Pharmacokinetic parameters and mechanism of action of an efficient anti-Aβ single chain antibody fragment

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Abstract

The success of the targeting of amyloid-β (Aβ) oligomers through immunotherapy in Alzheimer’s disease (AD) mouse models has not been translated into the clinics. The use of single-chain variable fragments (scFvs) has been proposed to prevent the potential severe effects of full-length mAbs by precluding crystallizable fraction-mediated microglia activation. The efficacy of scFv-h3D6, a bapineuzumab-derived anti-Aβ scFv, has been extensively proven. In this work, we compared scFv-h3D6-EL, an elongated variant of the scFv-h3D6, with its original version to assess whether its characteristic higher thermodynamic stability improved its pharmacokinetic parameters. Although scFv-h3D6-EL had a longer half-life than its original version, its absorption from the peritoneal cavity into the systemic compartment was lower than that of the original version. Moreover, we attempted to determine the mechanism underlying the protective effect of scFv-h3D6. We found that scFv-h3D6 showed compartmental distribution and more interestingly crossed the blood–brain barrier. In the brain, scFv-h3D6 was engulfed by glial cells or internalized by Aβ peptide-containing neurons in the early phase post-injection, and was colocalized with the Aβ peptide almost exclusively in glial cells in the late phase post-injection. Aβ peptide levels in the brain decreased simultaneously with an increase in scFv-h3D6 levels. This observation in addition to the increased tumor necrosis factor-α levels in the late phase post-injection suggested that the engulfment of Aβ peptide/scFv-h3D6 complex extruded from large neurons by phagocytic cells was the mechanism underlying Aβ peptide withdrawal. The mechanism of action of scFv-h3D6 demonstrates the effectivity of Aβ-immunotherapy and lays the background for other studies focused on the finding of a treatment for AD.

Introduction

The currently available therapies for Alzheimer’s disease (AD) include the use of cholinesterase inhibitors (donepezil, galantamine, and rivastigmine) that partially compensate the pathological reduction in acetylcholine and an NMDA receptor antagonist (memantine) that prevents...
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Abbreviations: Aβ, amyloid-beta; AD, Alzheimer’s disease; APP, amyloid precursor protein; AUC, area under the curve; BBB, blood–brain barrier; BCA, bicinchoninic acid; BSA, bovine serum albumin; DAPI, 4',6-diamino-2-phenylindol; EDTA, ethylenediaminetetraacetic acid; ELISA, Enzyme-Linked ImmunoSorbent Assay; Fc, crystallizable fraction; FcγR, Fc gamma receptor; mAb, monoclonal antibody; MAPT, microtubule-associated protein tau; mo, months-old; NTg, non-injection; scFv, single-chain variable fragment; monoclonal antibody; MAPT, microtubule-alpha; TBS, Tris-buffered saline; TNF-α, tumor necrosis factor-alpha; 1/2, half-life; Vi, variable light chain domain; 3xTg-AD, triple-transgenic mouse model.

the effect of the increased glutamate levels in the synaptic cleft [1]. Because these treatments palliate the symptoms of AD rather than targeting its underlying causes, the implementation of novel therapeutic strategies has become a necessity [2]. In this sense, several molecules have been designed for targeting amyloid-β (Aβ) peptide, the key component in AD [3,4]. Aβ peptide-directed immunotherapy is a promising approach because it focuses on capturing the Aβ peptide through active immunotherapy (by directing a patient’s immune response to different forms and/or fragments of the peptide) or through passive immunotherapy (by administering antibodies or their derivatives that directly arrest the Aβ peptide) [5,6]. Passive immunotherapy is a safer option than active immunotherapy because it can be stopped immediately in case of any adverse reaction [7]. Several clinical trials are currently ongoing in this regard [8–10]. Bapineuzumab (Pfizer/Janssen) was the first mAb to reach phase III clinical trials; however, the occurrence of vasogenic edema and microhemorrhage resulted in the suspension of the studies in 2012 [11]. Similarly, solanezumab (Eli Lilly), albeit resulted safe, showed a benefit that was not higher than that associated with the palliative acetylcholinesterase inhibitors drugs, and the studies were terminated [12]. Unfortunately, this has also recently been the case for aducanumab (Biogen Idec) [13]. However, other mAbs, such as gantenerumab (Hoffman-La Roche) and crenezumab (Genentech), are currently under phase III clinical trials [10].

Recombinant antibody fragments are reliable alternatives to the full-length antibodies from which they are derived [14,15]. These fragments retain the antigen-binding specificity of full-length mAbs and possess other interesting properties such as potential linkage to therapeutic payloads (enzymes or liposomes, etc.). The use of single chain antibody fragments (scFvs) is safer than that of full-length mAbs because scFvs lack the crystallizable fraction (Fc), which mediates microglia activation and subsequently induces pro-inflammatory cytokines and mediators secretion [11,16,17]. Similarly, the absence of the Fc fragment prevents scFvs from triggering of the complement system; therefore, they exhibit limited immunogenicity and prevent opsonization and antibody-dependent cell toxicity. Although scFvs have shorter half-lives than their corresponding full-length mAbs, they exhibit better tissue distribution, penetration, and clearance properties than the corresponding full-length mAbs [18].

