peptide was completely blocked by addition of excess peptide in solution, showing specific binding of M07 to the hTREM2 stalk region.

M07 efficiently activates TREM2-expressing cells in a Syk-dependent manner

In order to test activation of TREM2 signaling via agonistic anti-hTREM2 antibodies, we employed a p-Syk AlphaLISA assay, which allowed detection of human Syk phosphorylation that occurs upon ligand binding to human TREM2. HEK293 Flp-In cells stably expressing human TREM2 and human DAP12 were incubated with the antibody at different concentrations for 5 min at 37°C before Syk phosphorylation was determined. Treatment of HEK293 Flp-In hTREM2/hDAP12 cells with both variants of M07 resulted in strongly increased p-Syk activity (Fig. 2A). M07-TFN potently activated p-Syk, as demonstrated by a 30-fold increase over baseline. M07-WT treatment resulted in a tenfold increase of p-Syk.

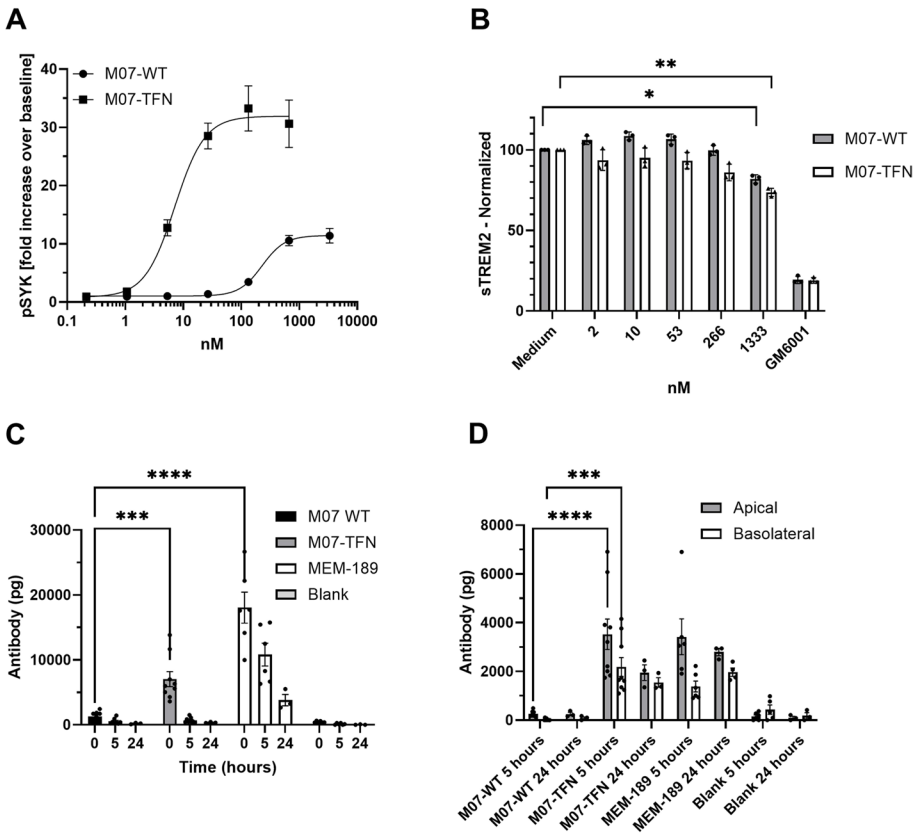

Fig. 2 M07 potently activates Syk signaling, decreased shedding and is shuttled across a human in vitro blood–brain barrier model **A** Titration curves of the anti-hTREM2 antibodies M07-WT and M07-TFN demonstrating strong activation of p-Syk signaling in HEK293 Flp-In hTREM2/hDAP12 cells. Data represent mean ± SEM ($n = 3$) **B** Titration of M07 antibodies for inhibition of TREM2 shedding in HEK293 Flp-In hTREM2/hDAP12 cells. M07-WT and M07-TFN inhibited TREM2 cleavage (one-way ANOVA; *, $p < 0.05$; **, $p < 0.01$). Each dot represents an independent experiment. The mean ± SEM is plotted ($n = 3$) **C** M07-TFN and anti-hTfR1 antibody MEM-189 were present in hCMEC/D3 lysates immediately after the pulse (0 h) with decreasing levels over time. M07-WT was not detected at significant levels. Two-way ANOVA; *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$; and ****, $p < 0.0001$. Each dot represents an independent experiment. The mean ± SEM is plotted **D** M07-TFN and MEM-189 were detected in the chase media of both the apical and basolateral compartments at 5 and 24 h. M07-WT, at both 5 and 24 h, was present at background levels similar to the blank. Two-way ANOVA; *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$; and ****, $p < 0.0001$. Each dot represents an independent experiment. The mean ± SEM is plotted

TREM2 shedding is partially reduced by M07 binding

TREM2 signaling is terminated by shedding of the extracellular domain of TREM2, resulting in sTREM2 release. Anti-TREM2 antibodies that bound to the TREM2 stalk region encompassing the cleavage site (His 157) block TREM2 cleavage, and thereby increase TREM2 signaling [26–28]. This potentiated the activating effect of agonistic anti-TREM2 antibodies on the TREM2 receptor.

M07 binds the stalk region of TREM2 and should therefore interfere with TREM2 cleavage. In order to test this, we measured sTREM2 levels in the supernatants of HEK293 Flp-In cells stably expressing human TREM2 and human DAP12 after incubation with M07 antibody variants for 24 h. sTREM2 levels were determined using an ELISA assay. sTREM2 concentrations, and therefore shedding of the receptor, decreased with increasing concentrations of M07 antibody (Fig. 2B). At a concentration of 1333 nM, M07-WT significantly decreased sTREM2 by 20%. Similar results were observed with M07-TFN. The ADAM-10/17 inhibitor GM6001, used as a positive control, mediated nearly full inhibition of TREM2 cleavage.

M07-TFN but not M07-WT exhibits brain shuttling in a human in vitro BBB model

In order to assess the brain shuttling capacity of the Fc domain modification on M07-TFN that confers hTfR1 binding (Supp. Figure 1), a BBB transwell model was established using the immortalized human brain endothelial cell line hCMEC/D3. The hCMEC/D3 monolayer exhibits weak barrier properties measured by transendothelial electrical resistance (TEER) and permeability to dextrans but expresses hTfR1 (data not shown). In order to rectify the leaky barrier, a pulse-chase

transcytosis assay was performed [29]. A commercially available mouse IgG1 antibody against TfR1 (clone MEM-189) was used as a positive control. After a 1 h "pulse", M07-TFN and anti-TfR1 antibody MEM-189 were detected at high levels in the cell lysates and decreased in a time-dependent manner (Fig. 2C). M07-TFN and MEM-189 but not M07-WT were observed in the media of the apical and basolateral compartments (Fig. 2D). Taken together, this suggests a human TfR1-binding dependent uptake of M07-TFN and MEM-189 followed by a release to either the basolateral (transcytosis) or apical compartment (recycling). Interestingly, the quantity of uptake (cell lysates) and kinetics (time-dependent release) appear to differ between M07-TFN and MEM-189 while the amount of antibody transcytosed and recycled is comparable.

M07 activates hiPSC-derived microglia and increases viability

In order to test how strongly M07-WT and M07-TFN antibodies activate hiPSC-derived microglia cells, we performed p-Syk AlphaLISA assays in this cell type. Antibody treatment of microglia cells with M07-WT and M07-TFN resulted in fourfold increases of p-Syk activity (Fig. 3A and B).

Next, we asked if stressed microglia cells are more viable when activated by M07 antibodies. Microglia stress was induced by decreasing the key cytokines present in the culture medium (M-CSF, IL-34 and TGFβ1) from 100 to 50%, 25% or 0%. Afterwards, ATP was measured as an indicator of viability (Fig. 3C). When the cells were not stressed (100% cytokines), no effects were observed upon treatment with the antibodies. However, when the cells were exposed to mild or high stress (50%, 25% or 0% cytokine concentration), microglia viability was strongly decreased. This effect was comparably rescued by M07-WT and M07-TFN. Treatment of the microglia cells with control IgG antibody did not result in any beneficial effect on cell survival.

Single dose of M07-TFN antibody shows expected peak serum levels and half-life in mice

The antibody M07-TFN was given to mice intravenously at a dose of 30 mg/kg body weight, and serum levels of M07-TFN were assessed by ELISA. Results are shown in Fig. 4. As typical for all pharmacokinetic studies in mice, peak serum values and half-life were estimated from apparent data. True peak values were much likely higher than those observed 4 h after dosing, but this could not be further investigated because sampling volumes and times in mice are limited by feasibility and animal protection laws. Serum levels declined with an estimated half-life of 12 h and were barely detectable by ELISA after 14 days.

