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MultiTEP Platform-based DNA Vaccines for alpha-Synucleinopathies: Pre-clinical Evaluation of Immunogenicity and Therapeutic Potency

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Abstract

We have previously demonstrated that anti-A β DNA vaccine (AV-1959D) based on our proprietary MultiTEP platform technology is extremely immunogenic in mice, rabbits, and monkeys.

Importantly, MultiTEP platform enables development of vaccines targeting pathological molecules involved in various neurodegenerative disorders. Taking advantage of the universality of MultiTEP platform, we developed DNA vaccines targeting three B cell epitopes (aa85–99, aa109–126, aa126–140) of human alpha-Synuclein (h α -Syn) separately, or all three epitopes simultaneously.

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Disclosure statement

MGA is a co-founder of Neuroimmune that licensed MultiTEP vaccine platform technology from the Institute for Molecular Medicine.

The remaining authors have no financial or commercial conflict of interests.

Authors' contribution

HD, KZ, DHC, MGA and AG conceived the study and participated in its design. HD, KZ, IP, KK, JP and NRS performed the experiments. MBJ generated the SH-SY5Y/h α -Syn cells. MBJ, EM and DHC helped with interpretation of data. HD, MGA and AG wrote the paper. All authors read and approved the final manuscript.

All four DNA vaccines (i) generate high titers of anti-h α -Syn antibodies; (ii) induce robust MultiTEP-specific Th cell responses without activation of potentially detrimental autoreactive anti-h α -Syn Th cells. Generated antibodies recognize misfolded h α -Syn produced by neuroblastoma cells, h α -Syn in the brain tissues of transgenic mouse strains and in the brain tissues of Dementia with Lewy Bodies (DLB) cases. Based on these results, the most promising vaccine targeting three B cell epitopes of h α -Syn simultaneously (PV-1950D) has been chosen for ongoing pre-clinical assessment in mouse models of h α -Syn with the aim to translate it to the human clinical trials.

Keywords

DNA Vaccines; MultiTEP platform; Anti- α -Synuclein Antibodies; B-cell mapping; T-cell mapping; Immunogenicity; Therapeutic potency

1. Introduction

Intracellular aggregates of alpha-Synuclein (α -Syn) accumulate as Lewy bodies (LB) and Lewy neurites (LN) in patients with Dementia with Lewy bodies (DLB) and Parkinson's disease (PD). However, synucleinopathy is not restricted to pure DLB and PD. Up to 50% of AD cases exhibit Lewy bodies, and the presence of LB pathology in AD is associated with a more aggressive disease course and accelerated cognitive dysfunction (McGeer and McGeer, 2008). Overlap of clinical and neuropathological features of AD and PD is observed in DLB (Kotzbauer et al., 2012; Obi et al., 2008). Likewise, co-occurrence of tau and α -Syn inclusions could be observed in Parkinson's disease with dementia (PDD), DLB, Lewy body variant of AD (LBVAD) and Guam-Parkinson-ALS dementia complex (Badiola et al., 2011; Forman et al., 2002; Giasson et al., 2003a; Marui et al., 2000). Furthermore, there are multiple reports demonstrating that amyloidogenic proteins can interact *in vivo* to promote an aggregation and accumulation of each other (Gallardo et al., 2008; Giasson et al., 2003b; Mandal et al., 2006; Tsigelny et al., 2008). Today medications may help control some symptoms of PD/DLB, but very few disease-modifying treatments have been developed yet (Fahn, 2005; Fahn et al., 2004). The era of vaccination against neurodegenerative disorders was initiated almost 16 years ago when a group of researchers from Elan Pharmaceuticals reported on effective clearance of extracellular amyloid pathology after immunizations of AD transgenic (Tg) mice with fibrillar beta-amyloid (A β ₄₂) peptide formulated in adjuvant (Schenk et al., 1999). Several years later Masliah *et al.* (Masliah et al., 2005) and Asuni *et al.* (Asuni et al., 2007) demonstrated that vaccination strategies could be viable not only for targeting extracellular, but also intracellular pathological human α -Syn (h α -Syn) and tau aggregates, respectively. More specifically, vaccination of h α -Syn transgenic mice mimicking certain aspects of PD/DLB, with recombinant h α -Syn protein induced therapeutically potent antibodies capable of reducing intracellular toxic h α -Syn aggregates. Notably, these antibodies generated by full-length protein recognized four epitopes spanning amino acids (aa) 85–99, 109–123, 112–126, and 126–138 of h α -Syn (Masliah et al., 2005). Later the same group of scientists demonstrated that administration of monoclonal antibodies (mAb) 9A4 specific to aa118–126 of h α -Syn reduced neurological deficits and improved behavior in the same mouse model of PD/DLB (line D) (Masliah et al., 2011)

while two other mAbs (1H7 & 5D12) mitigated neurodegeneration in the second mouse model (Thy1- α -Syn) mimicking PD (line 61) (Games et al., 2014). Later it was shown that short peptides mimicking aa sequence 110–130 of h α -Syn attached to Keyhole Limpet Hemocyanin (KLH) carrier (e.g. AFF 1 vaccine) also induced therapeutically potent antibodies in these two mouse models of PD/DLB, and the authors showed improvements in both motor function and memory in h α -Syn Tg mice (Mandler et al., 2014). Finally, active immunization with AFF 1 vaccine formulated in alhydrogel reduced h α -Syn accumulation, demyelination in the neocortex, striatum and corpus callosum, as well as neurodegeneration in mouse models of another synucleinopathy, multiple system atrophy (MBP- α -Syn Tg) (Games et al., 2014; Mandler et al., 2015; Mandler et al., 2014; Masliah et al., 2005; Masliah et al., 2011). Thus, anti-h α -Syn immunotherapy reduced the accumulation of pathological α -Syn in axons and synapses and alleviated motor and learning deficits in various models of α -synucleinopathies.

By providing genes encoding a specific antigen, DNA vaccination represents a unique alternative method of immunization and has properties that may be advantageous in developing a range of vaccines against a variety of pathogens and human diseases (Abdulhaqq and Weiner, 2008; Agadjanyan et al., 1998; Davtyan et al., 2014b; Ghochikyan, 2009; Kim et al., 1998; Li et al., 2012; Ugen et al., 2006). Previously, we demonstrated that an anti-A β DNA vaccine based on the MultiTEP platform technology (AV-1959D) generated very strong cellular and humoral immune responses in mice, rabbits, and monkeys without induction of potentially harmful autoreactive T cell responses (Davtyan et al., 2014a; Davtyan et al., 2014b; Evans et al., 2014; Ghochikyan et al., 2013). Importantly, at least in monkeys, this vaccine did not induce antibodies specific to MultiTEP (Davtyan et al., 2014a), suggesting that this vaccine platform may be beneficial in human clinical trials. To take advantage of this immunogenic and universal vaccine platform technology we decided to generate MultiTEP-based DNA vaccines targeting three different B cell antigenic determinants of h α -Syn, spanning aa85–99 (PV-1947D), aa109–126 (PV-1948D), aa126–140 (PV-1949D) separately, or simultaneously (PV-1950D). Here we present data demonstrating that none of the DNA epitope vaccines induced potentially harmful autoreactive Th cell responses, while all of them generated strong cellular responses to the vaccine platform. Importantly, anti-MultiTEP specific Th cells have activated B cells producing high titers of antibodies that recognized pathological forms of h α -Syn.

2. Material and methods

2.1. Mice

Female, 6–8 weeks old C57BL/6 mice (H2^b haplotype) were obtained from Jackson Laboratory. All animals were housed in a temperature and light-cycle controlled facility, and their care was under the guidelines of NIH and an approved IACUC protocol at UC Irvine.

2.2. Plasmids and peptides

The generation of plasmid encoding the MultiTEP platform composed of a string of twelve foreign Th epitopes was described previously (Ghochikyan et al., 2013). In this study, four different minigenes encoding three copies of B cell epitopes aa85–99, aa109–126, aa126–

140, or all three epitopes together [3×(aa126–140)+3×(aa109–126)+3×(aa85–99)], have been synthesized by GenScript Company and ligated with a gene encoding MultiTEP. The resulting genes were cloned into the pVAX1 vector (Invitrogen) designed to be consistent with current Food and Drug Administration (FDA) guidelines. Additionally, we have generated a plasmid encoding full-length ha-Syn fused with MultiTEP platform (pha-Syn-MultiTEP). Immunization-grade plasmids were purified by Aldevron, and the correct sequences of the generated plasmids were confirmed by nucleotide sequence analysis. Expression and secretion of the proteins encoded by generated DNA constructs were analyzed in the lysates and supernatants from transiently transfected Chinese hamster ovary (CHO) cells by western blotting (WB) as described previously (Movsesyan et al., 2008a; Movsesyan et al., 2008b), except that immunoblots were stained with anti-ha-Syn_{85–99}, anti-ha-Syn_{109–126} and anti-ha-Syn_{126–140} antibodies generated at the Institute for Molecular Medicine (IMM). Peptides spanning ha-Syn, aa85–99 (α-Syn_{85–99}), aa109–126 (α-Syn_{109–126}), aa126–140 (α-Syn_{126–140}), as well as 20mer overlapping peptides aa1–20 (α-Syn_{1–20}), aa16–35 (α-Syn_{16–35}), aa31–50 (α-Syn_{31–50}), aa46–65 (α-Syn_{46–65}), aa61–80 (α-Syn_{61–80}), aa76–95 (α-Syn_{76–95}), aa91–110 (α-Syn_{91–110}), aa106–125 (α-Syn_{106–125}), aa121–140 (α-Syn_{121–140}) and irrelevant peptide were synthesized by GenScript.

