Conformation-specific Antibodies Targeting Aggregated Forms of α-synuclein Block the Propagation of Synucleinopathy

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Abnormal aggregation of α-synuclein is a key element in the pathogenesis of several neurodegenerative diseases, including Parkinson’s disease (PD), dementia with Lewy bodies, and multiple system atrophy. α-synuclein aggregation spreads through various brain regions during the course of disease progression, a propagation that is thought to be mediated by the secretion and subsequent uptake of extracellular α-synuclein aggregates between neuronal cells. Thus, aggregated forms of this protein have emerged as promising targets for disease-modifying therapy for PD and related diseases. Here, we generated and characterized conformation-specific antibodies that preferentially recognize aggregated forms of α-synuclein. These antibodies promoted phagocytosis of extracellular α-synuclein aggregates by microglial cells and interfered with cell-to-cell propagation of α-synuclein. In an α-synuclein transgenic model, passive immunization with aggregate-specific antibodies significantly ameliorated pathological phenotypes, reducing α-synuclein aggregation, gliosis, inflammation, and neuronal loss. These results suggest that conformation-specific antibodies targeting α-synuclein aggregates are promising therapeutic agents for PD and related synucleinopathies.

Key words: Parkinson’s disease, Immunotherapy, Synuclein, Microglia

INTRODUCTION

Parkinson’s Disease (PD) is the second-most common age-related neurodegenerative disease after Alzheimer’s disease (AD). Selective loss of dopaminergic neurons in the substantia nigra pars compacta and α-synuclein inclusion bodies in the form of Lewy bodies are the pathological hallmarks of PD [1]. Although the cause of PD is unclear, a large body of evidence suggests that misfolding and aggregation of α-synuclein are crucial factors in the pathogenesis of the disease [2]. Synucleinopathies refer to a group of neurological diseases that are characterized by the presence of α-synuclein aggregates in neurons and glia in the brain as a pathological feature. These diseases include PD, dementia with Lewy bodies, and multiple system atrophy.

Post-mortem analyses have suggested that α-synuclein aggregation initiated at a few discrete brain regions, such as olfactory bulbs and some brain stem nuclei, spreads through wider brain areas as the disease progresses [3]. The pattern of α-synuclein spread correlates with the overall pattern of symptomatic progression, raising the possibility that the former is the underlying mechanism of disease progression [4]. In support of this idea, stereotaxic injection of preformed α-synuclein fibrils causes brain-wide spread of α-synuclein aggregates and manifests behavioral and pathological features of neurodegenerative disease in both mice and non-human primates [5-8]. Studies have suggested that direct neuron-to-neuron propagation is the mechanism by which aggregates spread through interconnected brain regions [9-11].
α-synuclein is a cytosolic protein that is abundant in presynaptic terminals [12]. However, a minor fraction of cellular α-synuclein is present in electron-dense vesicles and is released from cells via exocytosis [13, 14]. This secreted α-synuclein adopts both monomeric and aggregated forms. Subsequent studies have shown that extracellular α-synuclein aggregates disseminate to neighboring neurons [15, 16] and elicit inflammatory responses by glial cells [17, 18]. In humans, the levels of α-synuclein oligomers are elevated in both the plasma and cerebrospinal fluid of PD patients compared with control groups [19-21]. Based on these findings, it has been proposed that extracellular α-synuclein aggregates are the culprits that drive disease progression in PD [22]. Targeting these species of α-synuclein may thus be a promising strategy for delaying the progression of PD.

Here, we generated conformation-specific antibodies that preferentially recognize aggregated forms of α-synuclein relative to monomers. We showed that these antibodies promote phagocytosis of α-synuclein, thereby enhancing α-synuclein clearance from the extracellular space, and block cell-to-cell propagation of α-synuclein. When administered to a transgenic model of synucleinopathy, these antibodies ameliorated pathological phenotypes, reducing α-synuclein aggregation, gliosis, inflammation, and neuronal loss.

