Neuron-derived exosomes trigger a PD-L1-mediated broad suppression of T cells in Parkinson's disease

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Research article

Keywords: Exosome, Parkinson's disease, α-synuclein, T cell, PD-L1

Posted Date: October 5th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-2109216/v1

Abstract

Background

Adaptive immunity plays an important role in Parkinson's disease (PD). Multiple lines of evidence indicate a significant decrease in peripheral T cells in PD patients. Although this suppression impacts both overall immune and neuroimmune response in Parkinson's disease, there is currently no mechanistic explanation for this important phenomenon reported by many clinical studies.

Methods

The exosomes were isolated from culture media of cell lines overexpressing α-synuclein A53T mutant (A53T-syn), plasma of transgenic mouse expressing A53T-syn, and dopaminergic neuron-specific organoid derived from induced pluripotent stem cells of familial PD patients carrying A53T-syn mutation (termed neuron-derived exosomes or NDEs). Western blot was used to measure the expressions of exosome markers and transmission electron microscopy was used to confirm the morphology of purified exosomes. The CD4 + and CD8 + T cells were purified from mouse spleen using a negative selection method and the effects of NDEs on the cytokines production, activation, and proliferation of purified CD4 + and CD8 + T cells were assessed by flow cytometry. Purified naïve CD4 + T cells were used to examine the effects of NDEs on CD4 + T cell differentiation.

Results

Exosomes derived from all three sources suppressed IL-4 and INF-γ production by both purified CD4 + and CD8 + T cells and inhibited T cell activation and proliferation. The suppressed phenotype of T cells induced by NDEs was accompanied by a reduction of Th1-promoting transcription factor T-bet and Th2-promoting transcription factor GATA-3 in T cells. Consistently, NDEs isolated from plasma of A53T-syn mice and dopaminergic neuron-specific organoid carrying A53T-syn mutation also suppressed Th1 and Th2 differentiation of naïve CD4 + T cells. Mechanistically, the suppressed phenotype induced by NDEs isolated from PD models was associated with altered programmed death ligand 1 (PD-L1) level in T cells. Blocking PD-L1 with an anti-PD-L1 antibody or a small molecule inhibitor BMS-1166 reversed T cell suppression induced by A53T-syn exosomes.

Conclusions

Our study reveals the key role of neuron-derived exosomes in mediating the broad suppression of T cells observed in PD and provides the basis for exploring peripheral T cells in PD pathogenesis and as biomarkers or therapeutic targets for the disease.

Background

Adaptive immunity plays an important role in Parkinson's disease (PD) and peripheral T lymphocytes are key players in the pathogenesis of the disease [1, 2]. Recent evidence has shown that lower peripheral lymphocyte count is associated with increased risk of PD in two large-scale longitudinal cohorts [3, 4]. Several studies indicate that the percentages of peripheral CD3 + and CD4 + T cell are reduced in clinical PD patients [5–9]. In addition, PD patients exhibit a reduced activation of peripheral CD3+, CD4+, and CD8 + T cells [10, 11] and reduced numbers of naïve and regulatory CD4 + and CD8 + T cells [12, 13]. Consistent with these, the levels of IL-4, IL-6, IL-10, tumor necrosis factor, and IFN-γ are reported to be reduced in the plasma of PD patients [11]. Recent new evidence further reports that the levels of transcription factors important for CD4 + T cell differentiation, including *T-bet*, *STAT3*, and *STAT4*, are reduced in PD patients [14]. Therefore, the peripheral T cells in PD patients exhibit a suppressed phenotype in general although T cells infiltrating into the brain are cytotoxically activated [15–18]. However, the mechanism that underlies the broad suppression of peripheral T cells in PD remains elusive.

Exosome is a subtype of extracellular vesicles (EVs) with 30–170 nm diameter in size originated from multivesicular bodies, which are specialized endosomes containing membrane-bound intraluminal vesicles [19, 20]. Exosomes have been shown to deliver DNA, RNA, and proteins from the donor cells to acceptor cells, interstitial fluid, as well as blood circulation [19, 20]. In cancers, exosomal proteins and RNAs mediate anti-tumor T cell immunity and promote tumor metastasis [21–23]. Neuron-derived exosomes (NDEs) have also been shown to play an important role in multiple pathological processes and are associated with the clinical phenotypes of patients with neurodegenerative diseases, including Alzheimer's disease (AD), PD, dementia with Lewy bodies, and frontotemporal dementia/amyotrophic lateral sclerosis [24–29]. Previous studies have shown that NDEs can induce the seeding and transmission of neurotoxic proteins including α-synuclein [26, 28, 30, 31]. Moreover, the level of α-synuclein is significantly increased in plasma NDEs of PD patients [9, 32–35]. Reduction of exosome biogenesis significantly suppresses the pathological propagation of Aβ in AD and α-synuclein in PD [26, 30, 36]. Even though T cells from PD patients have been shown to respond to α-synuclein-derived peptides [37], it is not clear if and how exosomes modulate peripheral T cells in PD. In this study, we identified the role of NDEs derived from models of PD in mediating the broad suppression of peripheral T cells and revealed the mechanism underlying this inhibitory process.

Methods

Mice

A53T-syn mice were bred by crossing PITX3 $^{+/IRES2-tTA}$ mice with tetO-A53T transgenic mice. The protocols for the establishment of A53T-syn mice have been described previously [38]. C57/BL6J wild type mice were used as control. The mice were housed in a 12 h light/dark cycle and fed regular diet ad libitum. All mouse experiments were approved by the Institutional Animal Care and Use Committees of Emory university.

Reagents

The chemicals used in the study include recombinant mouse IL-2 protein (402-ML-020/CF, R&D Systems), Dynabeads™ mouse T-activator CD3/CD28 (11456D, Thermo Fisher Scientific), recombinant mouse IL-12 protein (419-ML-010/CF, R&D Systems), recombinant mouse IL-4 protein (404-ML-010/CF, R&D Systems), recombinant mouse TGF-β protein (763102, BioLegend), recombinant mouse IL-6 protein (406-ML-005/CF, R&D Systems), 2-Mercaptoethanol (M6250, M6250-100ML), 100 U/ml penicillin/streptomycin (15140122, Gibco™, Thermo Fisher Scientific), protease inhibitor cocktail (P2714, Sigma-Aldrich), bovine serum albumin (A1933-100G, Sigma-Aldrich), and BMS-1166 (S8859, Selleck).

Antibodies used in the study include anti-mouse CD63 mouse antibody (sc-5275, Santa Cruz Biotechnology), anti-mouse Alix antibody (sc-53540, Santa Cruz Biotechnology), anti-mouse TSG101 antibody (sc-7964, Santa Cruz Biotechnology), anti-mouse GAPDH antibody (sc-47724, Santa Cruz Biotechnology), anti-mouse L1CAM antibody (ab24345, Abcam), anti-mouse α-synuclein antibody (AHB0261, syn211, Thermo Fisher Scientific), anti-mouse programmed death ligand 1 (PD-L1) antibody (14-5983-82, MIH1, Thermo Fisher Scientific), anti-mouse Tyrosine Hydroxylase antibody (sc-25269, Santa Cruz Biotechnology), and anti-human Tyrosine Hydroxylase Polyclonal Antibody (OPA1-04050, Thermo Fisher Scientific). Monoclonal antibodies against mouse CD3 (clone 145-2C11), CD4 (clone GK1.5), T-bet (clone 4B10), PD-L1 (clone MIH7), IFN-γ (clone XMG1.2), Ki67 (clone 16A8), and PD-1 (clone 29F.1A12) were purchased from Biolegend; against mouse CD45 (clone 30F11), CD3 (clone REA641), CD8 (clone REA601), CD62L (clone MEL14-H2.100), CD25 (clone 7D4), IL-4 (clone BVD4-1D11), and CD44 (clone DB105) from Miltengy Biotec; and against mouse GATA-3 (clone TWAJ) from Bioscience.

Plasmids used in this study include pRK5 vector and pRK5-A53T-syn.