Single dose of M07-TFN antibody slightly improves cognitive decline in 5xFAD mice

Since the M07-TFN bound to mouse TREM2, we wanted to test effects of the antibody in an Alzheimer´s disease mouse model. In order to test the efficacy and safety of M07-TFN, we performed a single-application mouse study in 5xFAD mice with mixed gender and observation over 3 weeks. The study was conducted in a blinded fashion and we did not observe any weight loss or health issues in the treated animals. All, animals safely completed the study. To assess the effects of M07-TFN on brain function, learning and memory formation in 5xFAD mice, we performed Novel-Object-Recognition/

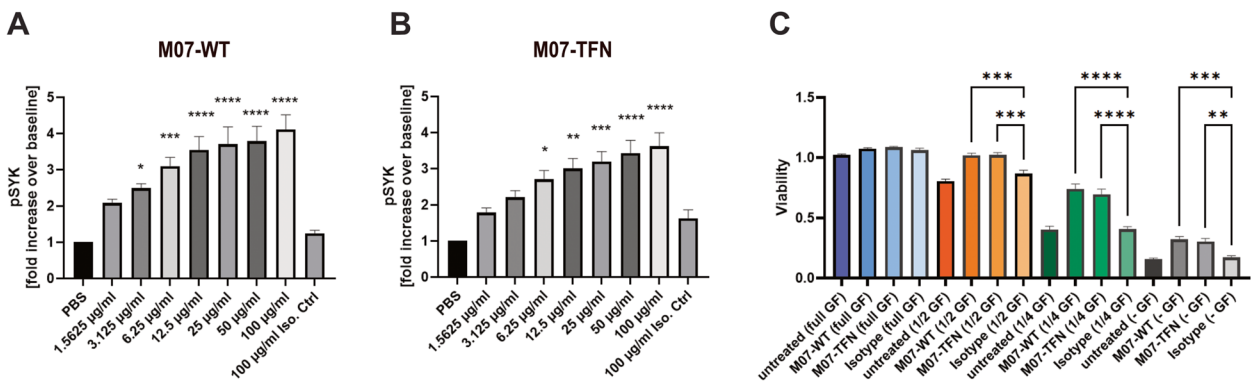

Fig. 3 M07 activates hiPSC-derived microglia cells and increases their viability **A** Titration of p-Syk signaling in hiPSC-derived microglia upon antibody treatment: One-way ANOVA; *, $p < 0.05$, **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$; Data represent mean ± SEM ($n = 5$) **B** Titration of p-Syk signaling in hiPSC-derived microglia upon antibody treatment: One-way ANOVA; *, $p < 0.05$, **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$; Data represent mean ± SEM ($n = 12$) **C** Microglia viability assay upon stimulation with M07-WT and M07-TFN antibody. One-way ANOVA; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$; Data represent mean ± SEM ($n = 3$)

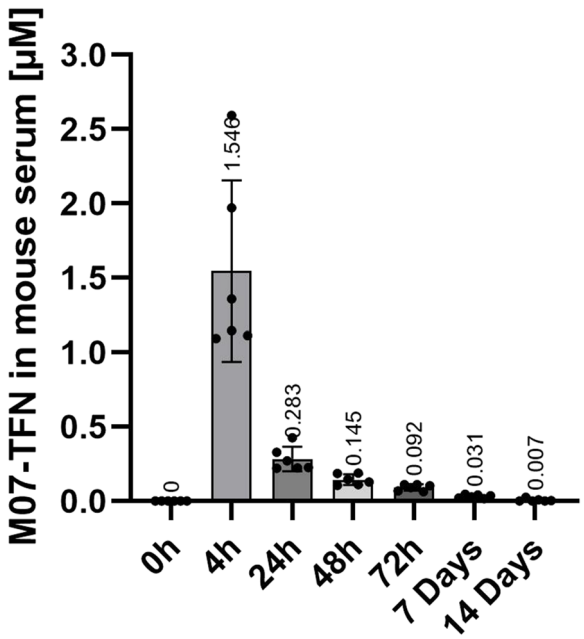

Fig. 4 Pharmacokinetic study of M07-TFN in 5xFAD mice. Serum concentrations of M07-TFN were determined in mice over time after injection of 30 mg/kg M07-TFN. 6 independent animals were used. Each dot represents one mouse. ELISA evaluation was repeated three times. Means are shown with standard deviations (SD)

Location (NOR/L) tests. The test could be used as a "pure" test of recognition memory to assess working memory. The NOR/L test was based on natural curiosity behavior of mice and did not involve positive or negative reinforcement, making it comparable to memory tests currently used in humans [30, 31].

The test setup consisted of a habituation and familiarization phase followed by the NOR/L trials (Fig. 5A, B). We did not observe differences upon antibody treatment in the NOR test after 3 weeks (Fig. 5C). In the NOL test, we observed a significant difference comparing animals treated with the higher M07-TFN dose (30 mg/kg) as compared to vehicle-treated animals (Fig. 5D). The animals treated with the higher dose of M07-TFN showed a clear preference to stay with the object at the original location, whereas those treated with the lower dose and vehicle controls did not. Those animals showed a mild preference to stay with the object at the novel location. The animals treated with the higher dose of M07-TFN remembered the original set-up and select the object at the 'old' location.

Sustained M07-TFN treatment attenuates cognitive decline in 5xFAD mice

The second in vivo study was a repeated dosing study and was designed to obtain efficacy data over a 12-week time course (based on studies by Fassler et al. 2021; Wang et al. 2020 [16, 17]) (Fig. 6A and B). We injected the antibody once per week, based on an antibody half-life of approximately 12 h and relevant serum levels up to 7 days, thus ensuring that the antibody is systemically present over the time course of the study. Therefore, repeated dosing was intended to increase TREM2 activation consistently, which was expected to have a strong effect on behavioral changes in these animals. Cognitive impairment was monitored monthly in order to determine at what time point effects on the short- and long-term memory of the mice can be observed and whether those increase over time. A number of behavioral tests were performed in order to assess cognitive decline. During the time course of the study, we did not observe any weight loss or health issues in the treated animals, so that all animals safely completed the study.

In the NOR/L test, we systematically acquired the traveled distance, mean speed and time mobile of the animals in order to determine changes in locomotor behavior. Over the time course of the study, the animals became less active (Supp. Figure 2 A-C). Evaluating these data sets, it became apparent that the M07-TFN treated animals stayed more active over time compared to control animals (Supp. Figure 2D and Fig. 6C). The traveled distances of the high dose M07-TFN-treated animals were significantly higher after 8 and 12 weeks compared to vehicle control (Fig. 6C). For the NOR test, we did not observe significant changes comparing the antibody-treated groups to control after 12 weeks, similar to the single administration study (Fig. 6D). In the NOL test, we observed that the animals treated with M07-TFN spent more time with the object at the novel location (Fig. 6E). At 8 weeks, this difference was not significant. Unfortunately, due to a shutdown of some lab premises, we could not acquire the values of the NOL at the 12-week time point.

In addition to the NOR/L test, we performed a Barnes Maze test to investigate spatial and long-term memory and learning. In order to test the short-term memory and to train the animals, several training runs were carried out per day on two consecutive days. Long-term memory was tested in two test runs, each 24 h apart (Fig. 7A). To complete the test, all groups tested took equally long in the beginning to find the flight box (Fig. 7B). Over the time course of the study, control animals took longer to find the box, whereas the antibody-treated groups performed better. The higher antibody dose-treated animals consistently stayed at the same level over 8 weeks and performed significantly better than controls after 8 weeks (Fig. 7C). Similarly, the animals treated with the lower antibody dose performed better as compared to controls. They demonstrated a tendency to need less time to find the flight box, though this was not statistically significant.

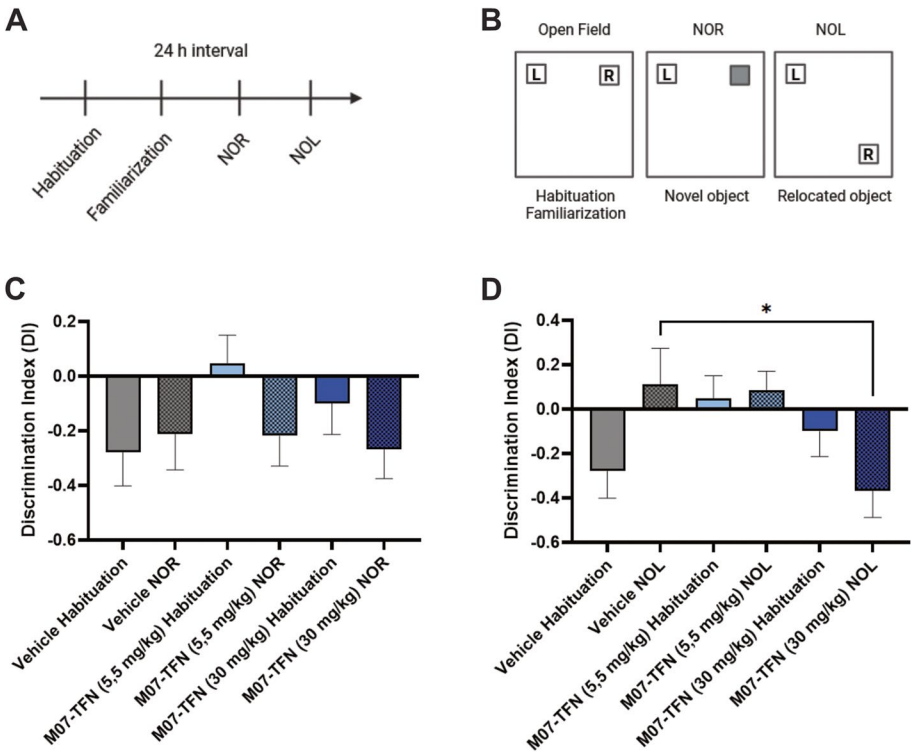

Fig. 5 Single application study of M07-TFN displays mild improvement of cognitive behavior in male 5xFAD mice **A** Timeline for Novel Object Recognition (NOR) and Novel Object Location (NOL) test setup with a 24 h delay between individual test phases **B** Scheme for Novel Object Recognition (NOR) and Novel Object Location (NOL) test. Mice were habituated to empty arenas and thereafter familiarized to two identical objects (L and R). Afterwards, a novel, equally attractive object was presented to the mice or the familiar object was moved to a new location (Relocated object) **C** Novel Object Recognition (NOR) after single antibody application followed by a 3 week chase. Animals in the different treatment groups showed similar preference for the familiar and novel object. DI = $(Time_{novel} - Time_{familiar})/(Time_{novel} - Time_{familiar})$; One-way ANOVA; Data represent the mean ± SEM (of n = 12 independently measured animals in each group) **D** Novel Object Location (NOL) after single antibody application followed by a 3 week chase. DI = $(Time_{novel} - Time_{familiar})/(Time_{novel} - Time_{familiar})$; One-way ANOVA; *, $p < 0.05$, Data represent the mean ± SEM (n = independently measured 12 animals in each group)

During the test, animals used different search strategies to find the flight box and were grouped into three main categories – Direct, Wall and Random (Fig. 7D). In the vehicle group, we observed a strong increase of the 'Random' search strategy after 8 weeks, whereas the high-dose animal group stayed relatively constant in using the 'Wall' strategy.