2.3. Immunizations

Mice were injected into both tibialis anterior muscles with 20µg per leg of the appropriate plasmid vaccine in 30µl PBS. Immediately after the DNA administration electrical pulses were applied using the AgilPulse™ device from BTX Harvard Apparatus, as previously described (Davtyan et al., 2014b). Briefly, the needle electrode made of two parallel rows of four 5-mm needles 0.3mm in diameter (1.5 × 4-mm gap) was inserted in such a way that the i.m. injection site was located between the two needle rows. The EP pulses were applied (“high amplitude, short duration” (450/0.05) two pulses and “low amplitude, long duration” (110/10) eight pulses) using the AgilPulse™ device from BTX Harvard Apparatus (Holliston, MA). The mice were immunized with indicated DNA vaccines on days 0, 14, 28 and 42. The sera were collected on 12th day after the third immunization (Day 40) and were used both for analyzing of the humoral immune responses and purification of polyclonal antibodies. On the 7th day after the fourth immunization (Day 49) mice were terminated, spleens were harvested, and splenocytes were used for detection of cellular immune responses. For the B and T cell epitope mapping studies, two groups of mice were immunized with either pha-Syn-MultiTEP plasmid followed by EP, or with the full-length ha-Syn protein (rPeptide) formulated with Advax^{CpG} adjuvant. Mice were vaccinated 4 times biweekly. Blood was collected 10 days after the 3rd immunization and sera were used for B cell epitope mapping. On the 7th day after the last immunization mice were terminated and T cell epitope mapping was performed on splenocytes (see below). In the second set of T cell epitope mapping experiment mice were immunized twice with full-length ha-Syn protein (rPeptide) and terminated 7 days after the second immunization.

2.4. Detection of IFN-γ-producing T cells and mapping of T cell epitopes

The analysis of cellular immune responses was performed in splenocyte cultures from individual animals by detection of T cells producing IFN-γ cytokine by ELISPOT (BD Biosciences, San Jose, CA, USA) as previously described (Cribbs et al., 2003; Davtyan et

al., 2014a; Davtyan et al., 2013; Petrushina et al., 2007). For T cell epitope mapping the splenocyte cultures collected from pha-Syn-MultiTEP- and full-length ha-Syn protein immunized mice were re-stimulated with 10µg/ml of each indicated peptide spanning ha-Syn or irrelevant peptide for 20 hours *in vitro*. Splenocyte cultures from mice immunized with different DNA epitope vaccines were re-stimulated *in vitro* with a cocktail of 12 peptides representing the Th epitopes in MultiTEP platform (Davtyan et al., 2014a) (2µg/ml of each peptide), ha-Syn protein or peptides(s) representing self B cell epitope(s) at 10µg/ml for 20 hours. The numbers of SFC per 10⁶ splenocytes were then counted.

2.5. Detection of the titer of anti-ha-Syn antibody and mapping of B cell epitopes

B cell epitopes were mapped by ELISA, as previously described (Ghochikyan et al., 2014). Binding of sera from pha-Syn-MultiTEP and ha-Syn protein immunized mice (at dilutions 1:1000 and 1:5000, respectively) to 20-mer overlapping peptides spanning ha-Syn was analyzed. The titers of total anti-ha-Syn antibodies, as well as antibodies specific to ha-Syn₈₅₋₉₉, ha-Syn₁₀₉₋₁₂₆ and ha-Syn₁₂₆₋₁₄₀ were detected by ELISA as described previously (Ghochikyan et al., 2014). Briefly, plates were coated with 1µg/ml of ha-Syn protein or the appropriate peptide. Sera from individual mice were diluted (from 1:1,000 to 1:2,560,000) and endpoint titers were calculated as the reciprocal of the highest sera dilution that gave a reading twice above the background levels of binding of non-immunized sera at the same dilution (cutoff). HRP-conjugated anti-IgG1, IgG2a^b, IgG2b and IgM specific antibodies (Bethyl Laboratories, Inc.) were used to characterize the isotype profiles of anti-ha-Syn antibodies in individual sera at dilution 1:1000.

2.6. Purification of anti-ha-Syn antibodies

Anti-ha-Syn antibodies from sera of mice immunized with PV-1947D, PV-1948D and PV-1949D epitope vaccines were purified by an affinity column (SulfoLink, ThermoFisher Sci.) using an immobilized α-Syn₈₅₋₉₉-C, α-Syn₁₀₉₋₁₂₆-C and α-Syn₁₂₆₋₁₄₀-C peptides (GenScript), respectively, as we previously described (Mamikonyan et al., 2007). Purified antibodies were analyzed via 10% Bis-Tris gel (ThermoFisher Sci.), and the concentrations were determined using a BCA protein assay kit (ThermoFisher Sci.).

2.7. Human α-Syn overexpressing cell line and Western blotting

SH-SY5Y neuroblastoma cells stably overexpressing human α-Synuclein (SH-SY5Y/ha-Syn) were generated by AMAXA nucleofection with a plasmid expressing wild type ha-Syn and puromycin resistance under control of a CAG promoter. Control (SH-SY5Y/WT) cells were concurrently produced by nucleofection with an empty vector. Stable cells were selected by treatment with puromycin for 6 weeks and ha-Syn expression confirmed by WB (data not shown). All SH-SY5Y cells were grown in DMEM supplemented with 10% FBS, in a 5% CO₂ atmosphere. Cell lysates were subjected to electrophoresis on NuPAGE 10% Bis-Tris gel in MES buffer under reducing conditions and electro-transferred onto a nitrocellulose membrane (GE Healthcare). Membranes were stained with purified anti-ha-Syn₈₅₋₉₉, anti-ha-Syn₁₀₉₋₁₂₆ and anti-ha-Syn₁₂₆₋₁₄₀ antibodies at concentration 1µg/ml and goat anti-mouse IgG-HRP (Santa Cruz Biotechnology). As a positive control, 0.5µg of full-length ha-Syn was loaded onto the gel.

2.8. Mouse brain extraction and Western blotting

Brain homogenates from h α -Syn tg (line D) mice were prepared using T-PER (Tissue Protein Extraction Reagent) buffer (ThermoFisher Sci.) with added protease inhibitor cocktail (Sigma). Brain homogenates from h α -Syn tg (line 61) mice were prepared using PDGF buffer (1mM HEPES, 5mM Benzamidine, 2mM 2-Mercaptoethanol, 3mM EDTA, 0.5mM Magnesium sulfate, 0.05% Sodium azide, pH=8.8) with added phosphatase and protease inhibitors (Calbiochem). Lysates were centrifuged (14,000 \times g for 1 hr at 4 $^{\circ}$ C), supernatants were collected, and protein concentrations were determined by BCA protein assay kit (ThermoFisher Sci). Striatal tissue lysate from a α -Syn knockout mouse (Jackson Laboratories #016123) was prepared along with experimental samples and it is present on every α -Synuclein Western blot as a negative control. Samples were subjected to electrophoresis on NuPAGE 4–12% Bis-Tris gel in MES buffer under reducing conditions and electro-transferred onto a nitrocellulose membrane (GE Healthcare). α -Syn was visualized by incubating with sera from mice immunized with PV-1947D, PV-1948D, PV-1949D or PV-1950D followed by HRP-conjugated anti-mouse IgG (Santa Cruz Biotechnology). Sera were used after normalization of antibody endpoint titers measured by ELISA. Mouse anti- α -Syn antibody against aa15–123 (1:1000; BD Biosciences) and Syn211 anti- α -Syn antibody (1:1000; EMD Millipore) served as positive controls. Sera from mice immunized with irrelevant antigen were used as a negative control.

2.9. Human brain extraction and Western blotting

Brain homogenates from DLB cases and control subjects were prepared using PDGF buffer with added phosphatase and protease inhibitors (Calbiochem). Samples were analyzed using WB performed as described above.

2.10. Preparation and analysis of α -Synuclein oligomers

Oligomers were prepared as described by Roberts *et al.* (Roberts et al., 2015). Briefly, oligomers were produced by incubating recombinant h α -Syn (rPeptide) at 1mg/ml (~70 μ M) with 30:1 M excess of 4-hydroxy-2-nonenal (HNE; EMD Millipore) at 37 $^{\circ}$ C for 18h. After the incubation, unbound aldehyde was removed with an Amicon 3kDA cut-off ultra-centrifugal unit (EMD Millipore). WB analysis was carried out as described above. 0.25 μ g h α -Syn monomers and oligomers were used in each well. Additionally, α -Syn was also visualized with monoclonal mouse anti- α -Syn antibody (aa15–123; 1:1000; BD Biosciences) and Syn211 anti- α -Syn antibody (1:1000; EMD Millipore).

2.11. Immunohistochemistry

The brain samples were obtained from the Brain Bank and Tissue Repository (MIND, UCI) as formalin-fixed blocks of anterior cingulate cortex of control normal brain and the brain of a human subject with established DLB pathology. The brains were processed for immunohistochemistry (IHC) by previously published methods (Ghochikyan et al., 2014; Petrushina et al., 2007) employing proteinase K pretreatment, and Lewy bodies (LBs) and Lewy neurites (LNs) were detected using sera from mice immunized with PV-1947D, PV-1948D, PV-1949D, and PV-1950D. The images were captured via Digital Whole Slide

Scanning System Aperio AT2 (HistoWiz, Brooklin, NY), and the photomicrographs of immunostained images at 40X magnification are presented in this paper.

2.12. Statistical Analysis

Statistical parameters (mean, standard deviation (SD), significant difference, etc.) were calculated using the Prism 6 software (GraphPad Software, Inc.). Statistically significant differences were examined using analysis of variance (one-way ANOVA) and Tukey's multiple comparisons post-test (a P value of less than 0.05 was considered significant).

3. Results and Discussion

3.1. Mapping of B and T cell epitopes of α -Syn after DNA- and protein-based immunizations and construction of vaccine plasmids

Previous epitope mapping studies showed that α -Syn/Tg mice responded to immunization with full-length α -Syn recombinant protein formulated in the adjuvant CFA/IFA with production of antibodies binding to the middle and C-terminal regions of this protein (aa85–99, aa109–123, aa112–126, and aa126–138) (Masliah et al., 2005). These epitope-mapping data became instrumental for future immunotherapeutic studies demonstrating that mAb specific to aa118–126 (Games et al., 2014) of α -Syn and antibodies against α -Syn mimotope of aa110–130 (Mandler et al., 2015) effectively reduce pathology and improve behavior in three transgenic mouse models of α -synucleinopathies.

