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α-Synuclein orchestrates Th17 responses as antigen and adjuvant in Parkinson's disease

Emi Furusawa-Nishii[1], Bataa Solongo[1], Kou Rai[1], Soichiro Yoshikawa[1], Asako Chiba[1], Ayami Okuzumi[2], Shin-ichi Ueno[2], Yasunobu Hoshino[2], Yoko Imamichi-Tatano[2], Haruka Kimura[2], Taku Hatano[2], Nobutaka Hattori[2,3] and Sachiko Miyake[1*]

Abstract

Recently, the role of T cells in the pathology of α-synuclein (αS)-mediated neurodegenerative disorders called synucleinopathies, including Parkinson's disease (PD), dementia with Lewy bodies (DLB), and multiple system atrophy, has attracted increasing attention. Although the existence of αS-specific T cells and the immunogenicity of the post-translationally modified αS fragment have been reported in PD and DLB, the key cellular subset associated with disease progression and its induction mechanism remain largely unknown.

Peripheral blood mononuclear cells (PBMCs) from synucleinopathy patients and healthy controls were cultured in the presence of the αS peptide pools. Cytokine analysis using culture supernatants revealed that C-terminal αS peptides with a phosphorylated serine 129 residue (pS129), a feature of pathological αS aggregates, promoted the production of IL-17A, IL-17F, IL-22, IFN-γ and IL-13 in PD patients compared with that in controls. In pS129 peptide-reactive PD cases, Ki67 expression was increased in CD4 T cells but not in CD8 T cells, and intracellular cytokine staining assay revealed the existence of pS129 peptide-specific Th1 and Th17 cells. The pS129 peptide-specific Th17 responses, but not Th1 responses, demonstrated a positive correlation with the Movement Disorder Society-Unified Parkinson's Disease Rating Scale (MDS-UPDRS) Part III scores. A similar correlation was observed for IL-17A levels in the culture supernatant of PBMCs from PD patients with disease duration < 10 years. Interestingly, enhanced Th17 responses to pS129 peptides were uniquely found in PD patients among the synucleinopathies, suggesting that Th17 responses are amplified by certain mechanisms in PD patients. To investigate such mechanisms, we analyzed Th17-inducible capacity of αS-exposed dendritic cells (DCs). In vitro stimulation with αS aggregates generated Th17-inducible DCs with IL-6 and IL-23 production through the signaling of TLR4 and spliced X-box binding protein-1 (XBP1s). In fact, the levels of IL-6 and IL-23 in plasma, and the XBP1s ratio in type 2 conventional DCs were increased in PD patients compared with those in controls.

Here, we propose the importance of αS-specific Th17 responses in the progression of PD and the underlying mechanisms inducing Th17 responses. These findings may provide novel therapeutic strategies to prevent disease development through the suppression of TLR4-XBP1s-IL-23 signaling in DCs.

Keywords synucleinopathy, α-synuclein specific T cells, TLR 4, spliced X-box binding protein, IL-23

*Correspondence:
Sachiko Miyake
s-miyake@juntendo.ac.jp
Full list of author information is available at the end of the article

Introduction

Inflammatory responses generated by resident brain cells and peripherally recruited immune cells are closely associated with neurodegeneration in neurodegenerative disorders, including Parkinson's disease (PD) [1–6]. Lymphocytes routinely survey the central nervous system (CNS), and disruption of the blood–brain barrier by peripheral inflammation allows more immune cells to migrate from the periphery to the brain [7–9]. Therefore, understanding disease-specific peripheral immune responses is important.

Synucleinopathies, including PD, dementia with Lewy bodies (DLB), and multiple system atrophy (MSA), are neurodegenerative disorders that result in the progressive loss of motor and/or cognitive functions accompanied by abnormal α-synuclein (αS) deposition. αS usually exists as a nontoxic monomer or tetramer [10, 11]; however, in brain regions affected by synucleinopathies, the αS structure changes from nontoxic monomers to toxic oligomers or fibrils. In these toxic αS aggregates, the serine 129 (S129) residue of αS deposits is highly phosphorylated [12–15]. Although PD, DLB, and MSA share the pathological feature of abnormal αS deposition in neurons and glial cells, the cell types and regions of the brain where αS accumulates differ among them, resulting in a wide spectrum of clinical phenotypes.

During the process of αS propagation and neuronal cell death, αS aggregates in neurons are released from cells [16, 17]. The presence of abnormally structured αS in patient serum supports the idea that αS aggregates circulate throughout the body [18–22]. Therefore, extracellular αS that travels from the CNS to the draining lymph nodes or that is in the peripheral circulation may activate the peripheral immune system and significantly influence the type and number of peripherally derived immune cells including T cells, which eventually infiltrate into the CNS. Since the identification of T cells in autopsied brains of synucleinopathy patients [4–6], the role of T cells in neurodegeneration has attracted increasing attention.

Recently, several studies have identified the presence of αS-specific T cells in the peripheral blood of LB disease patients [23–25]. The analysis of αS-specific T cells is valuable when evaluating disease-associated T cells. Although αS-specific Th1 responses have been observed at an early stage of the disease [24], the T cell subset involved in disease progression remains unclear. Analyses using PD animal and cellular models have demonstrated that, in addition to Th1 cells, Th17 cells contribute to the promotion of neurodegeneration [26–29]. Indeed, Th17 cells have been identified in autopsied brains of LB disease patients [25].

In this study, to identify αS-specific T cell responses associated with disease progression, we recruited patients with synucleinopathies or those in its preclinical phase: rapid eye movement sleep behavior disorder (RBD), and analyzed their peripheral αS-specific T cell responses. Consistent with previous reports, C-terminal αS peptides with a phosphorylated S129 residue (pS129) were a key fragment for the induction of αS-specific T cells. Although pS129 peptide-specific Th1, Th2, and Th17 responses were identified through cytokine measurement and/or cell-based analysis, pS129 peptide-specific Th17 responses, but not Th1 responses, were positively correlated with Movement Disorder Society-Unified Parkinson's Disease Rating Scale (MDS-UPDRS) Part III scores during the "ON" state. These observations suggest that pS129 peptide-specific Th17 responses are involved in disease progression in PD. Conversely, the detection of Th17 responses to pS129 peptides was significantly lower in MSA patients than in PD patients, suggesting that the PD-specific disease environment contributes to the generation of αS-specific Th17 responses. We further demonstrated that Th17-inducible dendritic cells (DCs) with IL-6 and IL-23 production were generated through the signaling of Toll-like receptor (TLR)4 and spliced X-box binding protein-1 (XBP1s). The expression of XBP1s in type 2 conventional DCs (cDC2s) and the IL-6 and IL-23 levels in plasma were elevated in PD patients, indicating that the enhanced XBP1s-IL-6/IL-23 pathway in DCs resulted in the amplification of Th17 responses in PD patients. These findings may provide novel therapeutic strategies to prevent the development of PD.

Materials and methods

Study design and participants

We recruited 47 patients with PD, 9 with PD with dementia (PDD)/DLB, 29 with MSA, 16 with RBD, and 29 healthy controls from 2022 to 2024 (Table 1, Supplementary Tables 1–7). The following clinical data were collected: age, sex, disease duration, Hoehn-Yahr stage, and MDS-UPDRS Part III score in an "ON" state. This study was approved by the Ethics Committee of Juntendo University (no. 2021100). Written informed consent was obtained from all participants before enrollment, in accordance with the Declaration of Helsinki. The diagnostic criteria for PD, MSA, and RBD were based on the Movement Disorder Society-sponsored PD clinical criteria [30], Gilman's criteria [31], and definitions and guidelines provided by the DLB Consortium [32], respectively. Patients with RBD were examined by polysomnography.

Sample collection

Peripheral blood mononuclear cells (PBMCs) and plasma were isolated from whole blood by density centrifugation

Table 1 Characteristics of the study participants

	Assay	Age(years), mean (s.d.)	Men, n (%)	Hoehn-Yahr stage, mean (s.d.)	Disease duration (years), mean (s.d.)
PD (n=47)	Peptide culture, Plasma cytokine measurement and/or XBP1s analysis in cDC2 (n=44)	63 (9)	19 (43)	2 (1)	9 (4)
	IP/RT-QuIC (n=3)	65 (4)	2 (67)	2 (1)	11 (5)
PDD/DLB (n=9)	Peptide culture (n=9)	75 (7)	6 (67)	3 (1)	8 (7)
	IP/RT-QuIC (n=3)	76 (4)	2 (67)	3 (2)	11 (10)
MSA (n=29)	Peptide culture (n=28)	64 (9)	9 (32)	3 (1)	3 (2)
	IP/RT-QuIC (n=3)	68 (2)	1 (33)	5 (0)	6 (1)
RBD (n=16)	Peptide culture	74 (5)	10 (63)	NA	7 (6)
HC (n=29)	Peptide culture, Plasma cytokine measurement and/or XBP1s analysis in cDC2 (n=16)	66 (10)	8 (50)	NA	NA
	mDC generation (n=13)	36 (8)	8 (62)	NA	NA
	IP/RT-QuIC (n=1)	86	0 (0)	NA	NA

using Histopaque-1077, in accordance with the manufacturer's instructions (Merck, Darmstadt, Germany). Freshly isolated PBMCs were used for cytokine measurement assays of peptide culture supernatants or CD14-positive cell isolation. PBMCs cryopreserved using CryoStor®CS10 (StemCell Technologies, Vancouver, Canada) were employed for intracellular cytokine staining assays or XBP-1 s staining assays using cDC2s. Serum samples were collected by centrifugation. Collected plasma and serum samples were immediately frozen at − 80 °C.