M07-TFN treatment reduces ß-Amyloid burden in cortical region of 5xFAD mice

At the end of the 12-week treatment period mice were euthanized, PBS perfused and brains were frozen after dissection. All histo-pathological studies were done in a blinded manner, such that the investigator did not know the treatment group of the respective animals. To evaluate whether sustained treatment with M07-TFN led to changes regarding ß-amyloid plaques or microglial number, the brains were sliced, and immunohistochemical stainings for ß-amyloid and the microglial marker PU.1 (*SPI1*: Spi-1 Proto-Oncogene) were performed. Similar to results reported in a reference study on the 5xFAD mouse model [32], we detected plaque structures in cortical regions after the 7-month paradigm used in this experimental setup (Fig. 8A). The 5xFAD mice treated with 30 mg/kg M07-TFN showed a significant reduction of the area of detected plaques normalized to the number of plaques compared to vehicle treatment (Fig. 8B), while the total area of detected plaques in mouse cortices displayed a significant reduction in mice treated with 10 and 30 mg/kg M07-TFN respectively (Fig. 8C). Of note, the total number of ß-Amyloid positive plaques did not significantly change in all three treatment groups (Fig. 8D). Assessment of the number of microglial cells positive for PU.1 in all three groups showed no significant differences after termination of the treatment paradigm (Fig. 8E).

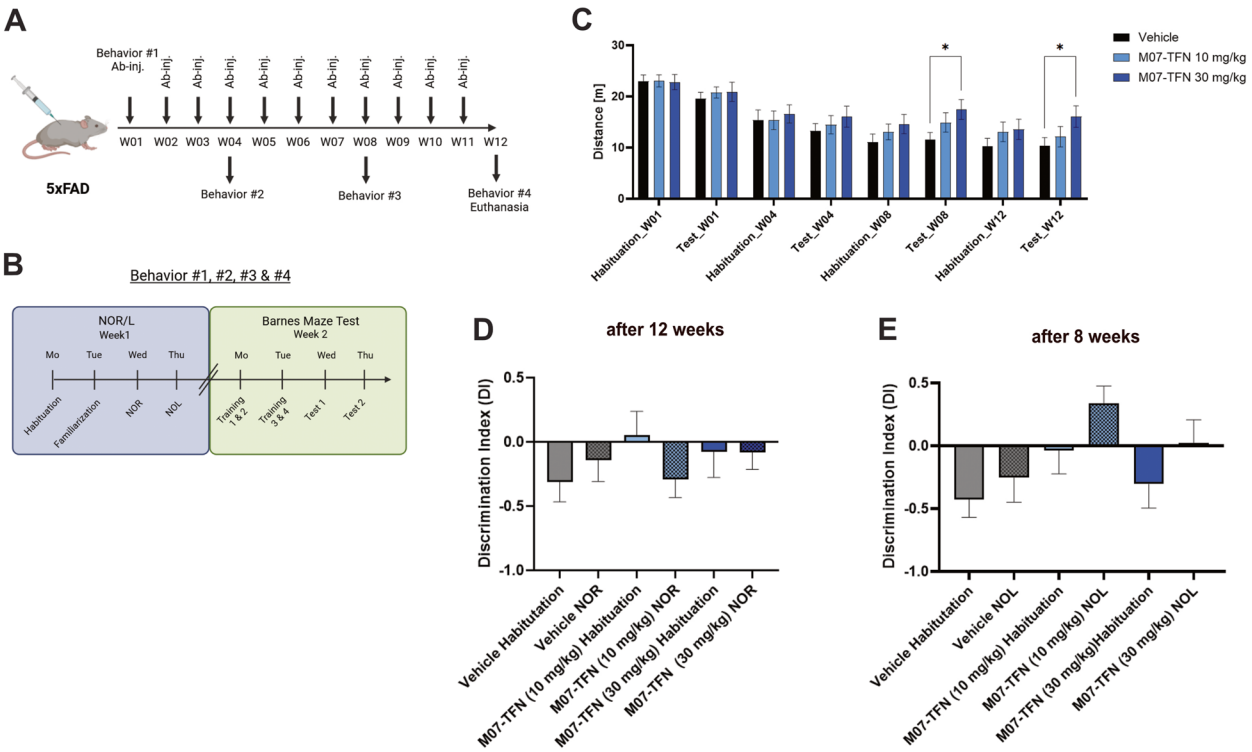

Fig. 6 12-Week study – NOR/NOL in mixed gender 5xFAD mice **A** Design of the mouse study. Equal numbers of male and female mice were included in all study groups, respectively. Behavior was assessed prior to the first antibody application (W01). The behavior tests were performed every 4 weeks. Legend: behavior = AD-specific behavioral tests; Ab-inj. = administration of antibody; W = week; 5xFAD = AD mouse model **B** Timing of the behavioral study. NOR/L was performed in the first week on 4 consecutive days followed by the Barnes maze experiment in the second week **C** Distance the animals travel during the NOR was measured and showed reduced activities in the vehicle-treated group as compared to the animals treated with 30 mg/kg M07-TFN antibody. One-way ANOVA; *, $p < 0.05$, Data represent the mean ± SEM (n = independently measured 12 animals in each group) **D** Novel Object Recognition (NOR) after weekly antibody application followed by a 12-week chase. DI = $(Time_{novel} - Time_{familiar})/(Time_{novel} - Time_{familiar})$; One-way ANOVA; Data represent the mean ± SEM (n = 12 independently measured animals in each group) **E** Novel Object Location (NOL) after weekly antibody application followed by an 8-week chase. DI = $(Time_{novel} - Time_{familiar})/(Time_{novel} - Time_{familiar})$; One-way ANOVA; Data represent the mean ± SEM (n = 12 independently measured animals in each group)

Safety study in 5xFAD mice including full necropsy and histo-pathological analysis (Vivo Science GLP study I48-001)

In an additional study, 24 5xFAD mice on black-six background of mixed gender were treated weekly with either 30 mg/kg M07-TFN or vehicle (PBS) IV for 12 weeks. Clinical evaluation and scoring on all work days did not reveal any relevant findings. Blinded analysis showed that white and red cell counts, differential cell counts, and platelets at study start, and after 4 and 8 weeks did not differ significantly between groups. Also, blinded analysis of liver and kidney retention parameters at study start and end (after 12 weeks) did not result in significant differences between groups. After euthanasia, major organs were harvested and blinded microscopic analysis found no systemic/local toxicologic effects of 30 mg/kg M07-TFN in these major organs. In conclusion, repeated intravenous injection of the antibody M07-TFN once per week for 11 weeks at a dosage of 30 mg/kg body weight was well tolerated by mice without microscopic evidence of local or systemic toxicity.

Discussion

We have identified novel fully human monoclonal anti-hTREM2 antibodies, and the best performing M07-TFN showed higher binding affinity to the extracellular domain of hTREM2 and stronger activation of hTREM2 signaling (p-Syk activation) than all previously described anti-hTREM2 antibodies, as outlined in Table 1. In addition, we observed a significant inhibition of hTREM2 shedding. M07-TFN further resulted in reduced plaque load, improved novel object location, and better spatial orientation and memory (Barnes Maze test) in vivo in a relevant Alzheimer´s disease mouse model. No hematological or other side effects occurred in mice in contrast to previously developed anti-hTREM2 antibodies such as TAK-920/DNL-919 which caused anemia in preclinical studies and humans (clinical trial NCT05450549 and