**MATERIALS AND METHODS**

**Materials**

The following antibodies were used in this study; α-synuclein monoclonal antibody (#610787, 1:1,500 dilution; BD Biosciences, San Diego, CA, USA), α-synuclein polyclonal antibody (#2642, 1:1,500 dilution; Cell Signaling Technology; Inc., Danvers, MA, USA), Tau5 (AH80042, 1 μg ml-1; Life Technologies), 6E10 (SIG-39320, 1 μg ml-1; BioLegend), phospho-S129 α-synuclein (#ab59264, 1:500 dilution; Abcam, Waltham, MA, USA), NeuN (#MAB377, 1:1,000 dilution; Merck Sigma, CA, USA), anti-GFAP (#ab7260, 1:500 dilution; Abcam), Iba-1 (#019-19741, 1:200 dilution; Wako, Osaka, Japan), and anti-IL-1β (#ab9722, 1:200 dilution; Abcam). Isopropyl-1-thio-β-D-galactopyranoside (IPTG), glycine, thioflavin T (Th T), and protease inhibitor cocktail were purchased from Sigma-Aldrich (St. Louis, MO, USA). HiTrap Q FF anion-exchange (#17-5053-01) and Superdex-200 gel-filtration columns (#17-5175-01) were purchased from GE Healthcare (Fairfield, CT, USA).

**Purification of recombinant α-synuclein**

α-synuclein protein was expressed in Escherichia coli BL21 (DE3) strain (#RH217; RBC Korea, Seoul, Korea) and was induced by incubating with 0.1 mM IPTG for 3 hours at 37°C after the absorbance of the culture medium at 600 nm (OD600) reached 0.6. Cells were pelleted, resuspended in 20 mM sodium phosphate buffer (pH 7.4), sonicated, boiled at 100°C for 20 minutes, and then centrifuged at 10,000×g for 10 minutes. α-synuclein in the resulting supernatant was purified by anion-exchange chromatography and Superdex-200 gel-filtration column chromatography, then dialyzed against distilled water and lyophilized. For monomer preparation, lyophilized α-synuclein was reconstituted in phosphate-buffered saline (PBS; #CAP08-050; GenDEPOT, Katy, TX, USA), followed by ultrafiltration using a 100,000 MWCO centrifugal device ( Pall, NY, USA).

**Fibrillation of α-synuclein**

α-synuclein (200 μm in PBS) was incubated at 37°C for 9 days with constant shaking at 1.050 r.p.m. in a Thermomixer C (#5382000015, Eppendorf, Hamburg, Germany).

**Mutagenesis**

A C-terminal, truncating α-synuclein mutation (1-119) was introduced into human wild-type α-synuclein (α-syn/pDualGC) with a QuikChange Site-Directed Mutagenesis Kit (#200522; Stratagene, La Jolla, CA, USA) using the following primer pair: 5’-ATT CTG GAA GAT ATG CCT GTG GAT TAA GAC AAT GAG GCT TAT GAA ATG CC-3’ (sense) and 5’-GGC ATT TCA TAA GCC TCA TTG TCT TAA TCC ACA GGC ATA TCT TCC AGA AT-3’ (antisense).

**Cell culture**

BV-2 microglial cells were maintained in Dulbecco’s Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS) and 100 units ml-1 penicillin and 100 units ml-1 streptomycin at 37°C in a humidified 5% CO2 environment and subcultured every 2 days. For propagation assays, stably transfected V1S and SV2 cells (230,000 cells each) were mixed on a coverslip and cultured for 3 days as described previously [23]. The effects of antibodies on the propagation of α-synuclein were assessed by adding antibodies (50 μg ml-1) to the V1S/SV2 co-culture the day prior to the assay.

**Dot blot**

Two-fold serial dilutions (from 50 ng) of both monomeric (M) and sonicated α-synuclein aggregated (A) forms of α-synuclein were spotted onto a nitrocellulose membrane. After completely drying, the membranes were wetted with PBS containing 0.05% Tween-20 (PBST) and blocked with 5% skim milk in PBST for 30 minutes at room temperature (RT). After a brief rinse, the blots were incubated with antibodies on a shaker at 37°C overnight. The
next day, the membranes were washed three times with PBST, then incubated for 1 hour with horseradish peroxidase (HRP)-conjugated goat anti-mouse antibodies (#170-6516; Bio-Rad, Hercules, CA, USA), diluted 1:3,000 in PBST containing 5% skim milk. After washing three times with PBST, the membranes were incubated with ECL solution (#RPN2232; GE Healthcare). Images were acquired using an Amersham Imager 600 (GE Healthcare) and Multi Gauge (v.3.0) software (Fujifilm, Akishima, Tokyo, Japan).