In 2000, Frenkel et al. designed the first anti-Aβ scFv based on the variable regions of an anti-Aβ IgM [19]. Since then, some other recombinant engineered molecules have been developed [20] and modified using stabilizing mutations [21–23], including scFv-h3D6, an scFv derived from bapineuzumab [24]. ScFv-h3D6 has been incorporated with different mutations to enhance its thermodynamic stability for improving its pharmacokinetic parameters and thus its therapeutic effect [25]. A two-residue elongation of the C-terminal domain (V\text{L}) of scFv-h3D6 produced scFv-h3D6-EL, which shows 25% higher thermodynamic stability than the original scFv (referred to as scFv-h3D6-EL in this manuscript for clarity) [26]. In vitro studies showed that both scFv-h3D6 molecules prevented Aβ peptide-induced cytotoxicity, with scFv-h3D6-EL providing improved results [25]. Single administration of scFv-h3D6-EL in young 3xTg-AD female mice ameliorated the first hallmarks of AD by reducing amyloid burden and improving cognitive disabilities [27–29]. Next steps involve the elucidation of the mechanism underlying the protective effects of scFv-h3D6 to demonstrate the therapeutic potential of Aβ-immunotherapy and to lay the background for other studies on AD pathology in mouse.

In this study, we examined whether the higher thermodynamic stability of scFv-h3D6-EL than that of scFv-h3D6-WT improved its pharmacokinetic parameters. For this, 5-month-old 3xTg-AD female mice were administered scFv-h3D6-EL or scFv-h3D6-EL and plasma concentration profiles for these proteins were determined for elucidating their main pharmacokinetic parameters. Furthermore, some other factors such as the capability of scFv-h3D6 to cross the blood–brain barrier (BBB), its colocalization with the Aβ peptide, the specific cell types
containing these molecules as well as its effect on tumor necrosis factor-α (TNF-α) levels were examined to determine the mechanism of action of scFv-h3D6.

**Materials and methods**

**ScFv-h3D6 expression and purification**

ScFv-h3D6 was recombinantly expressed in *Escherichia coli* and was purified, as described previously [24]. Lipopolysaccharide (LPS), a major endotoxin in gram-negative bacteria, was removed from the protein by using Detoxi-Gel Endotoxin Removing columns (ref. 20344, ThermoFisher Scientific, Waltham, MA, USA).

**Animals**

A triple-transgenic mouse model of AD (3xTg-AD mouse model) was initially engineered at the University of California, Irvine. For this, two independent transgene constructs encoding human APP<sub>Swe</sub> and tau<sub>P301L</sub> proteins under the control of the mouse Thy1.2 promoter were co-microinjected into single-cell embryos harvested from mutant homozygous PS1<sub>M146V</sub> knock-in mice [30,31]. APP<sub>Swe</sub> and PSEN1<sub>M146V</sub> mutations are associated with familial AD, whereas MAPT<sub>P301L</sub> mutation is associated with a familial form of frontotemporal dementia and not with AD. This mouse model is shown to reproduce the human amyloid and tau pathologies observed in patients with AD through similar regional and temporal patterns [31,32]. The animals used in the present work belonged to the 3xTg-AD colony, and to the corresponding non-transgenic (NTg) mice (B6129SF2), established by our group at the UAB Animal Facility. Founder animals were provided by The Jackson Laboratory, Bar Harbor, ME, USA. Only female were used because they exhibit a greater Aβ burden and larger behavioral deficits than age-matched males [33]. Furthermore, in contrast to males, females maintain the original phenotypic traits described (https://www.jax.org/strain/004807).

The animals were maintained under standard laboratory conditions such as temperature of 22 °C ± 2 °C, relative humidity of 55% ± 5%, 12/-12-h light/dark cycle starting at 08:00 a.m., and bedding made of wood chips, and were given food and water *ad libitum*. All the experiments were approved by the UAB Animal Research Committee and the Government of Catalonia (Protocol Number: CEEAH-3553) and were conducted in accordance with the legislation for the protection of animals used for scientific purposes (directive 2010/63/EU; BOE 2013/1337). All efforts were made to minimize suffering.

**Experimental design**

Two groups containing 24 randomly-distributed five-month-old 3xTg female mice each were intraperitoneally injected with 100 μg (~ 3.3 mg/kg) of scFv-h3D6-WT or scFv-h3D6-EL, diluted in 200 μl of vehicle (PBS-buffer, pH 7.4). These conditions were consistent with the already published experiments on scFv-h3D6-WT pharmacodynamics [27–29,34]. Animal sacrifices and sample collection were performed at the following time points p.i.: 0h (for PBS-administered mice), 1h, 5h, 10h, 1 day, 2 days, 5 days, and 8 days (n = 3).