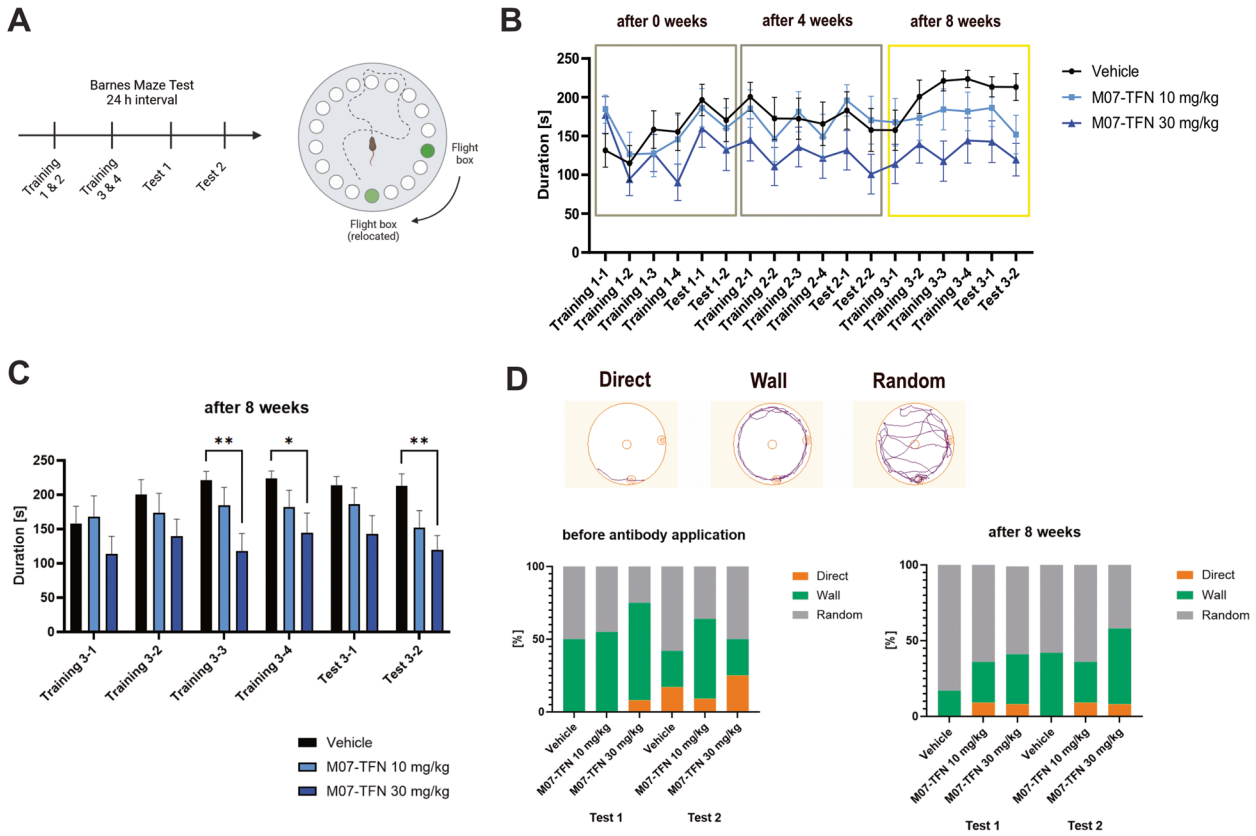

Fig. 7 12-week study—Barnes Maze in mixed gender 5xFAD mice **A** Scheme for Barnes Maze behavior testing. Mice were located at the middle of the platform ('start') and had to find a flight box located below one of the 20 holes. In the Training phase the box was located at the same location. For the Test situation the box was moved by 45°C (relocated flight box) **B** The time the animals needed to find the flight box for 0 (Training 1–1 to Test 1–2), 4 (Training 2–1 to Test 2–2) and 8 weeks (Training 3–1 to Test 3–2). Highlighted in the yellow box are the time points that are shown in **C** including statistical analysis **C** Statistical analysis of the 8-week time point of the Barnes Maze study. One-way ANOVA; *, $p < 0.05$, **, $p < 0.01$; Data represent the mean ± SEM ($n = 12$ independently measured animals in each group) **D** Representative images of strategies during the Barnes Maze study. Direct (mice move directly to the flight box), Wall (mice go through consecutive holes), Random (arbitrary pattern). Percentage of search strategies used to find the flight box of the Barnes maze test before the first antibody application and after 8 weeks

https://www.alzforum.org/therapeutics/dnl919). The beneficial effects in vivo coincided with significantly improved microglia viability in a hiPSC-derived human microglia cell stress assay.

Being a fully human anti-hTREM2 antibody, M07-TFN holds the promise of reduced immunogenicity for use in human patients compared to existing humanized anti-TREM2 antibodies (AL002c, Alector [16], ATV-TREM2 from Denali [15], hT2 AB/VGL101 from VigilNeuro [18], Ab18 [20], Ab-T1 [17]). Because these aforementioned antibodies were raised and generated in animal immune systems, less immunological complications can be expected from the fully human M07, especially upon repeated preventive or therapeutic applications in vivo in humans. The LALA modification in the Fc domain leads to reduced Fc receptor-dependent immune effects.

M07-TFN also showed best-in-class, approximately 30-fold activation of p-Syk over baseline in TREM2-overexpressing HEK293 cells (for comparison, please see Table 1). We also observed p-Syk activation in human microglia cells of up to fourfold, exceeding the twofold increase shown by van Lengerich et al. [15]. The selected antibody M07 was modified in the Fc domain in order to bind to TfR1, based on one of the published Fc domain modifications [25]. While higher p-Syk activation was observed with M07-TFN compared to M07-WT in HEK293, comparable p-Syk occurred in human microglia cells. This may be explained by simultaneous binding of M07-TFN to human TfR1 which is highly expressed in HEK293 cells. Binding of the antibody to TfR1 and TREM2 receptor may have increased cross-linking of the TREM2 and thus increased phosphorylation of Syk downstream. Simultaneous binding of M07-TFN to TREM2 and TfR1 will not affect shedding of the TREM2 extracellular domain, because blocking of the cleavage is mediated by direct binding of the antibody

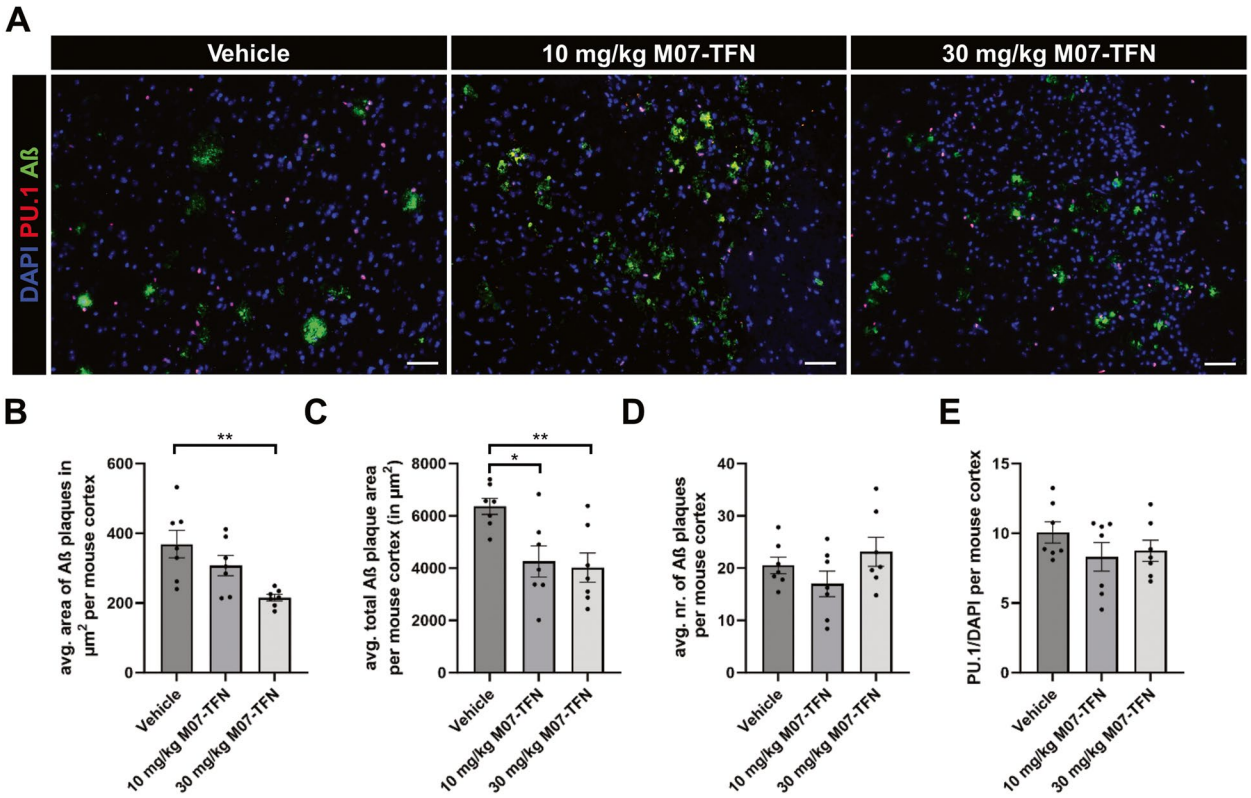

Fig. 8 Anti-TREM2 antibody M07-TFN attenuates ß-Amyloid burden in mixed gender 5xFAD mice **A** Representative immunohistochemical stainings of 5xFAD mice cortices displaying nuclei (DAPI), microglia (PU.1), and ß-Amyloid (Aß) after treatment for 12 weeks with either Vehicle (PBS), 10 mg/kg or 30 mg/kg M07-TFN **B** Quantification of average Aß plaque area normalized by the number of plaques per area of interest **C** Quantification of average total Aß plaque area per area of interest within mouse cortices **D** Quantification of average number of Aß plaques per area of interest within mouse cortices **E** Quantification of $DAPI^{+}PU.1.^{+}$ cells per area of interest within mouse cortices (**A**): Scalebar = 50 µm (**B**, **C**, **D**, **E**): Statistical analysis of sections from $n = 7$ independently measured animals per dose group. One-way ANOVA; *, $p < 0.05$, **, $p < 0.01$

to the cleavage site, which will prohibit shedding of the extracellular domain. In human microglia cells, a novel experimental setup allowed us to determine the effects of TREM2 antibodies on cell stress as a correlate of neurodegenerative diseases in vitro. This assay streamlines previous efforts to measure the effects of anti-TREM2 antibodies on survival performed in primary human macrophages [15]. In these investigations, we found that both M07-WT and M07-TFN significantly improved microglia viability.

Given that M07-TFN can bind human TfR1 in a bivalent manner, M07-TFN should be shuttled inside the CNS via TfR-mediated transport. A pulse-chase assay using human brain endothelial cells showed an uptake as well as release of M07-TFN but not M07-WT, indicating brain shuttling capacity of M07-TFN [25].

M07 did not only exhibit best-in-class affinity to human TREM2, but also exerted cross-reactivity to the mouse TREM2 stalk region, albeit at lower affinity. This feature allowed us to investigate the effects of M07-TFN directly in a 5xFAD mouse model of Alzheimer´s disease without the need for cross-breeding with human TREM2-overexpressing mice.

