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The Murine Version of BAN2401 (mAb158) Selectively Reduces Amyloid-β Protofibrils in Brain and Cerebrospinal Fluid of tg-ArcSwe Mice

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Abstract. Amyloid-β (Aβ) immunotherapy for Alzheimer's disease (AD) has good preclinical support from transgenic mouse models and clinical data suggesting that a long-term treatment effect is possible. Soluble Aβ protofibrils have been shown to exhibit neurotoxicity *in vitro* and *in vivo*, and constitute an attractive target for immunotherapy. Here, we demonstrate that the humanized antibody BAN2401 and its murine version mAb158 exhibit a strong binding preference for Aβ protofibrils over Aβ monomers. Further, we confirm the presence of the target by showing that both antibodies efficiently immunoprecipitate soluble Aβ aggregates in human AD brain extracts. mAb158 reached the brain and reduced the brain protofibril levels by 42% in an exposure-dependent manner both after long-term and short-term treatment in tg-ArcSwe mice. Notably, a 53% reduction of protofibrils/oligomers in cerebrospinal fluid (CSF) that correlated with reduced brain protofibril levels was observed after long-term treatment, suggesting that CSF protofibrils/oligomers could be used as a potential biomarker. No change in native monomeric $A\beta_{42}$ could be observed in brain TBS extracts after mAb158-treatment in tg-ArcSwe mice. By confirming the specific ability of mAb158 to selectively bind and reduce soluble Aβ protofibrils, with minimal binding to Aβ monomers, we provide further support in favor of its position as an attractive new candidate for AD immunotherapy. BAN2401 has undergone full phase 1 development, and available data indicate a favorable safety profile in AD patients.

Keywords: Alzheimer's disease, amyloid-β, antibody, BAN2401, biomarker, cerebrospinal fluid, immunotherapy, mAb158, oligomer, protofibrils

INTRODUCTION

Alzheimer's disease (AD) is a neurodegenerative disorder characterized by brain deposition of amyloid plaques and neurofibrillary tangles. Research over the past several decades support a critical role for amyloid-β $(A\beta)_{42}$ in this process [1, 2]. $A\beta_{42}$, a cleavage product of amyloid-β protein precursor (AβPP), is prone to aggregation and is a major constituent of amyloid plaques [3, 4]. Since a definitive correlation between amyloid plaques and degree of dementia in AD has not been established [5, 6], attention has turned to soluble aggregates of Aβ such as oligomers and protofibrils.

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A wide spectrum of soluble Aβ aggregates with pathogenic effects has been described (reviewed in [2]). These soluble aggregates display neurotoxicity and the ability to inhibit synaptic function *in vitro* and *in vivo* [7–15]. Further, amelioration of cognitive impairment in transgenic mouse models using Aβ immunotherapy has been demonstrated in the absence of amyloid plaque reduction, suggesting that soluble Aβ species are associated with memory impairment in these models [16, 17]. Perhaps more importantly, data from AD patients indicate that the pool of soluble brain Aβ species appears to be a better correlate of cognitive decline than amyloid plaque burden [18–20]. Similarly to the French mutation (AβPPV715M) [21], a familial form of early-onset AD caused by the Arctic mutation (AβPPE693G or $A\beta PP_{Arc}$) is characterized by reduced total Aβ levels compared to most other familial AD mutations. However, the Arctic mutation interestingly results in accelerated formation of Aβ protofibrils [22–24]. Indeed, Arctic mutation carriers that had developed the disease were negative for PiB PET, an imaging technique that detects insoluble fibrillar Aβ deposits, suggesting that protofibrils have a central role in the disease mechanism [25]. Aβ protofibrils, first described by Walsh et al. in 1997 [26], have been found to be the major Aβ species in the soluble pool of Aβ extracted from the brain of AD patients as well as tg-ArcSwe mice [27]. The fractions containing Aβ protofibrils also showed significant cell toxicity. The increased protofibril levels found in young tg-ArcSwe mice correlate with diminished spatial learning [28], supporting the *in vitro* findings of protofibril neurotoxicity. Therefore, Aβ protofibrils constitute a highly relevant target for disease intervention.

Current treatments for AD only ameliorate the symptoms of the disease. Therefore, novel therapeutic strategies are aimed at halting or attenuating disease progression, for example by inhibiting formation, or promoting clearance of $A\beta_{42}$. These therapies include inhibitors of BACE and γ-secretase enzymes that cleave Aβ from AβPP, and immunotherapeutic approaches utilizing antibodies to target Aβ. The BACE and γ-secretase inhibitors have suffered from toxicity and challenges with target specificity. For γ-secretase inhibitors, this has led to discontinuation of clinical trials due to worsening of cognitive decline in AD patients, and increased frequency of skin cancer [29, 30]. BACE1 inhibitors have thus far been well tolerated in phase I studies and their success in patients is still under evaluation, but this enzyme has several substrates that may result in toxicity after prolonged treatment [29, 31, 32]. Since the initial Aβ immunization studies performed by Schenk et al. (1999), numerous studies in transgenic mouse models of AD have shown a dramatic effect on plaque burden and cognition using both active immunization and passive transfer of Aβ antibodies [16, 17, 33–39].

A phase 2 clinical trial of $A\beta_{42}$ immunization (AN1792) was stopped due to symptoms consistent with meningoencephalitis in 6% of the patients. The cases of meningoencephalitis were independent of antibody titers [40]. Interestingly, significantly reduced cognitive decline was found in antibody responders compared to placebo-treated patients. This provides preliminary support for the potential long-term benefits of Aβ immunotherapy, though these studies were based on very small sample sizes in part due to the limited number of antibody responders [41–43]. Since then, passive administration of Aβ antibodies has been used as an alternative therapeutic approach that is believed to minimize the risk of a proinflammatory T-cell based response [44]. Monoclonal antibody administration has been safely employed for over 20 years with more than 25 antibodies currently in clinical use [45], supporting this approach. Clinical trials of bapineuzumab, a monoclonal antibody directed against fibrillar Aβ, were stopped because of imaging abnormalities and absence of clinical effects. However, pooled data from mild AD patients in two phase 3 studies of another monoclonal antibody targeting soluble Aβ, solanezumab, have shown some effects on cognitive measures indicating that beneficial effects of Aβ immunotherapy are possible [43, 46–50]. In this report, we examine the potential of BAN2401, an antibody with a unique target binding profile selective for Aβ protofibrils, to provide an effective treatment for AD.

Here we confirm that BAN2401, the humanized version of mAb158, exhibits a strong binding preference for soluble Aβ protofibrils compared to monomers. Further, we find that the mAb158-mediated selective reduction of brain Aβ protofibrils was mirrored by a similar lowering of protofibrils in cerebrospinal fluid (CSF) from tg-ArcSwe mice, indicating a possible use of CSF Aβ protofibrils as a clinical biomarker. These results indicate the potential for BAN2401 to effectively reduce brain Aβ protofibrils in AD patients.