**Sandwich ELISA**

96-Well ELISA plates were coated overnight at 4°C with the antibodies, 3A9, 9B11, 11F11 and 11F4 (used as capture antibodies) at 1 μg ml-1 in 50 mM carbonate buffer (pH 9.6). Plates were washed four times in PBST, after which SuperBlock T20 PBS blocking buffer (#37516; Thermo Fisher) was added and plates were incubated for 2 hours at RT with modest shaking. Plates were again washed four times in PBST, and samples and standards, freshly prepared by serial dilution of recombinant α-synuclein monomer or aggregate solutions, were loaded and incubated at RT for 2.5 hours with modest shaking. Recombinant α-synuclein monomers were always filtered through a 100,000 MWCO centrifugal device with pre-incubated complexes for 10 minutes at 37°C. The reaction was stopped by serial dilution of recombinant α-synuclein monomer or aggregate solutions, were loaded and incubated at RT for 2.5 hours with modest shaking. Recombinant α-synuclein monomers were always filtered through a 100,000 MWCO centrifugal device before use. After washing four times with PBST, 500 ng ml-1 of biotinylated reporter antibody in binding solution (1% bovine serum albumen [BSA] in PBST) was added and plates were incubated for 2 hours at RT with modest shaking. Plates were washed four times in PBST, after which avidin-conjugated peroxidase (#E2886; ExtrAvidin; Sigma-Aldrich) was added at a 1:5,000 dilution in binding solution and plates were incubated for 2 hours at RT. After washing the plates four times with PBST, 100 μl of substrate solution (3,3’,5’,5’-tetramethylbenzidine [TMB], #T4444; Sigma-Aldrich) was added to each well and plates were incubated for 10 minutes with shaking in the dark. The reaction was stopped by adding 50 μl of 2N H2SO4 and absorbance at 450 nm was measured using a spectrophotometer (BioTek, VT, USA).

**Uptake of α-synuclein aggregates in cells**

BV-2 microglial cells were split into 35-mm culture dishes on the day before the experiment. α-synuclein aggregates (0.4 μM), sonicated for the experiment, were pre-incubated with 5 μg ml-1 mouse IgG or α-synuclein antibodies for 5 minutes at RT. Cells were then washed twice with serum-free media and cultured with pre-incubated complexes for 10 minutes at 37°C.

**Preparation of whole-cell extracts**

Cells were placed on ice, then washed with ice-cold PBS, and lysed in extraction buffer (1% Triton X-100 and 1% [v/v] protease inhibitor cocktail [Sigma-Aldrich; #P8465] in PBS). Cell lysates were incubated on ice for 10 minutes and centrifuged at 16,000xg for 10 minutes. The Triton X-100-insoluble fraction (pellet) was resuspended in 1X Laemml sample buffer and sonicated briefly.

**Dual-cell BiFC cell-to-cell propagation assay**

Cells grown on poly-L-lysine-coated coverslips were fixed in PBS containing 4% paraformaldehyde and permeabilized with PBS containing 0.1% Triton X-100. Cells were mounted onto slide glasses in the presence of Prolong Gold Antifade Reagent (#P36931; Invitrogen) and nuclei were stained with TOPRO-3 iodide (#T3605; Invitrogen). An InCell 2200 Analyzer (GE Healthcare; USA) was used for image analysis. Twenty regions (~1,500 cells/region) were randomly selected from each slide.

**Western blotting**

Western blotting was performed as described previously [24]. Images were obtained and quantified using an Amersham Imager 600 (GE Healthcare) and Multi Gauge (v.3.0) software (Fujifilm).