**Sample collection**

The animals were anesthetized by making them inhale 1% isoflurane. Firstly, blood was collected by cardiac puncture and was supplemented with a complete protease inhibitor cocktail (ref. 5892970001, Roche) and 1 mM EDTA to prevent protein degradation and coagulation. Next, the samples were centrifuged at 20,230 × g and 4 °C for 10 min. The supernatant obtained was carefully recovered to prevent contamination from the interphase (white blood cells and
platelets) and centrifuged again. Finally, the supernatant was recovered and stored at -80˚C as blood plasma until further use. Next, the mice were guillotined and dissected, and their brains were quickly removed from their skulls, extensively rinsed in cold PBS, weighted, and dissected. Both hemispheres of the mouse brains were separately processed for histological and biochemical analyses.

**Protein extract preparation**

The cerebral hemispheres were mechanically broken in a cold tissue homogenization buffer (TBS [pH 7.6] supplemented with the protease inhibitor cocktail and 1 mM EDTA) by using a tissue homogenizer (Sigma-Aldrich, Saint Louis, MO, USA). After gentle sonication with one cycle of 35 s, at 35% duty cycle, and output 4 by using Dynatech Sonic Dismembrator ARTEK 300; BioLogics INC., Manassas, VI, USA), the samples were centrifuged at 100,000 x g and 4˚C for 1h. The supernatant obtained was stored at -80˚C as the extracellular fraction. The pellet obtained was suspended in a lysis buffer (1% Triton X-100 in homogenization buffer), sonicated and centrifuged similarly, and the supernatant obtained was stored at -80˚C as the intracellular fraction.

**Histological sections preparation**

The tissues were immersed in 4% paraformaldehyde for 36h at 4˚C. Next, the tissues were rinsed in PBS, dehydrated by immersing in a series of increasing concentrations of ethanol solutions (50%, 70%, 96%, and 100%), in xylene and 1:1 xylene-paraffin. Paraffin embedding was performed using the regular procedures from our laboratory. Coronal sections (10-μm thick) were mounted on Superfrost™ Plus microscope slides (ref. 4951PLUS4, Thermo Fisher Scientific) and stored at R.T. until further use. The region of analysis corresponded to the range of coordinates between Fig 43 (interaural 2.34 mm and bregma -1.46 mm) and Fig 48 (interaural 1.74 mm and bregma -2.06 mm) in a study by Franklin and Paxinos [35].

**Anti-scFv-h3D6 production**

**Animal immunization.** Anti-scFv-h3D6 polyclonal antibodies were produced by Cell Cultures, Antibody Production and Cytometry Core at the UAB. Two New Zealand rabbits were intradermally immunized with 400 μg of scFv-h3D6-WT protein conjugated with a Freund’s complete adjuvant. After the first immunization, the rabbits were administered three booster injections (at 21-day intervals) of 300 μg of scFv-h3D6-WT and Freund’s incomplete adjuvant. Blood samples were obtained from the rabbits to monitor serum-specific antibody levels by ELISA. Finally, total rabbit sera were collected 10 days after the fifth immunization.

**Specificity analysis.** Specificity of the anti-scFv-h3D6 polyclonal antibodies was determined by performing an ELISA standardized by the Cell Cultures, Antibody Production and Cytometry Core at the UAB. Briefly, all sera were analyzed using 96-well polystyrene plates (ref. 442404, Maxisorp, NUNC, Labclinics, Barcelona, Spain) coated with scFv-h3D6 diluted in 0.1 M carbonate buffer (pH 9.6). The ELISA plates were incubated with several dilutions of sera for 90 min and were washed extensively. Next, a peroxidase-conjugated anti-rabbit IgG to (ref. 170–6515, BioRad, Hercules, CA, USA) was added the plates to detect the antigen–antibody complexes. Immunodetection was performed using O-phenylenediamine dihydrochloride (OPD) Fast Substrate (ref. P9187; Sigma Aldrich), and absorbance was read at 450 nm by using a multilabel reader Victor3 (Perkin Elmer, Turku, Finland). S1 Fig shows the titration of the antibodies from the two animals. All subsequent experiments were performed using the hyperimmune serum obtained from rabbit 2 because it had a higher antibody titer than the serum obtained from rabbit 1.
**ScFv-h3D6 quantification**

ScFv-h3D6 levels were quantified by indirect competitive ELISA. For this, 96-well polystyrene plates (ref. 44-2404-21, Maxisorp, NUNC, Labclinics) were coated with scFv-h3D6 (10 ng per well from a 100 ng/mL dilution in PBS, pH 7.4) and were incubated overnight at 4°C. After several washes with 0.1% Tween-20 in PBS, the plates were blocked with 1% bovine serum albumin (BSA) in PBS for 30 min at 37°C and were washed again. Simultaneously, the samples (6 replicas for each mouse (n = 3) and time point) were incubated with the primary anti-scFv-h3D6 antibody (dilution 1:100,000) at 37°C for 1h and were added to the already blocked plates for 1h at 37°C. Next, the plates were washed again. The primary antibody was detected by incubating the plates with a peroxidase-conjugated anti-rabbit IgG secondary antibody (ref. 1662408, dilution 1:2000, BioRad) for 1h at 37°C and was revealed and visualized using the OPD Fast Substrate. Absorbance was read at 450 nm by using the multilabel reader Victor3. Values were obtained using specific calibration curves for scFv-h3D6-WT and scFv-h3D6-EL each. The titration took into account and annulated putative differences in the binding of the anti-scFv-h3D6 antibodies to the two molecules.