Here we compared the B cell antigenic determinant of α -Syn synthesized in eukaryotic mouse cells versus that produced in prokaryotic bacterial cells (e.g., DNA vaccine versus recombinant protein vaccine). Accordingly, we immunized mice of H2^b immune haplotype with plasmid encoding full-length of *ha-Syn* gene or recombinant α -Syn protein formulated in Advax^{CpG} adjuvant and then mapped the B cell epitopes using immune sera from vaccinated mice with a series of overlapping peptides covering the entire sequence of α -Syn protein (Fig. 1A, B). Both DNA and protein-based full-length α -Syn generated antibodies that bind strongly to the peptides spanning aa91–110 and aa106–125, but not to aa126–138, as was shown earlier by Masliah *et al.* (Masliah et al., 2005). Injection of a plasmid encoding *ha-Syn* also induced antibodies that weakly bound to aa46–65 and aa61–80 peptides, while administration of protein-based vaccine generated antibodies that bound weakly to peptide spanning aa31–40 of α -Syn. Of note, no natural anti- α -syn antibody had been detected in non-immunized mice. Prior to using these B cell epitopes for generation of MultiTEP platform-based vaccines, we tested an ability of each of the overlapping peptides to stimulate *in vitro* autoreactive Th cells in mice immunized with full-length α -Syn. These experiments demonstrated that regardless of the nature of the vaccine, DNA vs. recombinant protein, two peptides spanning aa76–95 and aa106–125 of α -Syn activated Th cells in immunized mice (Fig. 1C, D). Therefore, at least in mice of H2^b immune haplotype, one peptide (span aa106–125) of α -Syn possessed B and Th cell antigenic determinants. Previously, we showed that slightly smaller peptides, aa85–99 and aa109–126 of α -Syn did not possess Th cell epitopes (Ghochikyan et al., 2014), therefore it was suggested that aa76–85 and aa106–108 of α -Syn may be important for activation of Th cells. To test this possibility, we performed a separate study and tested Th cell responses in mice immunized

and boosted once with full-length ha-Syn protein formulated with adjuvant. The data showed that small peptides spanning aa85–99 and aa109–125 do not activate autoreactive Th cells (Fig. 2). Collectively, these results suggested that B cell epitopes spanning aa85–99 and aa109–126 at the C-terminus of ha-Syn are candidates for prototype PD/DLB vaccine development.

At the same time, data presented in Fig. 1 did not confirm the existence of B cell antigenic determinants in the ha-Syn region spanning aa126–140 as was shown after immunization of ha-Syn/Tg mice (line D) with recombinant ha-Syn protein formulated with CFA/IFA adjuvant (Masliah et al., 2005). Such differences could be explained by different strains of mice (wild-type mice of H2^b immune haplotype vs. ha-Syn/Tg mice of H2^{dx^b} immune haplotype), as well as by different adjuvants used for formulation of recombinant protein (Advax^{CpG} vs. CFA/IFA). Furthermore, we have previously demonstrated that the B cell epitope aa126–140 attached to one Th epitope (P30) and a carrier peptide is immunogenic. In fact, it induced higher titers of anti-ha-Syn antibodies than B cell epitopes aa85–99 and aa109–125 attached to P30 and the same carrier peptide (Games et al., 2014; Ghochikyan et al., 2014; Mandler et al., 2015; Masliah et al., 2005; Masliah et al., 2011). Therefore, based on these data and results published earlier (Games et al., 2014; Ghochikyan et al., 2014; Mandler et al., 2015; Masliah et al., 2005; Masliah et al., 2011), we decided to use B cell epitopes spanning aa85–99 and aa109–126 of ha-Syn, as well as the epitope aa126–140 for the generation of MultiTEP-based DNA vaccines.

3.2. Generation of MultiTEP platform-based DNA vaccines and analysis of their immunogenicity

Four plasmids were constructed containing the MultiTEP platform fused with three copies of B cell epitopes, aa85–99 (PV-1947D), aa109–126 (PV-1948D), aa126–140 (PV-1949D) separately or consecutively, but in reverse order [aa126–140 + aa109–126 + aa85–99 (PV-1950D)] (Fig. 3A). Expression of the plasmid construct was confirmed by analyzing the supernatants of transiently transfected CHO cells (Fig. 3B–D) using previously developed antibodies specific to the epitopes (Ghochikyan et al., 2014). We detected several discrete bands on immunoblotting with anti- α -Syn antibodies indicating that α -Syn-MultiTEP proteins are proteolytically processed in CHO cells at varying sites within the MultiTEP sequence. Although it would be interesting to know precisely where these cleavages occur, such experiments are beyond the scope of the current study.

To analyze the immunogenicity of these constructs we immunized mice with PV-1947D, PV-1948D, PV-1949D and PV-1950D vaccines and tested the cellular and humoral immune responses. All four DNA vaccines induced strong Th cell responses specific to the MultiTEP platform, but not self-peptides (aa85–99, aa109–126, aa126–140) or recombinant ha-Syn (Fig. 4A). Therefore, the data suggest that at least in mice of H2^b immune haplotype, PV-1947D, PV-1948D, PV-1949D and PV-1950D vaccines do not stimulate potentially harmful autoreactive Th cells specific to B cell epitopes used for construction of the plasmids (Fig. 4). As expected, these MultiTEP-specific Th cells supported production of high titers of antibodies specific to the ha-Syn B cell epitopes chosen for generation of constructs (Fig. 5). Notably, two vaccines, PV-1947D and PV-1949D (Fig. 5A, C) induced

significantly higher titers of antibodies specific to their corresponding peptides ($p < 0.001$ and $p < 0.05$, respectively) than PV-1948D vaccine (Fig. 5B). Similar results were obtained after immunization with PV-1950D targeting all three hA-Syn B cell epitopes where significantly higher titers of antibodies specific to aa85–99 and aa126–140 epitopes ($p < 0.01$ and $p < 0.001$, respectively), compared to aa109–126 epitope were detected (Fig. 5D).

In order to investigate the therapeutic potential of the antibodies induced by the DNA vaccines, we examined the binding of antibodies generated against the various B cell epitopes to hA-Syn recombinant protein. These analyses demonstrated that vaccinated mice produced very high titers of antibodies recognizing recombinant hA-Syn (Fig. 6A–D), and the titers of antibodies generated with PV-1947D and PV-1949D vaccines were significantly higher ($p < 0.01$ and $p < 0.05$, respectively) compared with PV-1948D vaccine (Fig. 6A, C vs. B). These differences in binding to full-length hA-Syn recombinant protein could be explained by stronger immunogenicity of B cell epitopes used for generation of PV-1947D and PV-1949D vs. PV-1948D (Fig. 5A–D), or better accessibility of aa85–99 and aa126–140 epitopes vs. aa109–126 in the vaccines to B cell receptors. Of note, the vaccine composed of three B cell epitopes of hA-Syn, PV-1950D induced significantly higher antibody titers specific to aa109–126 than PV-1948D ($p < 0.05$) (Fig. 6A–D). These data demonstrate that all three B cell epitopes, aa85–99, aa109–126 and aa126–140 are exposed on recombinant hA-Syn protein. Next, we tested accessibility of these epitopes in the protein produced by a human SH-SY5Y neuroblastoma cell line that was modified to stably overexpress hA-Syn. Western blot data presented in Fig. 6E–G show that in reducing conditions purified antibodies specific to all three B cell antigenic determinants bind very well to hA-Syn in lysates of hA-Syn-SH-SY5Y cells. Thus, the Th cells activated by the MultiTEP platform promoted production of antibodies against all three B cell antigenic determinants, aa85–99, aa109–126 and aa126–140 of hA-Syn that are exposed on recombinant hA-Syn protein from *E. coli* and hA-Syn synthesized in a human neuroblastoma cell line.

More than a decade ago Bard *et al.* showed that the isotype of the anti-A β antibody is important for *in vitro* clearance of pathology (Bard *et al.*, 2003). On the other hand, it was shown that full-length and F(ab')₂ fragments of the same antibody clear pathology in APP/Tg mice equally well (Bacsikai *et al.*, 2002). Recently, this finding was confirmed when Lee *et al.* demonstrated that the effector function of an anti-tau antibody Fc fragment was not required for the reduction of pathology *in vivo* (Lee *et al.*, 2016). Importantly, the authors of this elegant study showed that the antibody with an effector function not only promotes uptake of tau by microglia, but also triggers secretion of pro-inflammatory cytokines in these cells that could have harmful effects on neurons. One advantage of AgilPulse™ mediated delivery of our DNA vaccines for AD is that we can choose different routes of immunization, intradermal (i.d.) or intramuscular (i.m.). Previously we have reported that injections of AV-1959D into the skin of mice induced predominantly Th2-type of humoral responses, while i.m. injections of the same vaccine in the same strain of mice induced predominantly a Th1 type of humoral immune responses (Davtyan *et al.*, 2014b). Accordingly, we measured IgG1, IgG2a^b, IgG2b and IgM responses in mice vaccinated with DNA vaccines (Fig. 6H–K). As expected, i.m. immunizations with PV-1947D, PV-1948D, PV-1949D, and PV-1950D induced equal levels of antibodies of all three IgG isotypes, with predominant Th1 type of humoral immune response. Since anti-hA-Syn antibody isotypes

may be important for their efficacy, in our future studies we are planning to test different routes of vaccination (i.e., i.m. vs. i.d.) in mouse models of PD and DLB.