**Animal model and passive immunization**

A Tg mouse model overexpressing full-length human α-synuclein under control of the murine Thy1 promoter (mThy1-α-syn Tg, Line61) and wild-type littersmates were used for this study [25]. A total of 36 α-synuclein Tg and 6 non-Tg mice, all 3 months old and male, were used. Mice were intraperitoneally injected weekly with antibodies (10 mg kg-1) for 3 months (n=5-6 mice per group) and analyzed at the end of the immunization protocol. Brains and peripheral tissues were removed, and brain hemispheres were divided sagittally. The right hemibrains were post-fixed in phosphate-buffered 4% paraformaldehyde at 4°C for immunohistochemistry analysis. All animals were housed under a 12-hour light-dark cycle and had free access to food and water. Animal housing and treatment and tissue preparation were performed at the University of California San Diego (UCSD) under the outsourcing contract. All experiments described were approved by the animal subjects committee at the UCSD and were performed according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

**Immunohistochemistry**

Immunohistochemical staining was performed as described previously [26]. Briefly, post-fixed brains were coronally cut into 40-μm-thick sections with a vibratome (Leica VT 1000S; Leica Instruments, Nussloch, Germany). Floating sections were blocked by incubating with 4% BSA in PBS containing 0.1% Triton X-100 for 1 hour, then incubated overnight at 4°C with primary antibodies. Sections were washed with PBS containing 0.1% Triton X-100,
then incubated with secondary antibodies diluted 1:200 in PBS containing 0.1% Triton X-100. Signals were visualized using an ABC Elite kit (PK-6200; Vector Laboratories, Burlingame, CA, USA). Immunoreactivity was quantified by analyzing images of DAB-stained sections using a bright field digital ZEISS AX-10 microscope and the Image J program (NIH).

**Statistical analysis**

Values shown in figures represent mean±s.e.m. Statistical significance was determined by calculating p-values using paired, two-tailed Student's t-tests and one-way analysis of variance (ANOVA) with Tukey's post-hoc test using GraphPad Prism 7.04 (GraphPad Software Inc., La Jolla, CA, USA). Graphs were drawn using GraphPad Prism 7.04.

**RESULTS**

**Generation of conformation-specific α-synuclein antibodies**

To generate conformation-specific antibodies against α-synuclein aggregates, we immunized C57BL6 mice with fibrils generated from either C-terminally truncated or full-length α-synuclein (Fig. 1A). Both these fibrils exhibited typical amyloid fibril morphologies (Fig. 1B). These immunizations yielded 13 clones. Antibody isotype analysis showed that the antibodies generated were IgG1, IgG2a, IgG2b, or IgG3 (Fig. 1C). To evaluate the specificity of the antibodies for α-synuclein aggregates, we performed dot blot analyses, spotting two-fold serial dilutions (from 50 ng) of both monomeric (M) and fibril (F) forms of α-synuclein onto a nitrocellulose membrane. These analyses showed that 4 out of 13 antibodies exhibited strong selectivity for aggregated forms of α-synuclein (Fig. 1D, 1E). Of these antibodies, three—3A9, 9B11 and 11F11—were selected for follow-up study based on their stability, solubility, and binding specificity towards α-synuclein aggregates. Unlike these three antibodies, clone 11F4 recognized both monomeric and fibril forms of α-synuclein (Fig. 1D, 1E), and was chosen for comparison with the three aggregate-specific antibodies. In this paper, we refer to 11F4 as pan-α-synuclein antibody, which indicates the antibody that recognize α-synuclein regardless of conformation of the protein, hence recognizing both monomers and various forms of multimers including fibrils. To confirm the specificity of antibodies towards aggregates, we performed sandwich enzyme-linked immunosorbent assays (ELISAs), detecting serially diluted recombinant human α-synuclein proteins using biotinylated-7B7 as a detection antibody and each indicated antibody as a capture antibody. Whereas 11F4 detected both α-synuclein monomers and aggregates, 3A9, 9B11 and 11F11 preferentially recognized α-synuclein aggregates compared with the monomer (Fig. 1F).

Next, we investigated their cross-reactivity with other protein aggregates related to neurodegenerative diseases, spotting monomeric and fibrillar forms of α-synuclein, tau, and amyloid β on the membrane. As shown in Fig. 1G, none of the three conformation-specific antibodies cross-reacted with tau or amyloid β.