**Aβ peptide quantification**

Aβ42 peptide levels were quantified from 6 replicas for each mouse (n = 3) and time point and using a commercial amyloid-β 42 ELISA kit (ref. KMB3441, Invitrogen), according to the manufacturer’s protocol. Absorbance was measured at 450 nm by using the multilabel reader Victor3. Data obtained were normalized using the total amount of protein in each extract, as measured using the bicinchoninic acid (BCA) assay (ref. 23225, Pierce, ThermoFisher Scientific).

**TNF-α quantification**

TNF-α levels in hippocampal protein extracts were quantified from 6 replicas for each mouse (n = 3) and time point and using a commercial ELISA DuoSet kit (ref. DY410-05, R&D Systems, Minneapolis, MN, USA). Absorbance was measured using the multilabel reader Victor3. Because wavelength correction was not available, readings obtained at 540 nm were subtracted from those obtained at 450 nm. Next, the data obtained were normalized using the total amount of protein in each extract, as measured by the BCA assay.

**Fluorescent immunodetection and colocalization**

The tissue sections were deparaffined in xylene, hydrated in decreasing concentrations of ethanol solutions, and extensively washed in distilled water. Non-binding sites were blocked using 5% BSA in PBS containing 0.05% Tween-20 and 5% normal goat serum for 1h at room temperature. Next, the sections were incubated overnight at 4°C with the anti-scFv-h3D6 polyclonal antibody (dilution 1:100, produced by the Cell Cultures, Antibody Production and Cytometry Core, UAB), washed several times, and incubated with Alexa 488 fluorophore-conjugated goat polyclonal anti-rabbit secondary antibody (ref. ab150077, dilution, 1:200 Abcam, Cambridge, UK) for 1h at R.T. Finally, the sections were cover-slipped for microscopic observation with 4', 6-diamino-2-phenylindole (DAPI)-containing Vectashield solution (ref. D9542, Vector Laboratories, Burlingame, CA, USA) for fluorescent nuclei staining. Immunostaining controls were generated using a blocking buffer instead of the primary antibodies. The specificity of the anti-scFv-h3D6 antibody was determined based on the absence of immunofluorescent staining in non-injected controls (vehicle-injected mice corresponding to 0h p.i.). Double immunodetection of scFv-h3D6 and Aβ peptide was performed by incubating the samples with anti-scFv-h3D6 antibody and 6E10 mAb (ref. SIG-39320, dilution1:100, Covance
Signet, Princeton, NJ, USA), followed by incubation with Alexa 488-conjugated anti-rabbit and Cy3-conjugated goat polyclonal anti-mouse secondary antibodies (ref. AP124C, dilution 1:100, Chemicon, Millipore, Billerica, MA, USA), respectively.

**Epifluorescence and confocal microscopy**

Photographs were digitally captured using ProgRes C10Plus color video camera coupled with a Zeiss Axioskop microscope by using ProgRes Capture Pro program (Jenoptik, Jena, Germany). Confocal microscopy was performed at the Microscopy Facility of the UAB using a Spectral Fluoview-1000 device (Olympus, Shinjuku, Tokio, Japan) and capturing 5–10 optical sections at 1-μm intervals with an objective lens UPLSAO0. The obtained images were processed and assembled with Image J software (v. 1.43 u, NIH).

**Results**

**Comparison of the pharmacokinetic parameters of scFv-h3D6-WT and scFv-h3D6-EL**

The beneficial effects of a single intraperitoneal injection of 100 μg scFv-h3D6 have already been demonstrated in young 3xTg-AD female mice [27–29]. In this work, we analyzed the pharmacokinetic parameters of scFv-h3D6 by studying variations in the plasma concentrations of the original version of the molecule (scFv-h3D6-WT) and its elongated and thermodynamically more stable version (scFv-h3D6-EL) [24,26].

The plasma concentrations of these molecules were quantified at 0h, 1h, 5h, 10h, 1 day, 2 days, 5 days, and 8 days post-injection (p.i.) by performing competitive enzyme-linked immunosorbent assay (ELISA) by using specific calibration curves for each molecule. Values are presented by plotting a concentration–time curve (Fig 1A), from which the main pharmacokinetic parameters of these molecules were calculated (Fig 1B).