3.3. DNA vaccine-induced antibody was capable of binding to native ha-Syn

Next, we evaluated the specificity of antibodies generated with all four vaccines to ha-Syn expressed in the brains of two α -Syn/Tg mouse strains (line D and line 61) and their non-transgenic littermates (non-Tg). Line D expressing ha-syn under the regulatory control of the platelet-derived growth factor- β (PDGF β) promoter (Masliah et al., 2000) displayed abnormal accumulation of detergent-insoluble ha-syn and developed ha-syn-immunoreactive inclusion-like structures in cortical and subcortical regions of the brain. Line 61 mice expressing ha-syn under the mThy1 promoter showed higher level of expression than line D. In these mice ha-syn accumulates in cell bodies of pyramidal neurons in the neocortex and limbic system (Rockenstein et al., 2002). Of note, we used two different methods for preparation of the soluble fractions of brain homogenates (see Method section) and used brain extract of α -Syn knockout mice as a negative control (Fig. 7A–G). Brain homogenates were analyzed using immune sera from vaccinated mice (Fig. 7A–D), two commercial monoclonal antibodies, Syn211 (recognizing only ha-Syn) and anti-Syn15–123 (recognizing both ha-Syn and mouse α -Syn) (Fig. 7E, F), as well as immune sera from mice vaccinated with irrelevant antigen (Fig. 7G). This data demonstrated that antibodies generated by PV-1947D, PV-1948D, and PV-1949D bound to monomeric ha-Syn isolated from the brains of both strains of Tg mice (Fig. 7). However, antibodies generated by PV-1948D (aa109–126), as well as Syn211 mAb bound only to ha-Syn (Fig. 7B, F), while anti-aa85–99 and anti-aa126–140 antibodies induced by PV-1947D and PV-1949D along with anti-Syn15–123 mAb were capable of binding to both ha-Syn and mouse α -Syn (Fig. 7A, C, E). As expected from these data, the PV-1950D vaccine induced antibodies that also recognized both ha-Syn and mouse α -Syn (Fig. 7D). Of note, binding of antibodies to mouse α -Syn in brain homogenates of non-Tg littermates is significantly weaker than binding to ha-Syn in brain homogenates of both strains of Tg mice (Fig. 7). Previously it was reported that oligomeric forms of ha-Syn in the soluble fraction of brain homogenates of line D and line 61 mice (Games et al., 2014; Mandler et al., 2014; Masliah et al., 2005), as well as MBP- α -Syn Tg mice (Games et al., 2014; Mandler et al., 2015; Mandler et al., 2014; Masliah et al., 2005; Masliah et al., 2011) could be detected by WB. However, our WB analysis of brain extracts from both line D, line 61 and non-Tg littermates suggested that antibodies generated by all four vaccines recognized monomeric forms of ha-Syn, while high molecular weight bands are likely non-specific (Fig. 7). In fact, similar bands have been detected after analyzing the same homogenates with irrelevant immune sera (Fig. 7G), but not with commercial mAb that recognized only one non-specific high MW band in Line 61 Tg mice (Fig. 7E,F). To confirm these data, we tested the binding of these antibodies to the soluble fraction of brain homogenates of α -Syn knockout mice. As we expected, the same high molecular weight bands were visualized in lanes loaded with brain extracts of α -Syn knockout mice in all immunoblots (Fig. 7A–D). We further analyzed insoluble fractions of brain homogenates from ha-Syn/Tg and non-Tg mice. Similarly, to soluble fractions, all high-molecular weight bands detected by WB appeared to be non-specific and were detected in non-Tg animals (data not shown). Of note, at least one epitope used for generation of antibodies is the same that was described in (Games et al., 2014),

allowing to suggest that the difference between our data and results reported previously is likely related to the varying methods of detection of oligomers in brain homogenates. Therefore, we analyzed the reactivity of immune sera generated by PV-1947D, PV-1948D, PV-1949D, and PV-1950D to the monomeric and aggregated forms of recombinant h α -Syn generated by cross-linking with 4-hydroxy-2-nonenal (Roberts et al., 2015). Data demonstrated that all four vaccines induced antibodies that specifically recognized not only monomers, but also all types of h α -Syn oligomers generated *in vitro* (Fig. 7H–K). Interestingly, two control commercial anti- α -Syn antibodies (anti-Syn15–123 and Syn211) also recognized h α -Syn monomers and oligomers (Fig. 7L, M). Next, we tested the reactivity of antibodies to the pathological h α -Syn molecules in brains homogenates from DLB and control non-DLB cases. Our data showed that all immune sera recognized monomeric h α -Syn in the soluble fraction of brain homogenates (Fig. 8A–D). Notably, antibodies generated by PV-1947D and PV-1950D vaccine recognized h α -Syn stronger compared to PV-1948D and PV-1949D vaccines and it was slightly different from the results obtained with h α -Syn in brain homogenates of Tg mice (Fig. 7). Binding of immune sera from vaccinated mice to homogenates from postmortem DLB cases was compared to that with commercial mAb (Fig. 8E, F) and immune sera from mice vaccinated with irrelevant antigen (Fig. 8G). Data (Fig. 8) suggested that the method used for preparation of homogenates did not allow obtaining oligomers visualized in Western blot. Analyses of insoluble fractions of DLB and control brain homogenates also showed strong binding to h α -Syn monomers, whereas binding to high molecular weight bands was found to be non-specific as processing of blots with irrelevant serum produces the same high molecular weight bands (data not shown).

Therefore, in this study we could not detect α -Syn oligomers in brain extracts not only from two h α -Syn Tg mice strains, but also in three DLB cases via standard WB methods. A potential explanation of this phenomenon could be the instability of α -Syn oligomers leading to their disruption during sample preparation or electrophoresis. Such instability would indeed be expected, considering the evidence of functional polymerization of α -Syn, which would likely involve a functional de-polymerization as well (Burre et al., 2014; Kim et al., 2013; Wang et al., 2014). Therefore, α -Syn oligomers would be expected to be unstable and easily dissociable, to compensate for their high formation rate (Lashuel et al., 2013). In light of this, we decided to detect binding of antibodies generated by all four vaccines to pathological forms of h α -Syn (LBs, LNs) using the 40 μ m-thick sections of the anterior cingulate cortex of a DLB case compared to the similar region of a control non-DLB brain, in regard to a total Synuclein pathology of LBs and affected neuropil containing LNs. We observed a wide range of variation in positive specific staining for h α -Syn pathology. The strongest immunoreactivity with the most densely clustered LBs and pronounced LNs was observed with antibodies generated against PV-1950D, followed by PV-1949D, PV-1948D and PV-1947D, respectively (Fig. 9). The relative differences in immunoreactivity detected by IHC could be explained by better accessibility of h α -Syn_{126–140} versus h α -Syn_{85–99} and both versus h α -Syn_{109–126}, since in these studies we have used equal concentrations of antibodies based on ELISA data. Importantly, none of the antibodies recognized Synuclein-related pathology in the control brain sections (Fig. 9). Although all vaccines induced Abs that clearly recognize monomeric recombinant and

cellular α -Syn, we did not detect normal α -Syn in control brains since tissues were pretreated with proteinase K that may completely break down normal synuclein, while sparing aggregated synuclein. These data suggest that the PV-1950D vaccine targeting all three B cell epitopes of the α -Syn simultaneously may represent a feasible immunotherapeutic strategy, and we are currently testing the immunogenicity and efficacy of this DNA vaccine and its recombinant protein version (PV-1950R) in line D α -Syn Tg mice.

4. Conclusions

We have used a DNA vaccination strategy for the development of vaccines that target various individual B cell epitopes of α -Syn and several epitopes simultaneously. Taking advantage of our proprietary universal vaccine platform, MultiTEP, we generated four different vaccines and demonstrated their immunogenicity in wild-type mice. We have also demonstrated that our anti- α -Syn vaccines did not induce potentially harmful Th cell responses, generating instead the therapeutically relevant concentrations of α -Syn antibodies. Furthermore, testing of the therapeutic efficacy of these novel DNA epitope vaccines in Tg mouse models of α -synucleinopathies will facilitate translation of the most effective DNA vaccine to human clinical trials.

In fact, the only anti- α -syn active vaccine that is currently in clinical trial is PD01 (Affiris) composed of B cell epitope mimicking α -Syn_{110–130} linked to KLH and formulated in Alum [(Mandler et al., 2015; Mandler et al., 2014) and NCT01568099]. All three other similarly designed anti-A β vaccines from Affiris (AD01, 02, 03) have failed in clinical trials likely because they did not induce sufficient anti-A β antibody responses in vaccinated people, although they did generate undesirable strong antibody responses to the KLH. Our vaccination strategy based on the MultiTEP platform technology provides a unique opportunity to generate high antibody responses in the great majority of vaccinated subjects by activating broad repertoire of both naive and memory (previously generated in the human population in response to infection or vaccination by TT, HBV and Flu) Th cells that could be especially beneficial in the elderly population with immunosenescence. In this manuscript, we are reporting for the first time on immunogenicity of all four DNA vaccines based on this universal platform. Future efficacy studies will demonstrate which DNA vaccine described herein is most suitable for translation to clinical trials.

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Highlights

- Four novel MultiTEP platform based anti-h α -Syn DNA vaccines are developed
- All four vaccines generate high titers of anti-h α -Syn antibodies in mice
- Generated antibodies recognize pathological h α -Syn in the brain tissues of DLB cases

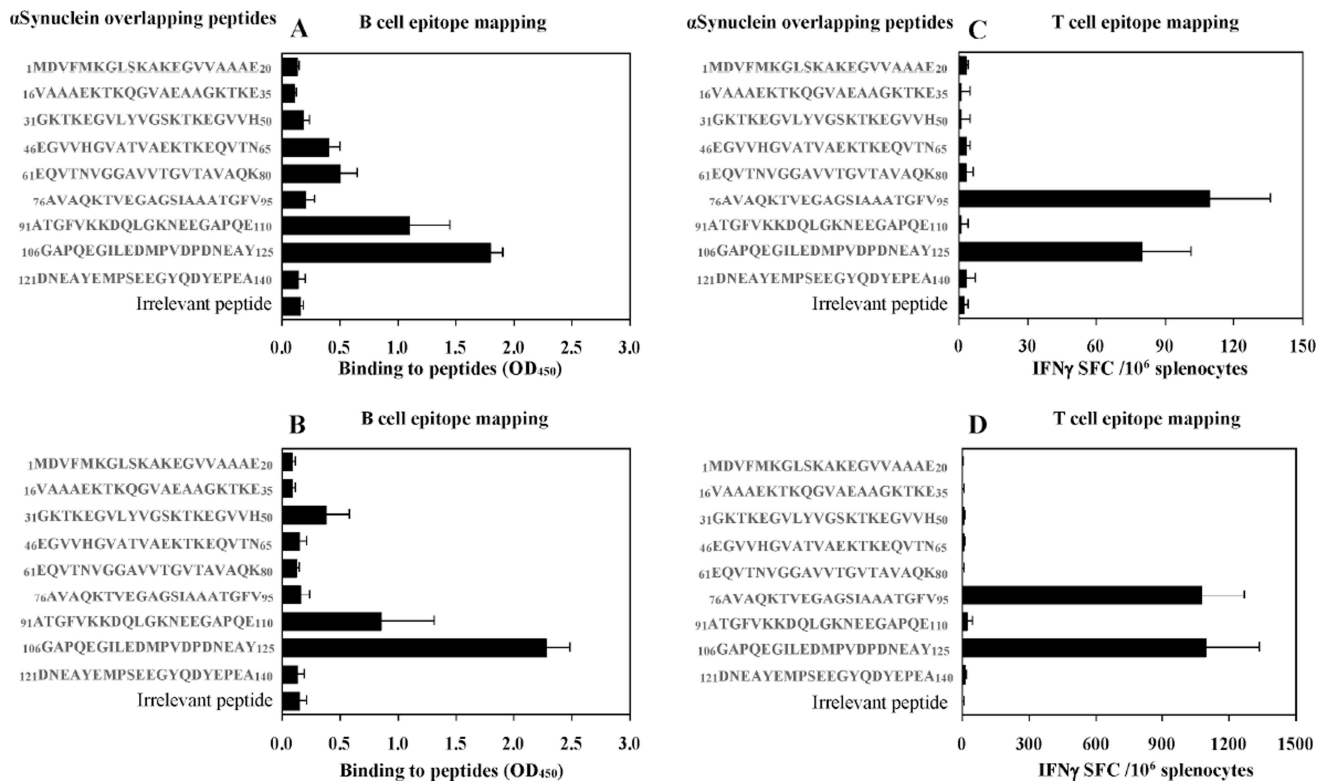


Fig. 1. Mapping of B cell and T cell epitopes of α -Syn in C57BL/6 mice immunized with plasmid encoding full length α -Syn protein followed by electroporation (**A, C**) or full length α -Syn recombinant protein formulated with Advax^{CpG} adjuvant (**B, D**). (**A, B**) B cell epitopes were mapped by analyzing the binding of immune sera (1:1000 for DNA immunized sera and 1:5000 for protein immunized sera) to overlapping α -Syn peptides or irrelevant peptide by ELISA. (**C, D**) Th cell responses were detected in splenocytes of immunized mice *in vitro* re-stimulated with the same overlapping peptides.