MATERIALS AND METHODS

Ethics statement

The use of human brain material was approved by the regional ethical committee in Uppsala (decision number 2009/089). Written informed consent was

obtained from all subjects involved in the study (or their relatives). All animal procedures were performed in accordance with the ethical committee in Stockholm (decision number N247/09), and applicable guidelines for animal experimentation and animal care were followed.

Aβ monomers and protofibrils

$A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$ peptides were purchased from American Peptide Company, CA, USA. Lyophilized peptides were dissolved to 100 μM in 10 mM NaOH. $A\beta_{42}$ protofibrils were prepared by diluting the $A\beta_{1\text{-}42}$ peptide to 50 μM in 0.1 M Phosphate buffer containing 0.3 M NaCl, pH 7.4. The preparation was incubated for 30 min at 37°C and then centrifuged at 16000× g for 5 min to pellet potential large aggregates. The supernatants were further purified from monomers by size exclusion chromatography (Superdex 75 column, GE Healthcare, Sweden) at a flow rate of 0.08 ml/min in 0.05 M Phosphate buffer, 0.15 M NaCl, pH 7.4, and protofibrils were collected in the void fraction as previously described [22, 26, 27], see Supplementary Figs. 1–3.

Antibodies

The protofibril-selective monoclonal mouse antibody mAb158 (IgG2a, BioArctic Neuroscience, Stockholm) was formulated in sterile PBS (pH 7.5) at a concentration of 2 mg/ml and has previously been characterized [51]. Its humanized form, BAN2401 (Eisai Co., Ltd., Tokyo) was formulated in sterile PBS (pH 7.5) at a concentration of 2 mg/ml.

Biacore analysis

BAN2401 and mAb158 binding to Aβ monomers and Aβ protofibrils were evaluated by surface plasmon resonance (SPR) analysis using a Biacore T100 instrument. For the antibody-Aβ protofibril interaction experiments, 80 resonance units (RU) $A\beta_{42}$ protofibrils were immobilized on a Biacore CM5 sensor chip surface using amine coupling chemistry. HBS-EP was used as running buffer and 0.78, 1.6, 3.1, 6.3, 13, 25, 50, and 100 nM antibody samples were passed over the sensor surface at a flow rate of 30 μl/min. Using this setup for the protofibril, each individual antibody binding event to the protofibril will be detected. The rate constants for the interaction were determined by fitting the SPR sensorgrams to a bivalent analyte model. For the antibody-Aβ monomer interaction experiments, 2000 RU antibody (mAb158 or BAN2401) were immobilized on a Biacore CM5 sensor chip surface using amine coupling chemistry. HBS-EP was used as running buffer and 0.061, 0.13, 0.25, 0.5, 1, 2, and 4 μM $A\beta_{1\text{-}40}$ monomer samples were passed over the sensor surface at a flow rate of 30 μl/min. The kinetic constants were determined by fitting the SPR sensorgrams to a 1 : 1 Langmuir binding model.

Inhibition ELISA

The half maximal inhibitory concentration (IC50) values of BAN2401 and mAb158 for Aβ protofibrils and Aβ monomers were determined using an inhibition ELISA in which antibodies were pre-incubated in solution with protofibrils or monomers and then transferred to Aβ coated ELISA plates [51]. When Aβ peptide is adhered to a substrate such as an ELISA plate, it behaves similarly to an Aβ protofibril in solution with respect to its ability to capture the protofibril-selective mAb158 antibody. $A\beta_{42}$ protofibrils were diluted to 500 nM in incubation buffer (Dulbecco's PBS containing 0.1% BSA and 0.05% Tween-20) and $A\beta_{1\text{-}40}$ monomers were diluted to 25 μM. The solutions were then further diluted in duplicates in an 11-step 3x serial dilution in 96-well storage plates, to generate a final volume of 30 μl per well. To each well, 30 μl of antibody (0.1 μg/ml) were added and the plates were incubated for 45 min at room temperature (RT) with shaking (900 rpm). The samples were then transferred to ELISA plates that had been coated with 0.5 μM $A\beta_{1\text{-}40}$ overnight at +4°C, and blocked with 1% BSA in Dulbecco's PBS (D-PBS). Samples were incubated for 25 min at RT without shaking, after which anti-mouse IgG-ALP (Mabtech, Sweden, 1 : 1000) or anti-human IgG-ALP (Mabtech, Sweden, 1 : 3000) was added for 45 min. Plates were developed with ALP substrate (pNPP tablets, Sigma Aldrich, USA) for 30–60 min and optical density was measured at 405 nm (Infinite M1000, Tecan, Switzerland).

Immunoprecipitation

Five AD brains, AD1 (Swedish mutation carrier), AD6, AD10, AD11, and AD19, and one control brain (C12) from Uppsala brain bank were included in this study. Brain samples, obtained from temporal cortex, were homogenized on ice using Dounce homogenizer (2 × 10 strokes) in 1 : 10 weight:volume Tris buffered saline (20 mM Tris, 137 mM NaCl,

pH 7.6, TBS) containing protease inhibitors (Complete mini, Roche). The samples were centrifuged at 16000 × g for 1 h and the supernatants were defined as TBS extracts. Immunoprecipitations were performed on the TBS extracts using mAb158, BAN2401, or a hIgG1 control antibody (SIGMA, 15154). Antibodies were covalently coupled to Tosyl-activated M280 Dynabeads™ (20 μg antibody/mg Dynabeads) according to the manufacturer's recommendations (Invitrogen). A DynaMag™-2 magnet (Invitrogen) was used in all steps where liquid was separated from beads. All incubations were performed with end-over-end rotation. In brief, coupling was performed overnight at 37°C, followed by blocking with 0.5% BSA in D-PBS (1 h at 37°C). Brain TBS extracts (50 μl) were diluted by adding 420 μl IP-buffer (D-PBS containing 0.1% BSA and 0.5% Tween-20). Antibody-coupled Dynabeads (30 μl) were added to the samples and the immunoprecipitation reactions were allowed to incubate for 2 h at RT. The Dynabeads were washed 4 × 10 min in IP-buffer followed by a final rapid wash in D-PBS and careful removal of all liquid. Immunoprecipitated Aβ was eluted and monomerized by adding 20 μl 70% formic acid (FA).

Aβ$_{42}$ ELISA on immunoprecipitated human brain samples

Total soluble Aβ$_{42}$ levels in TBS extracts denatured with 70% FA, and in immunoprecipitated samples (eluted in 70% FA), were measured using the high sensitive Human/Rat β Amyloid (42) ELISA Kit (Wako). Samples were neutralized using 19 parts 1 M Tris to one part denatured/eluted sample. Diluted samples were run in duplicate and manufacturer's protocol was followed.

Animal treatments

In a short-term study, 19 (13–14 months old) tg-ArcSwe mice [52] were equally distributed in three groups, treated for four weeks and sacrificed 24 h (mAb158) or 7 days (mAb158 and PBS) after the last injection. The PBS group and the 24 h group contained two female and four male mice, and the group sacrificed 7 days after the last injection contained three female and four male mice. In a long-term study, 111 (11–13 months old) tg-ArcSwe mice were equally distributed in two groups and treated for 13 weeks. The PBS group contained 27 females and 28 males, and the mAb158 treatment group contained 27 females and 29 males. Animals were sacrificed 7 days after the last injection. In both studies, animals received weekly intraperitoneal (i.p.) injections of either D-PBS (5 ml/kg) or 10 mg/kg mAb158. Four animals in the mAb158-treatment group of the long-term study developed anti-drug antibodies and were excluded from all subsequent analyses.