Cell Lines

HEK293T (ATCC, CRL-3216), H4 (ATCC, HTB-148), and human embryonic microglia clone 3 (HMC3, ATCC, CRL-3304) cells were purchased from American Type Culture Collection (ATCC). HEK293Tand H4 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/mL of penicillin, and 100 µg/mL of streptomycin. HMC3 cells were cultured in Modified Eagle's Medium (MEM) with 10% FBS, 100 U/mL of penicillin and 100 µg/mL of streptomycin. SN4741 cells were cultured in DMEM supplemented with 20% glucose and glutamine (200mM). H4 and HMC3 cells were maintained at 37°C in a 5% CO_2 in incubator and SN4741 cells were maintained at 33°C in a 5% CO_2 incubator.

Transfection

The cells were cultured until it reached 70%~90% fluency for transfection. The culture medium was changed with exosome-depleted culture medium by replacing the regular FBS with exosome-depleted FBS. Exosome-depleted FBS was prepared by ultracentrifuging the FBS for 16 h at 4°C and then filtered with a 100-nm filter. The culture media were collected for exosome purification 48 h after transfection.

Organoid Culture

Human iPSC lines carrying A53T mutant *SNCA* and A53T mutation-corrected *SNCA* were purchased from NINDS Human Cell and Data Repository. The protocols for the maintenance of iPSC lines and culture of midbrain organoids from human iPSC have been described in a previous study [39]. In brief, cultured human iPSC colonies were detached with Collagenase Type IV and washed with fresh stem cell medium 7 days after passage. From day 1 to day 4, the iPSC lines were cultured on an Ultra-Low attachment 6-well plate containing EB medium composed of DMEM:F12, 15% Knockout Serum Replacer, 1 x Glutamax, 1 x 2-Mercaptoenthanol, 100 nM LDN-193189, 10 μM SB-431542, 100 ng/ml SHH (Peprotech), 2 μM Purmorphamine (Stemgent), 100 ng/ml FGF-8 (Peprotech). On day 5, the EB medium was switched to SHH medium, containing DMEM:F12, 1 x N2 Supplement, 1 x Glutamax, 100 nM LDN-193189, 3 μM CHIR99021, 100 ng/ml SHH, 2 μM Purmorphamine, 100 ng/ml FGF-8. From day 7 to day 13, SHH medium was replaced with induction medium, containing DMEM:F12, 1 x N2 Supplement, 1 x Glutamax, 100 nM LDN-193189, 3 μM CHIR99021. On day 14, 10–20 organoids were cultured in SpinΩ with differentiation medium, consisting of Neurobasal, 1 x B27 Supplement, 1 x Glutamax, 1 x 2-Mercaptoenthanol, 20 ng/ml BDNF, 20 ng/ml GDNF, 0.2 mM Ascorbic Acid, 1 ng/ml TGFβ, and 0.5 mM c-AMP. The culture media of midbrain organoids were collected on day 28 when the DAergic neurons matured.

Exosome Extraction from Cell Culture Medium

The exosomes from cell culture were extracted using sequential centrifugation process as previously reported [40]. Briefly, the culture media were collected and subsequently subjected to sequential centrifugation steps at 300g for 5 min, 800g for 5 min, and 2,000g for 10 min to remove cells and cell debris. The supernatant was further centrifuged by 10,000g for 30 min at 4°C to remove large extracellular vesicles. The EVs were purified by ultracentrifugation at 110,000g and 4°C for 70 min, washed with 1 x PBS, and re-centrifugated under the same condition.

Neuronal Exosome Extraction from Mouse Plasma

Exosomes were isolated from mouse plasma using antibody-coated superparamagnetic microbeads following the protocol as previously described [33]. Isolated exosomes were fixed using a fixing buffer (4% paraformaldehyde (PFA) / 5% glutaraldehyde) for transmission electron microscopy (TEM) imaging. For detection of exosome markers using western blot, the exosome proteins were extracted by incubating the beads with 110 μL 0.1% BSA/PBS (pH7.4) containing Triton X-100 (1%) and protease and phosphatase inhibitors for 1 h at room temperature with gentle shaking.

Western Blot

Whole cell lysates were prepared in SDS containing sample buffer (31.5 mM Tris-HCl, pH 6.8, 10% glycerol, 1% SDS, and 0.005% bromophenol blue) and equal protein amounts of lysates (20–30 μg) were separated by 10–15% SDS-PAGE gel at 100 V for 1.5 h. Proteins were transferred to polyvinylidene difluoride membrane at 25 V for 1.5 h using a half-dry blotting system (Bio-Rad Laboratories). Blots were

probed by incubation with primary antibodies (1:1,000) overnight at 4°C, washed three times, and then incubated with the secondary antibodies (1:10,000) at room temperature for 1 h. Images were developed in an M35A X-OMAT Processor system (Kodak) using ECL reagent (GE Healthcare).

Transmission Electron Microscopy

Purified exosomes were fixed with 1 mL of 4% PFA for 5 min. Five - seven µL exosome suspension solution was loaded on the grid and incubated for 1 min. Twenty drops of filtered 1% uranyl acetate (UA) were applied to the EM grid. After removing the excess UA solution, the grid was rinsed with a drop of water, and placed on the table for 10 min at room temperature. The grid was imaged by TEM at 80 kV.

Isolation of Mouse Spleen Cells, CD4 + T Cells, and CD8 + T Cells

Single-cell spleen lymphocyte suspensions were obtained from spleens of 8-week-old A53T-syn transgenic mice or WT mice using the lymphocyte separation medium according to the manufacturer's instructions (CL5030, CEDARLANE). For the purification of CD4 + and CD8 + T cells, non-CD4 + cells and non-CD8 + cells were removed by negative selection using MojoSort Mouse CD4 T or CD8 T Cell Isolation Kits according to the protocols provided by manufacture (BioLegend). The purity of CD4 + T cells and CD8 + T cells was determined using flow cytometry. For isolation of naïve CD4 + T cells (CD4 + CD25- $CD44^{low}CD62L^{high}$), purified CD4 + T cells were further sorted using antibodies to mouse CD4-APC, CD25-FITC, CD44-PE, and CD62L-PE-Cy7 by flow cytometry. Purity of CD4 + CD25-$CD44^{low}CD62L^{high}$ T cells after isolation was over 97%.

Culture of Mouse Spleen Cells, CD4 + T Cells, and CD8 + T Cells

Isolated mouse spleen cells, CD4+, and CD8 + T cells were cultured in 24-well flat bottom plates (0.5×10^6 cells per well) in 0.5 mL of complete RPMI 1640 media supplemented with 10% exosome-depleted FBS, 200 mM L-glutamine, 100 U/ml penicillin/streptomycin and 55 mM 2-Mercaptoethanol (RP-10). Mouse T-Activator αCD3/αCD28 beads and 50 ng/ml recombinant mouse IL-2 were added to the culture medium for long term maintenance. The cells were cultured for 24 h before exosome treatment.

Differentiation Induction of Mouse Naïve CD4 + T Cells

Isolated mouse naïve CD4 + T cells were cultured in 24-well plates (0.5×10^6 cells per well) in 0.5 ml of complete RPMI 1640 media supplemented with 10% exosome-depleted FBS, 200 mM L-glutamine, 100 U/ml penicillin/streptomycin and 55 mM 2-Mercaptoethanol in the presence of Mouse T-Activator αCD3/αCD28 beads in addition to 50 ng/ml recombinant mouse IL-2. Cells were cultured under Th1 (50 ng/ml recombinant IL-12 and 10 mg/ml anti-IL-4), Th2 (50 ng/ml recombinant IL-4 and 10 mg /ml anti-IFN-γ), Th17 (10 ng/ml recombinant TGF-β, 100 ng/ml recombinant IL-6, 10 mg/ml anti-IFN-γ and 10 mg/ml anti-IL-4) or iTreg (10 ng/ml recombinant TGF-β) conditions. The naïve cells were cultured for 24 h under different conditions before exosome treatment.