Procedures

Peptides

αS peptides were synthesized by Toray Industries (Tokyo, Japan) as purified material (> 95% by reverse-phase HPLC). The peptides were 15-mers with 10 overlapping residues. Two to six 15-mer peptides were combined to create the αS peptide pool. The αS peptide pools contained the following amino acid sequences: αS peptide pool 1: aa1–40, αS peptide pool 2: aa31–46, αS peptide pool 3: aa36–75, αS peptide pool 4: 66–105, αS peptide pool 5: 96–125, and αS peptide pool 6: aa116–140. For three C-terminal 15-mer peptides (aa116–140), peptides with S129 phosphorylation were synthesized, and a peptide pool was created (αS peptide pool 7).

Peptide pools of *Candida albicans* mannoprotein MP65 protein (PepTivator® *Candida albicans* MP65, research grade) and cytomegalovirus (CMV) pp65 protein (PepTivator® CMV pp65, research grade) were obtained from Miltenyi Biotec (Bergisch Gladbach, Germany), and consisted of 15-mer sequences with 11 amino acid overlap.

Purification of recombinant α-synuclein and preparation of pre-formed fibrils

Recombinant monomeric human αS protein was purified from *Escherichia coli* BL21 harboring pRK172-α-synuclein (Y136-TAT), as previously reported [33]. Endotoxin levels measured by ToxinSensor™ Chromogenic LAL (GenScript, Piscataway, NJ, USA) were below the detection limit (0.0125 EU/μg). Protein concentrations were measured using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). Purified αS monomers were incubated at 37 °C in a shaking incubator (Eppendorf, Tokyo, Japan) for 5 days. Turbidity measurements were performed at an OD of 600. After 5 days, αS pre-formed fibrils were pelleted by spinning and suspended in PBS. Purified protein samples were kept at − 80 °C. Fibrils were sonicated in a water bath sonicator for 5 min for use in the DC stimulation assay.

Real-time quaking-induced conversion assay

The real-time quaking-induced conversion (RT-QuIC) assay using serum immunoprecipitated with anti-alpha-synuclein antibody [MJFR1] (ab138501; Abcam) was performed as previously reported [20]. After confirming that the RT-QuIC was negative in the healthy donor sample and positive in the patient samples, DCs were stimulated

with × 1000 diluted RT-QuIC products without thioflavin T. These RT-QuIC products were sonicated for 3 min in a water bath sonicator prior to DC stimulation. Endotoxin levels of × 1000 diluted RT-QuIC products measured by ToxinSensor™ Chromogenic LAL (GenScript) were less than 0.03 EU/ml.

Culture with peptides

A total of 1×10^6 PBMCs were cultured in 96-well plates in TexMACS medium (Miltenyi Biotec) containing αS-, *C. albican*s, or CMV peptide pools (0.6 μM) at 37 °C in a 5% CO_2 incubator. PBMCs under unstimulated conditions were cultured in medium only. On day 3, human recombinant IL-2 (PeproTech Inc., Rocky Hill, NJ, USA) was added at a final concentration of 1 ng/mL, and cultured until day 7. For intracellular cytokine staining assays, half of the medium was also changed on days 7 and 10. On day 14, PBMCs were restimulated with 6 μM pS129 peptide for 6 h with BD GolgiPlug™ (1/1000 dilution; BD Biosciences, San Jose, CA, USA).

Generation of human monocyte-derived DCs.

CD14-positive cells were isolated from PBMCs of healthy donors using magnetic column separation (MS columns; Miltenyi Biotec) with CD14 MicroBeads (Miltenyi Biotec), in accordance with the manufacturer's instructions. CD14-positive cells of > 90% purity were used in the experiments.

After purification, CD14-positive monocytes were seeded at a density of 5×10^5 cells/ml in 24-well plates and cultured for 5 days in RPMI 1640 medium containing 10% FCS, 2 mM L-glutamine, 100 U/ml pen-strep, and 50 μM 2-ME, supplemented with 50 ng/ml recombinant human GM-CSF (BioLegend) and 50 ng/ml recombinant human IL-4 (PeproTech). On day 3, half of the medium was changed. Monocyte-derived DCs (mDCs) expressing > 90% of CD209 were used in the experiments.

DC culture

The generated mDCs were resuspended in RPMI 1640 medium containing 10% FCS, 2 mM L-glutamine, 100 U/ml pen-strep, and 50 μM 2-ME. Then, 1×10^5 mDCs were cultured in 96-well plates in the presence or absence of αS or RT-QuIC products.

For inhibitor experiments, a TLR4 inhibitor (TAK-242), TLR1/2 inhibitor (TLR2-IN-C29), or XBP-1 s inhibitor (STF-083010) was purchased from Selleck Chem (Houston, TX, USA). LPS (10 ng/ml; Sigma-Aldrich, St. Louis, MO, USA) and Pam3CK4 (1 μg/ml; InvivoGen, San Diego, CA, USA) were used as positive controls for TLR4 and TLR1/2 stimulation, respectively. Two hours before αS or TLR agonist stimulation, the following concentrations of inhibitors were added: TAK-242, 60 μM; TLR2-IN-C29, 120 μM; and STF-083010, 120 μM. Vehicle containing the same concentration of DMSO was used as a control.

Cytokine measurements

Cytokine concentrations in culture supernatants were measured using the LEGENDplex™ HU Th Cytokine Panel (12-plex) with a V-Bottom Plate V02 (BioLegend) or the LEGENDplex™ Human Inflammation Panel 1 with a V-Bottom Plate (BioLegend). The protocol was performed in accordance with the manufacturer's instructions and data were analyzed using the LEGENDplex™ online analysis platform (https://legendplex.qognit.com/). If cytokine production was below the detection limit, the detection limit was used as the measured value. For the peptide culture assay, the fold change in cytokine production after peptide stimulation was calculated relative to the cytokine production in medium alone. Values ≤ 1 were defined as 1. In addition, the concentration of cytokines after peptide stimulation minus the concentration in medium alone was calculated. Values of less than ≤ 0 were defined as 0.

Flow cytometry

Cells were incubated with Zombie Yellow or Zombie Aqua Fixable Viability Dye (BioLegend), and Fc receptor blocking solution (BioLegend), and then stained with monoclonal antibodies (mAbs) against human cell surface antigens. After washing, cells were fixed and permeabilized in accordance with the manufacturer's instructions. BD Cytofix/Cytoperm Plus was used for intracellular cytokine expression analyses. For other analyses, eBioscience™ FOXP3/Transcription Factor Straining Buffer Set (Invitrogen) was used. Subsequently, intracellular staining was performed. Details of the antibodies are provided in the Supplementary material. Data were acquired using a flow cytometer (LSR Fortessa; BD Biosciences) and analyzed using FlowJo software, v10.8.0 (Tree Star, Ashland, OR, USA). To evaluate the pS129 peptide-mediated elevation of cytokine expression, the ratio of IFN-γ- or IL-17A-expressing CD4 T cells in the pS129 peptide stimulation minus the ratio in the unstimulated condition (Δ) was calculated. Values of less than ≤ 0 were defined as 0.

Statistical analysis

All data were analyzed using GraphPad Prism 10.0 (GraphPad, La Jolla, CA, USA). The significance of differences between groups was analyzed using appropriate tests, as indicated in the figure legends. Student's *t*-test was used to analyze parametric differences in age between patients and controls, and Pearson's chi-square

test was used for sex ratio statistics (Supplementary Tables 1, 3–7).

Results

Immunogenic α-synuclein peptides enhance cytokine production

We established an in vitro experimental system to detect αS-specific T cells using PBMCs from PD patients and age-matched controls (Table 1, Supplementary Table 1). To identify immunodominant peptides, we examined cytokine production in the culture supernatants of PBMCs stimulated with αS peptides and calculated the fold change compared with that of unstimulated PBMCs. The fold changes of IL-17A, IL-17F, IL-22, IFN-γ, IL-13, IL-5, IL-6, IL-9, IL-10, and TNF-α levels secreted by PBMCs from PD patients were significantly greater than those from healthy controls when stimulated with aa116-140 peptides with pS129, suggesting that C-terminal pS129 peptides are immunodominant (Fig. 1A, Supplementary Fig. 1, Supplementary Fig. 2A). In addition to the C-terminal pS129 peptides, cytokine production was increased in PBMCs from PD patients compared with that from controls in the presence of the following αS peptide pools: aa1–40 peptides: IL-17A, IL-22, IL-9, and