Tissue sampling

At animal study termination, CSF was collected from cisterna magna and immediately frozen on dry ice. The amount of blood contamination of CSF samples was evaluated by visual inspection and graded on a scale from zero to two, with zero indicating no visible blood contamination and two indicating clearly visible blood contamination. Blood was sampled from the heart and collected in EDTA tubes (BD), and plasma was prepared. The mice were transcardially perfused with physiological saline at body temperature, brains were collected and after removal of olfactory bulbs and cerebellum, the left hemisphere was immediately frozen on dry ice. The success of the perfusion was assessed by visual inspection and each brain was graded on a scale from zero to two, with zero indicating no visible blood contamination and two indicating clearly visible blood contamination. Frozen brains were homogenized on ice using Dounce homogenizer (2 × 10 strokes) in 1 : 10 weight:volume TBS containing protease inhibitors (Complete mini, Roche) followed by either 16000× g or 100000× g centrifugation. The supernatants were defined as TBS extracts.

mAb158 sandwich ELISA

The mAb158 sandwich ELISA, previously described in [51], specifically detects Aβ protofibrils [27] without interference from Aβ monomers or mAb158 at levels present in brain TBS extracts from tg-ArcSwe mice analyzed here. ELISA plates were coated with 2 μg/ml mAb158 in D-PBS overnight at +4°C and blocked with 1% BSA in D-PBS for 90 min. TBS extracts were incubated in duplicate for 2 h at 22°C with shaking (900 rpm), thereafter biotinylated mAb158 (0.5 μg/ml) was added and the plates were incubated for another hour. Streptavidin-HRP (Mabtech, Sweden 1 : 5000) was used as detection agent (45 min incubation). Plates were developed with TMB substrate and the reaction was stopped after 15 min by the addition of 2 M H_2SO_4. Optical density was measured at 450 nm and sample concentrations were calculated from an Aβ$_{42}$ protofibril standard curve using a 4-parameter equation.

82E1 sandwich ELISA

82E1 is an antibody that detects the N-terminus of β-secretase cleaved AβPP. In the 82E1 sandwich ELISA described here, 82E1 is used for both capture and detection. Hence, the 82E1 sandwich ELISA detects Aβ oligomers/protofibrils (dimers to protofibrils) without binding Aβ monomers or interference by mAb158 at levels present in CSF from tg-ArcSwe mice analyzed here (see Supplementary Fig. 4). White 384-well plates (Greiner bio-one) were coated overnight at +4°C with 0.25 μg/ml of 82E1 antibody (IBL, Japan) diluted in D-PBS. After blocking in 1% BSA in D-PBS, CSF samples were diluted 1 : 50 in incubation buffer and added in duplicate (25 μl/well) and incubation was carried out overnight at +8°C. Biotinylated 82E1 antibody (0.25 μg/ml) was added and plates were incubated for 2 h at RT, followed by detection using streptavidin-HRP, diluted 1 : 4000 (45 min incubation at RT). Plates were developed with SuperSignal substrate (Thermo Scientific) and luminescence was measured after shaking the plate for 1 min. Sample concentrations were calculated from an $A\beta_{42}$ protofibril standard curve using a 4-parameter equation.

ELISA analysis of mAb158 in plasma and brain

The levels of mAb158 in TBS brain extracts and plasma samples were measured using a direct ELISA. ELISA plates were coated with 0.5 μM $A\beta_{1\text{-}40}$ in PBS at +4°C overnight. The ELISA plates were blocked with protein-free blocking buffer (Thermo Fisher Scientific, USA). Samples were diluted in incubation buffer and incubated on the plate for 90 min. Detection was carried out for 1 h using an HRP-conjugated goat anti-mouse IgG (Southern Biotech, 1 : 10000). Plates were developed with TMB substrate (Neogen, USA) and the reaction was stopped after 15 min by the addition of 2 M H_2SO_4. Optical density was measured at 450 nm and sample concentrations were calculated from a mAb158 standard curve using a 4-parameter equation in the Magellan software (Tecan, Switzerland). The mAb158 levels in TBS extracts were converted to nanogram antibody per gram brain tissue using a multiplication factor of 10 to account for the 10-fold dilution that is introduced when the tissue is homogenized in TBS. This measurement of mAb158 does not discriminate between free mAb158 or mAb158 potentially bound to Aβ. Animals with a perfusion grade of >0 were excluded from ELISA analyses of mAb158 concentration in brain ($n = 2$ from short-term study, $n = 11$ from long-term study), to avoid overestimating the degree of mAb158 brain penetrance.

Analysis of $A\beta_{1\text{-}42}$ in mouse brain

Soluble $A\beta_{1\text{-}42}$ was measured in 16000× g brain TBS extracts from the long-term study that had either been subjected to denaturation/monomerization by boiling in 1% SDS or had been left untreated (native). ELISA plates were coated with 0.5 μg/ml polyclonal rabbit anti-$A\beta_{42}$ (BioArctic Neuroscience, Sweden) in D-PBS overnight at +4°C and blocked with 1% BSA in D-PBS for 90 min. TBS extracts were added in duplicate and incubated for 2 h at RT. Biotinylated 82E1 antibody (0.25 μg/ml) was added and plates were incubated for 1 h. Plates were developed with TMB substrate for 15–30 min and the reaction was stopped by adding 2 M H_2SO_4. Optical density was measured at 450 nm and sample concentrations were calculated from an $A\beta_{42}$ standard curve using a 4-parameter equation. This measurement of $A\beta_{42}$ does not discriminate between free $A\beta_{42}$ or $A\beta_{42}$ bound to mAb158.

Statistical analysis

All statistical analyses were performed using GraphPad Prism version 6.02 for Windows (GraphPad Software, San Diego, CA, www.graphpad.com). Unpaired two-tailed *t*-test of mAb158-treated versus placebo-treated mice was performed at a significance level of $p < 0.05$. Correlation analysis was performed using a two-tailed Pearson correlation. One-way ANOVA with Tukey's multiple comparison test was performed at a significance level of $p < 0.05$. IC50 values were computed using non-linear regression with variable slope (four parameters) of the log (inhibitor) versus normalized response using $y = 0$ as 0%.