**Conformation-specific antibodies enhance clearance of extracellular α-synuclein aggregates and block cell-to-cell propagation**

In our previous study, we showed that extracellular α-synuclein aggregates can be internalized and degraded by lysosomes, and further demonstrated that microglia are the cell type most efficient in clearing extracellular α-synuclein [27, 28]. As shown in Fig. 2A, our previous study also showed that treatment with antibodies against α-synuclein enhanced uptake of extracellular α-synuclein in BV-2 microglial cells (Fig. 2A) [29]. To examine the effects of conformation-specific antibodies on the uptake of extracellular α-synuclein aggregates in microglial cells, we preincubated α-synuclein fibrils (0.4 μM) with either 5 μg ml-1 of control IgG or antibodies against α-synuclein for 5 minutes at room temperature and then treated BV-2 cells with α-synuclein fibril-antibody solution for 10 minutes at 37°C. Of the three conformation-specific antibodies isolated, 9B11 significantly increased uptake of extracellular α-synuclein fibrils in BV-2 cells (Fig. 2B, 2C). To rule out the measurement of cell surface-attached α-synuclein, we washed the cells with an acidic solution to remove the cell surface-bound α-synuclein fibrils and analyzed the image with confocal microscopy. The findings were consistent with those obtained from western blotting (Fig. 2B, 2C). The results showed that 9B11 significantly increased the levels of the internalized α-synuclein fibrils (Fig. 2D, 2E).

Antibodies can also interfere with cell-to-cell propagation of α-synuclein aggregates [23, 30]. To evaluate the ability of antibodies to block this propagation, we used the dual-cell BiFC propagation assay system developed in our previous study [23]. The dual-cell BiFC system, composed of two cell lines, V18 and SV2, allows α-synuclein propagation between these cell types to be monitored by the appearance of fluorescent spots. When added to this propagation-assay system, all antibodies tested significantly reduced the number of BiFC-positive cells (Fig. 2F, 2G), indicating that conformation-specific antibodies effectively inhibit cell-to-cell propagation of α-synuclein.

**Passive immunization with aggregate-specific antibodies reduces α-synuclein deposition, neurodegeneration, gliosis, and inflammation**

To examine the effects of the aggregate-specific antibodies, 3A9,
Conformation-specific Antibodies against α-synuclein Alleviates Synucleinopathy

Fig. 1. Characterization of α-synuclein antibodies. (A) Antigens for immunization for production of antibodies. (B) Ultrastructural image of the proteins in (A). (C) Information on immunogens and isotypes of resulting antibody clones. Conformation-specific antibodies were generated by immunization of C57BL6 mice with either full-length α-synuclein or C-terminally truncated α-synuclein. (D) Specificity for monomer (M) or fibril (F) in dot blots. (E) Preference for aggregated forms of α-synuclein based on (D). The relative affinity towards M and F forms is indicated by the number of ‘<’ symbols. ‘M <<< F’ antibodies were selected for further assay. (F) Affinity of antibodies for monomer and aggregated forms of α-synuclein, determined by sandwich ELISA. (G) Cross-reactivity with amyloid β42 and tau proteins. Two-fold serial dilutions (from 50 ng) of both monomeric and aggregated proteins were spotted onto a nitrocellulose membrane. Syn-1, Tau5 and 6E10 antibodies were used as positive controls for α-synuclein, tau, and amyloid β42 respectively.
Fig. 2. Conformation-specific antibodies enhance the clearance of extracellular α-synuclein aggregates in BV-2 cells and block cell-to-cell propagation. (A) Proposed mechanism of action of antibodies in the uptake of extracellular α-synuclein and cell-to-cell propagation. (B) Internalization of α-synuclein fibrils in the presence of the indicated antibodies in BV-2 microglial cells. (C) Amount of internalized α-synuclein, quantified and normalized to the levels of β-actin. (D) Immunofluorescence images of α-synuclein fibrils in BV-2 cell after acid wash. Nuclei was stained by DAPI. The close-up panel: Magnified images of the boxed area. Scale bars: 20 μm. (E) Fluorescence puncta from the internalized α-synuclein fibril in (D) were quantified. Puncta was indicated with white arrowheads. One hundred cells per each coverslip were analyzed from three independent experiments. (F) Blocking effects of antibodies against the propagation of α-synuclein. BiFC-positive inclusions are indicated with white arrowheads. Bottom panels: Magnified images of the boxed area. Images were acquired with an InCell analyzer 2200. (G) Quantification of BiFC-positive cells in (F). All data are expressed as means±s.e.m. (n>10,000 cells per experiment (three independent experiments were performed); one-way ANOVA with two-sided Tukey’s post hoc test).
9B11 and 11F11, together with those of the pan-α-synuclein antibody, 11F4, on the accumulation of α-synuclein in vivo, we administered these antibodies intraperitoneally into α-synuclein transgenic (Tg) mice, and performed immunohistochemical analyses using antibodies against phosphorylated α-synuclein (pS129) and total α-synuclein (Fig. 3). Passive immunization with each of the above antibodies significantly reduced pS129 α-synuclein levels in the parietal cortex of α-synuclein Tg animals (Fig. 3A, 3C). A trend towards reduced pS129 was observed in the prefrontal cortex of antibody-treated animals, although this difference did not reach statistical significance (Fig. 3B). Similar trends were also observed in the hippocampus, with 11F11 producing a significant decrease in pS129 levels in the CA1 region, and 11F4 and 3A9 producing significant decreases in the CA3 region (Fig. 3D, 3E). Immunohistochemical analyses of total α-synuclein showed reductions in α-synuclein deposition in the prefrontal cortex, parietal cortex, and CA1 and CA3 regions of the hippocampus (Fig. 3G–3I). In the case of total α-synuclein, 9B11, 11F11 and 11F4 antibodies were more effective in reducing α-synuclein deposition than the 3A9 antibody (Fig. 3G–3I). To verify these immunohistochemical data, we have performed western analysis with the brain extracts and found that although only the 11F11 showed statistically significant effect, all the antibodies exhibited trends toward reducing the levels of the total α-synuclein (Fig. 3K, 3L). To determine the levels of fibril forms of α-synuclein in the brain extracts, we also performed dot blot analysis with the FILA-4 antibody, an antibody specific for fibrillar α-synuclein [31]. Again, the levels of α-synuclein fibrils in the brain extracts were consistently reduced with the conformation-specific antibody treatments, although only the 3A9-treated group reached the statistical significance (Fig. 3M, 3N).