As predicted by our previous in vitro investigations, M07-TFN resulted in improved novel object location memory and better spatial orientation and short- and long-term memory (Barnes Maze test) in vivo in the Alzheimer´s model, both after single administration and even more so, after repeated dosing over 12 weeks. From the NOR/L test, we concluded that brain function is not affected by application of the antibody since the animals had no problems with object recognition nor spatial perception, which speaks for intact structures of the visual system as well as the hippocampal formation, which plays an important role in learning and memory formation. In addition, we observed a normal activity of the animals indicating a normal motor and sensory cortex function. Over the time course of the study, we observed that the animals became less active. This was expected, since the animals got used to the set-up and simultaneously their health declined during the progressive study. The undisturbed motor activity can be attributed to the fact that

Table 1 Comparison of published agonistic anti-TREM2 antibody characteristics. Comparison of biochemical features as well as in-vitro assays of published agonistic anti-TREM2 antibodies

Publication	Species specificity	TREM2 binding affinity	Anti-TREM2 Antibody	Company	pSYK activation (HEK cells)	Assays in microglia cells (primary, iPS-derived) or BMDM (Bone marrow derived macrophages)
Wang et al. 2020	Human	EC50 = 360 pM	AL002c	Alector Therapeutics / Abbvie	Yes / 2,3 fold	not performed
Price et al. 2020	Mouse	n.a.	AL002a	Alector Therapeutics / Abbvie	not performed	not performed
Fassler et al. 2021	Mouse and Human	KD = 5,73 nM (human) 18,63 nM (mouse)	Ab-T1	-	Yes - Western Blot for pSYK	prim. Mouse microglia - Aß uptake increased
Ellwanger et al. 2021	Human	EC50 = 222 pM	T2AB	Amgen - currently owned by Vigil Neuro	Yes - Western Blot for pSYK	BMDM - viability (increased), pSYK (increased); Neuronal debris uptake in MIC
Zhao et al. 2022	Mouse	EC50 = 79,4 nM	Ab18	-	Yes / 8 fold	prim. Mouse microglia - Aß uptake increased
Jain et al. 2022	Mouse	n.a.	AL002a	Alector Therapeutics / Abbvie	Yes / 18 fold	BMDM - phagocytosis of AD-tau unchanged, pSYK in BMDM (3 fold increase)
van Lengerich et al. 2023	Human	KD = 2,64 nM; EC50 = 2,7 nM	ATV:TREM2	Denali Therapeutics / Takeda	Yes - Western Blot for pSYK	micorglia - phagocytosis assay, increase with ATV-TREM2 antibody but not with anti-TREM2 antibody
This study	Mouse and Human	EC50 = 0,093 nM (human)	M07-TFN	ISAR Bioscience	Yes / 30 fold	iPSC-Microglia, viability assay and pSYK assay (4 fold increase)

Table 2 Animal studies performed with published agonistic anti-TREM2 antibodies. Comparison of behavioral studies performed with published agonistic anti-TREM2 antibodies. n.a. = not addressed, EPM = Elevated Plus Maze, RAWM = Radial Arm Water Maze, MWM = Morris Water Maze, NOR/L = Novel object recognition / location

Publication	Antibody dose	Treatment duration	Dosing interval	Mouse model	Mouse gender	Behavioral tests on Cognition
Wang et al. 2020	30 mg/kg	12 weeks	weekly	5xFAD	n.a.	Improved elevated plus maze (EPM)
Price et al. 2020	50 mg/kg	14 weeks	weekly	5xFAD	male only	Improved (NOR/L & Radial arm water maze (RAWM))
Fassler et al. 2021	10 mg/kg	12 weeks	2 weeks	5xFAD	female only	Improved (NOR/L & Morris water maze (MWM))
Ellwanger et al. 2021	30 mg/kg	10 days	3 days	5xFAD	n.a.	not performed
Zhao et al. 2022	20 mg/kg	14 weeks	weekly	5xFAD	female only	Improved (EPM and fear-conditioning)
Jain et al. 2022	80 mg/kg	12 weeks	weekly	5xFAD	female & male	not performed
van Lengerich et al. 2023	10 mg/kg	8 days	single dose	5xFAD	n.a.	not performed
This study	10 and 30 mg/kg	14 weeks	weekly	5xFAD	female & male	Improved Barnes Maze; NOR/L no significant change

no abnormalities in the gait pattern were detected during the course of the disease and during treatment. The speed of locomotion and the phases between mobile and immobile episodes did not change significantly between the individual groups. These observations are supported by findings on 5xFAD mice in which influences on motor activity were only observed in later phases of disease starting at an age of 9 months, which can be explained by reduced reflexes, influences on the skeletal muscle or loss of balance [33]. Animals in the vehicle group showed disease progression and poorer performance in the Barnes maze from week 8 onwards, which was also reflected in a longer test duration. The treatment groups, on the other hand, showed stable and thus improved performances

in the Barnes testing compared to the vehicle group. We therefore assume that the data demonstrate a positive effect of the treatment, particularly with regard to disease progression. We observed improved novel object memory indicating that M07-TFN application improved recognition of familiar objects. An overview of the outcomes of our studies compared to previous investigations on anti-human TREM2 antibodies is shown in Table 2.

In the Barnes Maze, we did not observe any adverse effects of antibody application on short- and long-term memory as well as spatial perception. Again, this required an intact function of different brain regions, such as motor cortex and hippocampus, which were unaffected by the antibody M07-TFN. On the contrary, 5xFAD animals treated with the anti-hTREM2 antibody displayed improved performance in the Barnes Maze already after 8 weeks. They spent less time locating the flight box and in addition showed improved search strategies for the box. To locate the flight box in the maze, the animals used visual cues located around the arena and the arrangement of exits to orient themselves and find the right escape route. The 'Random' strategy, however, relies less on spatial information and can be impulsive or random. Animals using this strategy could simply try different outcomes without remembering previous experiences or the spatial arrangement. This search strategy is the least effective since it is not based on a systematic approach. In the vehicle group, we observed a strong increase in the 'Random' search, whereas antibody-treated animals showed a tendency to use the ‚Wall ' strategy.

In comparison to previous cognition studies of anti-TREM2 antibodies in 5xFAD mice, our studies were performed in a blinded fashion and mice with both sexes were used in order to obtain more reliable and applicable data. NOR/L tests were performed in other studies [17, 21] and similarly to our results antibody-treated animals showed an improvement in object recognition. The Barnes Maze can be compared to the Morris Water Maze (MWM) previously used in another study [17] since both tests investigate the same neuronal pathways. Similar to Fassler et al. [17], we observed an improved performance of 5xFAD animals treated with anti-TREM2 antibody. The difference between the two mazes is the different support for the animals to reach the escape zones. While in the MWM, the animals have to reach the zone by swimming – which is a greater stressor/trigger for the animals than in the Barnes Maze in which the animals are induced to flee into the dark box by bright light. The results of both tests can be compared since they examine the same neuronal networks. However, since the stressor in the Barnes maze is lower, more animals may be needed to find small effects. The Barnes Maze test has the advantage that it is less susceptible to stress as compared to the MWM and more transferable to the humans. Studies using anxiety-related behavioral tests [16, 20] such as the Elevated Plus Maze or fear-conditioning test cannot be compared to our results. In addition, these tests do not reflect the pathology of Alzheimer's disease very well and are not clinically relevant.

After termination of the 12 weeks experimental setup, the mice were euthanized and brains were removed for further investigation of potential changes regarding pathological Alzheimer´s disease hallmarks such as plaque formation and number of microglia as seen in 5xFAD mice upon treatment with agonistic anti-TREM2 antibodies [17, 18, 34, 35]. In line with the outcome of the behavioral tests, we show an improvement in ß-amyloid burden. A significant reduction of plaque area in cortical regions was observed in the low and high doses of M07-TFN exposure while no substantial changes regarding plaque nor microglia number have been observed. It is noteworthy to mention that future studies should address the mode of action of M07-TFN treatment. While no reduction in the number of plaques was identified, plaque area decreased, supporting the notion of agonistic TREM2 antibodies facilitating the compaction of plaques, which results in inert ß-amyloid aggregates lacking neurotoxic properties as seen in studies using agonistic TREM2 antibodies in a 5xFAD context [16, 41].

Taken together, we demonstrate the beneficial effects of prolonged TREM2 activation via the agonistic antibody M07-TFN while ß-amyloid aggregation was already ongoing. Further development of the brain-targeting monoclonal antibody M07-TFN for earlier and later stages of Alzheimer´s disease is warranted. No side effects occurred in mice in contrast to previously developed anti-hTREM2 antibodies.

Limitations of the study

Administration of fully human anti-human TREM2 antibodies in mice may elicit mouse anti-human Fc antibodies. Unfortunately, we did not sample enough blood volumes to investigate the occurrence of such anti-antibodies in mouse sera. These investigations, however, will be included in follow-up studies.

Also, the binding affinities of M07-WT and M07-TFN were lower against mouse TREM2 than hTREM2, albeit in the range of other anti-hTREM2 antibodies which showed effects in 5xFAD mice (for comparison, please see Table 1). Yet, these effects will have to be substantiated by further studies in hTREM2 knock-in mice which will be carried out in the near future. These studies should also investigate binding of M07-TFN to brain lysates of such mice, and of TREM-2 knock-out mouse

and human brain lysates, as well as try to determine M07-TFN brain concentrations after IV administration.

Further studies will also investigate the effects of M07-TFN in phagocytosis assays.

Methods

Agonist antibody generation

Using phage display technology, a human immunome-derived antibody library [36, 37] was screened for binding to a peptide antigen of the human TREM2 stalk region. Antibodies were screened based on binding to human TREM2, and clones of interest were reformatted onto human effectorless human IgG1-LALA backbones for material generation and further evaluation of cell binding potency and functional impact to TREM2 signaling. Agonists were identified by their ability to activate p-Syk on HEK293 Flp-In cells over-expressing hTREM2 and hDAP12.

The antibody candidate M07 was modified in order to mediate binding to TfR1. Therefore, out of several published mutations [25], we selected one specific mutation cassette which was introduced into the CH3 domain of both Fc regions [32]. In detail, the WT amino acid (AA) sequence aa384-NxQPENNxxxxxxxxxxxxxxxxxxxxxxxDxxRxxxxN-aa421 was exchanged for aa384-YxTEWSSxxxxxxxxxxxxxxxxxxxxxTxxExxxxF-aa421.