RESULTS

BAN2401 and mAb158 bind selectively to Aβ protofibrils analyzed by surface plasmon resonance

The binding characteristics of BAN2401 and its murine version mAb158 were evaluated by SPR. Aβ protofibrils were immobilized on the sensor chip and antibodies passed over the surface to allow the antibodies to bind individually to the protofibrils. Since each antibody will give rise to two linked binding events in this setting, a bivalent analyte model was used for evaluation. This model allows for determination of an

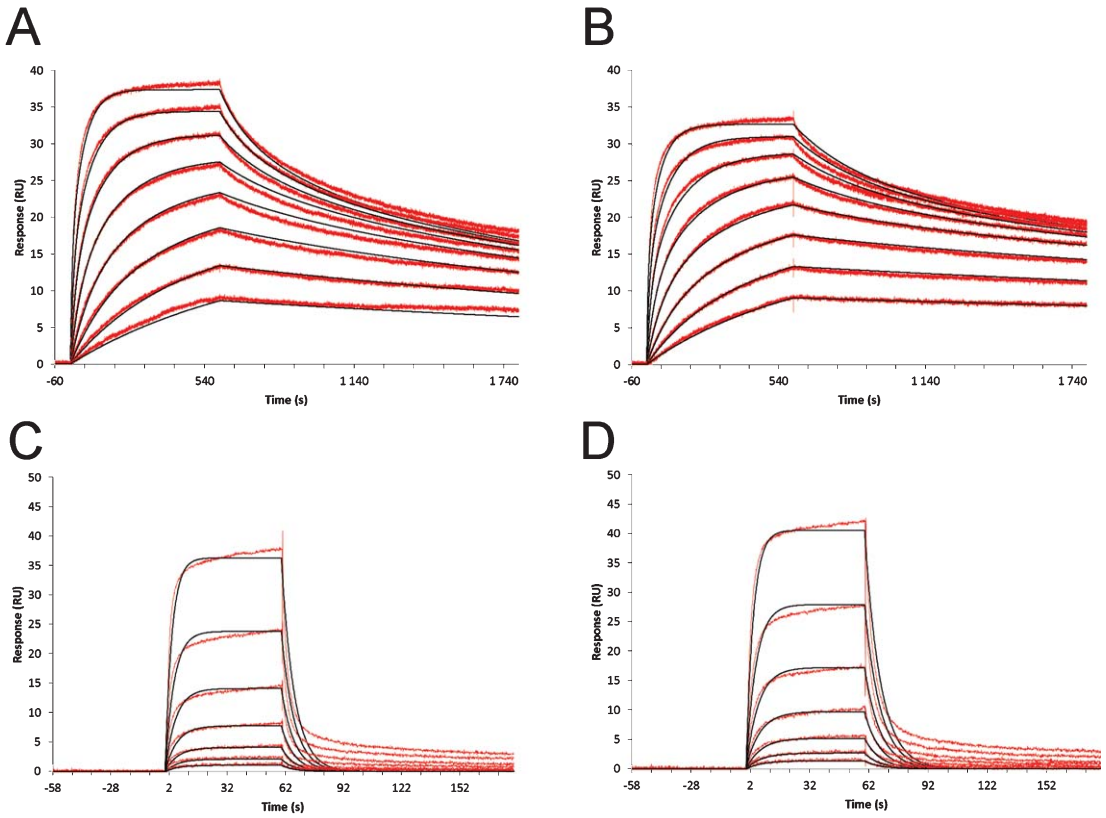

Fig. 1. mAb158 and BAN2401 show stronger binding to $A\beta_{42}$ protofibrils than to Aβ monomers. SPR sensorgrams from mAb158 antibody (A) and BAN2401 antibody (B) interactions with $A\beta_{42}$ protofibrils. In the experiments, 80 RU $A\beta_{42}$ protofibril was immobilized on the chip surface and 0.78, 1.6, 3.1, 6.3, 13, 25, 50, and 100 nM antibody samples were passed sequentially over the chip surface. The experimental data are shown in red, while the black curves represent fitting to a bivalent analyte model, assuming two binding events for each molecule. The rate constants for the initial binding were $k_{a1} = 5.1 \times 10^5$ 1/Ms and $k_{d1} = 0.0032$ 1/s for mAb158 and $k_{a1} = 6.6 \times 10^5$ 1/Ms and $k_{d1} = 0.0013$ 1/s for BAN2401, calculated from duplicate analyses. The apparent equilibrium dissociation constants, K_{D1} (only taking the first interaction of two into account), were 6.3 nM for mAb158 and 2.0 nM for BAN2401. SPR sensorgrams from mAb158 (C) and BAN2401 (D) interactions with $A\beta_{1\text{-}40}$ monomer. In these experiments, 2000 RU antibody was immobilized on the chip surface and 0.061, 0.13, 0.25, 0.5, 1, 2, and 4 μM $A\beta_{1\text{-}40}$ monomer samples were passed sequentially over the chip surface. The experimental data are shown in red, while the black curves represent curve fitting to a 1 : 1 Langmuir binding model. The kinetic constants were calculated from duplicate analysis, for mAb158 $k_a = 3.6 \times 10^4$ 1/Ms, $k_d = 0.15$ 1/s and $K_D = 4.2$ μM; and for BAN2401 $k_a = 3.8 \times 10^4$ 1/Ms, $k_d = 0.12$ 1/s and $K_D = 3.3$ μM.

apparent equilibrium dissociation constant (K_D) for the first interaction. A regular K_D cannot be determined since the second binding event is dependent on the first. However, the apparent K_D for the first interaction (K_{D1}) can be used for approximate comparisons. As seen in Fig. 1A (mAb158) and 1B (BAN2401), a slow dissociation from Aβ protofibrils was observed for both antibodies, demonstrating strong binding to the protofibril. The apparent K_D (K_{D1}) was found to be in the low nanomolar range for both mAb158 and BAN2401. As seen in Fig. 1C (mAb158) and 1D (BAN2401), a rapid dissociation of Aβ monomer was observed for both antibodies and the K_D were found to be 4.2 and 3.3 μM for mAb158 and BAN2401, respectively. Although it is not possible to directly compare the affinities obtained from the different evaluation models, it is clear that both antibodies show considerably stronger binding to Aβ protofibrils compared to monomeric Aβ.

BAN2401 and mAb158 bind selectively to Aβ protofibrils analyzed by inhibition ELISA

To verify the SPR results, we compared the binding characteristics of BAN2401 and mAb158 in solution using an inhibition ELISA. The antibodies were incubated with Aβ protofibrils and monomers at increasing concentrations in solution. By measuring the amount of unbound antibody present in solution, we achieved an indirect measurement of antibody binding to soluble