Exosome Treatment

For most of exosome treatment experiments, 5 μg exosomes were added into the culture medium of mouse splenocytes or purified CD4 + or CD8 + T cells for 48 h (The final concentration of exosome in medium is 10 μg/mL). To observe the dose-effect relationship, varying amounts of exosome (20–30 μg/mL) were used. For differentiation assay, cells were treated with exosomes for 96 h.

Blocking of PD-L1

To block PD-L1 and PD-1 interaction, anti-PD-L1 antibody (4 μg/ml for final concentration; clone MIH-1; Thermo Fisher Scientific) [41] or BMS-1166 (1μM for final concentration; S8859, Selleck) [42], was added into the CD4 + and CD8 + T cell culture media before the treatment of exosomes.

Flow Cytometry

Cytokines, transcription factors, and surface markers of T cells were evaluated by flow cytometry with a FACSCanto II Cell Analyzer (BD Biosciences) following the recommended protocols by eBioscience™. To detect intracellular expression of INF-γ, IL-4, and IL-17A in CD4 + or CD8 + T cells, cells were first treated with 1μg/mL ionomycin (Sigma), 50 ng/ml phorbol 12-myristate 13-acetate (PMA, Sigma), and 2 ug/mL Brefeldin A (BioLegend) for 4–6 h at 37°C before flow cytometry analysis.

Immunocytochemistry

DA organoids differentiated from iPSC were cultured on the glass coverslip (Corning Incorporated) with 24-well plates and stained for immunocytofluorescence as described [39]. Images were acquired with an Olympus DP70 fluorescent microscopy (Olympus Corporation, Japan).

Statistics

Statistical analysis was performed with GraphPad Prism software (Version 8). A two-tailed unpaired Student t test was used for statistical analysis of two groups. One-way analysis of variance (ANOVA) with Tukey's multiple comparisons test was used to compare the means of three or more groups. Two-way ANOVA followed by Tukey's multiple comparisons test was used to evaluate data with two factors. A p-value < 0.05 was considered statistically significant.

Results

Exosomes derived from cells expressing A53T α-synuclein suppress T cells

To clarify the role of exosomes in regulating T cell function in PD, we over-expressed α-synuclein A53T mutant (A53T-syn) in mouse midbrain dopaminergic progenitor SN4741, human neuroglioma H4, and microglial HMC3 cell lines, and prepared exosomes (referred as purified exosomes or Exo) from their culture media using sequential centrifugation processes [40]. The purified exosomes were shown to have

typical cup-shaped morphology and size by TEM as previously reported [40] (Fig. S1A) and expressed exosome markers, such as Alix, CD63, and TSG101 in total cell lysates and purified exosome lysates of transfected cells were confirmed with western blot (Fig. S1B-D). A53T-syn expression was significantly increased in the total cell lysates and purified exosomes of A53T-syn-overexpressing SN4741, H4, and HMC3 cells compared to cells with control vector overexpression (Fig. S1B-D).

To investigate the effects of A53T-syn Exo on CD4 + and CD8 + T cells, we purified CD4 + and CD8 + T cells from mouse spleens using a negative selection method [43] and incubated them with A53T-syn Exo isolated from media of SN4741 cells, and assessed cytokine production by flow cytometry. This analysis showed that A53T-syn Exo derived from media of SN4741 cells significantly reduced the IL-4 + and INF-γ + percentages for both CD4 + and CD8 + T cells compared with control Exo (Fig. S1E-F). In addition, A53T-syn Exo derived from media of either H4 or HMC3 cells exhibited similar inhibitory effects on the cytokine production of CD4 + and CD8 + T cells (Fig. S1G-H). These findings indicate that A53T-syn Exo negatively regulates the cytokine production of both CD4 + and CD8 + T cells. Given the similar effects of A53T-syn Exo isolated from different cell culture media, we chose SN4741 cells as the primary exosome source for the subsequent investigation.

NDEs from A53T α-synuclein transgenic mice suppress T cells

Since α-synuclein A53T mutant dysregulates neuronal biogenesis of exosomes, we tested the effects of NDEs on T cells. We followed an experimental protocol as shown in Fig. 1A. We initially isolated NDEs from the plasma of wild type mice (WT-NDEs) and A53T α-synuclein transgenic mice (A53T-NDEs) using the method as previously described [33]. Analysis of exosomal markers revealed that similar to Exo isolated from cells, plasma WT-NDEs and A53T-NDEs expressed exosomes markers CD63 and TSG101 but the levels of Alix and α-synuclein were undetectable (Fig. 1B). We incubated the spleen cells, purified CD4 + T cells, and purified CD8 + T cells with plasma WT-NDEs and A53T-NDEs. The analysis showed that compared to WT-NDEs, A53T-NDEs significantly reduced the percentage of CD45 + CD3 + T cells when incubated with murine spleen cells (Fig. 1C). Moreover, this reduction was caused primarily by a significant decrease of CD8 + T cell but not CD4 + T cells (Fig. 1C). Interestingly, consistent with the results shown in Fig. S1, A53T-NDEs significantly reduced the percentages of CD4 + and CD8 + T cells expressing either IL-4 or INF-γ when incubated with purified CD4 + or CD8 + T cells (Fig. 1D-E). Based on these results, we further evaluated whether A53T-NDEs reduced the activation or proliferation of CD4 + and CD8 + T cells. We found that the percentages of activated CD4+ (CD4 + CD44+) and CD8+ (CD8 + CD44+) T cells were significantly reduced by A53T-NDEs compared to WT-NDEs (Fig. 1F-G). Furthermore, we also observed a decline in the percentages of CD4 + Ki67 + and CD8 + Ki67 + T cells with the treatment of A53T-NDEs compared to WT-NDEs (Fig. 1H-I). These findings demonstrate that plasma A53T-NDEs induce a T cell phenotype that is consistent with a broad suppression of both CD4 + and CD8 + cells.

NDEs isolated from media of human iPSC-derived dopaminergic neurons carrying A53T mutation suppress T

cells

To strengthen the findings above, we tested the effects of NDEs isolated from culture media of human iPSC-derived dopaminergic neurons on T cells. For this study, we prepared NDEs from media of dopaminergic neurons derived from human iPSC carrying A53T mutation (A53T-DA neurons) or DA neurons of the same iPSC line with the A53T mutation corrected by CRISPR-Cas9 (WT-DA neurons). We first showed that WT-DA neurons and A53T-DA neurons derived from these two human iPSCs expressed robust levels of tyrosine hydroxylase (TH) and CD63 as assessed by immunocytofluorescence (Fig. 2A). Surprisingly, the level of soluble α-synuclein was lower in A53T-DA neurons compared to WT-DA neurons (Fig. 2B). We isolated exosomes from the culture media of WT-DA and A53T-DA neurons (WT-DA Exo and A53T-DA Exo, respectively) and confirmed that they expressed comparable high levels of CD63 and TSG101 but low levels of Alix and α-synuclein (Fig. 2C). We then compared the effects of WT-DA Exo and A53T-DA Exo on T cells and found that compared to WT-DA Exo, exposure of purified murine CD4 + or CD8 + T cells to A53T-DA Exo significantly reduced the percentages of CD4 + IL-4+, CD4 + INF-γ+, CD8 + IL-4+, and CD8 + INF-γ + T cells (Fig. 2D-E). The suppression of cytokines induced by A53T-DA Exo was also accompanied by the reduction of T cell activation and proliferation since the percentages of CD4 + CD44+, CD8 + CD44+, CD4 + Ki67+, and CD8 + Ki67 + T cells were all significantly reduced by A53T-DA Exo (Fig. 2F-I). Thus, A53T-syn Exo from human iPSC-derived DA neurons also causes a general suppression of T cells.