Both molecules were absorbed from the intraperitoneal cavity into the blood before 1h p.i. (Fig 1A) and showed a similar profile. However, a higher peak was initially observed for scFv-h3D6-WT than for scFv-h3D6-EL, indicating enhanced absorption of the original molecule. Following the pronounced initial peak, a second peak appeared at ~24h p.i., suggesting a two-phased absorption mode or a compartmental distribution of both molecules. The higher absorption of scFv-h3D6-WT than that of scFv-h3D6-EL affected the remaining pharmacokinetic profile, with area under the curve (AUC), which were measured using a trapezoidal rule for n-1 subintervals from 0 to the last time point, of scFv-h3D6-WT being five-fold higher than those of scFv-h3D6-EL (Fig 1B). However, scFv-h3D6-EL exhibited a longer half-life than the scFv-h3D6-WT, with the values for scFv-h3D6-EL being almost two-times higher than those for scFv-h3D6-WT, which was consistent with the improved stability of scFv-h3D6-EL observed in biophysical analyses and in cell cultures [25,26]. Because of the difference in the absorption of scFv-h3D6-WT and scFv-h3D6-EL, subsequent experiments were performed using the original version of the molecule.

**ScFv-h3D6 can cross the blood–brain barrier**

To further determine whether scFv-h3D6 could penetrate the central nervous system, we determined the concentration of this molecule in the brain at different time points p.i. by competitive ELISA with protein extracts and by fluorescent immunodetection with histological sections. ScFv-h3D6 concentration in the brain protein extracts over time p.i. produced a main peak at the very beginning (~1h p.i.) and a slightly smaller peak later (~48h p.i.), after which it decreased progressively until the end of the experiment (8 days p.i.; Fig 2A). These
Fig 1. Plasma concentrations of scFv-h3D6-WT and scFv-h3D6-EL. (A) Plasma concentrations of scFv-h3D6-WT and scFv-h3D6-EL were measured by competitive ELISA at 0h (for vehicle-injected mice), 1h, 5h, 10h, 1 day, 2 days, 5 days, and 8 days p.i. The continuous line indicates the scFv-h3D6-WT profile, and the dashed line the scFv-h3D6-EL profile. Insets below the plot are magnifications for early (0-25h) and late (25-192h) p.i. times. The concentrations are expressed as micrograms of scFv-h3D6 per milliliter of the plasma and are represented as means and SEM. (B) Comparative pharmacokinetic parameters of scFv-h3D6-WT and scFv-h3D6-EL were quantified using the concentration–time curve; Cmax (maximum plasma concentration, μg/mL), Tmax (time to reach Cmax, h), AUC (area under the curve), and t₁/₂ (half-life, h).
ScFv-h3D6 pharmacokinetics and mechanism of action

A

![Graph showing concentration of ScFv-h3D6 in nervous tissue over time](image1)

B

![Graph showing concentration of ScFv-h3D6 in plasma and nervous tissue over time](image2)

C

![Images of anti-scFv-h3D6 staining and DAPI](image3)
results were compared with the plasma concentration of scFv-h3D6 to determine the temporality of its distribution through the central nervous system (Fig 2B). Consistently, the first peak of scFv-h3D6 in the plasma overlapped the first peak in the brain, suggesting that the fast absorption of the molecule from the intraperitoneal cavity into the blood coincided with its early distribution to other tissues such as the central nervous system. The increased plasma concentration of scFv-h3D6 at ~24h, which produced a second peak in its plasma profile, produced a second peak in its central nervous system profile at 48h p.i. These observations can be explained by the progressive re-distribution of plasma scFv-h3D6 (probably more slowly than its initial distribution because of its decreased concentration).

Fig 2C illustrates the immunodetection of scFv-h3D6 in the amygdalar region of a mouse sacrificed at 5h p.i. as a representative of its early entry into the central nervous system. A fluorescent signal was observed throughout the tissue, with increased signal intensities observed mainly around the blood vessel walls and in the cell body of both large and small cells (Fig 2C, bottom).

The first location suggests the accumulation of the molecule through the BBB as a momentary influx into/efflux from the central nervous system or because of its deposition tendency along the BBB. Detection of the fluorescent signal in large cells indicated that scFv-h3D6 was internalized by large neurons, and detection of a strong signal intensity in the small cells indicated that scFv-h3D6 was internalized by glial cells or small neurons (see Section 2.5). The fact that the signal was observed within the cell body indicated that the penetration of scFv-h3D6 into the brain was actual and was not associated with the presence of a small amount of blood remaining in the vessels.