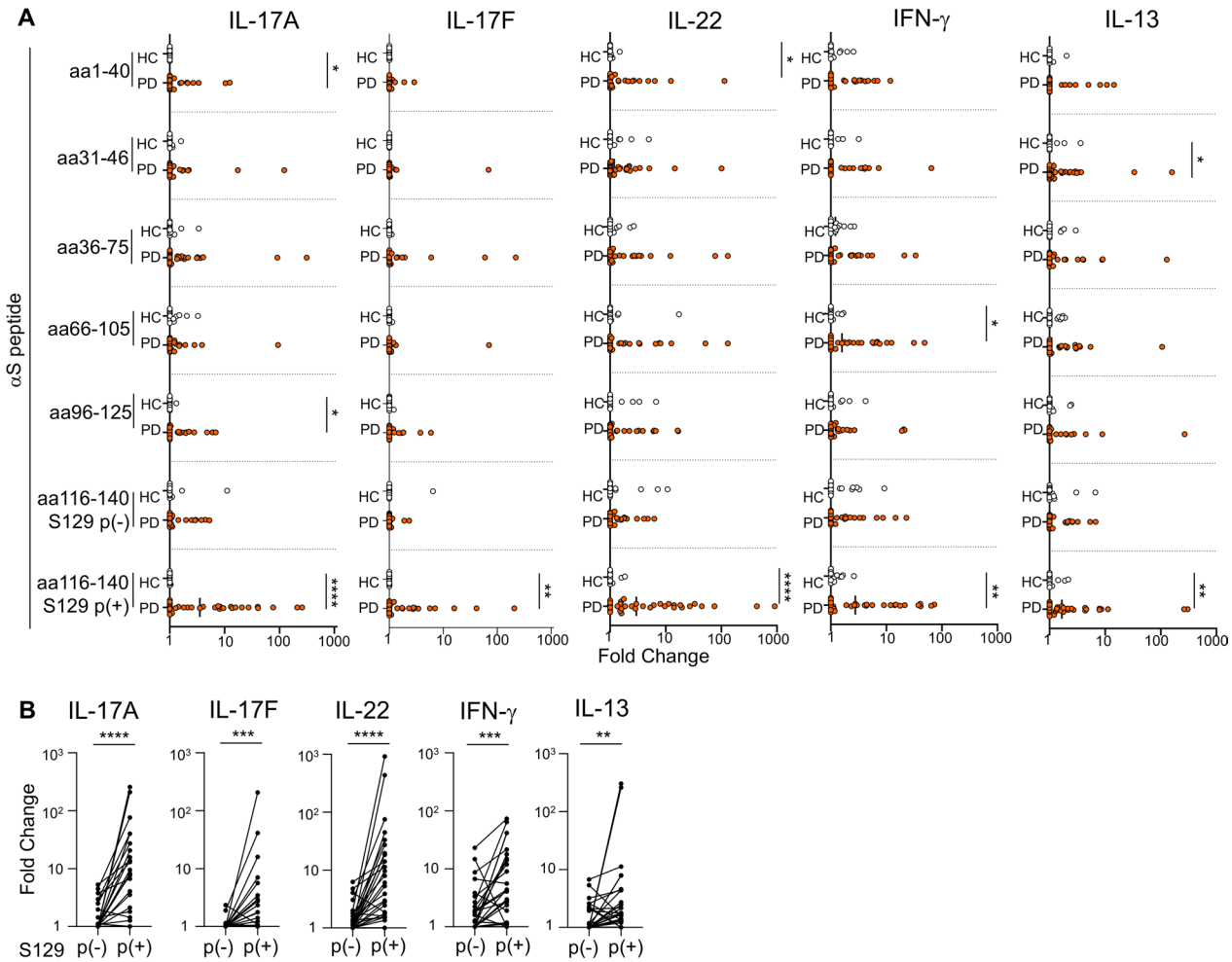

Fig. 1 Stimulation with C-terminal α-synuclein peptides with phosphorylated 129 serine residue increases T cell-related cytokine production in PD patients. PBMCs from PD patients and age-matched controls were cultured for 7 days in the presence or absence of α-synuclein (αS) peptide pools [controls: n = 15, PD: n = 33 for S129 p(+) peptides, controls: n = 15, PD: n = 31 for other αS peptides]. Clinical information is shown in Supplementary Table 1. IL-17A, IL-17F, IL-22, IFN-γ, and IL-13 concentrations in the culture supernatant were measured. The fold change in cytokine production upon αS peptide stimulation was calculated relative to cytokine production in medium alone. (**A**) The fold change in cytokine production by the indicated αS peptide stimulation. The line represents the median. (**B**) Comparison between aa116–140 S129 p(−) peptide stimulation and aa116–140 S129 p(+) peptide stimulation (PD: n = 31). HC, healthy control; p(−), without phosphorylation; p(+), with phosphorylation. Each dot indicates the value of one individual. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. P-values were determined by the Mann–Whitney *U*-test (**A**), or the Wilcoxon matched-pairs signed-rank test (**B**)

TNF-α; aa31–46 peptides: IL-13, IL-9, and TNF-α; aa66–105 peptides: IFN-γ, IL-2, and IL-10; and aa96–125 peptides: IL-17A. These results indicated the importance of the C-terminal pS129 peptides. Among the 12 cytokines measured, we focused on IL-17A, IL-17F, IL-22, IFN-γ, and IL-13 because they are produced by T cells and showed highly significant fold changes and increased cytokine levels in PBMCs from PD patients compared with the levels from controls (Fig. 1A, Supplementary Fig. 2B). Although the levels of IL-17A, IL-17F, IL-22, IFN-γ, and IL-13 in PBMCs from PD patients were elevated compared with those from controls after stimulation with pS129 peptides, particularly marked differences in fold change were observed for the Th17 cell-related cytokines IL-17A and IL-22 (Fig. 1A).

Next, to assess whether there were functional differences in T cell responses between PD patients and controls, we analyzed cytokine production in response to CMV pp65 and *Candida albicans* MP65 peptides (Supplementary Table 1). These peptides, like the αS peptides, are 15 amino acid length and elicit $CD4^+$ and $CD8^+$ T cell responses [34, 35]. Indeed, stimulation with CMV or *C. albicans* peptides increased Ki67 expression in CD4 T and CD8 T cells (Supplementary Fig. 3). The analysis of the fold changes in cytokine production in response to CMV stimulation revealed no significant differences in IL-17A, IL-17F, IL-22, IFN-γ, IL-13, IL-5, IL-2, IL-6, IL-9, IL-10, TNF-α, and IL-4 levels between PD patients and controls (Supplementary Fig. 4A). These results were in accordance with a previous report [36]. Conversely, in response to *C. albicans* peptides, the levels of Th17 cell-derived cytokines, including IL-17A, IL-17F, and IL-22, as well as IL-6, were significantly higher in PD patients than in controls, whereas the levels of IFN-γ, IL-13, IL-5, IL-2, IL-9, IL-10, TNF-α, and IL-4 were comparable (Supplementary Fig. 4B). Enhanced Th17 responses in PD patients were observed for pS129 αS peptides as well as *C. albicans* peptides, suggesting that the Th17 response is important.

Next, we analyzed the changes in cytokine levels associated with phosphorylation at the S129 residue of the C-terminal αS peptides in PD patients. Compared with PBMCs cultured with C-terminal peptides with no phosphorylation at the S129 residue, phosphorylation at the S129 residue of the C-terminal peptides markedly increased the production of IL-17A, IL-17F, IL-22, IFN-γ, and IL-13 in the PBMCs of PD patients (Fig. 1B). These results suggest that the post-translational modification of the S129 residue by phosphorylation conferred antigenicity on the C-terminal fragment and induced αS-specific T-cell responses in the PBMCs of PD patients.

Cell-based identification of C-terminal pS129 peptide-specific T cells in PD patients

To determine whether CD8 T cells and/or CD4 T cells responded to the pS129 αS peptides, and to confirm that this assay assessed pS129 peptide-specific T cell responses, we analyzed proliferative responses and T cell-related cytokine expression upon stimulation with pS129 peptides. These analyses were performed using T cells from PD patients that showed reactivity to pS129 peptides (Supplementary Table 2). As shown in Fig. 2A–D, stimulation with pS129 peptides increased Ki67 expression in CD4 T cells, but not in CD8 T cells. The CD4 T cell-biased responses were unique to αS peptides because stimulation with CMV or *C. albicans* peptides increased Ki67 expression in CD4 T and CD8 T cells (Supplementary Fig. 3). Next, we analyzed pS129 peptide-induced IFN-γ, IL-4, and IL-17A expression in CD4 T cells to confirm the presence of pS129 peptide-specific Th1, Th2, and Th17 cells at the cellular level in this assay. Of 14 cases in which T cell reactivity to pS129 peptides was observed (≥0.1% increased cytokine expression compared with that without stimulation), IFN-γ, IL-4,

(See figure on next page.)