We then examined the effects of these antibodies on neurodegeneration, gliosis, and inflammation. The Tg model used in this study showed neuronal loss in the hippocampus CA1 region (Fig. 4A, 4C) as also reported previously [32]. Treatment with α-synuclein antibodies prevented neuronal loss, as assessed using the NeuN antibody, with 11F11 and 11F4 being more effective than the other two antibodies (Fig. 4A, 4C). Next, we examined astrogliaosis and microgliosis in the prefrontal cortex, parietal cortex, and hippocampus. Immunohistochemical analyses using an antibody against glial fibrillary acidic protein (GFAP) showed reductions in astroglial cells in both the parietal cortex and the hippocampus in Tg mice treated with α-synuclein antibodies compared with those treated with control IgG antibodies (Fig. 4D–4F). Likewise, immunohistochemical analyses using an antibody against Iba1 (ionized calcium binding adaptor molecule 1) showed reduced microglial cell numbers in the same brain regions in antibody-treated animals compared with IgG control-treated animals (Fig. 4G–4I). The effects on microgliosis were more pronounced in the prefrontal cortex than in the hippocampus. Finally, we examined brain inflammation by measuring levels of the pro-inflammatory cytokine, IL-1β, in the hippocampus. IL-1β levels in Tg mice treated with antibodies were significantly reduced compared with those in mice treated with control IgG (Fig. 4J, 4K).

**DISCUSSION**

In this work, we generated α-synuclein antibodies by immunization of C57BL6 mice with either full-length or C-terminally truncated α-synuclein fibrils. Of the 13 antibodies generated, four (2E3, 3A9, 9B11 and 11F11) were determined to be specific to the fibril forms of α-synuclein. Among these antibodies, we selected three (3A9, 9B11 and 11F11) for further characterization. None of these three antibodies cross-reacted with monomeric α-synuclein, nor did they react with other neurodegenerative disease-related proteins (i.e., amyloid β42 and tau). Notably, all of these conformation-specific antibodies effectively blocked the propagation of α-synuclein aggregates between neuronal cells. In addition, 9B11 enhanced the uptake of α-synuclein fibrils in BV-2 microglial cells, underscoring the ability of this antibody to enhance clearance of extracellular α-synuclein aggregates. Furthermore, passive immunization with each conformation-specific antibody ameliorated neuropathological features of α-synucleinopathy in a Tg mouse model, reducing α-synuclein aggregates, gliosis, inflammation and neuronal loss. These results suggest that, by virtue of their ability to effectively neutralize the pathogenic functions of α-synuclein aggregates, the conformation-specific antibodies developed here have potential for use as therapeutic agents.