ScFv-h3D6 is internalized by cells

Next, we quantified scFv-h3D6 levels in the extra- and intracellular brain compartments to further determine the process underlying its internalization. Protein extracts of samples obtained at the same time points p.i. were analyzed by competitive ELISA. Fig 3A shows scFv-h3D6 concentration in the extra- and intracellular compartments over time. The first peak in scFv-h3D6 concentration in the extracellular compartment was almost two-times higher than its first peak in the intracellular compartment; however, differences in scFv-h3D6 concentration between these compartments were not as significant at the remaining time points p.i. This suggested that the early inflow of scFv-h3D6 into the central nervous system promoted its initial accumulation in the extracellular compartment and subsequent distribution in both extra- and intracellular compartments.

Comparative superposition of both the extra- and intracellular profiles of scFv-h3D6 facilitated the interpretation of our results (Fig 3B). The first peak in scFv-h3D6 concentration in the extracellular compartment (~1h p.i.) indicated its penetration into the central nervous system. The first peak in scFv-h3D6 concentration in the intracellular compartment appeared later (~5h p.i.) and coincided with its reduction in the extracellular levels, suggesting its internalization. A second and faint peak in scFv-h3D6 concentration appeared at ~48h p.i. in both...
compartments, which may have been caused by the influx of scFv-h3D6 from the plasma at 24h p.i., as suggested in the previous section (Section 2.2, Fig 2B). Thereafter, scFv-h3D6 concentration progressively decreased in both compartments, indicating its clearance. Thus, scFv-h3D6 concentration in the intracellular compartment was higher than that in the extracellular compartment throughout the late phase p.i., suggesting a cell-mediated mechanism underlying scFv-h3D6 clearance in the brain.

Compartmentalization of scFv-h3D6 and Aβ peptide in the central nervous system

Because the therapeutic effect of scFv-h3D6 is mediated by a reduction in Aβ peptide burden [28,36], we examined the interaction between these both molecules in terms of compartmental distribution (extra- and intracellular compartments) and temporary evolution (by comparing their concentrations over time). First, scFv-h3D6 and Aβ peptide were immunodetected in the histological sections of a mouse sacrificed at 5h p.i., which coincided with the main peak in scFv-h3D6 concentration in the intracellular compartment (Fig 4A). A strong signal for both molecules was detected in the intracellular compartment; however, not all cells showing the scFv-h3D6 signal contained the Aβ peptide. Moreover, scFv-h3D6 was immunodetected within large and small cells, whereas the Aβ peptide was mostly detected in only large cells.

Aβ burden was also quantified using a commercial ELISA kit and was compared with the scFv-h3D6 concentration profiles in both extra- and the intracellular compartments. Fig 4B and 4C shows Aβ peptide concentration over time p.i., and its comparison with the scFv-h3D6 concentration. The first response to the treatment is shown in detail in panels showing the magnification of the 0–12h p.i. interval (see insets). Aβ peptide concentration was higher in the intracellular compartment than in the extracellular compartment at all the analyzed time points p.i., which was consistent with the early stage of the disease in the 5-mo-old 3xTg-AD mouse model [37,38]. The extracellular Aβ peptide concentration exhibited a pronounced decline tendency during the early phase p.i. (0–10h) and a subsequent progressive decrease later (from 2 days p.i.), which overlapped with the main first peak and the faint second peak in scFv-h3D6 concentration, respectively (Fig 4C). Aβ peptide concentration in the intracellular compartment reduced pronouncedly after the first peak in scFv-h3D6 concentration (0–5h p.i.). Interestingly, Aβ peptide concentration in the intracellular compartment increased at 10h and 5 days p.i., which coincided with the decrease in Aβ peptide concentration in the extracellular compartment, suggesting the engulfment of extracellular Aβ peptide.

Colocalization of scFv-h3D6 and Aβ peptide in different cell types

Although scFv-h3D6 and Aβ peptide partially colocalized at the early phase after the treatment (at 5h p.i., as shown in the previous section; Fig 4A), we found that scFv-h3D6 was localized
Fig 4. Colocalization of scFv-h3D6 and Aβ peptide in the brain. (A) Colocalization of scFv-h3D6 and Aβ peptide in a 10-μm-thick brain section (at 5h p.i.). Scale bar, 100 μm. (B) Extracellular and intracellular levels of Aβ peptide and scFv-h3D6 over time p.i.: 0h (for vehicle-injected mice), 1h, 5h, 10h, 1 day, 2 days, 5 days, and 8 days. The inset corresponds to the amplification of the initial profiles up to 12h p.i.
within both large and small cells whereas the Aβ peptide signal was only localized within the large cells. Fig 5A shows a representative panoramic image obtained by confocal microscopy of a mouse sacrificed 5 days p.i. to determine scFv-h3D6 and Aβ peptide colocalization and cell types involved at advanced time points p.i. Fig 5B–5D shows the cellular casuistry observed in the panoramic image, namely, (B) solitary cells, (C) couple of adjacent cells, and (D) cells

AntiscFv-h3D6 6E10 DAPI Merged

Fig 5. ScFv-h3D6 and Aβ peptide visualization 5 days p.i. by confocal microscopy. Colocalization of scFv-h3D6 and Aβ peptide in a 10-μm-thick brain section (at 5 days p.i.). (A) Panoramic image. Continued-line square in the merged figure shows an example of a large cell containing both scFv-h3D6 and Aβ peptide signals, which have been magnified in (B). Dashed-line square shows an example of a couple closely related cells, which have been magnified in (C). White arrow indicates the area next to the blood vessel, which have been magnified in (D). Scale bars: 50 μm in (A) and 10 μm in (B), (C), and (D).