Fig. 2 Evaluation of pS129 αS peptide-specific T cells in PD patients. PBMCs from PD patients were cultured in the presence or absence of C-terminal pS129 peptides (Supplementary Table 2). (**A**–**D**) PBMCs (n = 6) cultured for 7 days were subjected to Ki67 staining assay. Gating strategies are shown in Supplementary Fig. 3A. Representative dot plots and $Ki67^+$ ratio in CD8 T cells (**A**, **B**) or CD4 T cells (**C**, **D**) are shown. (**E**–**J**) PBMCs (n = 14) cultured for 14 days were subjected to intracellular cytokine staining assay. Gating strategies are shown in Supplementary Fig. 5. Representative dot plots and ratios of IFN-γ-expressing CD4 T cells (**E**, **F**), IL-4-expressing CD4 T cells (**G**, **H**), and IL-17A-expressing CD4 T cells (**I**, **J**) are shown. In graphs (**F**), (**H**), and (**J**), solid lines indicate differences in cytokine expression ≥ 0.1% between pS129 peptide stimulation and no stimulation. Dotted lines indicate < 0.1%. (**K**–**P**) Intracellular cytokine staining data (n = 14) were analyzed. Representative dot plots of IL-17A and IFN-γ expression in CD4 T cells from a Th1 > Th17 patient (**K**) or a Th17 > Th1 patient (**L**) are shown. (**M**, **P**) The ratio of IFN-γ- or IL-17A-expressing CD4 T cells after pS129 peptide stimulation minus the ratio under unstimulated conditions (Δ) was calculated. Then, the indicated correlation was evaluated. (**Q**, **R**) The pS129 peptide-mediated fold change of IL-17A levels in culture supernatant of PBMCs from PD patients (n = 33) was analyzed. These data sets were from Fig. 1. The correlation of IL-17A fold change in PD with disease duration ≤ 10 years and MDS-UPDRS Part III scores is shown in (**Q**) (n = 21). The difference of IL-17A fold change between patients with disease duration ≤ 10 years (n = 21) and ≥ 11 years (n = 12) is shown in (**R**). No, no stimulation; p(+), pS129 peptides. Each dot indicates the value of one individual. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. P-values were determined by the Wilcoxon matched-pairs signed-rank test (**B**, **D**), or the Mann–Whitney *U*-test (**R**). Correlations were analyzed using Spearman's correlation analysis (**M**–**Q**)

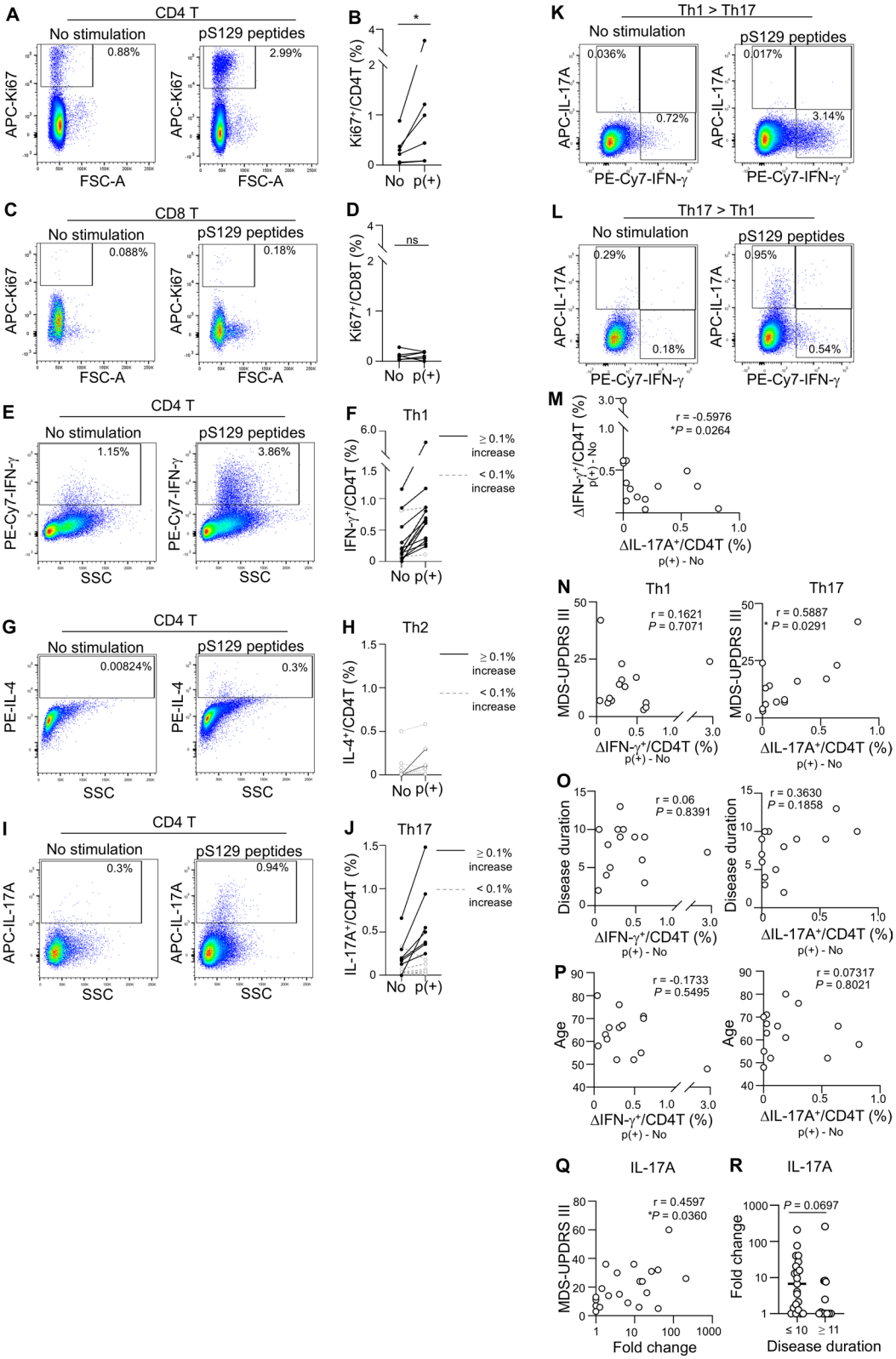

Fig. 2 (See legend on previous page.)

and IL-17A expression in CD4 T cells was increased in 12, 2, and 7 cases, respectively (Fig. 2E–J, Supplementary Fig. 5, Supplementary Table 8). Although the detection of IL-4-expressing CD4 T cells was more challenging than that of IFN-γ- or IL-17A-expressing CD4 T cells, we identified pS129 peptide-specific T cells using an intracellular cytokine staining assay.

Next, we analyzed the pS129 peptide-mediated elevation of IFN-γ, IL-4, or IL-17A expression in CD8 T cells and $CD3^-$ non-T cells (Supplementary Fig. 6A–L). Whereas IL-17A expression was not increased in CD8 T cells or $CD3^-$ non-T cells, in some cases IFN-γ expression was increased in CD8 T cells and $CD3^-$ non-T cells, and IL-4 expression was increased in $CD3^-$ non-T cells. However, when the results of intracellular cytokine staining were combined, CD4 T cells were shown to have responded more frequently to the pS129 peptides compared with CD8 T cells and $CD3^-$ non-T cells (Supplementary Table 8).

Next, we focused on pS129 peptide-specific Th1 and Th17 responses, which were frequently identified in the intracellular cytokine staining assay, and analyzed their interrelationships. In all 14 cases described above, pS129 peptide stimulation increased IFN-γ and/or IL-17A expression by more than 0.1%. IFN-γ or IL-17A expression was increased mainly in the $IFN\text{-}\gamma^+IL\text{-}17A^-$ fraction or $IFN\text{-}\gamma^-IL\text{-}17A^+$ fraction, respectively, although $IFN\text{-}\gamma^+IL\text{-}17A^+$ cells were rarely observed (Fig. 2K, L). Moreover, we found a negative correlation between pS129 peptide-mediated IL-17A and IFN-γ expression in CD4 T cells (Fig. 2M). These results suggest that pS129 peptide-specific T cell responses are Th17 skewed in some patients and Th1 skewed in others. Finally, we analyzed the relationship between pS129 peptide-specific Th17 or Th1 responses and clinical information including disease severity, disease duration, and age. Our analysis revealed a positive correlation between MDS-UPDRS Part III scores in an "ON" state and pS129 peptide-specific Th17 response (Fig. 2N–P). A similar correlation was observed for IL-17A levels in the culture supernatant of PBMCs from PD patients with disease duration ≤ 10 years, although there was a trend toward a decrease in IL-17A production when disease duration exceeded 11 years (Fig. 2Q, R). These results suggest that pS129 peptide-specific Th17 responses play an important role in the progression of the disease. Furthermore, it is notable that, even under *C. albicans* peptide stimulation, positive correlations were observed between MDS-UPDRS Part III score and IL-17F level (Supplementary Fig. 6M) and between Hoehn-Yahr stage and IL-17A or IL-17F level (Supplementary Fig. 6N). These results suggest that the mechanisms by which Th17 responses are amplified operate in PD patients.