Two mechanisms have been proposed to explain how immunotherapy against α-synuclein works. First, antibodies block the cell-to-cell propagation of α-synuclein by capturing extracellular α-synuclein secreted from neurons [23, 30]. Second, antibodies aid the clearance of extracellular α-synuclein by promoting uptake and degradation of the protein by microglia [29]. This latter mechanism is mediated by Fcγ receptors, which recognize α-synuclein-antibody complexes; Fcγ-mediated uptake appears to result in faster delivery of cargoes to lysosomes than occurs with the normal α-synuclein uptake pathway [29]. In the current study, we showed that at least one conformation specific antibody, 9B11, significantly increased the clearance of extracellular α-synuclein fibrils by microglial cells. The antibodies may influence in the microglial degradation of α-synuclein fibrils in addition to the uptake. In our previous study [29], we have shown that α-synuclein antibodies indeed accelerated the intracellular trafficking of the internalized fibrils to lysosomes. The clearance rate is determined...
Fig. 3. Passive immunization reduces α-synuclein deposition in mThy-1-α-synuclein Tg mice. mThy-1-α-syn Tg mice and non-Tg littermates were injected with aggregate-specific antibodies and a pan-α-synuclein antibody (10 mg kg⁻¹, i.p.) weekly for 3 months. (A) Representative images of phosphorylated α-synuclein (pS129) staining in the prefrontal cortex, parietal cortex, and hippocampal regions. (B–E) Optical density measurements in the prefrontal cortex (B), parietal cortex (C), and hippocampal regions (D, E). (F) Representative images of total-α-synuclein staining in the prefrontal cortex, parietal cortex, and hippocampal regions. (G–J) Optical density measurements in the prefrontal cortex (G), parietal cortex (H), and hippocampal regions (I, J). Scale bar, 200 μm. Data are expressed as means±s.e.m. (n=5–6 per group; #p<0.05 vs. WT+3A9 group, *p<0.05 vs. TG+IgG group; one-way ANOVA with two-sided Tukey’s post hoc test). Pfcx, prefrontal cortex; Pacx, parietal cortex; HP, hippocampus. (K–L) Western blotting of brain tissue extracts. (M–N) Dot blotting of brain tissue extracts. FILA-4 antibody, an antibody specific for fibrillary α-synuclein were used. Data were analyzed by one-way ANOVA with two-sided Tukey’s post hoc test. *p<0.05 ***p<0.0001 vs. TG+IgG group.
Fig. 4. Passive immunization reduces neurodegeneration, gliosis, and inflammation in mThy-1-α-synuclein Tg mice. (A) Representative images of mature neuron staining in the prefrontal cortex and hippocampus. (B, C) Optical density measurements in NeuN-immunopositive areas of the prefrontal cortex and hippocampus. (D) Representative images of astroglia staining in the parietal cortex and hippocampal regions. (E, F) Optical density measurements in GFAP-immunopositive areas of the parietal cortex and hippocampus. (G) Representative images of microgliosis staining in the prefrontal cortex and hippocampus. (H, I) Optical density measurements in Iba-1-immunopositive areas of the prefrontal cortex and hippocampus. (J) Representative images of IL-1β cytokine staining in the hippocampus. (K) Optical density measurements in IL-1β-immunopositive areas of the hippocampus. Scale bar, 200 μm (G) and 100 μm (J). Data are expressed as means±s.e.m. (n=5–6 per group; *p<0.05 vs. WT+3A9 group, **p<0.05 vs. TG+IgG group; one-way ANOVA with two-sided Tukey’s post hoc test). Pfcx, prefrontal cortex; Pacx, parietal cortex; HP, hippocampus.
not only by the rate of uptake but also by the rate of the intracellular degradation. Antibodies have shown to increase both uptake and degradation rates, hence increasing the overall clearance rate. Consistent with this, Gustafsson et al. [33] showed that mAb47, an antibody selective for oligomer/prototibril forms of α-synuclein, enhanced the uptake of extracellular α-synuclein via Fcγ receptors. As for the role of antibodies in blocking cell-to-cell propagation, our present study reports that conformation-specific antibodies effectively decrease this process for α-synuclein. These blocking effects have also been shown with antibodies against oligomers and fibrils of α-synuclein by El-Agnaf and colleagues [34]. Taken together, our findings indicate that the mechanism of action of conformation-specific antibodies includes interference with cell-to-cell propagation and increased clearance of α-synuclein, actions akin to those of pan α-synuclein antibodies.