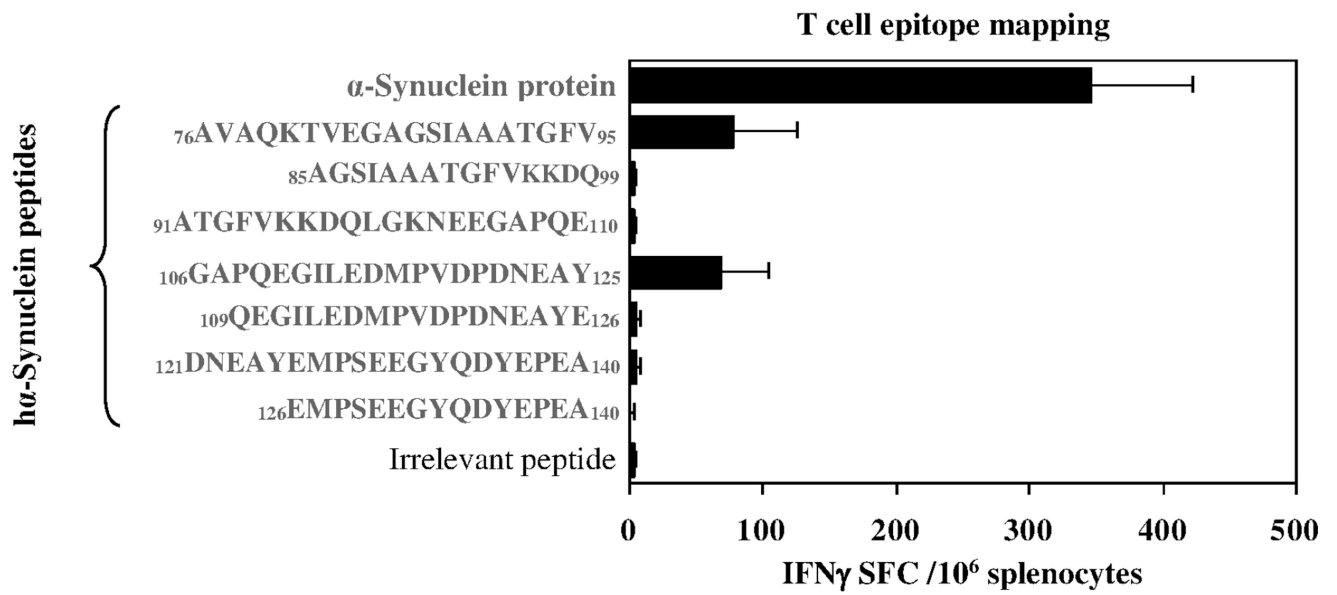


Fig. 2.

Peptides spanning aa85–99 and aa109–126 do not possess a T helper cell epitope. Th cell responses specific to indicated peptides were detected in mice immunized and boosted once with recombinant h α -Syn protein.

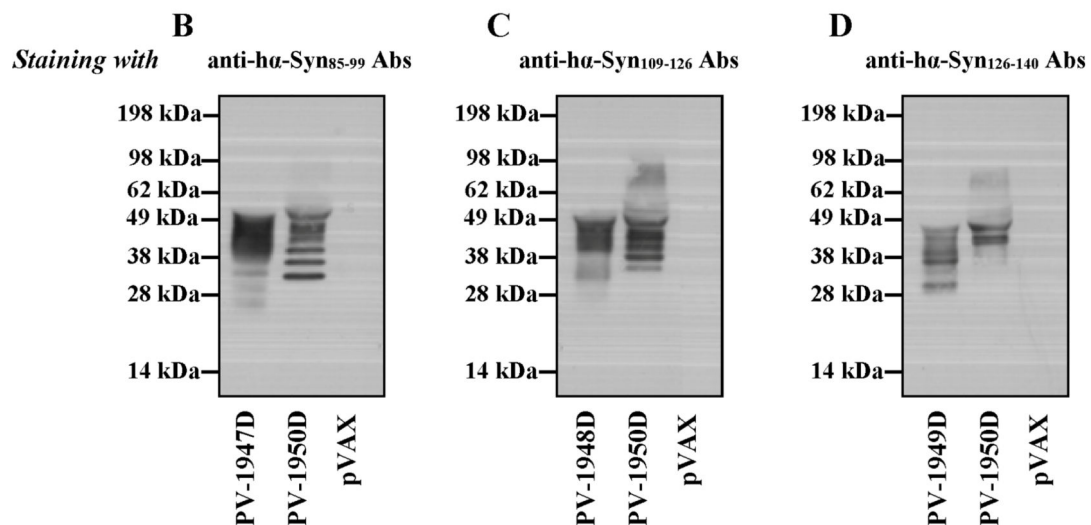
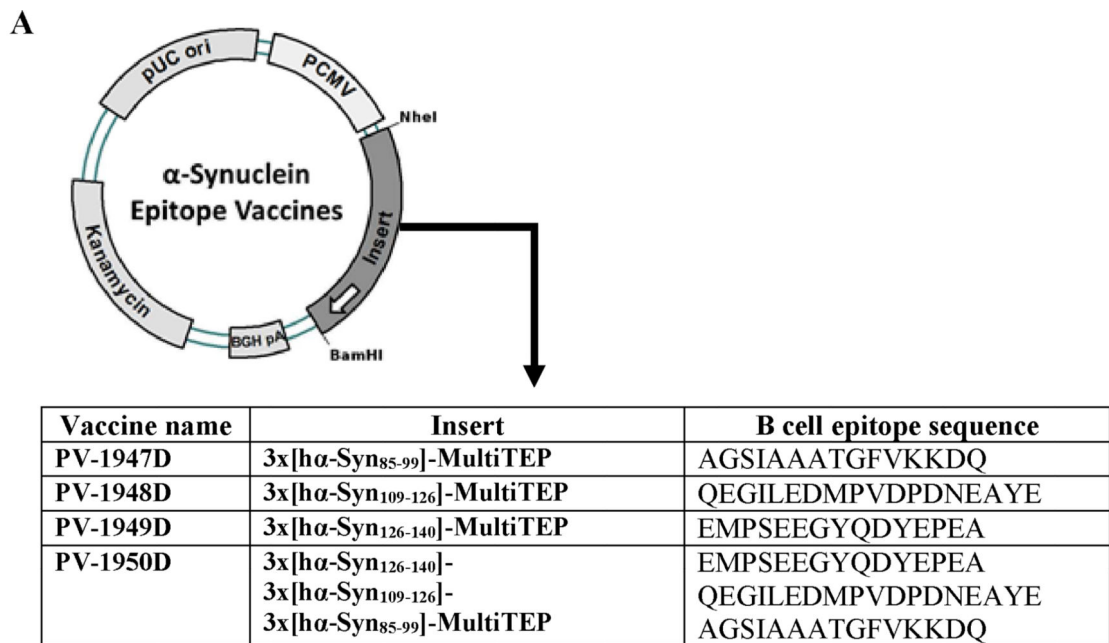


Fig. 3. Schematic representation of DNA h α -Syn vaccine constructs and expression in transfected CHO cells. **(A)** Strategy for cloning genes encoding several h α -Syn B cell epitope/s fused with MultiTEP into the pVAX1 vector and schematic representation of PV-1947D, PV-1948D, PV-1949D, and PV-1950D constructs. **(B–D)** The expression of PV-1947D **(B)**, PV-1948D **(C)**, PV-1949D **(D)**, and PV-1950D **(B–D)** and secretion of protein was demonstrated by WB of conditioned media of transiently transfected CHO cells. Proteins were visualized by staining with anti-h α -Syn₈₅₋₉₉ Abs **(A)**, anti-h α -Syn₁₀₉₋₁₂₆ Abs **(B)**, and anti-h α -Syn₁₂₆₋₁₄₀ Abs **(C)** followed by HRP conjugated anti-mouse IgG.