αS-mediated functional alterations of DCs

Recently, circulating αS levels have attracted attention as a biomarker reflecting disease progression in PD patients [37, 38]. Therefore, to investigate the mechanism by which pS129 peptide-specific Th17 responses increase in PD patients, we focused on the αS-mediated functional alterations of DCs. For antigen-specific CD4 T cells to be generated in the periphery, naive CD4 T cells must interact with immunostimulatory DCs [39]. The immunostimulatory capacity of DCs is dependent upon the upregulated expression of major histocompatibility complex molecules and co-stimulatory molecules, including CD86, and the secretion of T cell-polarizing cytokines. IL-1β, IL-6, and IL-23 produced by activated DCs are important for the induction and maintenance of Th17 cells [40]. Damage-associated molecular patterns (DAMPs) are well-known inducers of immunostimulatory DCs [39, 41]. Although several studies have reported that extracellular αS acts as DAMPs to activate innate immune cells via TLR signaling [42–44], few studies have examined whether DCs exposed to αS exhibit Th17-inducible properties. Moreover, the intracellular signaling involved remains unknown. To answer these questions, we added αS to mDCs generated from healthy donors and analyzed DC activation and Th17-inducible cytokine production. Low concentration (10 μg/ml) of the αS monomer or αS pre-formed fibril stimulation did not alter the level of DC activation defined as $HLA\text{-}DR^{high}CD86^+$, but high concentration (50 μg/ml) of αS fibril stimulation significantly increased the $HLA\text{-}DR^{high}CD86^+$ ratio of $CD209^+$ mDCs compared with no stimulation (Fig. 3A, B, Supplementary Fig. 7). In culture supernatants, the production of IL-1β, IL-6, and IL-23 was increased by fibril stimulation compared with no stimulation. In addition, IL-23 production was increased by fibril stimulation compared with monomer stimulation (Fig. 3C). These results indicate the contribution of αS fibrils to the generation of Th17-inducible DCs. Whereas DC-derived IL-23 is important for the survival and maintenance of Th17 cells, DC-derived IL-12 is involved in the survival and maintenance of Th1 cells [45]. Notably, high-dose αS fibril stimulation produced more IL-23 than IL-12 (Fig. 3D).

Recently, the presence of pS129 αS aggregates in serum has been reported [46]. Therefore, we investigated the effects of post-translational modification by the phosphorylation of αS aggregates on the Th17-inducible capacity of DCs. Referencing a previous report of polo-like kinase 3 (PLK3) [47], a serine-threonine kinase that phosphorylates only the S129 residue of αS, we prepared phosphorylated αS aggregates using PLK3 and confirmed S129 phosphorylation by western blotting (Supplementary Fig. 8A). As shown in Fig. 3A, B, stimulation with

low-dose non-phosphorylated αS fibrils did not increase the HLA-DR^{high}CD86^{+} ratio of mDCs compared with the same dose of monomers or no stimulation. However, even at low doses, PLK3-treated αS fibrils increased the HLA-DR^{high}CD86^{+} ratio and the production of IL-6 and IL-23, compared with stimulation with PLK3-untreated low-dose αS fibrils (Supplementary Fig. 8B–D). Additionally, the impact of PLK3-treated monomers on DCs was evaluated (Supplementary Fig. 9A). PLK-treated αS monomers exhibited a slight, but non-significant increase in the proportion of activated DCs (Supplementary Fig. 9B, C). Conversely, no discernible difference in IL-6 levels was observed between the two groups (Supplementary Fig. 9D). IL-23 and IL-1β levels were below the detection limit under both conditions. These results suggest that the phosphorylation of αS aggregates increased the Th17-inducible ability of DCs.

Next, we verified that the functional alteration of DCs observed in pre-formed fibril stimulation was replicated in DCs stimulated with PD-derived peripheral αS seeds. PD-derived αS seeds, but not a healthy donor-derived control product, increased the ratio of CD86-expressing DCs and the production of IL-1β, IL-6, and IL-23 (Fig. 3E–G). These results demonstrated that, when DCs were exposed to extracellular αS aggregates, they were activated and produced cytokines critical for the induction of Th17 cells. Indeed, IL-23 and IL-6 levels were significantly increased in plasma of PD patients compared with those in controls (Supplementary Fig. 10, Supplementary Table 3).

αS was reported to activate TLR1/2 and TLR4; therefore, we examined whether TLR1/2 and TLR4 were involved in αS-mediated DC activation leading to the induction of Th17 cells. As a positive control, we investigated the inhibitory effects of the TLR1/2 or TLR4 inhibitors, TLR2-IN-C29 and TAK-242, respectively, on the activation and/or cytokine production of DCs stimulated with PamCSK4 or LPS, respectively. Treatment with TAK-242 and αS fibrils inhibited the ratio of HLA-DR^{high}CD86^{+} DCs and the production of IL-6 and IL-23 compared with αS fibrils alone (Fig. 3H, I). These results indicate that TLR4 is involved in αS-mediated DC activation and Th17-inducible cytokine production. In contrast to the significant inhibitory effects of TAK-242 on IL-23 production, we found no significant difference in the suppression of IL-23 and IL-1β production by TLR2-IN-C29, although the inhibitory effects on IL-6 production were significant (Supplementary Fig. 11). These results indicate that TLR4 activation upon αS fibril stimulation is an important pathway involved in IL-6 and IL-23 production, as well as DC activation.

Involvement of TLR4-XBP-1s signaling in the induction of Th17-inducible cytokines

Next, we examined the signals downstream of TLR4 that are involved in the production of the Th17-inducible cytokines, IL-6 and IL-23. Recently, several studies have reported that TLR4 signaling and ER stress responses are interconnected [48], resulting in induction of the Th17-inducible cytokines IL-6 and IL-23 [49]. The inositol-requiring enzyme 1 (IRE1)-mediated pathway is a well-known ER stress pathway that contributes to the production of IL-6 and IL-23. IRE1 possesses an endoribonuclease domain that mediates the unconventional splicing of the mRNA encoding the transcription factor XBP-1s [50]. Because XBP-1s promotes the induction of IL-6 and IL-23, we investigated the contribution of XBP-1s to the αS fibril-mediated increase of IL-6 and IL-23 production. We evaluated the expression of XBP-1s in αS-stimulated mDCs. αS fibril stimulation increased the expression of XBP-1s in mDCs compared with no stimulation or αS monomer stimulation (Fig. 4A, B).

Next, we examined whether XBP-1s expression in DCs from PD patients was increased. We focused on type 2 conventional DCs (cDC2s), which prime CD4 T cells [51]. XBP-1s expression in cDC2s was increased

(See figure on next page.)

Fig. 3 Functional alterations of α-synuclein-stimulated DCs. Monocyte-derived DCs (mDCs) generated from healthy subjects (Table 1) were used for the following assays. (**A**–**D**) mDCs were cultured in the presence or absence of the indicated concentrations of αS monomers or fibrils for 24 h. (**A**, **B**) The HLA-DR^{high}CD86^{+} ratio in CD209^{+} mDCs was analyzed. Gating strategies are shown in Supplementary Fig. 7. A representative contour plot is shown in (**A**) and the HLA-DR^{high}CD86^{+} ratio in CD209^{+} mDCs (n = 8) is shown in (**B**). (**C**, **D**) IL-1β, IL-6, and IL-23 concentrations in culture supernatants stimulated as indicated (n = 8) are shown in (**C**) and a comparison of IL-23 and IL-12 levels after high-dose αS fibril stimulation (n = 8) is shown in (**D**). (**E**–**G**) mDCs (n = 4) were cultured for 48 h in the presence of 1000-fold diluted αS seeds. Three αS seeds derived from PD patients (#1–#3) and one control product derived from a healthy donor (control) were used for stimulation. (**E**, **F**) CD86 expression in CD209^{+} mDCs was analyzed. A representative histogram is shown in (**E**) and the CD86^{+} ratio in CD209^{+} mDCs is shown in (**F**). (**G**) IL-1β, IL-6, and IL-23 concentrations in culture supernatants were measured. (**H**, **I**) mDCs were treated with a TLR4 inhibitor, TAK-242 (60 μM), or control vehicle for 2 h prior to 24 h stimulation with high-dose (50 μg/ml) αS fibrils (n = 6) or LPS (n = 4–5). The HLA-DR^{high}CD86^{+} ratio in CD209^{+} mDCs is shown in (**H**), and IL-1β, IL-6, and IL-23 concentrations in culture supernatants are shown in (**I**). stimu, stimulation; mono, monomer; TAK, TAK-242. Each dot indicates the value of one individual. Data represent the mean ± SD (**B–D, F–I**). *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. P-values were determined by the Friedman test (**B**, **C**, **F**, **G**) or Wilcoxon's matched-pairs signed-rank test (**D, H, I**)