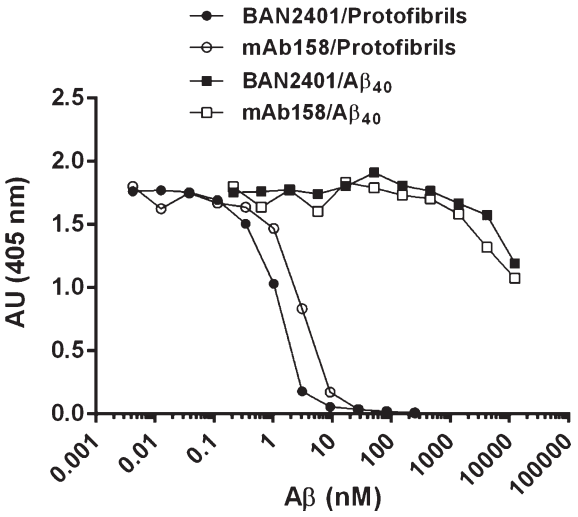

Fig. 2. BAN2401 and mAb158 are highly selective for protofibrils over monomers by inhibition ELISA. ELISA plates were coated with 0.5 μM $A\beta_{1\text{-}40}$. Binding of BAN2401 or mAb158 to increasing concentrations of $A\beta_{1\text{-}40}$ monomers ($A\beta_{40}$) or $A\beta_{42}$ protofibrils (Protofibrils) were performed in solution, before the mixture was added to the ELISA plate. Free BAN2401 or mAb158 was allowed to bind to the plate and detection was performed using an ALP-conjugated anti-human IgG antibody for BAN2401 and ALP-conjugated anti-mouse IgG antibody for mAb158. The concentration of $A\beta_{42}$ protofibrils and $A\beta_{1\text{-}40}$ monomers in the inhibition ELISA are expressed as nM of monomeric Aβ (x-axis). Protofibril inhibition of binding to the Aβ coat was pronounced for both BAN2401 and mAb158 (IC50 = 1.1 nM for BAN2401/Protofibrils versus 2.6 nM for mAb158/Protofibrils), while monomer ($A\beta_{40}$) inhibition of antibody binding to the Aβ coat was considerably less efficient for both BAN2401 and mAb158 (IC50 ~40000 nM for BAN2401/$A\beta_{40}$ versus 20000 nM for mAb158/$A\beta_{40}$). The IC50 values for monomer binding were calculated by extrapolation of the curve.

protofibrils and monomers. The antigen concentration required to inhibit half of the maximum signal in the inhibition ELISA was defined as IC50, which was used as an estimate of the antibody's binding preference for Aβ protofibrils or monomers [53]. Both BAN2401 and mAb158 displayed highly selective binding for Aβ protofibrils over monomeric Aβ (Fig. 2). Both antibodies exhibited an IC50 in the low nM range for protofibrils, while the IC50 for monomer ($A\beta_{1\text{-}40}$) was considerably higher (IC50 >20000 nM for both antibodies). These results are consistent with data obtained by SPR.

BAN2401 and mAb158 immunoprecipitate large soluble aggregates of $A\beta_{42}$ in AD brains

To confirm that BAN2401 and mAb158 were able to bind to their target in human AD brain, the antibodies were used for immunoprecipitation in 16000× g brain TBS extracts from five human AD brains and one age-matched control brain, followed by ELISA analysis of $A\beta_{42}$ levels. As shown in Fig. 3, complete immunoprecipitation of the total soluble $A\beta_{42}$ present in TBS extracts was accomplished with mAb158 and BAN2401, as shown by nearly identical levels of $A\beta_{42}$ in the eluted immunoprecipitates and the total denatured $A\beta_{42}$ levels (30–100 pmol/g) measured in the 16000× g brain TBS extracts. To verify that the efficiency of the immunoprecipitation reaction, the $A\beta_{42}$ levels in the supernatants from two of the patients (AD6 and AD11) were also measured. The supernatants contained less than 10% of the total $A\beta_{42}$ measured, supporting the conclusion that the vast majority of the $A\beta_{42}$ was immunoprecipitated using BAN2401 and mAb158. No Aβ was immunoprecipitated from the control brain by mAb158 or BAN2401 (Fig. 3), and the human IgG1 control antibody did not immunoprecipitate Aβ from any of the samples (data not shown).

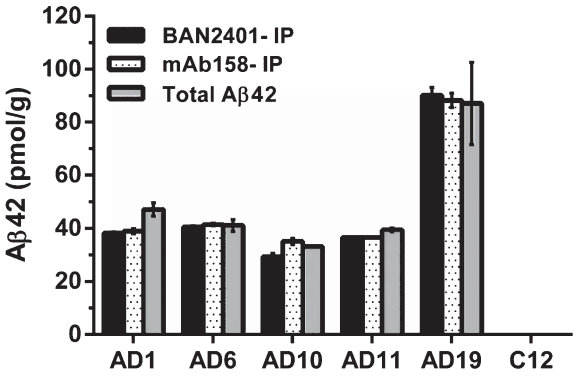

Fig. 3. BAN2401 and mAb158 successfully immunoprecipitated soluble aggregated $A\beta_{42}$ from human AD brain. Immunoprecipitation was performed using BAN2401 (BAN2401-IP) and mAb158 (mAb158-IP) in 16000× g TBS extracts from five human AD brains (AD1, AD6, AD10, AD11 and AD19) and one age matched control brain (C12). Total $A\beta_{42}$ levels were measured in the eluted immunoprecipitates and in denatured TBS extracts using Wako $A\beta_{42}$ ELISA. All soluble $A\beta_{42}$ in the TBS extracts was immunoprecipitated using both BAN2401 and mAb158, whereas no $A\beta_{42}$ could be immunoprecipitated from the control brain (LOD = 0.63 pmol/g).

Long-term administration of mAb158 in tg-ArcSwe mice reduces Aβ protofibril levels in brain and CSF

In order to evaluate whether the protofibril-selective mAb158 antibody could bind and clear soluble Aβ protofibrils *in vivo*, we performed weekly antibody administrations to tg-ArcSwe mice for 13 weeks (long-term study) and sacrificed the animals 7 days after the last injection. A significant 42% reduction of

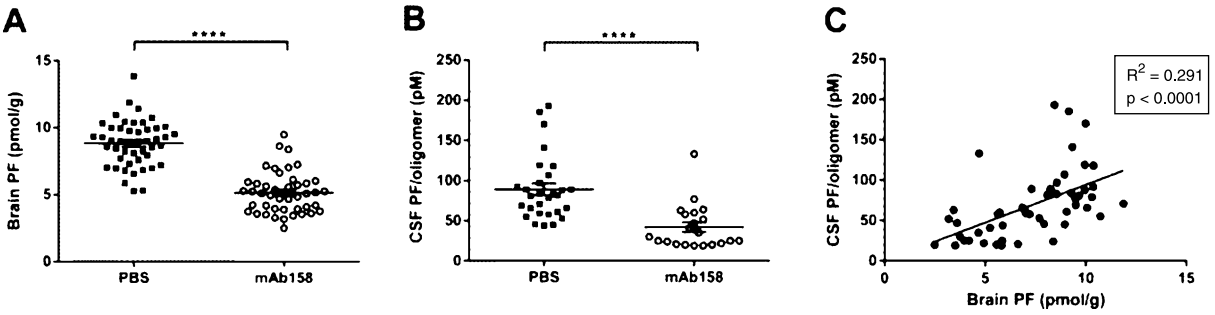

Fig. 4. Long-term treatment with mAb158 reduces Aβ protofibril levels in brain and CSF of tg-ArcSwe mice. A) Aβ protofibril (PF) levels in 16000× g TBS brain extracts were measured with the mAb158 sandwich ELISA after long-term treatment with mAb158. A significant 42% reduction in brain protofibril levels was detected in mice receiving long-term treatment with mAb158, compared to placebo-treated mice (*t*-test, $p < 0.0001$). Mice were sacrificed 7 days after the last injection. B) Aβ protofibril/oligomer levels in CSF were measured with the 82E1 sandwich ELISA. A significant 53% reduction in protofibril/oligomer levels was detected in mAb158-treated mice, compared to placebo-treated mice (*t*-test, $p < 0.0001$). C) A highly significant correlation was observed between protofibrils in brain and protofibrils/oligomers in CSF (Pearson's rank correlation, $p < 0.0001$, $R^2 = 0.291$). Protofibril levels are expressed as molarity of the monomeric $A\beta_{1\text{-}42}$ subunit (4.5 kDa)±SEM.