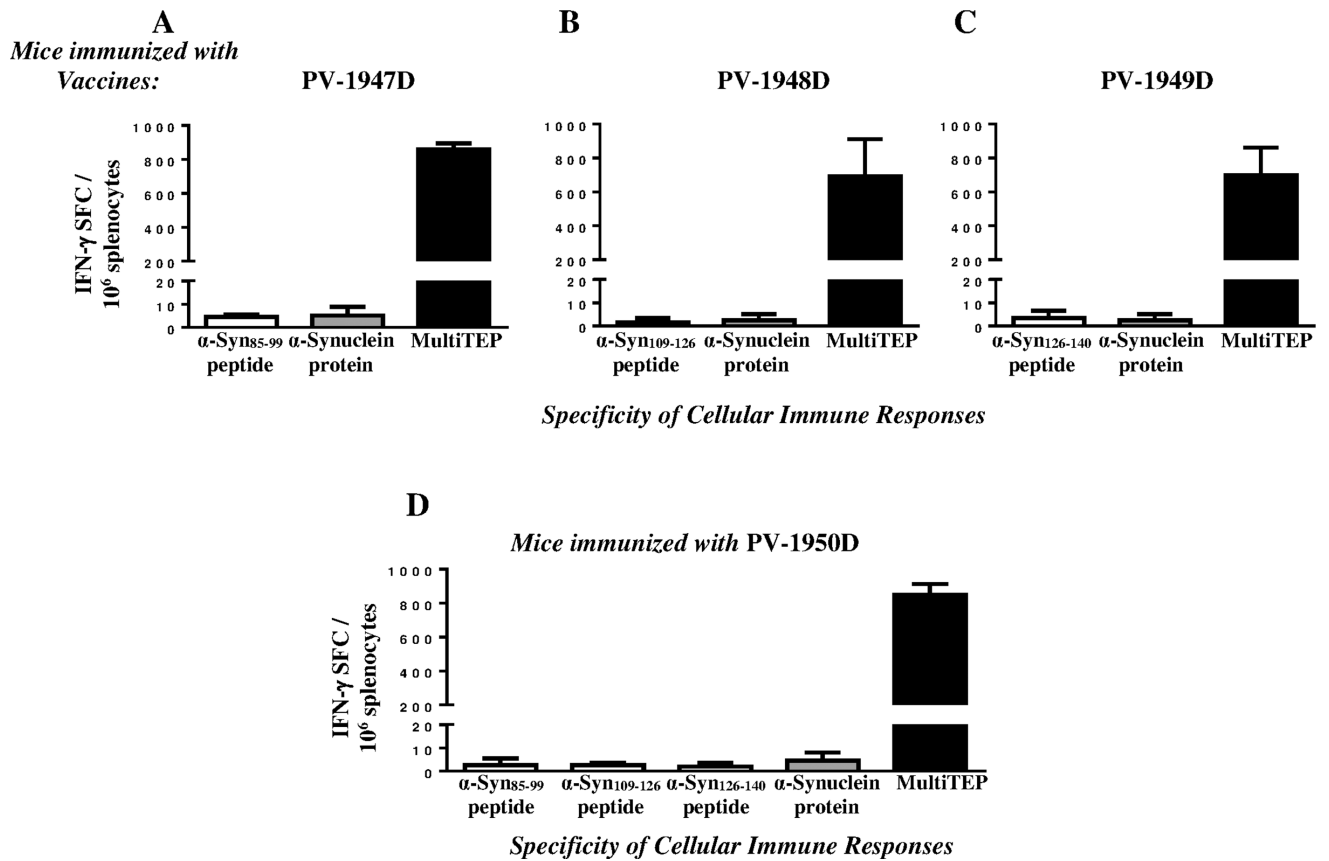


Fig. 4. Immunization with PV-1947D (3ha-Syn₈₅₋₉₉-MultiTEP; **A**), PV-1948D (3ha-Syn₁₀₉₋₁₂₆-MultiTEP; **B**), PV-1949D (3ha-Syn₁₂₆₋₁₄₀-MultiTEP; **C**), and PV-1950D (3ha-Syn₁₂₆₋₁₄₀-3ha-Syn₁₀₉₋₁₂₆-3ha-Syn₈₅₋₉₉-MultiTEP; **D**) vaccines did not induce potentially detrimental autoreactive Th cells, but induced strong Th cell responses specific to the MultiTEP platform. Numbers of IFN- γ producing T-cells were calculated by ELISPOT in splenocyte cultures obtained from immunized animals. Bars represent average \pm SD (n=4 per group).

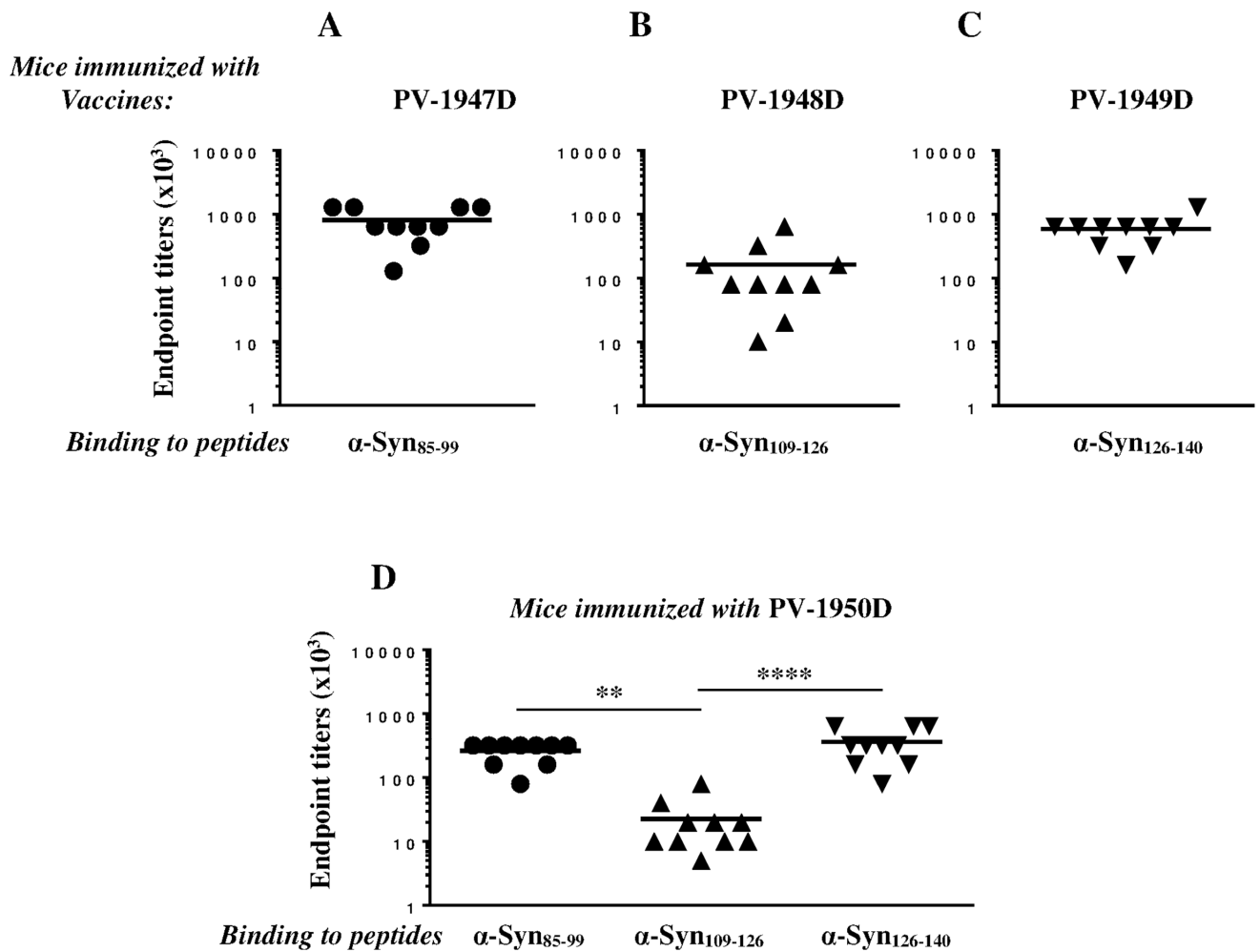


Fig. 5. DNA vaccines PV-1947D (3ha-Syn₈₅₋₉₉-MultiTEP; **A**), PV-1948D (3ha-Syn₁₀₉₋₁₂₆-MultiTEP; **B**), PV-1949D (3ha-Syn₁₂₆₋₁₄₀-MultiTEP; **C**), and PV-1950D (3ha-Syn₁₂₆₋₁₄₀-3ha-Syn₁₀₉₋₁₂₆-3ha-Syn₈₅₋₉₉-MultiTEP; **D**) induced high titers of antibodies specific to appropriate peptide/s. Endpoint titers of antibodies specific to ha-Syn₈₅₋₉₉, ha-Syn₁₀₉₋₁₂₆, ha-Syn₁₂₆₋₁₄₀ peptides were detected in sera collected after the third immunization with indicated vaccines by ELISA. Lines indicate the mean values of antibody titers (** $P < 0.01$, **** $P < 0.0001$).