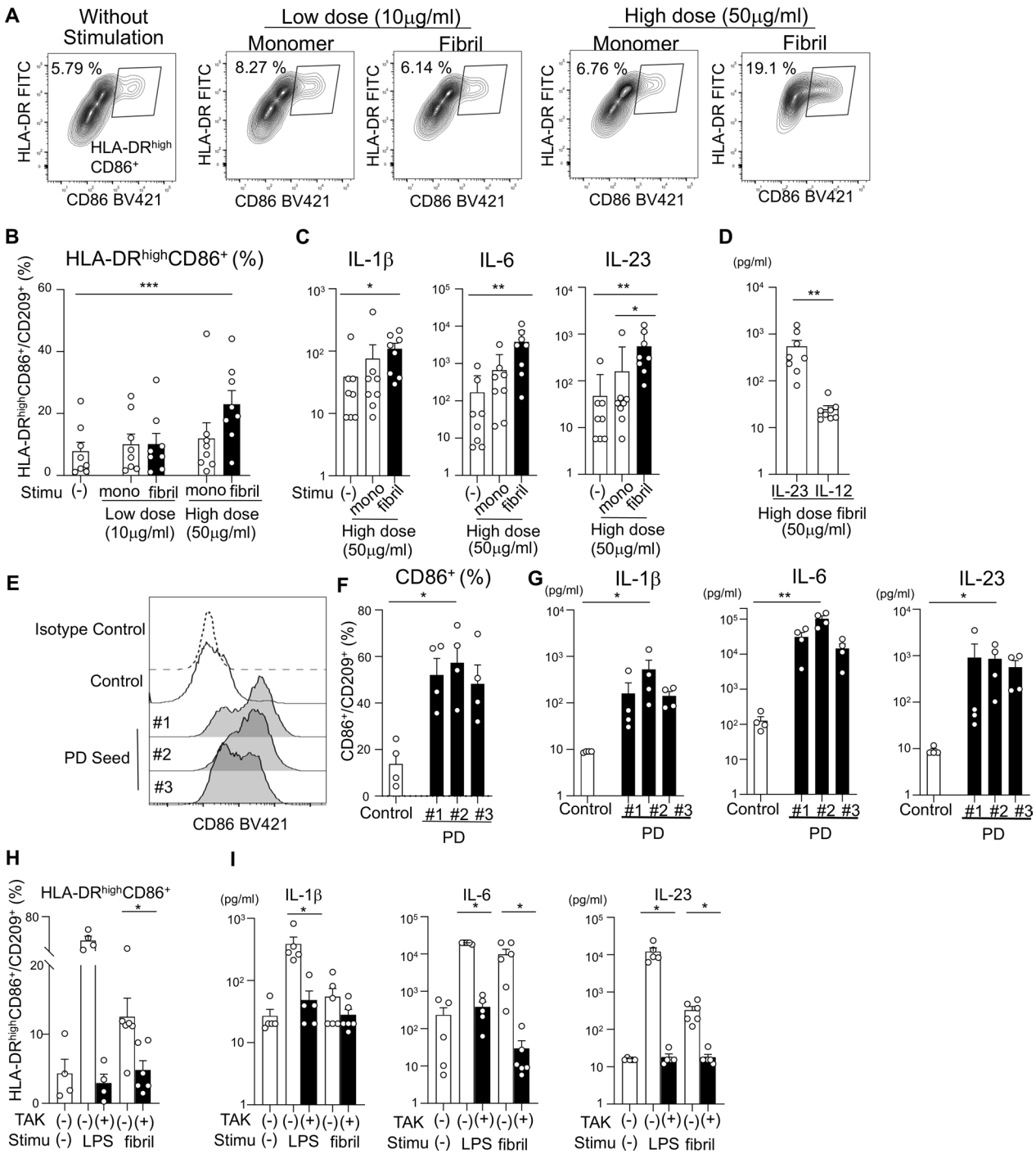

Fig. 3 (See legend on previous page.)

in PD patients compared with that in controls (Fig. 4C, Supplementary Fig. 12, Supplementary Table 4). The administration of TAK-242 with αS fibrils suppressed XBP-1s expression in an in vitro culture of mDCs (Fig. 4D, E), indicating that αS-mediated TLR4 signaling contributes to XBP-1s expression. The suppression of IL-6 and IL-23 production by an XBP-1s inhibitor, STF-083010, confirmed that TLR4-XBP-1 s signaling was linked to the αS fibril-mediated increase of Th17-inducible cytokine production (Fig. 4F, G).

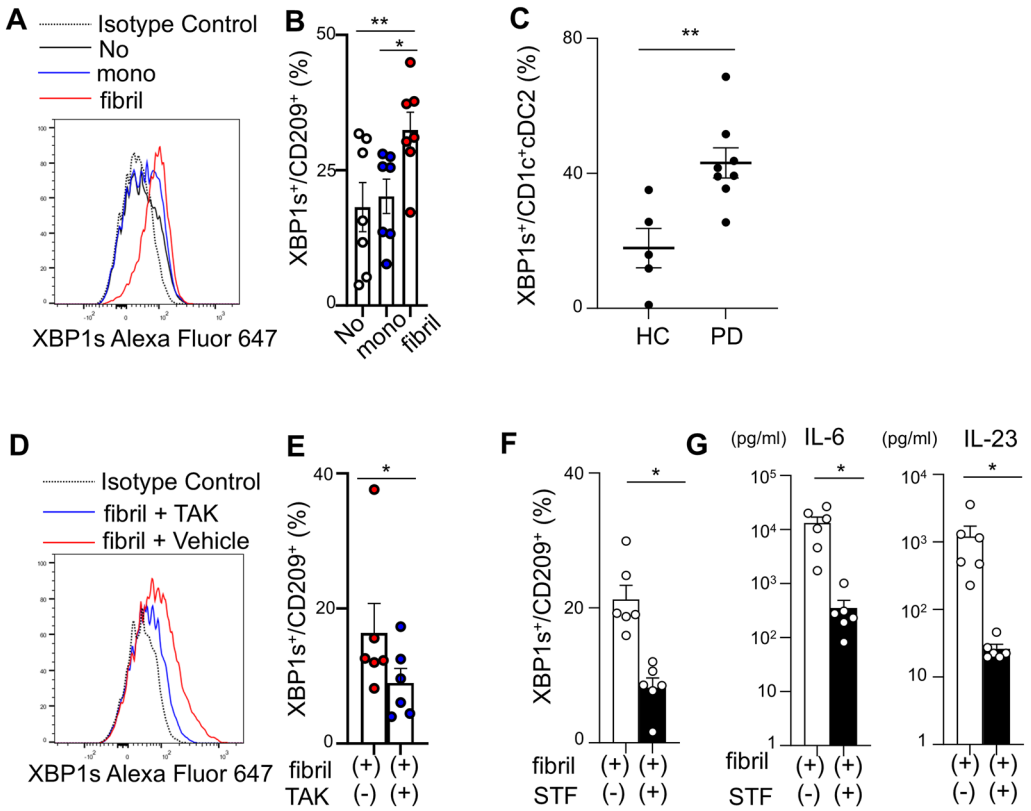

Fig. 4 XBP-1s expression in α-synuclein-stimulated DCs. (**A**, **B**) mDCs generated from healthy subjects (Table 1) were cultured in the presence or absence of high-dose (50 μg/ml) αS monomers or fibrils for 24 h. XBP-1s expression in $CD209^+$ mDCs was analyzed. Gating strategies are shown in Supplementary Fig. 7. A representative histogram is shown in (**A**) and the XBP-1 s^+ ratio in $CD209^+$ mDCs (n = 7) is shown in (**B**). (**C**) Cryopreserved PBMCs from PD patients (n = 8) and age-matched controls (n = 5) were subjected to flow cytometric analysis (Supplementary Table 4). Gating strategies are shown in Supplementary Fig. 12. The XBP-1 s^+ ratio in $CD1c^+$ type 2 conventional DCs (cDC2s) is shown. (**D**–**G**) mDCs generated from healthy subjects (Table 1, n = 6) were treated with a TLR4 inhibitor, TAK-242 (60 μM) (**D, E**), or an IRE1-XBP-1s inhibitor, STF-083010 (120 μM) (**F**, **G**), for 2 h prior to 24 h stimulation with high-dose (50 μg/ml) αS fibrils. Vehicle containing the same concentration of DMSO was used as a control. (**D**–**F**) XBP-1s expression in $CD209^+$ mDCs was analyzed. A representative histogram is shown in (**D**), and the XBP-1s^+ ratio in $CD209^+$ mDCs is shown in (**E**, **F**). (**G**) IL-6 and IL-23 concentrations in the culture supernatants were measured. mono, monomer; TAK, TAK-242; STF, STF-083010; XBP-1s, spliced X-box binding protein-1. Each dot indicates the value of one individual. Data represent the mean ± SD. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. P-values were determined by the Friedman test (**B**), the Mann–Whitney *U*-test (**C**), or Wilcoxon's matched-pairs signed-rank test (**E**–**G**)

Differential T cell responses in PD, DLB, and MSA

Finally, we investigated peripheral αS-specific T cell immunity in patients with synucleinopathies other than PD, and patients in the preclinical phase of synucleinopathies, RBD (Supplementary Tables 5–7). We analyzed whether the C-terminal pS129 fragment was important for the induction of cytokine production in PBMCs from patients with PD, as well as PDD/DLB and MSA. In the analysis focused on IL-17A, IL-17F, IL-22, IFN-γ, and IL-13, stimulation with C-terminal pS129 peptides increased the fold changes of IL-17A, IL-22, IFN-γ, and IL-13 in PBMCs from PDD/DLB patients, and the fold changes of IL-17A and IFN-γ in PBMCs from MSA patients, compared with those in age-matched controls (Fig. 5A). For cytokine production other than the above five cytokines, stimulation with C-terminal pS129 peptides increased the fold changes of IL-6, IL-10, and TNF-α in PBMCs from PDD/DLB patients and MSA patients compared with those in age-matched controls (Supplementary Fig. 13). In addition to the C-terminal pS129 peptides, stimulation with aa1-40 αS peptides increased IL-17A and TNF-α levels, and aa31-46 αS peptides increased TNF-α levels in MSA patients (Supplementary Fig. 13, 14).

To investigate whether reactivity to αS peptides could be detected before the onset of synucleinopathy, we examined reactivity to αS peptides in RBD patients. Although the fold changes of IL-22 and IFN-γ increased slightly upon the stimulation of PBMCs with C-terminal pS129 peptides, we did not observe a significant increase in cytokine levels in RBD patients compared

with those in controls upon stimulation with αS peptides (Fig. 5A, Supplementary Fig. 15). These results suggest that αS-specific T cell responses in the periphery are not present in RBD patients.