brain protofibrils was detected in mAb158-treated mice compared to placebo-treated mice (Fig. 4A). To assess the levels of Aβ protofibrils in CSF, the 82E1 sandwich ELISA was used to avoid interference from the treatment antibody. This assay differs from the mAb158 sandwich ELISA used for brain protofibrils in that the 82E1 assay also detects smaller soluble Aβ species (dimers to protofibrils). A 53% reduction of protofibrils/oligomers was detected in CSF from mAb158-treated mice compared to placebo (Fig. 4B). The levels of brain protofibrils and CSF protofibrils/oligomers in individual tg-ArcSwe mice displayed a highly significant correlation indicating a relationship between protofibrils in the brain and CSF compartments (Fig. 4C). The treatment effect was also evaluated in 100000× g brain TBS extracts and was similar to the effect observed in the 16000× g TBS extracts (46% reduction in protofibril levels after long-term treatment with mAb158 compared to placebo, data not shown). This result was consistent with our previous findings [54]. To ensure that these results were not limited to a transgenic model carrying the Arctic mutation, long-term treatment with mAb158 was performed in Tg2576 mice, resulting in a 27% reduction in brain protofibril levels after treatment with 12 mg/kg mAb158, and a 40% reduction after treatment with 24 mg/kg mAb158 (see Supplementary Fig. 6).

Short-term administration of mAb158 in tg-ArcSwe mice reduces Aβ protofibril levels in brain and CSF

To investigate whether shorter treatment duration with mAb158 could also clear soluble Aβ protofibrils in tg-ArcSwe mice, we performed weekly antibody administrations for 4 weeks (short-term study). Further, the mice were sacrificed 24 h or 7 days after the last injection to allow us to identify potential changes in CSF protofibril levels at two time-points after antibody administration. Short-term treatment with mAb158 resulted in a significant 42% reduction of Aβ protofibrils in brain TBS extracts from mAb158-treated mice sacrificed 7 days after the last injection, compared to placebo-treated mice (Fig. 5A). A trend toward protofibril reduction was observed in brain of mAb158-treated mice sacrificed 24 h after the last injection and in CSF protofibrils/oligomers of mice sacrificed at 7 days and 24 h, although these reductions did not reach significance (Fig. 5B). Consistent with the findings after long-term treatment, a significant correlation was seen between brain protofibrils and CSF protofibrils/oligomers after short-term treatment (Fig. 5C). The protofibril reductions observed in TBS extracts after short-term treatment with mAb158 were verified with the 82E1 sandwich ELISA. A 42% reduction in brain protofibrils/oligomers was observed in mice sacrificed 24 h after the last injection, and a 48% reduction in mice sacrificed 7 days after the last injection. The mAb158 sandwich ELISA analysis correlated strongly with the 82E1 sandwich ELISA analysis in TBS extracts from this study ($p < 0.0001$, $R^2 = 0.923$, see Supplementary Fig. 5).

mAb158 reaches the brain and correlates with reduced brain protofibril levels in tg-ArcSwe mice

To verify that mAb158 reached the brain of tg-ArcSwe mice, antibody levels in brain and plasma at the time of sacrifice were measured. Long-term treatment resulted in 0.2% brain penetrance of

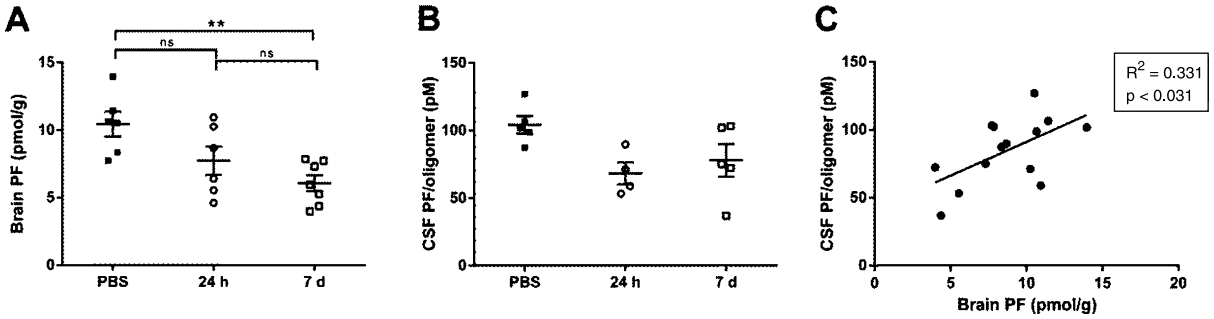

Fig. 5. Short-term treatment with mAb158 reduces Aβ protofibril levels in brain and CSF of tg-ArcSwe mice. A) Aβ protofibril (PF) levels in 16000× g TBS brain extracts from tg-ArcSwe mice after short-term treatment with mAb158 sacrificed 24 h or 7 days (7 d) after the last injection were measured with the mAb158 sandwich ELISA. A significant 42% reduction in protofibrils was detected in mAb158-treated mice sacrificed 7 days after the last injection, compared to PBS-treated mice (also sacrificed 7 days after the last injection, one-way ANOVA $p = 0.0076$, followed by Tukey's multiple comparison test). A trend towards reduction in brain protofibrils was observed 24 h after the last mAb158 injection, but this reduction was non-significant. B) Aβ protofibril/oligomer levels in CSF were measured with the 82E1 sandwich ELISA. A trend towards reduction in protofibril/oligomer levels was detected in mAb158-treated mice, compared to untreated mice, but this reduction did not reach significance (one-way ANOVA $p = 0.054$). C) A highly significant correlation was observed between protofibrils in brain and protofibrils/oligomers in CSF ($p = 0.031$, $R^2 = 0.331$). Protofibril levels are expressed as molarity of the monomeric $A\beta_{1\text{-}42}$ subunit (4.5 kDa)±SEM.