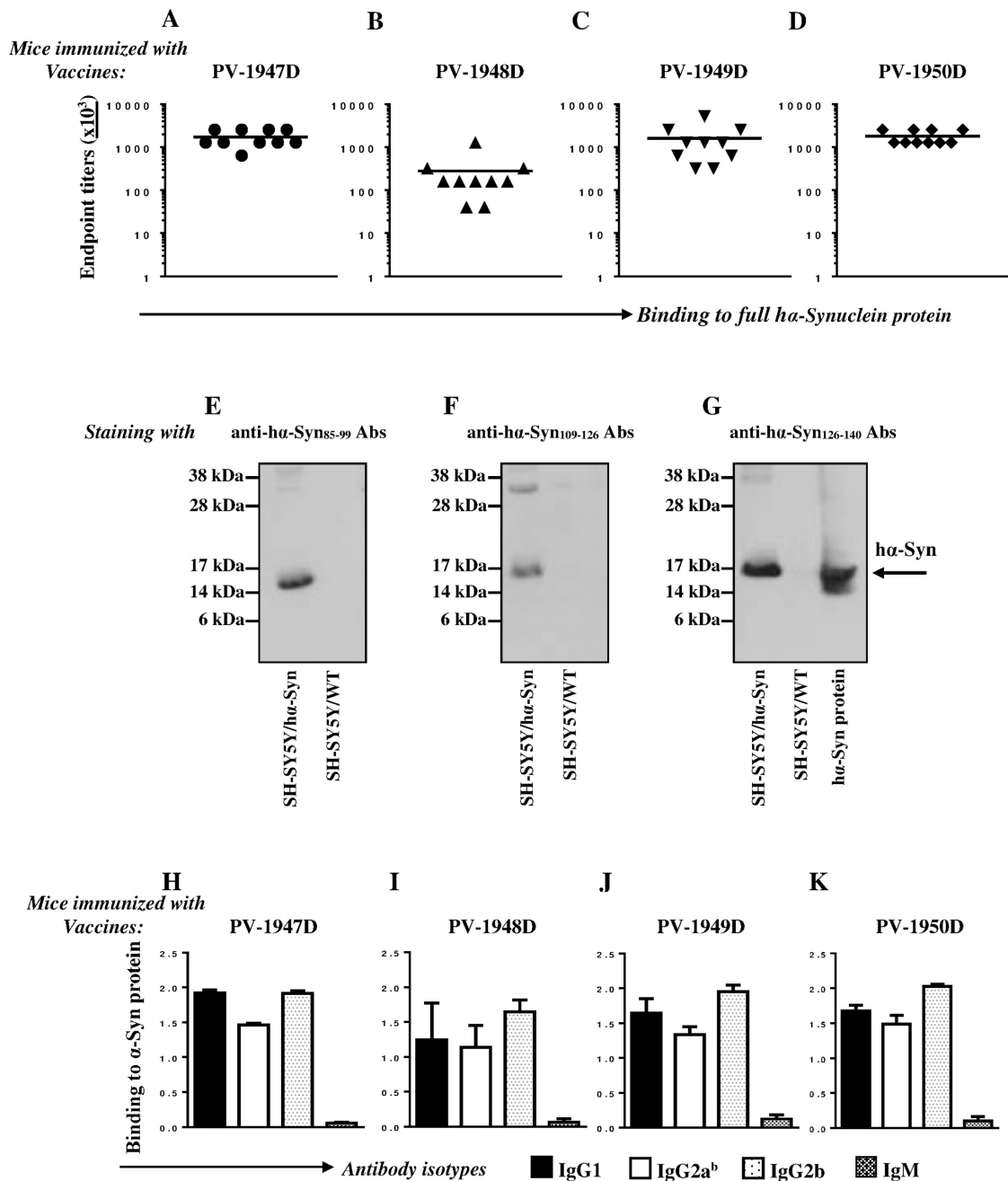


Fig. 6. DNA vaccines induced IgG1, IgG2a^b and IgG2b antibodies specific to recombinant ha-Syn and ha-Syn synthesized by SH-SY5Y/ha-Syn cells. (A–D) Endpoint titers of antibodies specific to recombinant ha-Syn protein were detected in sera collected after the third immunization with PV-1947D (p3ha-Syn₈₅₋₉₉-MultiTEP; A), PV-1948D (p3ha-Syn₁₀₉₋₁₂₆-MultiTEP; B), PV-1949D (p3ha-Syn₁₂₆₋₁₄₀-MultiTEP; C), PV-1950D (p3ha-Syn₁₂₆₋₁₄₀-3ha-Syn₁₀₉₋₁₂₆-3ha-Syn₈₅₋₉₉-MultiTEP; D). (E–G) Staining of SH-SY5Y/ α -Syn and SH-SY5Y/WT control cell lysates with purified anti-ha-Syn antibodies: anti-ha-Syn₈₅₋₉₉ (E), anti-ha-Syn₁₀₉₋₁₂₆ (F), and anti-ha-Syn₁₂₆₋₁₄₀ (G). Full recombinant ha-

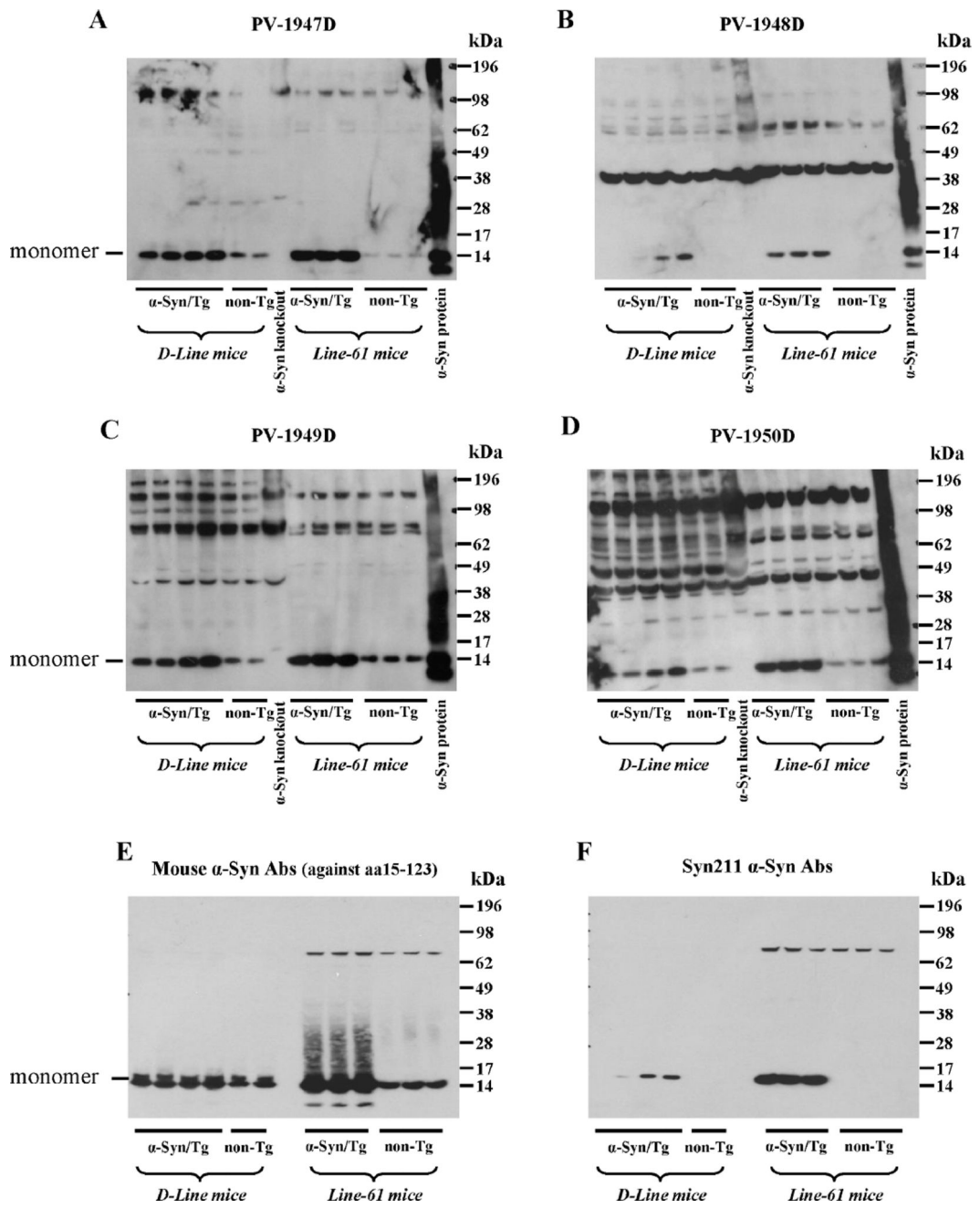
Synuclein protein was loaded as a positive control. **(H–K)** Isotypes of antibodies generated by DNA vaccines. Sera from individual mice were diluted 1:1000.

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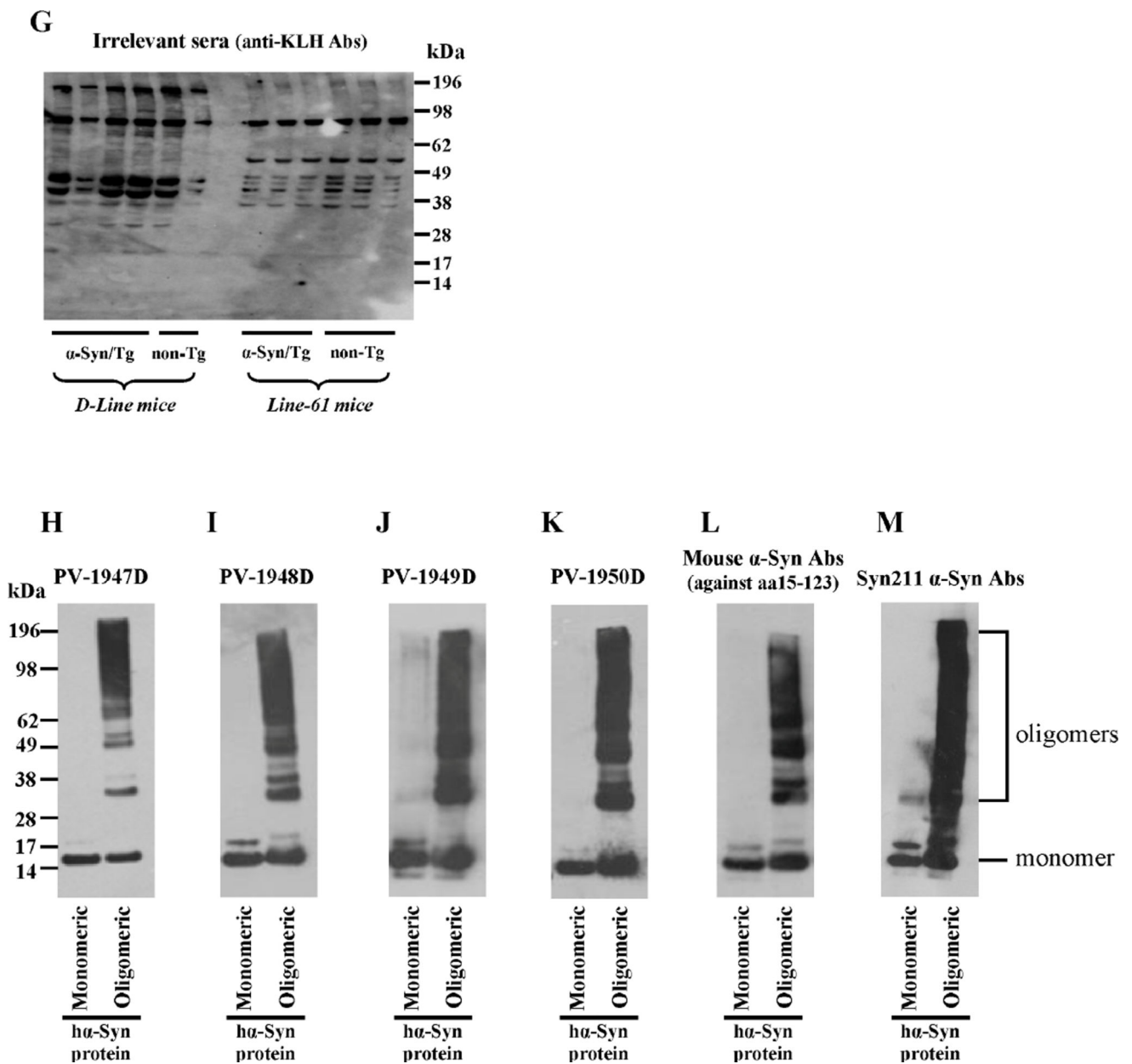


Fig. 7. DNA vaccines induced antibodies that recognize ha-Syn monomers in brain homogenates from ha-Syn/tg mice (line D and line 61) and both monomers and oligomers generated *in vitro* from recombinant protein. Brain homogenates from non-Tg mice and α -Syn KO mice were used as controls. Staining was done with sera collected from mice immunized with different DNA vaccines: PV-1947D (p3ha-Syn₈₅₋₉₉-MultiTEP; **A**), PV-1948D (p3ha-Syn₁₀₉₋₁₂₆-MultiTEP; **B**), PV-1949D (p3ha-Syn₁₂₆₋₁₄₀-MultiTEP; **C**), PV-1950D (p3ha-Syn₁₂₆₋₁₄₀-3ha-Syn₁₀₉₋₁₂₆-3ha-Syn₈₅₋₉₉-MultiTEP; **D**). Of note, PV-1948D immunized sera recognized only ha-Syn, while all other DNA vaccines induced antibodies that also recognized mouse α -Syn (**A–D**). Mouse anti- α -Syn antibody against aa15–123; (**E**) and Syn211 anti- α -Syn antibody (**F**) were used as positive controls, and irrelevant immune sera

served as a negative control (**G**). Oligomers and monomer were prepared from recombinant protein and immunostained with the same immune sera (**H–K**) or commercial mouse α -Syn antibody (**L**) and Syn211 anti- α -Syn antibody (**M**).