Next, we compared Th-related cytokine levels upon stimulation with pS129 peptides in PD, PDD/DLB, and MSA patients. The expression levels of IFN-γ in response to pS129 peptides did not differ between patients with PD, PDD/DLB, and MSA (Fig. 5B), and they remained at a higher level than that observed in age-matched controls (Fig. 5A). These observations suggest that there is no discernible difference in IFN-γ responsiveness to pS129 peptide among PD, PDD/DLB, and MSA. However, trends in IL-17A and IL-22 levels in response to pS129 peptides differed between PBMCs from PD, PDD/DLB, and MSA patients. The fold changes of IL-17A and IL-22 were lower in PBMCs from MSA patients than those from PD patients, and the fold change of IL-22 was lower in PBMCs from MSA patients than in those from PDD/DLB patients (Fig. 5B). These results indicated that the Th17 responses in MSA patients were lower than those in LB disease patients.

We investigated the effects of PD-, PDD/DLB-, or MSA-derived αS seeds on the generation of Th17-inducible DCs using serum-derived αS seeds for stimulation. Supplementary Fig. 16 shows that mDCs stimulated with patient-derived αS seeds had higher CD86 expression, and IL-1β, IL-6, and IL-23 production than mDCs stimulated with a healthy donor-derived control product. CD86 expression levels were comparable between mDCs stimulated with PD-derived, PDD/DLB-derived, or MSA-derived αS seeds (Fig. 5C). However, regarding cytokine production, PDD/DLB-derived αS seeds induced significantly less IL-1β and IL-23 production than PD-derived αS seeds (Fig. 5D). In addition, MSA-derived αS seeds induced less IL-1β and IL-23 production than PD-derived αS seeds, although this did not reach significance. These results suggest that qualitative and quantitative variations in αS seeds in synucleinopathies may affect the Th17-inducible capacity of DCs.

Discussion

In this study, we showed that T cells from synucleinopathy patients responded to C-terminal pS129 αS peptides. Of those PD patients who had reactivity to the pS129 peptide, a negative correlation was observed between Th1 and Th17 responses, suggesting that pS129 peptide-specific T cell responses are Th17-skewed in some patients and Th1-skewed in others. Enhanced Th17 responses were observed in patients with progressive clinical symptoms. Recently, elevated circulating αS levels have attracted attention as a potential biomarker that reflects disease progression [37, 38]. Considering this, our research focused on αS-mediated functional alterations in DCs as a potential mechanism by which enhanced Th17 responses are observed in PD patients. Results from in vitro experiments showed that αS fibrils induced IL-23 production in DCs through TLR4-XBP-1 s signaling, suggesting that αS aggregates released from neurons and other cell types not only serve as antigens to elicit T cell responses, but also enhance αS-specific Th17 responses via adjuvant effects on DCs. Finally, we demonstrated that IFN-γ production induced by pS129 peptides was similar between PD, PDD/DLB, and MSA patients. However, Th17 responses to pS129 peptides were lower in MSA patients than in PD patients. Therefore, the PD-specific disease environment may amplify the generation of pS129 peptide-specific Th17 cells.

Among the αS peptides, the C-terminal pS129 peptides were the most effective at inducing cytokines in PD patients, suggesting that these C-terminal pS129 αS fragments are highly immunogenic. These observations were consistent with previous reports [23, 25]. Because stimulation with pS129 peptides significantly increased Th-related cytokine levels compared with stimulation using unphosphorylated αS peptides, the phosphorylation

(See figure on next page.)

Fig. 5 Differential responses to C-terminal phosphorylated S129 peptides among synucleinopathies. (**A**, **B**) PBMCs from patients or controls were cultured in the presence or absence of C-terminal phosphorylated S129 peptide pools (aa116–140) for 7 days. PBMCs were isolated from the following participants: PD (n = 33), dementia with Lewy bodies or PD with dementia (PDD/DLB, n = 8), multiple system atrophy (MSA, n = 28), rapid eye movement sleep disorder (RBD, n = 16), and healthy controls (n = 15). The data sets of PD and HC were from Fig. 1. The indicated cytokine concentrations in the culture supernatants were measured, and the fold change was calculated. Each dot indicates the value of one individual. The line represents the median. (**A**) Comparisons between patients and age-matched controls. The following numbers of age-matched controls were used: vs. PD: n = 15, vs. PDD/DLB: n = 5, vs. MSA: n = 15, and vs. RBD: n = 8 (Supplementary Tables 1, 5–7). (**B**) Comparisons between PD, PDD/DLB, and MSA. (**C**, **D**) mDCs derived from healthy donors (n = 3) were cultured in the presence of 1000-fold diluted αS seeds for 48 h. Individual values are shown in Supplementary Fig. 16. The representative value of each seed was determined by averaging values obtained from three donor-derived mDCs. Comparisons were conducted between PD seeds (#1–#3), PDD/DLB seeds (#1–#3), and MSA seeds (#1–#3). The $CD86^+$ ratio in $CD209^+$ mDCs is shown in (**C**). IL-1β, IL-6, and IL-23 concentrations in culture supernatants are shown in (**D**). Data represent the mean ± SD. HC, healthy control. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. P-values were determined by the Mann–Whitney *U*-test, in (**A**) and the Kruskal–Wallis test in (**B**–**D**)

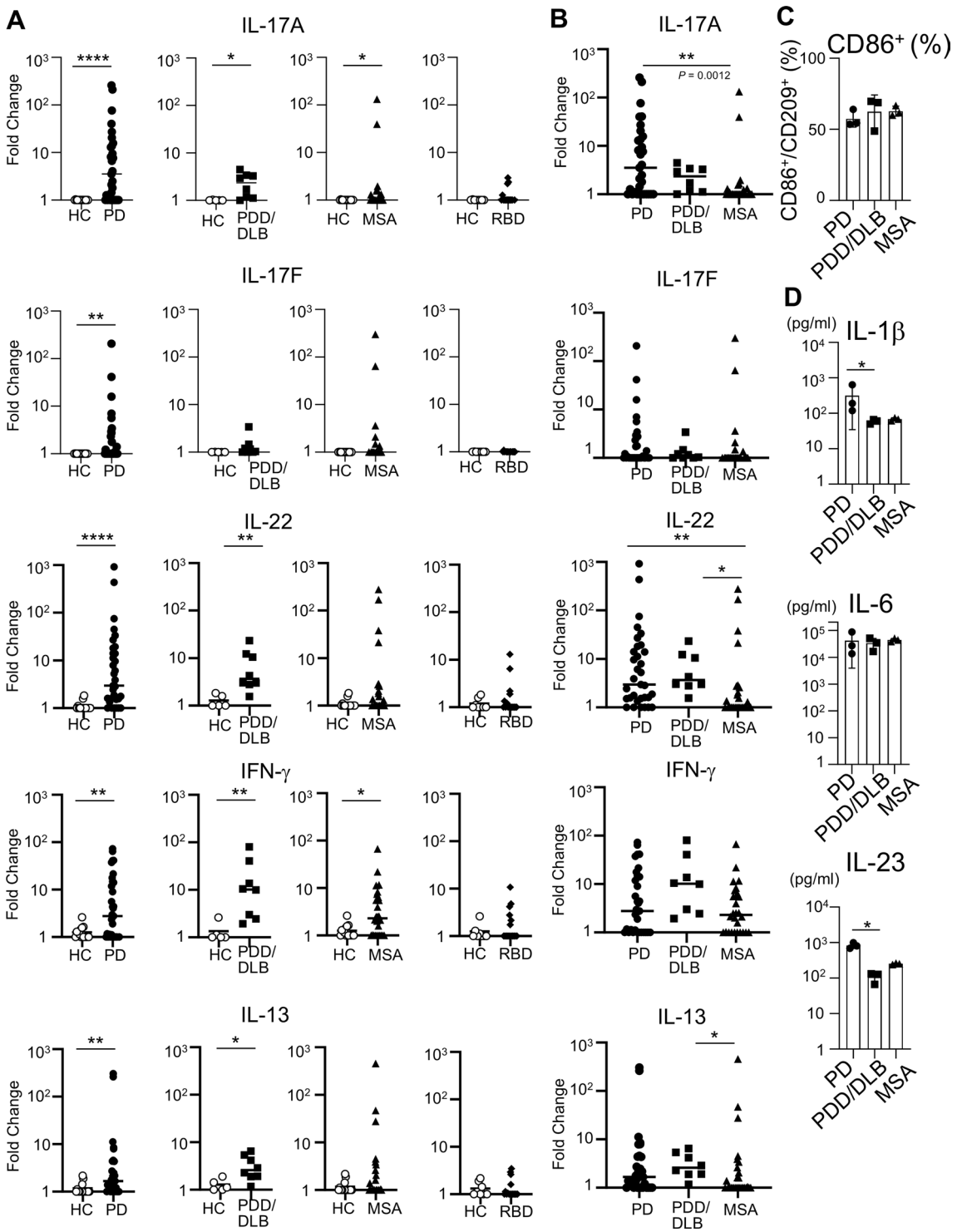

Fig. 5 (See legend on previous page.)

of S129 may provide a neoepitope that allows escape from negative selection in the thymus. Similar concepts have been proposed in studies of autoimmune diseases such as celiac disease and type 1 diabetes, showing that post-translational modifications generate neoepitopes that stimulate CD4 T cells [52].