A53T-syn Exo suppresses the differentiation of CD4 + T cells into Th1 and Th2 lineages

Because of the general inhibitory effects of A53T-syn Exo on T cells, we assessed the key transcription factors that are known to control the differentiation of CD4 + T cells. We examined whether Th1-promoting transcription factor T-bet and Th2-promoting transcription factor GATA-3 were altered by A53T-syn Exo in purified CD4 + T cells. This analysis showed that A53T-syn Exo isolated from culture media of SN4741 or H4 cells significantly reduced the percentages of CD4 + T-bet + and CD4 + GATA-3 + T cells compared to control Exo (Fig. S2A-B). Similarly, plasma A53T-NDEs and A53T-DA Exo also decreased the percentages of CD4 + T-bet + and CD4 + GATA-3 + T cells (Fig. S2C-D).

To further establish whether A53T-syn Exo modulated T cell differentiation, we assessed the effects of A53T-syn Exo on murine naïve CD4 + T cells under Th0, Th1, and Th2 conditions. Under Th0 condition, we found that plasma A53T-NDEs significantly reduced the percentages of CD4 + IL-4 + and CD4 + INF-γ + T cells (Fig. 3A), suggesting an inhibition of differentiation into both Th1 and Th2 lineages. Furthermore, we showed that plasma A53T-NDEs reduced the differentiation of naïve CD4 + T cells into either Th1 or Th2 T cells under their respective conditions (Fig. 3B-C). Similar to A53T-syn NDEs, our analysis showed that A53T-DA Exo and A53T-syn Exo exhibited similar inhibitory effects on the differentiation of naïve CD4 + T cells under Th0, Th1, and Th2 conditions (Fig. 3D-F and Fig. S3A-C). As shown in Fig. S4, the suppressions of Th1 and Th2 differentiation by plasma A53T-NDEs and A53T-DA Exo was also

accompanied by a significant reduction of CD4 + T-bet + and CD4 + GATA-3 + T cells under Th0, Th1, and Th2 conditions (Fig. S4A-H). Therefore, A53T-syn Exo represses the differentiation of naïve CD4 + T cells to either Th1 or Th2 lineage.

A53T-syn Exo increases the level of PD-L1 in T cells

Previous studies have shown that tumor-derived exosomes containing PD-L1 significantly suppress CD8 + T cells, leading to the impairment of antitumor immunity in cancer patients [22]. We therefore, assessed the level of PD-L1 on exosomes. Compared to control exosomes, we found no significant change in PD-L1 level associated with A53T-syn Exo prepared from culture media of SN4741 cells, plasma of mice, and iPSC-derived DA neurons (Fig. S5A-E), indicating that the suppressed T cell phenotype induced by A53T-syn Exo may not be caused directly by exosomal PD-L1. We then examined whether A53T-syn Exo affected PD-L1 in T cells and found that exposure of purified CD4 + or CD8 + T cells to A53T-syn Exo isolated from SN4741 and H4 cells, plasma of mice, or iPSC-derived DA neurons all significantly increased the percentages of CD4 + PD-L1 + and CD8 + PD-L1 + T cells (Fig. 4A-D). More importantly, we analyzed IL-4, INF-γ, T-bet and GATA-3 in PD-1 + T cells and found that A53T-syn Exo from SN4741 cells and plasma A53T-NDEs significantly reduced the expressions of these cytokines and transcription factors in PD-1 + T cells (Fig. S6A-D). These results suggest that A53T-syn Exo induces PD-L1 on T cells, which correlates with a general suppression of PD-1 + T cells.

Blocking of PD-L1 reverses T Cell suppression induced by A53T-syn Exo

To demonstrate whether PD-L1 was causally associated with T cell suppression induced by A53T-syn Exo, we inhibited PD-L1 and PD-1 binding in T cells with BMS-1166, a small molecule inhibitor of soluble PD-L1 [42]. This analysis showed that BMS-1166 significantly reversed the inhibitory effects of A53T-syn Exo from SN4741 cells and plasma A53T-NDEs on CD4 + IL-4+, CD4 + INF-γ+, CD8 + IL-4+, and CD8 + INF-γ + T cells (Fig. 5A-H). To strengthen the results of PD-L1 inhibitor study, we blocked PD-L1 in T cell cultures using an anti-PD-L1 antibody reported to inhibit PD-L1/PD-1 signaling [41]. This analysis showed that addition of anti-PD-L1 antibody (4 μg/mL) significantly reversed the suppression of CD4 + IL-4+, CD4 + INF-γ+, CD8 + IL-4+, and CD8 + INF-γ + T cells induced either by A53T-syn Exo isolated from SN4741 cells (Fig. S7A-D) or by plasma A53T-NDEs (Fig. S7E-H). Our findings demonstrate that blocking of PD-L1 rescues T cell suppression induced by A53T-syn Exo.

Discussion

Our findings in this study reveal that exosomes isolated from *in vitro* and *in vivo* PD models cause a broad suppression of T cells. This inhibition involves PD-L1/PD-1 pathway. We show that exosomes isolated from multiple A53T-syn models including SN4741 cells expressing A53T mutant, plasma of A53T-syn transgenic mice, and DAergic neurons derived from human iPSC carrying A53T mutation all suppress the cytokine production by CD4 + and CD8 + T cells. At cellular level, A53T-syn Exo exerts this

general suppression, in part, by inhibiting Th1 and Th2 differentiation of naïve CD4 + T cells. Furthermore, molecularly, A53T-syn Exo induces the expression of PD-L1 on T cells and PD-L1/PD-1 signaling is required to mediate T cell suppression. These findings highlight the role of PD-associated Exo in the broad dysregulation of T cells in the disease and offer a mechanistic basis for exploring peripheral T cells as biomarkers and therapeutic targets for PD.

Previous studies have shown that the NDEs from PD patients carry pathological proteins, such as α-synuclein and tau [24, 33, 34, 44, 45]. Moreover, exosomal α-synuclein and tau proteins derived from PD patients have been proposed as potential biomarkers for clinical diagnosis and prediction of disease progression [28, 32, 34, 44–46]. However, the immune regulatory role of NDEs in neurodegenerative diseases has not been defined. Although the PD-derived exosomes containing α-synuclein have been reported to activate peripheral monocytes [47], the effects of NDEs on peripheral T cells in neurodegeneration have not been investigated and remained completely undefined. Our current investigation reveals that NDEs from A53T-syn models exert a striking and broad suppressive effect on the proliferation, differentiation, activation, and cytokine production of T cells. Collectively, these effects direct T cells away from an activated pro-inflammatory phenotype in general. Our findings provide a mechanistic explanation for multiple studies reporting the heterogeneity and reduced T cells in peripheral immune phenotypes in PD. For example, it has been shown that peripheral CD3 + T cells are significantly reduced and inactivated while neutrophils, NK cells, and monocytes are abnormally increased and activated in PD patients [5–8, 10, 11, 47–49]. Our results that NDEs from multiple A53T-syn models reduce cytokines production by both purified CD4 + and CD8 + T cells are especially interesting in light of the report that the plasma levels of IL-4 and INF-γ in PD patients are reduced [11]. Furthermore, the broad suppressive effects of NDEs are completely consistent with the reported dysregulation of multiple subtypes of T cells in PD. For example, our finding that activation of purified CD4 + T cells is significantly reduced by NDEs from A53T-syn models may offer a mechanism underlying disease progression since a reduction of activated CD4 + T cells has been associated with the advancement of PD [10, 11, 49]. Our finding that A53T-syn Exo suppresses both naïve and matured CD8 + T supports previous studies reporting decreases of naïve CD8 + T cells and cytotoxic CD8 + T cells in PD [9, 49, 50]. Several studies have noted a decrease of Th1 cells and Th1-promoting transcription factors including *T-bet*, *STAT3*, and *STAT4* in PD patients [14, 51], which is consistent with our finding that A53T-syn NDEs reduce T-bet level and suppress Th1 differentiation. Thus, the effects of A53T-syn NDEs identified in the current study explain multiple deficiencies of T cells observed in PD patients.