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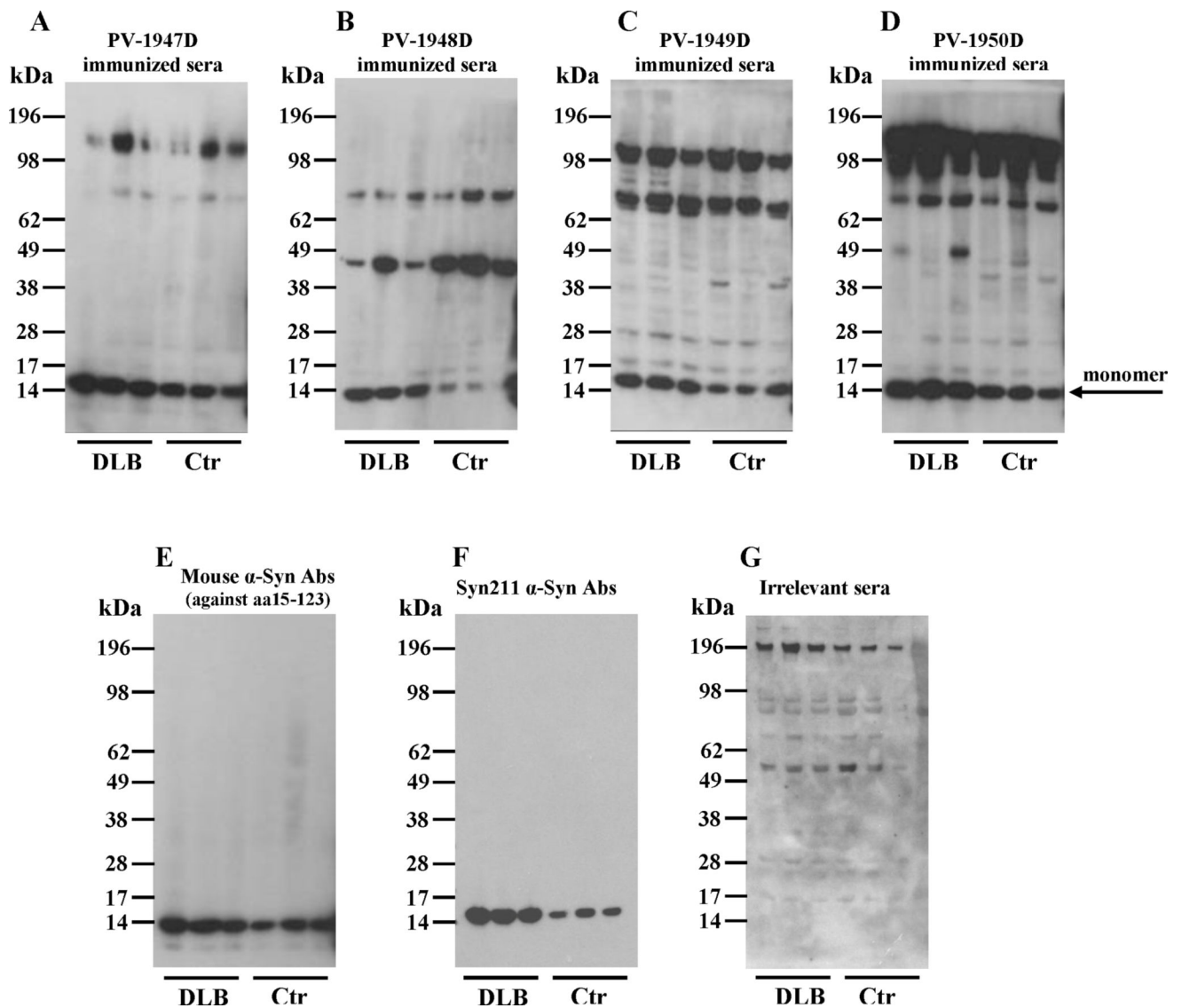
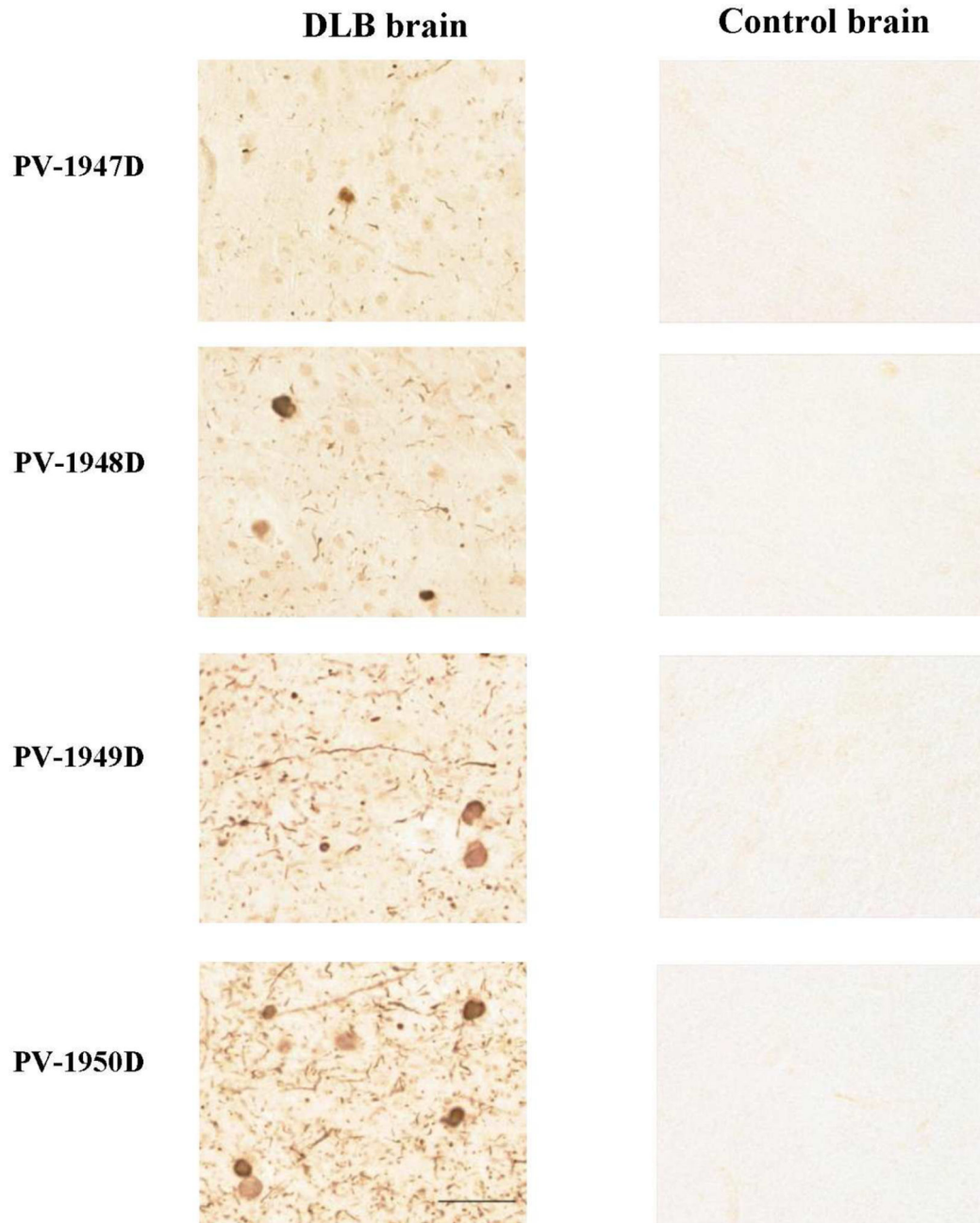


Fig. 8. DNA vaccines induced antibodies that recognize ha-Syn in DLB brain homogenates. Staining was done with sera collected from mice immunized with different DNA vaccines: PV-1947D (p3ha-Syn₈₅₋₉₉-MultiTEP; **A**), PV-1948D (p3ha-Syn₁₀₉₋₁₂₆-MultiTEP; **B**), PV-1949D (p3ha-Syn₁₂₆₋₁₄₀-MultiTEP; **C**), PV-1950D (p3ha-Syn₁₂₆₋₁₄₀-3ha-Syn₁₀₉₋₁₂₆-3ha-Syn₈₅₋₉₉-MultiTEP; **D**). Mouse anti- α -Syn antibody against aa15-123 (**E**) and Syn211 anti- α -Syn antibody (**F**) were used as positive controls, and irrelevant sera was used as a negative control (**G**). DLB –Dementia with Lewy Bodies brain extract; Ctr – Non-DLB brain extract.

**Fig. 9.**

Antibodies induced by DNA vaccines and full-length ha-Syn protein recognize LBs and LNs in the anterior cingulate gyrus of DLB case, but not in the similar region of a control brain, showing the range of variation in the positive specific staining for total Synuclein pathology. Most pronounced and densely clustered LBs and LNs were observed in DLB brain sections stained with antibodies generated through vaccination with PV-1950D (p3ha-Syn₁₂₆₋₁₄₀-3ha-Syn₁₀₉₋₁₂₆-3ha-Syn₈₅₋₉₉-MultiTEP), followed by PV-1949D (p3ha-Syn₁₂₆₋₁₄₀-MultiTEP), PV-1948D (p3ha-Syn₁₀₉₋₁₂₆-MultiTEP) and PV-1947D (p3ha-

Syn₈₅₋₉₉-MultiTEP), respectively. Photomicrographs depict immunoreactive profiles at 40x original magnification, scale bar = 50 μ m.

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