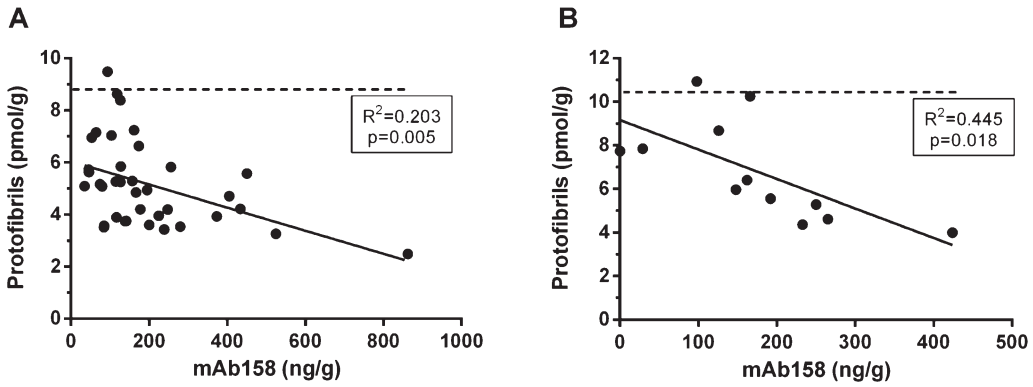

Fig. 6. mAb158 reaches the brain and correlates with reduced protofibril levels in tg-ArcSwe mice. mAb158 levels in plasma and brain (TBS extracts) were measured by ELISA. Brain samples with remaining blood contamination after PBS perfusion (by visual inspection) were excluded from analysis. Brain protofibril levels (mean: 5.2 ± 1.6 pmol/g) correlated negatively with brain mAb158 concentrations (mean: 197.3 ± 165.9 ng/g) in tg-ArcSwe mice that had received long-term treatment with mAb158 (Pearson's rank correlation, $p = 0.005$, $R^2 = 0.203$, A), and in tg-ArcSwe mice that had received short-term treatment with mAb158 (mean brain protofibril levels 6.8 ± 2.3 pmol/g, mean brain mAb158 concentrations 174.4 ± 113.1 ng/g, Pearson's rank correlation, $p = 0.018$, $R^2 = 0.445$, B). The dotted line in A and B indicates the average protofibril level of the placebo-treated groups in each respective study (8.8 pmol/g protofibrils in A and 10.4 pmol/g protofibrils in B). Brain penetrance from the plasma compartment was calculated by dividing brain mAb158 concentration (mAb158 concentration per gram brain tissue) by plasma mAb158 concentration (mAb158 concentration per milliliter plasma), assuming a density of 1 g/ml for both plasma and brain, as previously reported [68]. For animals that had received long-term treatment with mAb158, brain penetrance was 197.3 ng/g / 99000 ng/ml = 0.2% at the time of sacrifice. For animals that had received short-term treatment with mAb158, brain penetrance was 168.2 ng/ml / 156833 ng/ml = 0.1% for the group sacrificed 24 h after the last injection and 180.7 ng/g / 79857 ng/ml = 0.2% for the group sacrificed 7 days after the last injection.

mAb158 from the plasma compartment in samples taken 7 days post-dosing. The brain levels of mAb158 showed a significant negative correlation with the levels of brain protofibrils, suggesting an exposure-dependent relationship (Fig. 6A). In the short-term study, the brain penetrance from the plasma compartment was 0.1% in the 24 h group and 0.2% in the 7-day group, and also here a strong negative correlation was seen between brain levels of mAb158 and protofibrils (Fig. 6B).

Long-term treatment with mAb158 reduces soluble aggregated Aβ levels in brain

To determine whether treatment with mAb158 affected total brain levels of $A\beta_{42}$, we measured $A\beta_{42}$

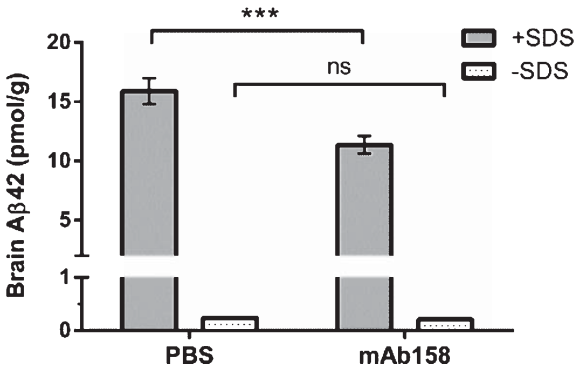

Fig. 7. Long-term treatment with mAb158 reduces SDS-denatured $A\beta_{1\text{-}42}$ levels in brain from tg-ArcSwe mice. $A\beta_{1\text{-}42}$ levels were measured in 16000× g brain TBS extracts by ELISA after boiling in 1% SDS (+SDS) or in the absence of SDS pre-treatment (-SDS). A significant 29% reduction in SDS-denatured $A\beta_{1\text{-}42}$ was detected in the mice that received mAb158, compared to placebo-treated mice (*t*-test, $p < 0.001$). No treatment effect on native monomeric $A\beta_{1\text{-}42}$ levels (-SDS) was found.

by ELISA in native 16000× g TBS extracts and in 16000× g TBS extracts that had been boiled in 1% SDS. As this SDS pre-treatment denatures aggregated Aβ species and can also dissociate other types of Aβ-protein interactions, it allows us to measure total $A\beta_{42}$ in the brain. In native TBS extracts, monomeric Aβ species would be detected in this ELISA. A significant 29% reduction of total $A\beta_{42}$ in SDS-denatured TBS extracts was observed in mice that had received long-term treatment with mAb158 compared to placebo (Fig. 7). Notably, in native TBS extracts, no change in monomeric $A\beta_{42}$ could be found after mAb158-treatment. Taken together, these results indicate the specific ability of mAb158 to selectively bind and reduce soluble Aβ protofibrils *in vitro* and *in vivo*, with minimal binding to native Aβ monomers.

DISCUSSION

Here, we demonstrate that both BAN2401 and its murine version, mAb158, exhibit high selectivity for soluble Aβ protofibrils over Aβ monomers. Further, we provide evidence that the Aβ protofibril target of these antibodies is present in brains from AD patients. We have also used mAb158 for long- and short-term treatment of tg-ArcSwe mice, and found that mAb158 reaches the brain from the plasma compartment and that the treatment leads to reduced levels of soluble Aβ protofibrils in brain extracts in an exposure-dependent manner. Importantly, we demonstrate that the reduction of brain protofibrils in tg-ArcSwe mice was mirrored by a reduction of protofibrils/oligomers in CSF, indicating the use of CSF protofibrils/oligomers as a biomarker for treatment effect. To our knowledge, this is the first report of a correlation between Aβ protofibrils in brain and CSF.

Interestingly, near complete immunoprecipitation of the total $A\beta_{42}$ found in TBS extracts from AD patients was achieved with the protofibril-selective antibodies mAb158 and BAN2401. This suggests that the majority of the soluble $A\beta_{42}$ species found in TBS extracts are in the form of soluble aggregates. Similarly, when subjecting brain TBS extracts from tg-ArcSwe mice to denaturation by boiling in 1% SDS, the majority of the soluble pool of $A\beta_{42}$ in these brain extracts were found to be in aggregated form. These results are consistent with the findings of Sehlin and colleagues who found that most Aβ in soluble brain extracts from AD patients and tg-ArcSwe mice were high molecular weight species matching the size of the 80–500 kDa synthetic Aβ protofibrils [27]. mAb158 displayed a treatment effect on total $A\beta_{1\text{-}42}$ in SDS-denatured brain extracts from tg-ArcSwe mice, but not on the low levels of monomeric Aβ in native TBS extracts, supporting the conclusion that the *in vivo* target is large soluble aggregated forms of $A\beta_{42}$. However, one potential caveat to these experiments is that denaturation of TBS brain extracts could dissociate other Aβ-protein complexes or release Aβ bound to membranes, thereby increasing the levels of monomeric Aβ available for detection by the $A\beta_{42}$ ELISA. Further characterization is necessary to conclusively prove that the majority of soluble brain $A\beta_{42}$ consists of aggregated species.