The primary model used in our study is exosomes derived from A53T-syn conditions. Previous studies have revealed that the expression of Leucine-rich repeat kinase 2 (LRRK2) was increased in T cells [52] and G2019S *LRRK2* mutant significantly suppresses Th17 cell differentiation [53], indicating that *LRRK2* (G2019S) also regulates T cell immunity. The *LRRK2* G2019S mutation has also been shown to alter astrocyte-to-neuron communication via extracellular vesicles [54]. In addition, *LRRK2* mutation has been shown to alter exosome contents [54]. Furthermore, the mutations of *GBA* (glucocerebrosidase) and *ATP13A2* have been reported to increase α-synuclein secretion through exosomes [55, 56]. Together with these, our results raise the possibility that exosomes derived from neural cells may be involved in

regulating T cells under a broad range of pathogenic conditions associated with mutations in *SNCA*, *LRRK2*, *GBA*, or *ATP13A2* in PD.

Accumulating evidence have shown that exosomes containing PD-L1 released by cancer cells inhibit anti-tumor immunity and blocking of PD-1 or PD-L1 has remarkable therapeutic benefit for many types of cancer, such as melanoma, non-small-cell lung cancer, and breast cancer [22, 23, 57–60]. But whether the role of exosome-mediated immune checkpoint in neurodegenerative diseases remain unexplored. We found no significant change of PD-L1 in A53T-syn Exo compared to control Exo. Thus, the level of exosomal PD-L1 does not appear to explain the T cell immunosuppression induced by A53T-syn Exo. Instead, our data show that exposure to A53T-syn Exo increases the expression of PD-L1 in T cells. This is consistent with several studies showing that tumor-derived exosomes modulate PD-L1 levels in immune cells [31, 61]. Since inhibiting PD-L1 by either an antibody or the small molecule inhibitor BMS-1166 can alleviate the inhibitory effects of NDEs on T cells in our experimental paradigms, it supports a model in which PD-L1/PD-1 expressed by T cells mediates NDE-induced suppression of T cell immunity in PD. This is clinically significant, considering low lymphocyte count is associated with increased PD risk and worse disease progression [3, 4, 6, 7, 51] and the regulation of T cell profiles with sargramostim (granulocyte-macrophage colony-stimulating factor) has been shown to improve motor functions in PD patients [62, 63]. Whether PD-1/PD-L1 blockade is sufficient to alter pathology and affect cognition in mouse models of AD or related diseases remains to be clarified [64–67]. Moreover, PD-1 deficiency has been shown to aggravate motor dysfunction in MTPT model of PD [68]. Thus, the role of PD-L1/PD-1 axis in the pathogenesis and treatment of PD requires careful assessment. Our findings clearly highlight the possibility of inhibiting PD-L1/PD-1 pathway as a therapeutical strategy to alleviate broad T cell deficiency and delay the progression of PD.

Although we showed that A53T-syn Exo induces T cell suppression in a PD-1/PD-L1 dependent manner. The molecular mechanisms underlying the increase of PD-L1 in T cells induced by A53T-syn Exo are still unknown. The expression of PD-L1 is controlled by multiple mechanisms [69]. For example, cytokines, microRNAs, and noncoding Y RNA contained in exosomes have been shown to increase PD-L1 expression in immune cells [31, 70–72]. NDEs extracted from plasma or cerebrospinal fluid of PD patients are shown to contain various pathogenic proteins [24, 33, 34, 44, 45]. Given that there is no research on the role of NDEs in the regulation of T cell immunity, one important future direction is to decipher the components in A53T-syn Exo and signaling events trigged in T cells responsible for regulating T cell PD-L1 expression and subsequent responses.

Conclusions

Our findings reveal that NDEs isolated from *in vitro* and *in vivo SNCA* A53T mutant PD models exert a broad suppressive effect on T cells. These results suggest that NDEs derived from *SNCA* A53T mutant PD models repress the immune functions of multiple subpopulations of T cells, resulting in an aberrant immune state susceptible to infection and autoimmunity. Importantly, this general suppression is mechanistically trigged by exosome-mediated PD-1/PD-L1 signaling in the recipient T cells. Thus,

exosomes modulate T cell immunity via PD-1/PD-L1 pathway both in cancer and neurodegeneration, which broadens our understanding of the biology of exosomes. Our study provides the first explanation for an important long-standing clinic observation in PD patients and is the first report on exosome-mediated immune checkpoint in neurodegenerative conditions based on our literature search. It offers a basis for exploring peripheral T cells as biomarkers and therapeutic targets for PD and opens several new areas of investigation that will impact PD, other neurodegenerative diseases, and neural stress conditions.

Abbreviations

AD: Alzheimer's disease; ANOVA: One-way analysis of variance; ATCC: American Type Culture Collection; A53T-syn: α-synuclein A53T mutant; Aβ: β-amyloid; DMEM: Dulbecco's Modified Eagle's Medium; Exo: Exosome; FBS: Fetal bovine serum; HMC3: Human embryonic microglia clone 3; LRRK2: Leucine-rich repeat kinase 2; L1CAM: L1 Cell Adhesion Molecule; MEM: Modified Eagle's Medium; NDE: Neuron-derived exosome; PD: Parkinson's disease; PD-1: Programmed death 1; PD-L1: Programmed death ligand 1; TEM: Transmission electron microscopy; TH: Tyrosine Hydroxylase; Th1: T helper 1; Th2: T helper 2.

Declarations

Supplementary Information

The online version contains supplementary material.

Ethics approval and consent to participate

All mouse experiments were approved by the Institutional Animal Care and Use Committees of Emory university.

Consent for publication

All authors have approved of the consents of this manuscript and provided consent for publication.

Availability of supporting data

The data of this study are available from the corresponding author on reasonable request.

Competing interests

The authors declare no competing interest.

Funding

This work was supported in part by grants R01NS107505 and R01NS095269 to Z.M.

Author Contributions

Z.C., J.L., and Z.M. designed research; Z.C., G.L., and C.X. performed research; Z.C., Z.M., and C.X. analyzed data; Z.C. and Z.M. wrote the paper; C.X., and Z.W. provided material; and Z.C., J.L., and Z.M. edited the manuscript.All authors read and approved the final manuscript.

Acknowledgements

The authors wish to thank Dr. Solomon Snyder at Department of Neuroscience, Johns Hopkins University for critical discussion. This work was supported in part by grants R01NS107505 and R01NS095269 to Z.M.