CHO cell culture and antibody production

CHO-S cells, which are commercially available as part of the Freedom CHO-S Kit (Thermo Fisher cat. #A13696-01), were thawed and grown in cell culture as recommended in the manufacturer´s instructions using recommended media (CD FortiCHO™ Medium cat #10,887,640 ThermoFisher supplemented with 8 mM L-Glutamine and Puromycin (20 µg/ml) and Methotrexate (200 nM) for selection). Gene transfer of pCHOv1 plasmids containing the respective heavy and light chain sequences under the control of two different hybrid CMV promotors was carried out according to manufacturer´s instructions. Supernatant of the stable transfected cells was harvested, sterile-filtered and the antibody purified.

In brief, supernatant (~ 5 L) was loaded onto a HiTrap® MabSelect SuRe™ column (Cytiva cat # 11–0034–95) with the help of ÄKTApure system (Cytiva Life Sciences). The column was washed with 10 column volumes of binding buffer (20 mM sodium phosphate; pH =7.2) and the antibody was eluted with elution buffer (0.1 M sodium citrate; pH =3). Fractions containing protein were pooled, neutralized 1:1 (with 1 M HEPES containing 300 mM NaCl) and dialyzed against 1× PBS overnight. Dialyzed sample was concentrated using centrifugal filter devices (Amicon Ultra cat # UFC901024). Protein concentration was determined spectrophotometrically and set to 1 mg/ml.

Stably transfected cells were selected in CD FortiCHO Medium followed by subcloning by limited dilution according to commercial manufacturer's specifications in the "Freedom™ CHO-STM Kit" User guide.

HEK293 cell culture

HEK293 Flp-In cells overexpressing hTREM2/hDAP12 were cultured in Dulbecco's modified Eagle's medium (DMEM) with GlutaMAX I, supplemented with 10% (v/v) fetal calf serum (FCS), 1% (v/v) Pen/Strep and 200 µg/ml Hygromycin B.

Agonist p-Syk AlphaLISA assay

Phosphorylated Syk (p-Syk) was measured using the AlphaLISA SureFire Ultra p-Syk Assay Kit (Revvity ALSU-PSYK-A500) following the manufacturer's instructions. Briefly, HEK293 Flp-In cells overexpressing human TREM2 and human DAP12 were plated in 50 µl media at a density of 50.000 cells/well in a 96-well plate, coated with poly-D-Lysine (1 µg/ml), and incubated overnight at 37°C in a cell culture incubator.

Antibody was prepared in serial dilutions in full DMEM Medium and cells were washed 3× with PBS before 50 µl of the antibody mixture was added to the cells. Antibody mix and negative control (DMEM only) were allowed to incubate for 5 min at 37°C. After incubation, medium was removed and cells were lysed with 50 µl lysis buffer supplemented with phosphatase inhibitor (VWR) for 10 min on a plate shaker (~ 350 rpm). 30 µl of lysate were then used for further incubation steps with acceptor and donor beads (each for 1 h) following analysis using a CLARIOstar^{Plus} Plate Reader (BMG Labtech).

For measuring human iPSC-derived microglia cells, a 96-well plate was pre-coated with antibody overnight at 4°C. On the following day 50.000 cells/well were seeded in medium directly into the antibody solution and centrifuged onto the plate at 300 ×g for 2 min. After a subsequent incubation for 10 min at 37°C, medium-antibody solution was removed and cells were lysed and measured as described above.

ELISA-based binding assay for anti-TREM2 antibody

Anti-TREM2 antibody binding to the human extracellular domain of TREM2 (hTREM2) (Sino Biological, 11,084-H08H) or human extracellular domain of TREM1 (hTREM1) (Sino Biological, 10,511-H08H) or human TREM2 peptide sequence of the stalk region (hTREM2p) (Biosyntan: DAGDLWFPG-TTDS-C–OH) or mouse TREM2 peptide sequence (mTREM2p) (Biosyntan: DAGDLWVPEESSSFEGAQVEHSTSRNC-Amid) were quantified in an ELISA assay.

All procedures were performed at room temperature and incubations were done on a microtiter plate shaker. ELISA plates were coated with 60 µl/well hTREM2 or hTREM1 or hTREM2p or mTREM2p in coating buffer ($NaHCO_3$; pH 9.6) at a concentration of 0.5 µg/ml for 1 h.

The coated plates were washed three times with PBS-T (PBS, 0.1% Tween-20), blocked with 100 µl/well of blocking solution (PBS-T, 5% milk powder) for 1 h, and washed again three times with PBS-T. Antibodies were set to 1 µg/ml and further diluted 1:3, 1:10, 1:30, 1:100, 1:300 and 1:1000 with PBS before they were transferred as 60 µl/well to the blocked ELISA plates and incubated for 1 h. The plates were washed three times with PBS-T and incubated with goat anti-human IgG HRP (Jackson Immunoresearch, #109–035–098) diluted in PBS-T 1:10.000 for 1 h. After washing three times with PBS-T, 100 µl/well of TMB substrate (Thermo Scientific, #34,029) was added and finally stopped with 100 µl/well stopping solution (1 M H_2SO_4) when the colorimetric reaction was saturated in the highest dilution. OD was determined at a wavelength of 450 nm with a reference wavelength of 620 nm in a plate reader (SpectraMax i3xl).

ELISA-based detection assay for anti-TREM2 antibody in mouse serum samples

Mouse blood was sampled and centrifuged. Samples (0 h, 4 h, 24 h, 48 h, 72 h, 7 days and 14 days) were diluted in PBS: 1:1.500, 1:5000, 1:1.500, 1:1.500, 1:1.500, 1:200, 1:10 and used as triplicates. ELISA plates were coated with 60 µl/well hTREM2p in coating buffer ($NaHCO_3$; pH 9.6) at a concentration of 0.5 µg/ml for 1 h. ELISA was performed as described above.

ELISA-based quantification of soluble TREM2 in cell culture supernatants

To quantify levels of sTREM2 in conditioned media, an ELISA was performed using the Meso Scale Discovery Platform. Briefly, streptavidin-coated small spot 96-well plates were blocked overnight at 4°C in blocking buffer (3% bovine serum albumin, 0.05% Tween-20 in PBS, pH 7.4). Plates were incubated with 0.125 µg/ml biotinylated polyclonal goat anti-human TREM2 capture antibody (BAF1828, R&D) diluted in blocking buffer. After washing three times with washing buffer (0.05% Tween-20 in PBS, pH 7.4) plates were incubated with samples and standard diluted in sample buffer (1% bovine serum albumin (BSA) and 0.05% Tween-20 in PBS, pH 7.4) for 2 h at RT. If samples were antibody treated, samples and standard were previously mixed 9:1 with denaturing buffer (200 mM Tris–HCL pH 6.8, 4% (w/v) SDS, 40% (v/v) glycerol, 2% (v/v) β-mercaptoethanol, 50 mM EDTA) and boiled at 95°C for 5 min to dissociate TREM2 antibodies from sTREM2. Plates were washed three times with washing buffer before incubation for 1 h at RT with 1 µg/ml mouse anti-human TREM2 antibody (B-3 SCBT-373828). After washing the plates three times with washing buffer, plates were incubated with a SULFO-TAG-labeled anti-mouse secondary antibody (MesoScaleDiscovery, R32 AC-5) for 1 h at RT. After three additional washing steps and one time with PBS, 1× Meso Scale Discovery Read buffer was added and the light emission at 620 nm after electrochemical stimulation was measured with a Meso Scale Discovery Sector Imager 2400 reader.

Differentiation of human iPSC-derived Microglia (hiMGL)

We differentiated hiMGL from iPSCs as described [38] with modifications to improve efficiency and yield: When hiPSCs were 70–90% confluent, they were split 1:100–200 onto Geltrex-coated 6-well plates for the HPC differentiation using EDTA to get around ~ 30 small colonies per well. Cells were fed with 2 ml of HemA medium (HPC differentiation kit, StemCell Technologies) on day 0 and half-fed with 1 ml on day 2. Media was switched to 2 ml of HemB on day 3 with half-feeds on days 5 and 7 and 1 ml added on top on day 10. On day 12 HPCs were collected as non-adherent cells to either freeze or continue with the microglia differentiation. HPCs were frozen at 1 million cells per mL in BamBanker (Wako). They were then thawed directly onto Geltrex-coated 6-well plates with 1 million cells evenly distributed among 6 wells in 2 ml hiMGL media with 25 ng/ml M-CSF, 100 ng/ml IL-34, and 50 ng/ml TGFβ1 added fresh. 1 ml of media was added on top every other day. During the microglia differentiation, the cells were split 1:2 every 6–8 days depending on confluency. A very similar differentiation protocol was published recently [39]. We did not use CD200 and CX3CL1 as this did not seem to have an effect on hiMGL gene expression, as determined by Nanostring analysis (data not shown). hiMGL was used for experiments on day 16 of the differentiation.

Viability assays

hiPSC-derived microglia cells were plated in a 96-well plate at a density of 25.000 cells per well with various cytokine concentrations (full Cytokine mix [25 ng/ml M-CSF, 100 ng/ml IL-34, and 50 ng/ml TGFβ1], ¼ Cytokine mix [5 ng/ml M-CSF, 25 ng/ml IL-34, and 12,5 ng/ml TGFβ1] or no Cytokines). Agonistic anti-TREM2 antibody or Isotype control antibody was added at a concentration of 20 µg/ml. 48 h after seeding the cell viability was determined using CellTiter-Glo reagent (Promega) according to the manufacturer´s instructions. In brief, ATP-standard was prepared and 100 µl CellTiter-Glo Reagent (Volume equal to the culture medium present per well) added per well. Contents were mixed for 2 min

on an orbital shaker and 170 µl of the lysed cells transferred to a white 96-well plate. The plate was incubated at room temperature for 10 min and the luminescent signal recorded using a plate reader.