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located around blood vessels. Fig 5B shows a large solitary cell containing both scFv-h3D6 and Aβ peptide. Fig 5C shows a couple of adjacent cells conformed by a large cell with a very low signal for both scFv-h3D6 and Aβ peptide and a small cell with strong signal for both scFv-h3D6 and Aβ peptide. Fig 5D shows a couple of cells located around the blood vessels with a higher signal for scFv-h3D6 than for the Aβ peptide.

Cell types were determined using a recently reported algorithm, based on the cytological features [39]. Thus, large cells were defined as large neurons because of their large-sized and lightly stained nucleus, with an “empty” appearance, and small cells were defined as glial cells because their small and darkly stained nucleus. Similarly, endothelial cells were clearly distinguished based on the shape of their nuclei wrapping around blood vessels. Although specific immunostaining is the most suitable methodology to classify cell types, we are confident that glial cells are actual because we previously showed them by glial fibrillary acidic protein (GFAP) and ionized calcium binding adaptor molecule 1 (Iba1) immunostaining [38].

Thus, the overall data suggested that in the early phase p.i. scFv-h3D6 was internalized by large neurons containing the Aβ peptide and was engulfed by glial cells as a part of the clearance process. Interestingly, in the late phase p.i., both scFv-h3D6 and Aβ peptide were strongly immunodetected within solitary large neurons but were almost negligible when these large neurons were accompanied by adjacent glial cells; however, a strong signal for scFv-h3D6 and Aβ peptide was detected in these glial cells. This suggested that phagocytic glial cells engulfed the intraneuronal Aβ peptide/scFv-h3D6 complex extruded into the extraneuronal compartment by large neurons. Fig 5D shows a couple of endothelial cells located around blood vessels containing both scFv-h3D6 and Aβ peptide. This indicates the involvement of endothelial cells in the flux of scFv-h3D6 and the Aβ peptide/scFv-h3D6 complex across the BBB.

Effect of scFv-h3D6 treatment on TNF-α levels

Because glial cells are involved in the clearance of scFv-h3D6 and the Aβ peptide/scFv-h3D6 complex and it is known that direct stimulation of glial cells may produce an early and critical response in terms of TNF-α levels increase [40], we measured levels of this cytokine in both extra- and intracellular compartments (Fig 6). We found that TNF-α levels increased in the intracellular compartment as a very early punctual response (only at ~1h p.i.). However, TNF-α levels increased during the late phase in both compartments, which was consistent with the occurrence of the clearance process in this phase.

Discussion

At present, only palliative treatment is available for AD, which interferes with the symptoms rather than the causes of AD and therefore cannot preclude the dramatic deterioration of patients with AD [2,8,9]. Determination of disease-modifying treatments involves enormous efforts and considerations for determining appropriate targets and mechanisms of action [41]. Molecular engineering and treatment designing play a key role in determining the suitability of a therapy. ScFv-h3D6 is already known to improve the first hallmarks of AD [27–29]. Moreover, molecular redesigning produced an elongated and thermodynamically more stable version of scFv-h3D6, i.e., scFv-h3D6-EL [25,26]. In the present study, we determined the pharmacokinetic parameters of scFv-h3D6 (scFv-h3D6-WT) and compared them with those of the thermodynamically improved version (scFv-h3D6-EL) to test whether the in vitro benefits of greater stability [25,26] were maintained in vivo. ScFv-h3D6-WT showed better absorption from the intraperitoneal cavity into the blood than scFv-h3D6-EL, whereas scFv-h3D6-EL exhibited a longer half-life than scFv-h3D6-WT. This indicated that the improved stability of scFv-h3D6-EL translated into its in vivo performance but its modified structure hindered its
absorption, thus reducing its potential effects. Moreover, these results also highlighted the reciprocal requirement between pharmacokinetic parameters and molecular/treatment designing because, for example, a different administration route could be used to improve scFv-h3D6-EL absorption [42].
Determination of the mechanism of action of a drug is also important for its molecular design in improving the efficiency and safety of the drug. ScFv-h3D6 prevents neuron loss and improves cognitive impairment in young 3xTg-AD female mice by reducing intracellular Aβ peptide levels [27–29]. However, the mechanism underlying the reduction in Aβ peptide levels is unclear. At least three mechanisms have been proposed to explain the reduction in amyloid burden in the brain by anti-Aβ antibodies. The first mechanism suggests that the antibodies enter the brain and disassociate amyloid fibrils by binding to the peptide [43–45], i.e., the N-terminal region of Aβ peptide in the case of scFv-h3D6. The second mechanism suggests that the antibodies cross the BBB and opsonize amyloid fibrils to activate the immune system, thus enhancing clearance (via microglia activation and Fc-mediated phagocytosis) [46–48].