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Figures

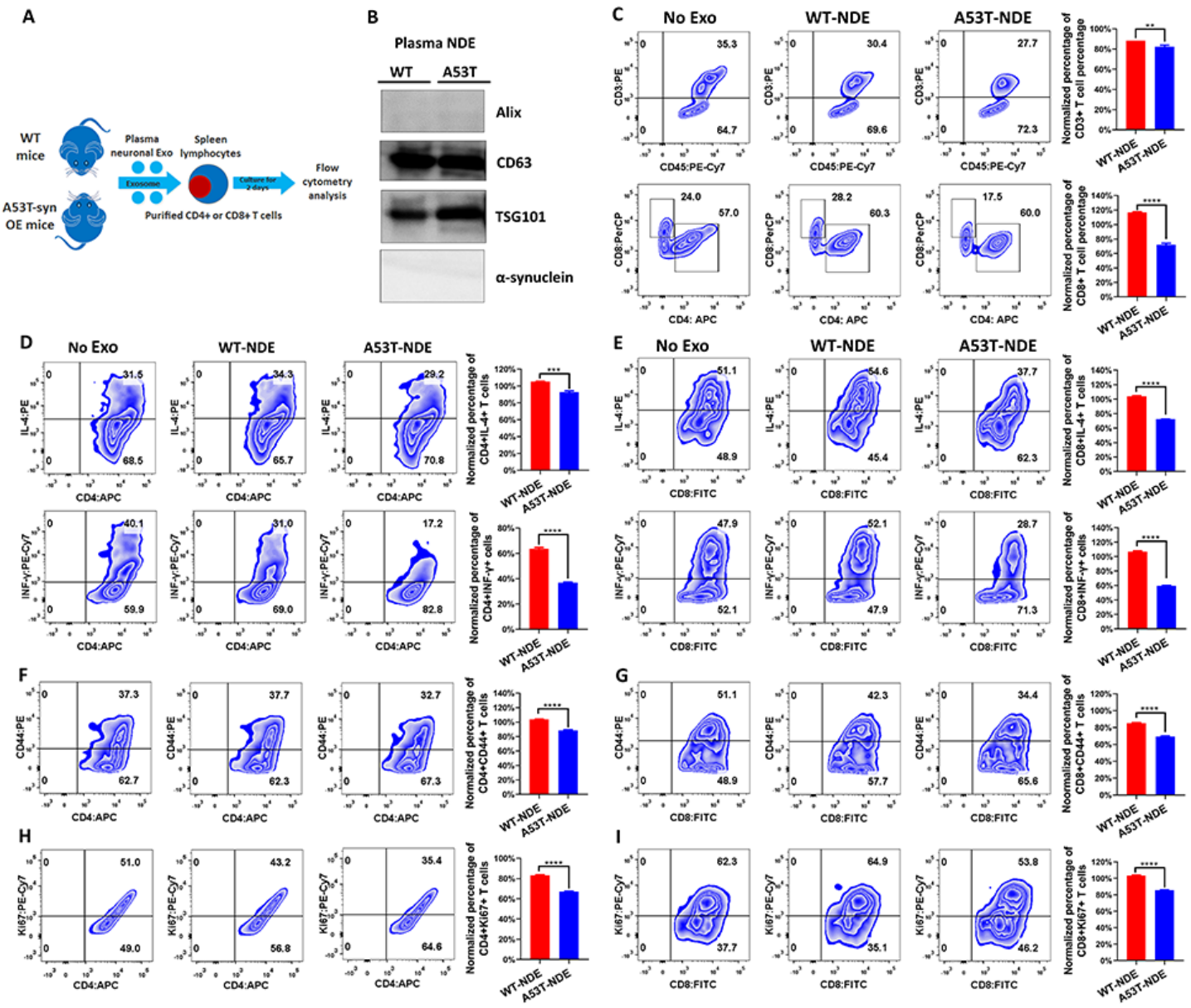

Figure 1

NDEs isolated from the plasma of A53T-syn transgenic mice suppress T cells. **A** Experimental protocol for treatment of isolated spleen lymphocytes or CD4+ or CD8+ T cells with NDEs from the plasma of A53T-syn transgenic mice (A53T-syn overexpression [OE] mice). **B** The expressions of exosome markers in NDE isolated from the plasma of A53T-syn transgenic mice. **C** A53T-NDEs reduce the percentages of CD3+ and CD8+ T cells of total spleen lymphocytes. Total mouse spleen lymphocytes were treated with NDEs (10 μg/mL; WT-NDE and A53T-NDE are purified from the plasma of WT mice or A53T α-synuclein transgenic mice, respectively) and cultured for 48 h before flow cytometry analysis. The percentages of cells expressing CD3 or CD8 were determined by flow cytometry. **D-I** A53T-NDEs reduce the percentages of CD4+INF-γ+, CD4+IL-4+ T, CD8+INF-γ+, and CD8+IL-4+ T cells (**D-E**), the percentages of CD4+CD44+ and CD8+CD44+ T cells (**F-G**), and the percentages of CD4+Ki67+ and CD8+Ki67+ T cells (**H-I**). Experiments were carried out using similar protocols as described in **C**. The significance of differences between

control group and A53T group was calculated by unpaired Student t test: $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

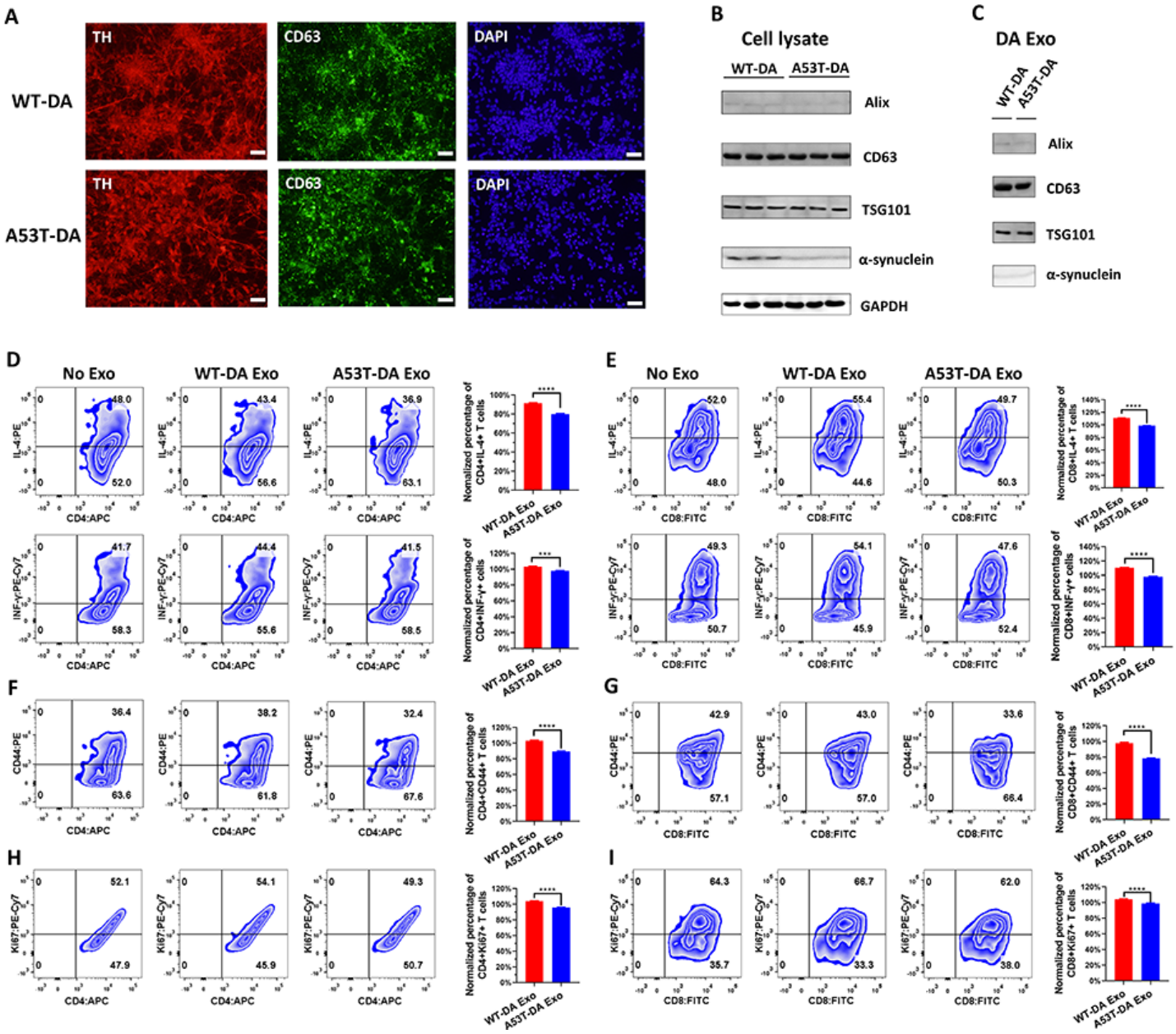

Figure 2

Exosomes isolated from DAergic neurons differentiated from human iPSCs with A53T-syn mutation suppress T cells. **A** The expressions of TH and CD63 in WT-DA and A53T-DA neurons derived from human iPSCs. The scale bar =50 μm. **B-C** The expressions of exosome markers and α-synuclein in total cellular lysates (**B**) and purified exosomes (**C**) of DAergic neurons differentiated from iPSC. **D-E** A53T-DA Exo reduces the percentages of INF-γ+ and IL-4+ T cells in both CD4+ (**D**) and CD8+ (**E**) T cells. CD4+ or CD8+ T cells isolated from mouse spleens were treated with or without exosomes (10 μg/mL; A53T-DA Exo and WT-DA Exo are exosomes prepared from DAergic neurons differentiated from human iPSCs with A53T-syn mutation and isogenic control, respectively) for 48 h. The percentages of cells expressing IL-4 or INF-

γ were determined by flow cytometry. **F-I** A53T-DA Exo reduces the percentages of CD4+ CD44+ (**F**), CD8+CD44+ (**G**), CD4+Ki67+ (**H**), and CD8+Ki67+ (**I**) T cells. Experiments were carried out using similar protocols as described in **D**. The significance of differences between control group and A53T group was calculated by unpaired Student t test: ***$p < 0.001$, ****$p < 0.0001$.