Culture of immortalized brain endothelial cell line hCMEC/D3

Immortalized human cerebral microvascular endothelial cell line hCMEC/D3 was purchased from Biozol (Clone ID: CEDCLU512-6). Maintenance culture of hCMEC/D3 cells was performed on collagen I (Merck #08–115) coated 10 cm dishes (50 µg/ml for 1 h at room temperature) in microvascular endothelial cell growth medium 2 (Lonza #CC-3202).

Transcytosis pulse-chase assay

In preparation for pulse-chase transcytosis assays, hCMEC/D3 cells were split onto Transwell inserts with 3.0 µm pores (Corning #3462). Transwells were coated with collagen I (50 µg/ml) for 1 h at RT. The collagen I solution was aspirated and the transwells were blocked with PBS + 1% BSA (Merck #A2153) overnight at 4°C or 1 h at room temperature (RT). The hCMEC/D3 cells were washed with PBS (VWR #21–040-CV) before being incubated with TrypLE (Thermo Fisher #12,563,011) for 5 min at 37°C. After incubation, microvascular endothelial cell growth medium 2 (EGM2 MV) media was used to stop the enzymatic reaction and the cells were collected and counted. The transwells were washed once with PBS to remove any residual bovine serum albumin (BSA) and then 70.000 cells were seeded on the apical side of the transwells in 400 µl of EGM2 MV. The basolateral side was filled with 1600 µl of EGM2 MV. After 3 days, the media was changed to EGM2 MV AM (EGM2 MV assay media) containing 2.5% serum and ¼ VEGF (vascular endothelial growth factor), hEGF (human epidermal growth factor), and bFGF (basic fibroblast growth factor). After 3 additional days, the pulse-chase transcytosis assay was performed.

To begin the pulse-chase transcytosis assay, 2 µg/ml of either M07-WT, M07-TFN, or commercial antibody clone MEM-189 (mouse IgG1 anti-hTfR1, Biozol #EXB-11–353-C100) was added to the apical side of the transwell in 400 µl of EGM2 MV AM and 1600 µl of fresh EGM2 MV AM was replenished in the basolateral compartment. A blank transwell without cells was always included and treated with M07-WT. After one hour, the media was collected from both the apical and basolateral compartments and the cells and blank were washed 3 times for at least 3 min with PBS with Ca^{2+} and Mg^{2+} (Thermo #14,040,117) containing 2.5% FBS (from Lonza EGM2 MV kit #CC-3202) at RT. Washes were collected and analyzed to ensure all excess antibody was removed. After washing, one transwell was lysed from each condition by adding 500 µl of RIPA (radioimmunoprecipitation assay) buffer (Carl Roth # 23 T1.3) directly to the cells and collecting for analysis. After the PBS from the final wash was aspirated, fresh EGM2 MV media was added (400 µl apical and 1600 µl basolateral) and the cells were placed in the incubator. After either 5 or 24 h, media was collected from both of the transwell compartments for analysis. Cells were then washed again 3 times for at least 3 min with PBS with Ca^{2+} and Mg^{2+} and 2.5% FBS and then lysed with RIPA buffer.

Sandwich ELISA to analyze transcytosis pulse-chase samples

Transparent 96-well Maxisorp plates were coated overnight with 1 µg/ml of Goat Anti-Human IgG, Fcγ fragment specific (Dianova #109–005–098) in PBS for the human IgG ELISA and Goat Anti-Mouse IgG (subclasses 1 + 2a + 2b + 3), Fcγ Fragment Specific (Dianova #115–005–164) in PBS for the mouse IgG ELISA. Wells were washed 3 times with PBS + 1% BSA and then blocked with PBS + 1% BSA. Transcytosis samples along with serial dilutions and standards were incubated for 90 min. This was followed by 4 washes with PBS + 1% BSA. For the human IgG ELISA, 50 ng/µl of Goat F(ab')2 anti-Human IgG(F(ab')2)-Biotin (Dianova #109–066–097) diluted in PBS + 1% BSA was incubated in each well for 90 min and for the mouse IgG assay 50 ng/µl of Goat F(ab')2 anti-Mouse IgG(F(ab')2)-Biotin (Dianova #115–066–072) was substituted. Following 6 washes with PBS + 1% BSA, 50 ng/ml for the human IgG ELISA and 100 ng/ml for the mouse IgG assay of streptavidin peroxidase (Biozol #VEC-SA-5004) diluted in PBS + 1% BSA + 0.05% Tween-20 was incubated in the wells for 15 min. After 4 washes with PBS + 1% BSA, TMB substrate (Thermo Scientific, #34,029) was added to the wells. After 5–10 min the reaction was stopped with 1 M H_2SO_4 and the OD was determined at a wavelength of 450 nm with a reference wavelength of 620 nm in a plate reader (SpectraMax i3xl). Antibody concentration was determined by comparison to the standard curve and absolute amount was quantified given that 500 µl of RIPA buffer was added to lyse the cells and 400 µl and 1600 µl of media were added in the apical and basolateral compartments respectively.

Streptavidin-linked TfR1 ELISA

Transparent 96-well Maxisorp plates were coated overnight with 10 µg/ml of streptavidin (MedChemExpress # HY-P3152) in coating buffer ($NaHCO_3$; pH 9.6). After 3 washes with (PBS + 1% BSA), biotinylated hTfR1 (Acro Biosystems #TFR-H82E5-25 µg) was diluted to 1 µg/ml in PBS + 1% BSA and added to each well for 1 h. Following 3 more washes with PBS + 1% BSA, the antibodies

(M07-WT, M07-TFN, MEM-189) were added in serial dilutions to the plate and incubated for 1 h. The wells were washed 3 times with PBS + 1% BSA and incubated with goat anti-human IgG HRP (Jackson Immunoresearch #109-035-098) or anti-mouse IgG HRP (Promega #W4021) diluted 1:10.000 in PBS + 1% BSA for 1 h. Wells were washed with PBS + 1% BSA 3 times and TMB substrate (Thermo Scientific, #34,029) was added. This reaction was stopped with 1 M H_2SO_4 and the OD was determined at 450 nm in a plate reader (SpectraMax i3xl).

Measurement of the TEER (transendothelial electrical resistance) of the hCMEC/D3 monolayer

Cells (hCMEC/D3) were cultured on transwells as described for the pulse-chase transcytosis assay. The transwells were placed inside a CellZscope (nanoAnalytics) and 820 µl of EGM2 MV was placed in the apical compartment followed by 1480 µl in the basolateral compartment (metal electrode). The measurement was started and data for the capacitance and resistance of the cell layer was obtained. Once a barrier was formed given by a capacitance between 0.5 and 5 µF/cm^2, the media was changed to EGM2 MV AM (¼ EGF, FGF, VEGF).

Permeability assay of the hCMEC/D3 monolayer

Cells (hCMEC/D3) were cultured on transwells as described for the pulse-chase transcytosis assay. To begin the experiment, 1 mg/ml of FITC-labelled dextran (4 kDa, 40 kDa, or 150 kDa) was added to the apical compartment in 400 µl of Hanks' balanced salt solution (Thermo Fisher # 14,175,095) and 1600 µl of Hanks' balanced salt solution (HBSS) was added to the basolateral compartment. After 1 h, a sample was collected from the bottom compartment and analyzed in a plate reader (SpectraMax i3xl) with an excitation of 498 nm and emission of 518 nm. Samples were compared to samples taken from the basolateral compartment of a coated blank transwell.

Animals

All animal experiments were permitted by the local Ethics board in accordance with European and German animal welfare legislations (5.1–231 5682/LMU/BMC/CAM). The most recent version of the Animal Research: Reporting In Vivo Experiments (ARRIVE) guidelines was stricly followed. 16-week-old male/female 5xFAD mice (B6.Cg-Tg(APPSwFlLon,PSEN1*M146L*L286V)6799 Vas/Mmjax (Nr: 034848 Jackson Laboratories, USA) were housed in IVC cages (Blue line, Type II long, Tecniplast, Germany) in groups of 3 to 5 individuals. For the single-dosing study, only male mice were used. For the repeated dosing studies (including the safety study), equal numbers of male and female mice were used in all study groups. All mice were housed under specified pathogen-free conditions. Room temperature and relative humidity ranged from 20–22°C to 45–55%. The light intensity in the room was set to 300 lx. The light cycle was adjusted to 12 h light: 12 h dark period. Room air was exchanged 11 times per hour and filtered with HEPA-systems. Hygiene monitoring of the facility was performed every three-month based on the recommendations of the FELASA-14 working group. All animals had free access to water and food (irradiated, 10 mm pellet; 1314P, Altromin, Germany). The cages were equipped with nesting material (5 × 5 cm, Nestlet, Datesand, UK), a red corner house (Tecniplast, Germany) and a rodent play tunnel (7.5 × 3.0 cm, Datesand, UK). Soiled bedding was replaced every 7 days. Breeding, behavioral experiments and tissue collection were performed under the animal license: AZ 03–22–026.

Pharmacokinetic study

5xFAD mice of mixed gender were investigated after single intravenous administration of 30 mg/kg M07-TFN and subsequent blood sampling of 100 µL each after 4 h, 24 h, 48 h, 72 h, 7 days and 14 days. Serum concentrations of M07-TFN were determined by ELISA, as described above.

Behavior analysis

Behavorial studies have been established for a long time and are commonly used to test behavioral components of AD mice [40] and other neurological diseases [41]. Between individual testing phases, animals were kept in their familiar housing cages. AnyMaze Software (Stoelting Co., USA) and automated activity monitors were used to track the locomotor activity of mice and to analyze the data, if not stated otherwise. In all behavior experiments, the experimenters were not visible to the animals. For this purpose, partitions with distant cues (colorful geometric shapes) were placed away from the edges of the platforms at heights visible from the platform surface. Behavioral experiments were performed as previously described [31]. Figure 6B shows a schematic overview on the timing of the behavioral studies.