Our results show that the efficacies of antibodies on microglial clearance and cell-to-cell propagation does not match. The biological effects of the antibodies are dictated not only by the affinity to the antigen but also by the interaction between the antibodies and specific cell types. Neurons and microglia express different repertoire of Fc receptors [35], which would result in cell type-specific responses to various antibodies. In the microglial clearance assay, 9B11 showed the strongest effect. The reason for this is not clear. However, the fact that 9B11 is IgG3 while other conformation-specific antibodies tested are IgG2b might have influenced the efficiencies of these antibodies on the interaction with microglia. Unlike in microglia, the cell-to-cell propagation assay performed in a neuronal cell line showed a consistent result among the antibodies. The antibodies used in these tests have similar affinity to α-synuclein fibrils. Therefore, we speculate that the effects of antibodies on cell-to-cell propagation are determined mainly by the antibody-antigen affinity, whereas those on microglial clearance are determined by both antibody-antigen affinity and the antibody subtypes.

There is limited amount of direct evidence for cell-to-cell propagation in α-synuclein tg mice. However, several PFF injection experiments have been performed in α-synuclein tg models [6, 7, 36-40]. These experiments clearly showed accelerated α-synuclein aggregation in various brain regions, suggesting that cell-to-cell propagation of α-synuclein probably occurs in the tg models. Another evidence for cell-to-cell propagation in α-synuclein tg model comes from the experiment where propagation blocker reduced α-synuclein pathology in a tg model. In this case, administration of anti-toll like receptor 2 (TLR2) antibody to an α-synuclein tg mouse model significantly reduced the deposition of α-synuclein [41]. TLR2 has been shown to be a neuronal receptor mediating the cell-to-cell propagation of α-synuclein [41]. This effect of the propagation receptor strongly suggests that cell-to-cell propagation of α-synuclein occurs in the transgenic mice.

In this study, conformation-specific antibodies were generated using both full-length and C-terminally truncated forms of α-synuclein fibrils. Among the antibodies generated, four showed high specificity for fibrils, three of which were raised from C-terminally truncated α-synuclein fibrils, suggesting that immunization with C-terminally truncated α-synuclein fibrils is more likely to generate conformation-specific antibodies. Using solid-state NMR together with electron microscopy and atomic force microscopy, it has been shown that the β-sheet-rich α-synuclein fibril core corresponded to residues 38–98 [42, 43]. This core region is highly structured, whereas the C-terminal region (residues 96–140) outside the core region is flexible and unfolded. Therefore, antibodies raised against the C-terminal region would not distinguish aggregates from monomers, probably explaining why immunization with C-terminally truncated α-synuclein fibrils was more effective in generating aggregate conformation-specific antibodies.

A growing body of evidence supports the beneficial effects of passive immunization against α-synuclein in animal models of synucleinopathy [29, 30, 44-46]. The more recent of these studies suggest that passive immunization with fibril- or prototibril-specific antibodies is also effective in ameliorating disease phenotypes in animal models [34, 47, 48]. Our present study is the first to compare the efficacies of conformation-specific antibodies with those of a pan-α-synuclein antibody. Our results demonstrate that the conformation-specific antibodies are as effective as a pan-α-synuclein antibody in ameliorating the neuropathological features of a synucleinopathy model, including α-synuclein aggregation, gliosis, neuroinflammation, and neuronal loss. This suggests that monomer recognition is not required for the success of passive immunization. The conformation-specific antibodies do not recognize the normal α-synuclein, and thus would not interfere with the normal function of α-synuclein. Therefore, the use of conformation-specific antibodies that do not cross-react with monomers would have clear benefits in avoiding adverse effects of passive immunization by preserving the normal functions of monomeric α-synuclein proteins. In conclusion, immunotherapy with conformation-specific antibodies raised against α-synuclein fibrils could be effective in treating α-synuclein-related diseases, such as PD, without interfering with the normal functions of α-synuclein proteins.

ACKNOWLEDGEMENTS

We are grateful to Eliezer Masliah (currently at NIA, Bethesda,
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