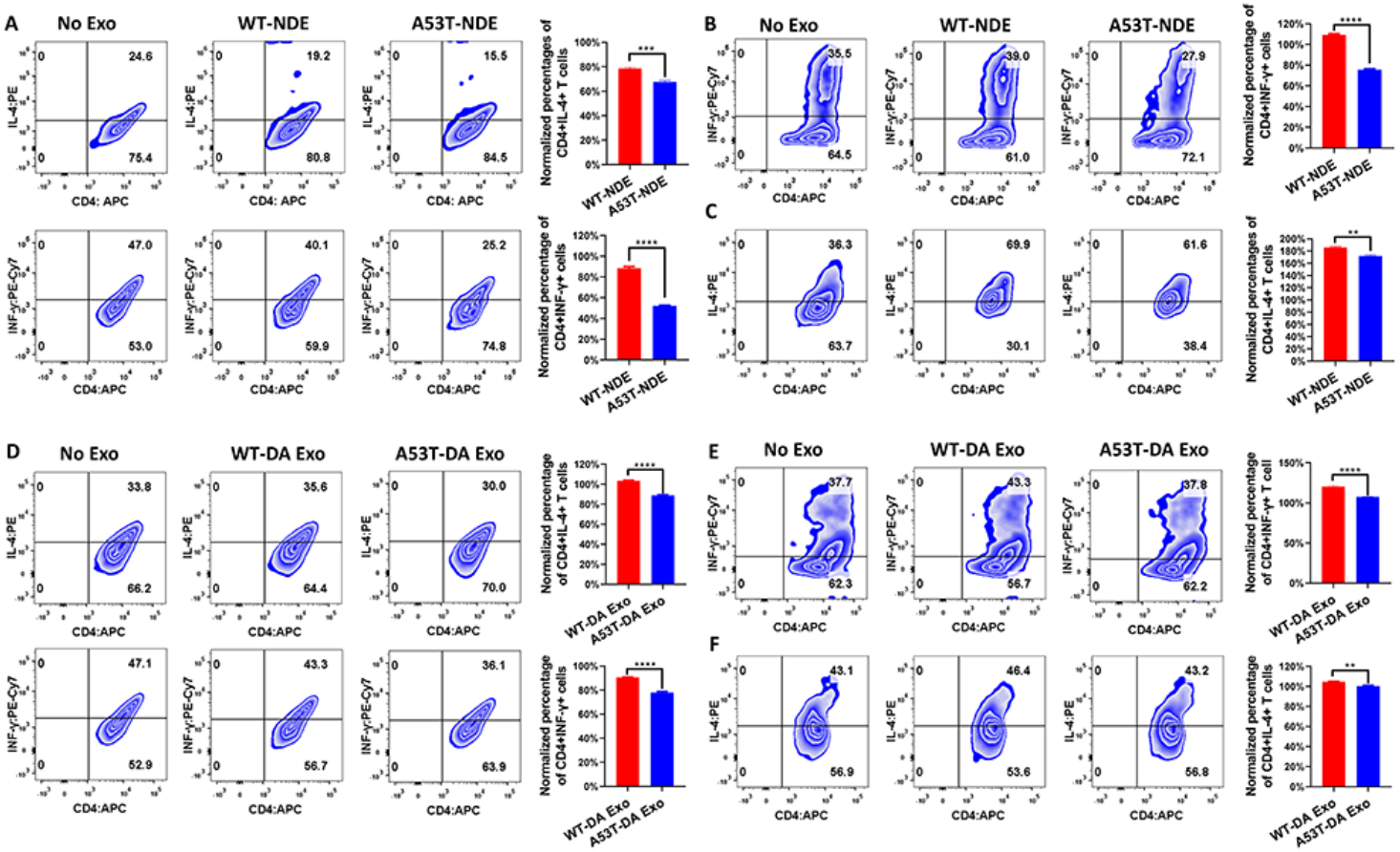

Figure 3

NDEs isolated from the plasma of A53T-syn transgenic mice and culture media of human iPSC-derived A53T-DA neurons inhibit Th1 and Th2 differentiation. **A** A53T-syn NDE reduces the percentages of CD4+IL-4+ and CD4+INF-γ+ T cells under Th0 conditions. Naive CD4+ T cells isolated from mouse spleens were treated with or without exosomes (10 μg/mL; WT-NDE and A53T-NDE are purified from the plasma of WT mice or A53T α-synuclein transgenic mice, respectively) for 96 h. The percentages of cells expressing IL-4 or INF-γ were determined by flow cytometry. **B-C**A53T-syn NDE reduces the percentages of CD4+ T cells with differentiated Th1 or Th2 markers (INF-γ and IL-4, respectively) under either Th1 (**B**) or Th2 (**C**) differentiation condition. Naïve CD4+ T cells were cultured under Th1 (50 ng/ml recombinant IL-12 and 10 mg/ml anti-IL-4) or Th2 (50 ng/ml recombinant IL-4 and 10 mg /ml anti-IFN-γ) conditions for 24 h. Then cells were treated with or without exosomes (10 μg/mL; WT-NDE and A53T-NDE are purified from the plasma of WT mice or A53T α-synuclein transgenic mice, respectively) for 96 h. The percentages of cells expressing IL-4 or INF-γ were determined by flow cytometry. **D**A53T-DA Exo reduces the percentages of CD4+IL-4+ T cell and CD4+INF-γ+ T cells under Th0 conditions. Experiments were carried

out using similar protocols as described in **A** except the source of exosomes. **E-F**A53T-DA Exo reduces Th1 and Th2 differentiation under Th1 (**E**) and Th2 (**F**) condition. Experiments were carried out using similar protocols as described in **B** and **C** except the source of exosomes. The significance of difference between control group and A53T group was calculated by unpaired Student t test: $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