Open field (OF): For habituation, mice were placed in the center of the 50 × 50 cm OF box and were allowed to freely investigate the testing environment for 4 min. Locomotor activity was investigated using AnyMaze Software (Stoelting Co., USA).

Novel object recognition/location assays (NOR/L): NOR/L tests were performed in the already familiar open field environment. Testing consisted of habituation and familiarization trials on the first day with a 24-h delay, a NOR assay on day three and NOL assay on day four. As

described previously, mice were placed in the center of the OF box and were allowed to investigate the objects for 4 min for each trial. During familiarization trials, two identical objects (identical in size, shape and color) were placed in the same location of the OF box. For NOR, one of the objects was replaced by a novel one differing in size, shape and color in the same location. NOL was performed on day four with one novel object and one familiar object in the opposite corner of the OF box. Based on the total time spent with the familiar and novel object, the discrimination index (DI) was calculated. For familiarization, a discrimination index was calculated comparing the left to the right object, respectively. Positive values indicate that the animal has spent more time with the novel object (resp. the object at the novel location), negative that it spent more time with the familiar object (resp. object at the familiar location).

Barnes maze: The Barnes maze platform (Stoelting Europe, Ireland) consisted of a circular surface (diameter 91 cm) with 20 circular holes (diameter 5 cm) around its circumference. The table surface was brightly lit by overhead lightning (900 lx), under one hole was a "flight box" (diameter 5 cm, depth 6 cm, length 15 cm of the flight box). On the first day of the trial, animals were accustomed to the flight box without getting to know the entire set-up (2 min/mouse). Mice were placed in the center of the platform (starting point) for a total test duration of 4 min. The first test run took place 24 h after the habituation. For each trial, mice had 240 s to find the area around the flight box and hide in the flight box. Recording was stopped either when the mouse entered the flight box or automatically after 240 s. The mice completed 2 test runs on each of the first two test days, and on test days 3 and 4, one each with a different position of the flight box. Before the test runs, the cage with the animals was placed in a dark box so that the bright light above the platform acted as an aversive stimulus. Extra maze cues were used and all surfaces were carefully cleaned with water after each individual. The search strategy used by the animals during the test is grouped in 3 categories—Direct, Wall and Random. 'Direct' included animals that found the box instantly when put on the platform. The 'Wall' category included animals that walked along the rim of the platform to search for the box. This strategy is based on spatial orientation and memory.

Injections and tissue sampling

Injections of test substances or vehicle (PBS) as control were carried out using a 30-gauge cannula. The test substances were administered once intraperitoneally or weekly intravenously (total volume of 5 ml/kg) over 12 weeks. Antibody M07-TFN was diluted to a final concentration of 2 or 6 mg/ml in 1× PBS. 5xFAD animals with mixed gender ($n = 12$ per treatment group) aged 4 months received M07-TFN antibody or vehicle control at a dose of 5.5, 10, or 30 mg/kg/week for 3 or 12 weeks. At the end of the experiment, animals were euthanized by Ketamine (150 mg/kg, Serumwerk Bernburg, Germany) and Xylocaine (15 mg/kg, WDT, Garbsen, Germany) injection followed by PBS perfusion and subsequently the brain was dissected and immediately frozen on dry ice using Tissue-Tek® O.C.T.™ Compound medium (Sakura Finetek, Germany) and stored at −80 degrees until processing.

Immunohistochemistry, image acquisition, and quantification

All histopathological studies were done in a blinded manner, such that the investigator did not know which treatment group was associated with each analyzed image. After freezing, brains were cut into 5 µm thick sagittal sections using a Thermo Fisher cryotome (Model HM 355S), collected on coverslips, and stored at −20°C until further use. Due to technical restraints, $N = 7$ animal brains were available from each treatment group for further sectioning. For immunohistochemical analyses, 5 µm sections were fixed with 4% PFA and subsequently blocked with goat serum blocking buffer (2% goat serum, 0.05% Tween-20 in 0.01 M PBS pH 7.2–7.4) and stained with primary antibody (β-Amyloid, 6E10, Biolegend, #803,004 and PU.1, Cell Signaling Technology, #2258) in staining buffer (1% bovine serum albumin, 0.1% gelatin from cold water fish skin, 0.5% Triton X-100 in 0.01 M PBS pH 7.2–7.4) over night at 4°C. Sections were washed in 1xPBS and staining with secondary antibody in antibody buffer (0.05% Tween-20 in 0.01 M PBS pH 7.2–7.4) was performed in staining buffer for 1 h at room temperature. After washing, tissue sections were stained with DAPI for 10 min at RT and mounted using ProlongTM Gold Antifade reagent. Images were acquired using an EVOS M7000 imaging system. Five images of the cortical region were taken per slide using a 20 ×objective at 2048 ×2048 pixel resolution. CellProfiler software [42] and ImageJ were applied for blinded image analyses. Automated detection of nuclear signals (DAPI as proxy for total cell number and PU.1 as marker for microglial cells) was performed using the Otsu thresholding method. The proportion of $PU.1^+$ cells was determined as the fraction of total cell number (all $DAPI^+$ cells). Plaque number and plaque area (marked by ß-Amyloid) were determined using ImageJ's area selection tool and counter plugin. Immunohistochemical analysis of average area of Aß plaques in μm^2 per mouse cortex was performed by determining the area of plaques per image followed by division by the number of plaques detected per image across five images taken per mouse cortex. Average total

Aß plaque area per mouse cortex in μm^2 was determined by measurement of the total ß-Amyloid positive area per image divided by the number of images taken per mouse cortex.

Safety study in 5xFAD mice including full necropsy and histo-pathological analysis (vivo Science GLP study I48-001)

Additionally, the safety of M07-TFN was studied in 5xFAD mice with a genetic background of C57BI/6 J over 12 weeks. 24 mice were administered either 30 mg/kg body weight M07-TFN dissolved PBS or PBS alone as vehicle control (12 mice in each group; [male/female: 6/6] in each group) once per week over 11 weeks via intravenous injection. The injection volume was 5 ml/kg body weight.

A clinical health monitoring was conducted by experienced veterinarians on all work days. Blood sampling at study start, and after 4 and 8 weeks were used to analyse white and red cell counts, differential cell counts (including lymphocytes, monocytes, neutrophils, granulocytes, basophils, eosinophils, mean cell volumes, platelets, haemoglobin content and relative proportions, and haematocrit). Additional samplings at study start and end (after 12 weeks) allowed for blinded analysis of GST, AST, creatinine, and urea. Blood sampling was only possible for either blood cell counts or serum values, not for both, at one time due to maximum sampling volumes according to animal protection rules. After euthanasia, a full necropsy at end of in-life were conducted and major organs were harvested, including heart + aorta + lung + oesophagus + trachea, spleen and liver, mandibular lymph node, bone marrow (from sternum) and kidneys + adrenal glands. The necropsied organs were fixed in formalin, then sent to Vivo Science, Göttingen, Germany, for subsequent histological preparation followed by blinded histopathological examination by an experienced pathologist according to principles of good laboratory practice (GLP).

Statistical analysis

Values shown in the figures are presented as mean ± SEM unless otherwise mentioned. If no other test of significance is indicated, for statistical analysis of two groups of samples the unpaired, two-tailed student´s t-test was performed. For comparison of more than two groups, one-way ANOVA and Dunnett`s post hoc test were used. Statistical analysis was performed using Prism 11. For comparison of more than two groups with two or more features, two-way ANOVA with Tukey's multiple comparison test was performed. Statistical significance was set at *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$; and ****, $p < 0.0001$.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13195-025-01759-x.

Supplementary Material 1. Supplementary Figure 1. Binding of M07-TFN but not M07-WT to TfR1 ELISA with streptavidin and biotinylated hTfR1. OD450 values at several concentrations are plotted. M07-TFN and MEM-189 (anti-hTfR1 antibody) bound hTfR1, whereas M07-WT only resulted in background signals.

Supplementary Material 2. Supplementary Figure 2. Animals become less active in mixed-gender 5xFAD mice **A** Distance which the animals traveled during the habituation to the two objects in the arena was reduced over time since they got accustomed to the set-up. **B** The speed at which the animals are moving during the habituation to the two objects in the arena was reduced over time. **C** The time the animals were mobile during the habituation to the two objects in the arena was reduced over time. **D** The distance the animals traveled during the Habituation and NOR was measured and showed reduced activities in the vehicle-treated group as compared to the animals treated with 10 and 30 mg/kg M07-TFN antibody.

Acknowledgements

We are grateful to the members of the Neurodegeneration Team at ISAR Bioscience for continual discussion and for critical reading of the manuscript.

Authors' contributions

M. K., J. F., A. J., J. K., B. F., B. P., M. C. conceived some experiments, carried out and analysed data. B. P., M. U. and M. C. also conceived experiments, wrote the manuscript text and prepared figures. All authors reviewed the manuscript.

Funding

This work was supported by a grant from the Bundesministerium für Bildung und Forschung (BMBF – 03LW0472 K).

Data availability

Data is provided within the manuscript or supplementary information files. The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

All animal experiments were permitted by the local Ethics board in accordance with European and German animal welfare legislations (5.1–231 5682/LMU/BMC/CAM). The most recent version of the Animal Research: Reporting In Vivo Experiments (ARRIVE) guidelines was strictly followed.

Consent for publication

Not applicable.

Competing interests

Several authors are employees of the biotech company ISAR Bioscience which is, however, owned (100%) by a non-profit foundation.

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Received: 23 January 2025 Accepted: 7 May 2025
Published online: 22 May 2025

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