The third mechanism (known as the sink hypothesis) is based on the peripheral Aβ peptide sequestering by the administered antibodies that modifies the brain–periphery equilibrium, thus promoting Aβ peptide efflux from the brain into the plasma [49]. All the three mechanisms are supported by in vivo data. Moreover, it has been described that epitope specificity of the Aβ-antibodies determines their mode of action [50]. An additional hypothesis, the masking hypothesis, by which binding of anti-Aβ scFvs to Aβ would be sufficient to reduce neurotoxicity [51], cannot be discarded. However, this does not seem to be the case for scFv-h3D6 since it was intracellularly colocalized with the Aβ peptide in this work.

In PDAPP mice, peripherally injected m3D6 mAb enters the brain and binds to amyloid plaques to induce Fc-gamma receptor-mediated microglia phagocytosis of the Aβ deposits [48]. The humanized version of the m3D6 mAb, i.e., bapineuzumab, also crosses the BBB and binds to a variety of Aβ species in the brain. However, other mAbs (such as solanezumab and crenezumab) inefficiently bind to the Aβ peptide in the human brain despite their efficacy in mice probably because of the lack of specificity due to cross-reactivity with other proteins showing epitope overlap [52]. In this work, we observed that scFv-h3D6 crosses the BBB as expected because the low molecular weight of scFvs is suggested to facilitate their passage across the BBB [23] and because other scFvs have already been detected in the brain [53].

Once in the brain, scFv-h3D6 was internalized by large neurons or engulfed by glial cells because it was immunodetected in both cell types. It is more likely that the neurons internalizing scFv-h3D6 are those containing the Aβ peptide because both molecules were colocalized within large neurons. Interestingly, the intracellular scFv-h3D6 signal showed a punctuate pattern similar to that observed for the Aβ peptide. Because we previously showed that Aβ colocalized with cathepsin D, a marker for intracellular vesicular structures [38], and here we show colocalization of Aβ and scFv-h3D6, it is tentative to suggest the vesicular-mediated internalization of scFv-h3D6. However, future studies are needed to determine the mechanism underlying the internalization of this molecule, which could be related to the already demonstrated neuronal internalization of the Aβ peptide involving lipid rafts and lipid raft-associated receptors or other modes [54].

Glia cells mainly contained scFv-h3D6 but not the Aβ peptide in the early phase post-injection, and both scFv-h3D6 and Aβ peptide in the late phase post-injection, thus highlighting potential mechanisms for a couple of processes, including scFv-h3D6 clearance in the brain and scFv-h3D6-mediated Aβ peptide withdrawal. On the one side, glial cells containing only scFv-h3D6, which were mainly observed in the early phase post-injection (~5h p.i.), may explain glial engulfment of scFv-h3D6 as the pharmacokinetic mechanism underlying its clearance. On the other side, glial cells containing both scFv-h3D6 and Aβ peptide, which were observed in the late phase post-injection (~5 days p.i.), may explain glial engulfment of the extruded Aβ peptide/scFv-h3D6 complex as the mechanism underlying Aβ peptide withdrawal. The Aβ peptide/scFv-h3D6 complex extruded from the intraneuronal compartment and its subsequent engulfment by glial cells could contribute importantly to neuronal homeostasis and survival. In addition, brain TNF-α levels showed an
acute peak in the very early phase p.i. (~1h p.i.) and a progressive increase in the late phase p.i. (from ~5 days p.i. up to the end of the experiment), suggesting an initial response corresponding to scFv-h3D6 engulfment and later phagocytosis of the Aβ peptide/scFv-h3D6 complex [55].

In conclusion, determination of the pharmacokinetic profile of scFv-h3D6 showed that the thermodynamically stable scFv-h3D6-EL had a longer half-life than the original scFv-h3D6-WT as expected but showed strikingly impaired absorption. Moreover, the pharmacokinetic profiles of both scFv-h3D6-EL and scFv-h3D6-WT and the subsequent brain analyses of scFv-h3D6-WT showed that scFv-h3D6 crossed the BBB and entered neurons to capture the Aβ peptide, which was likely cleared from the brain through the engulfment of the Aβ peptide/scFv-h3D6 complex by glial cells.

Supporting information

S1 Fig. Titration of the anti-scFv-h3D6 antibodies. (A) Sera from the two immunized rabbits were analyzed. Pre-serum (dashed lines) and hyperimmune serum (continuous lines) from rabbit 1 (on the left) and rabbit 2 (on the right). (B) A standard curve fitting to a 4PL curve. Antibody titer (1/IC_{50}) in the hyperimmune serum from rabbit 2 (dashed line) was higher than that in the serum from rabbit 1 (continuous line) and therefore was selected for performing subsequent experiments.

(TIF)

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