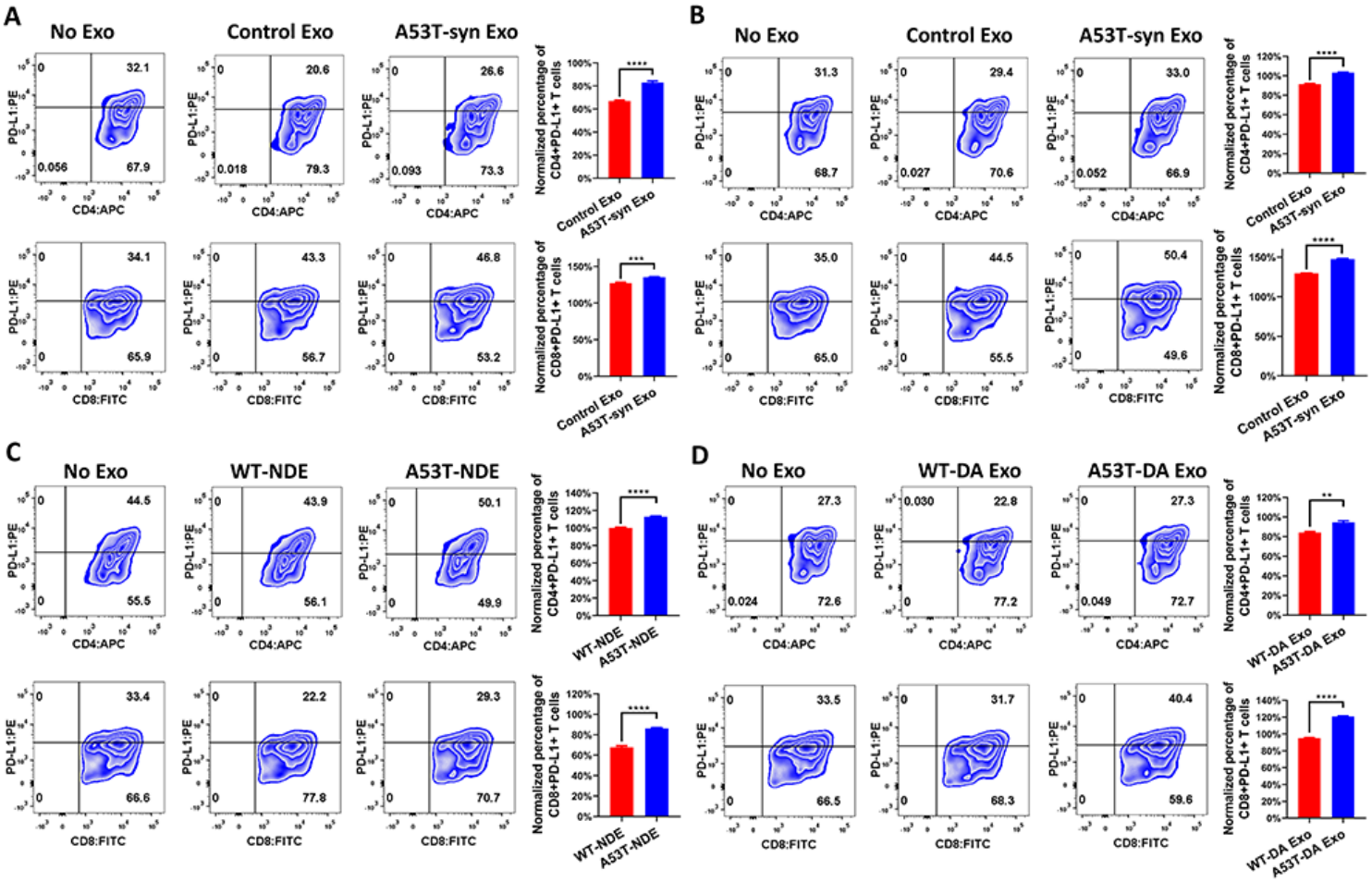

Figure 4

A53T-syn Exo increases the expression of PD-L1 in CD4+ and CD8+ T cells. **A-B** A53T-syn Exo from culture media of SN4741 (**A**) and H4 (**B**) cells increases the percentages of CD4+PD-L1+ and CD8+PD-L1+ T cells. Purified CD4+ or CD8+ T cells isolated from mouse spleens were treated with or without exosomes (10 μg/mL; control Exo and A53T-syn Exo are exosomes prepared from cells without or with A53T α-synuclein overexpression, respectively) for 48 h. The percentages of cells expressing PD-L1 were determined by flow cytometry. **C-D** A53T-NDE from A53T-syn transgenic mice (**C**) or A53T-DA Exo from human iPSC-derived DA neurons (**D**) increases the percentages of CD4+PD-L1+ and CD8+PD-L1+ T cells. Experiments were carried out using similar protocols as described in **A** except the source of exosomes. The significance of differences between control group and A53T group was calculated by unpaired Student t test: $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

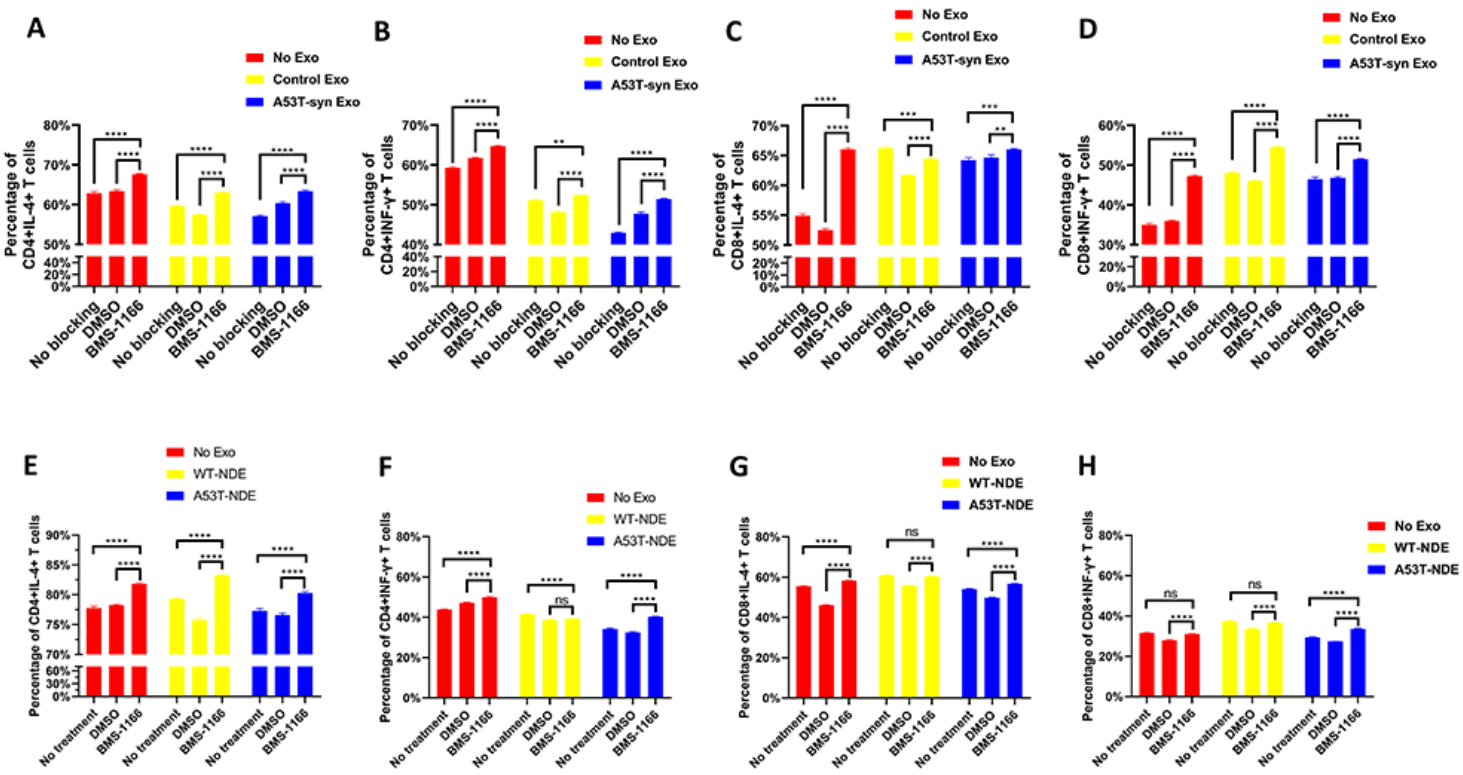

Figure 5

Blocking of PD-L1 with BMS-1166 reverses the immune suppression induced by A53T-syn exosomes. **A-H**Blocking of PD-L1 with BMS-1166 reverses the suppression of IL-4 and INF-γ in CD4+ (**A**, **B**, **E**, and **F**) or CD8+ (**C**, **D**, **G**, and **H**) T cells induced by A53T-syn Exo from SN4741 cells (**A-D**) or by the plasma NDEs from A53T-syn transgenic mice (**E-H**). Purified CD4+ or CD8+ T cells isolated from mouse spleens were treated with or without exosomes (10 μg/mL; control Exo and A53T-syn Exo are exosomes prepared from cells without or with A53T α-synuclein overexpression, respectively. WT-NDE and A53T-NDE are purified from the plasma of WT mice or A53T α-synuclein transgenic mice, respectively) for 48 h. BMS-1166 (1μM for final concertation) was added into the CD4+ and CD8+ T cell culture media before the treatment of exosomes. The percentages of cells expressing IL-4 or INF-γ were determined by flow cytometry. The significance of differences among three groups was assessed by two-way ANOVA followed by Tukey's multiple comparisons test: $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

Supplementary Files

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