In both the long-term and the short-term mAb158 treatment studies in tg-ArcSwe mice, a significant treatment effect on brain protofibrils was found, and this effect correlated to brain mAb158 concentration suggesting an exposure-dependent treatment effect. A strong treatment effect on brain protofibril levels could be observed after long- and short-term treatment in the subgroup sacrificed 7 days after the last injection. Ideally, a non-selective, Aβ-specific control antibody (such as 6E10) would have been used for comparison in these studies. A previous study has compared preventive treatment with mAb158 to 1C3, a non-protofibril selective antibody that binds the N-terminus of Aβ, in tg-ArcSwe mice. This study resulted in significant reductions in brain protofibril levels using mAb158, but not 1C3 [54]. In addition to the brain protofibril clearance, a reduction in CSF protofibrils that correlated to brain protofibrils was observed in the long-term study. However, a trend toward brain and CSF protofibril reductions could be shown in the subgroup of mice

that had received short-term treatment and was sacrificed 24 h after the last injection. It is possible that administration of mAb158 for 4 weeks is near the threshold of the exposure level or the time necessary to robustly clear protofibrils from brain and CSF. Nonetheless, the consistent correlations between brain and CSF protofibril levels observed in both studies shown here provide a compelling case for a relationship between Aβ protofibrils in brain and CSF, at least in the tg-ArcSwe mouse model. Since soluble Aβ oligomers and protofibrils may be upstream from neurodegeneration and plaque formation [18–20, 55], early treatment could be pivotal to disease intervention. Therefore, a biomarker such as Aβ protofibrils in CSF would be of importance both to monitor treatment response and assist with early diagnosis of AD patients.

There is increasing interest in antibodies that bind oligomeric forms of Aβ, and a number of other groups are developing oligomer-binding antibodies for use in AD immunotherapy studies [38, 56–59]. The binding characteristics and selectivity of these antibodies could be instrumental both to the safety profile and the ability to selectively clear the correct target. It is challenging to determine antibody binding affinities for various Aβ aggregates. In our experience, antibody binding to various conformational states of Aβ can change when these Aβ species are adhered to a substrate, such as in immunoblot and dot blot. Therefore, antibody binding to oligomeric forms of Aβ is best performed in solution, where it is possible to discriminate between different forms of Aβ. Although many of the antibodies under development are suggested to bind oligomers, the nature of their binding characteristics is still largely unclear. PFA1, a protofibril-binding antibody, has been shown to react to protofibrils but also to fibrils by SPR and competition ELISA, and to oligomers and monomers by immunoblot [58, 60]. The non-fibrillar oligomer-specific antibodies described by Kayed and Rasool and colleagues [38, 61] are described as oligomer-specific but are primarily characterized by dot blot and immunoblot. The MABT antibody (crenezumab) described by Adolfsson et al. [59] has been shown to bind $A\beta_{42}$ monomers, oligomers, and fibrils by ELISA, but it is unclear whether the antibody interaction with these Aβ species occurs in solution. A-887755 is an oligomer-specific Aβ antibody generated against $A\beta_{20\text{-}42}$ globulomers [56]. This antibody is shown to recognize an SDS-stable pool of oligomers in brain extracts from AD patients and transgenic mice, but the antibody binding characterization comparing its binding to monomers and fibrils is performed by dot blot. The binding of gantenerumab, an antibody generated utilizing phage display, was characterized by SPR and this antibody binds Aβ fibrils and oligomers with slightly higher affinity over Aβ monomers [57]. The mAb158 and BAN2401 antibodies have approximately 1000-fold higher selectivity for Aβ protofibrils over monomers in solution across different experimental platforms, which has not been shown, to our knowledge, by other groups. Further, binding studies performed by Magnusson et al. [62] have shown that mAb158 displays approximately three-fold higher binding affinity to Aβ protofibrils over fibrils, and when saturated this binding was 10–15 fold higher for protofibrils over fibrils. This group has also shown diffuse binding of radiolabeled mAb158 in brain parenchyma, suggesting that the primary target of this antibody is soluble protofibrils.

Many of the oligomer-binding antibodies mentioned here have shown amyloid plaque clearance [38, 57, 59] as well as improved performance in memory-related tasks in transgenic mouse models of AD [38, 56, 59]. We have not yet evaluated the effect of mAb158-treatment on memory-related tasks in the tg-ArcSwe mouse model, however, it would be of great interest to study whether amelioration of cognitive impairment can be achieved in the absence of plaque clearance as previously observed by Dodart et al. and Kotilinek et al. [16, 17]. mAb158 has previously been shown to inhibit fibril formation *in vitro* [54], and when administered preventively to tg-ArcSwe mice prior to amyloid plaque onset, plaque formation was inhibited. These data suggest that sustained clearance of protofibrils can prevent plaque formation. However, when mAb158 was administered to tg-ArcSwe mice after amyloid plaque onset, reduced protofibril levels were observed but this reduction was not accompanied by significant changes in amyloid plaque burden in this model of early-onset AD [54]. There is some data to suggest that antibodies targeting amyloid plaques may be associated with microhemorrhage and could increase cerebral amyloid angiopathy [63–67]. We hypothesize that an antibody that is selective for protofibrils with minimal monomer or AβPP binding [51] has a higher possibility of reaching and clearing its target when not competing with monomeric Aβ species in plasma or brain. The selectivity of mAb158/BAN2401 for Aβ protofibrils over amyloid plaques and Aβ monomers could contribute to a favorable safety profile of BAN2401 in the clinic, and data from an early clinical study of BAN2401 in AD patients have indicated that this antibody is well tolerated and associated with a beneficial safety profile [49].

In summary, we have confirmed that mAb158 and BAN2401 selectively bind to the toxic soluble Aβ protofibrils with high preferential binding compared to soluble monomers. We have shown target engagement of mAb158 after treatment of transgenic mice, and seen an effect of treatment in terms of reduced levels of protofibrils in brain and CSF. A clinically relevant biomarker of AD is of importance for a disease modifying treatment in AD patients, and CSF Aβ protofibrils could potentially fill that function. BAN2401 has undergone a full phase 1 development and is currently being evaluated in clinical phase 2b (Lannfelt et al., unpublished). The unique target profile of this antibody offers a novel approach for Aβ immunotherapy which will hopefully provide a successful treatment for AD.

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Authors' disclosures available online (http://www.j-alz.com/disclosures/view.php?id=2401).

SUPPLEMENTARY MATERIAL

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