As a signature of the peripheral T cell responses in PD patients, we found a positive correlation between pS129

peptide-specific Th17 responses and MDS-UPDRS Part III scores. In the intracellular staining assay, the disease duration associated with the 14 samples used was 8 ± 3 years; however, in the assay using the culture supernatant of the pS129 peptide culture, 12/33 cases had a disease duration exceeding 10 years. Analysis of IL-17A levels in culture supernatant in response to pS129 peptides showed a positive correlation between MDS-UPDRS Part III score and IL-17A levels in cases with disease duration of 10 years or less, but IL-17A levels tended to decrease in cases with disease duration of more than 11 years. These results suggest that the pS129 peptide-specific Th17 response may play an important role in the progression of PD within 10 years of disease onset. αS-specific Th17-cell responses may become quiescent after a decade of disease progression, at which point neurodegeneration is fully established [53]. Another possibility is that pS129 peptide-specific Th17 cells accumulate in the CNS in very advanced disease with a long duration. Further research to identify αS-specific T cells in CNS may reveal why pS129 peptide-specific Th17 responses decline in patients with a disease duration of 11 years or more.

We investigated the mechanism behind the increased Th17 responses in PD patients. Given the growing interest in elevated circulating αS levels as a potential biomarker for disease progression [37, 38], we investigated the function of extracellular αS as a DAMP. Although αS is an antigen that induces T cell responses, another important role suggested by studies using microglia or astrocytes is the activation of innate immunity via TLR signaling [42–44]. However, the effects of αS as a DAMP on the induction of Th17 responses and their intracellular mechanisms have not been reported. The present study showed that αS fibrils activated TLR4 and increased the production of Th17-inducible cytokines, including IL-6 and IL-23, in addition to the induction of co-stimulatory molecules. Furthermore, αS fibril stimulation produced more IL-23 than IL-12, suggesting that αS fibrils as DAMPs make an important contribution to Th17-cell induction. Indeed, we observed a disease progression-dependent elevation in Th17 responses not only to αS peptides but also to *C. albicans* peptides, which are potent inducers of Th17 responses and are suitable for assessing Th17 responses. In addition to our observations, several studies of antigen non-specific Th17 responses revealed that advanced PD patients exhibit increased Th17 responses [54, 55]. The increase in plasma IL-23 levels may be related to this observation. These observations, together with reports showing elevated αS levels in advanced PD patients [37, 38], suggest that increased peripheral αS levels associated with disease progression may enhance the ability of DCs to induce Th17 responses, leading to augmented Th17 responses. The transcription factor XBP-1s was reported to be involved in the regulation of IL-6 and IL-23 expression in DCs [50], and we found that XBP-1s expression was upregulated in αS fibril-stimulated DCs and suppressed by the inhibition of TLR4 signaling. These results indicate that XBP-1s expression was induced upon TLR4 activation via αS fibril stimulation. XBP-1s is an unfolded protein response (UPR)-associated molecule for ER homeostasis that is induced via IRE1α, an ER transmembrane protein sensor. Furthermore, IRE1-XBP-1s signaling is involved in the regulation of IL-6 and IL-23 expression [50]. Recently, TLR4 signaling and ER stress responses were reported to be interconnected, and TLR4 activation induced XBP-1s expression even in the absence of UPR [48]. In our study, TLR4 signaling was involved in αS fibril-mediated XBP-1s expression, suggesting that TLR4 activation rather than UPR may contribute to XBP-1s expression. Furthermore, inhibition of the IRE1-XBP-1s pathway decreased IL-23 production, indicating that the TLR4-XBP-1s pathway contributes to αS fibril-mediated IL-23 production. The increased expression of XBP1s in cDC2s from PD patients and the increased levels of IL-6 and IL-23 in plasma from PD patients support the idea that the αS aggregates-TLR4-XBP1s pathway promotes IL-6 and IL-23 production in DCs from PD patients. The TLR4-XBP-1s-IL-23 pathway in αS-exposed DCs may serve as a novel target to modulate Th17 responses.

Finally, we compared the characteristics of αS-specific T cells between PD, PDD/DLB, and MSA patients with different αS propagation profiles, and RBD patients in the preclinical phase. In RBD patients, αS peptide stimulation induced little cytokine production, whereas IL-22 and IFN-γ production to S129 peptides was slightly increased in some patients. One explanation for this is that the limited temporal progression of the αS pathology in the prodromal phase may fail to produce sufficient amounts of αS aggregates to generate αS-specific T cells. Unlike RBD patients, increased IFN-γ production induced by pS129 peptides was observed in all post-onset synucleinopathy patients, including those with PD, PDD/DLB, or MSA. These results suggest that post-onset synucleinopathy patients have αS-specific T cells. However, there were differences in Th17 responses between synucleinopathies. Compared with PD patients, significantly lower Th17 responses were observed in MSA patients, suggesting that the PD-specific disease environment contributes to the generation of αS-specific Th17 responses. Although relatively lower Th17 responses were observed in PDD/DLB patients than in PD patients, this did not reach

statistical significance. This may be due to limitations in the number of PDD/DLB patients recruited to this study.

In this human study, we propose the importance of αS-specific Th17 responses in PD. There were differences in the levels of αS-specific Th17 responses between patients with PD, PDD/DLB, and MSA, despite all of these being αS-mediated diseases. In PD, pS129 peptide-specific Th17 responses were closely related to disease progression. Moreover, we identified the TLR4-XBP1s-IL-23 pathway in αS aggregate-stimulated DCs as the mechanism involved in promoting Th17 responses. Therefore, when future studies elucidate the role of pS129 peptide-specific Th17 cells in neurodegeneration, novel therapies targeting the regulation of Th17 cells can be developed. Elucidating the characteristics of αS-specific T cells associated with disease progression and their induction mechanisms might provide clues for developing novel therapeutic strategies for synucleinopathies.

Conclusions

In conclusion, our results show that pS129 αS peptide-specific Th17 responses in the periphery are positively correlated with disease progression, suggesting that the importance of pS129 peptide-specific Th17 responses in the progression of PD. As a candidate mechanism for promoting Th17 responses, we found the TLR4-XBP1s-IL-23 pathway in αS aggregate-stimulated DCs.

Abbreviations

αS	α-synuclein
CNS	Central nervous system
CMV	Cytomegalovirus
DAMP	Damage-associated molecular pattern
DLB	Dementia with Lewy bodies
IRE1	Inositol-requiring enzyme 1
mDC	Monocyte-derived dendritic cell
MDS-UPDRS	Movement Disorder Society-Unified Parkinson's Disease Rating Scale
MSA	Multiple system atrophy
PD	Parkinson's disease
PBMC	Peripheral blood mononuclear cell
PLK3	Polo-like kinase 3
RBD	Rapid eye movement sleep behavior disorder
RT-QuIC	Real-time quaking-induced conversion
XBP1s	Spliced X-box binding protein-1
cDC2	Type 2 conventional DC

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12974-025-03359-w.

Supplementary material 1.

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Author contributions

S.M. conceived the study. E.-F.N., S.Y., A.C., N.H., and S.M. designed the experiments. E.-F.N., B.S., K.R., and Y.I. performed cell cultures, cytokine measurements, and flow cytometric analyses. A.O. and H.K. performed the purification of recombinant αS, the preparation of pre-formed fibrils, and IP/RT-QuIC. E.-F.N. performed αS phosphorylation and western blotting. A.O., S.U., Y.H., T.H., and N.H. obtained patient samples. E.-F.N., B.S., and K.R. analyzed the data. E.-F.N. and S.M. wrote the paper.

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Data availability

The datasets used and/or analyzed for the current study are available from the coresponding author upon reasonable request.

Declarations

Ethics approval and consent to participate

This study was approved by the Ethics Committee of Juntendo University (no. 2021100). Written informed consent was obtained from all participants before enrollment.

Consent for publication

Not applicable.

Competing interests

Outside of the submitted work, NH received grants from Asahi Kasei Medical Co., Ltd., SNBL, Ltd., FP Corp., and Eisai Co., Ltd.; funds for contract research from Cell Source Co., Ltd., MJFF, and MDS; and reports donations to the department, endowed research departments, and joint collaborative research departments from Sumitomo Pharma Co., Ltd., Otsuka Pharmaceutical, Co., Ltd., Takeda Pharmaceutical Co., Ltd., Kyowa Kirin Co., Ltd., Sunwels Co., Ltd., Eisai Co., Ltd., Nihon Medi-physics Co., Ltd., Abbott Japan LLC, AbbVie GK, Medtronic, Inc., Boston Scientific Japan K.K., Ono Pharmaceutical Co., Ltd., Mitsubishi Tanabe Pharma Co., Zebra Co., Ltd., Kowa Co., Ltd., Parkinson Laboratories Co., Ltd., and Ohara Pharmaceutical Co., Ltd.

Author details

[1]Department of Immunology, Juntendo University, Faculty of Medicine, Bunkyo-ku, Tokyo, Japan. [2]Department of Neurology, Juntendo University Faculty of Medicine, Bunkyo-ku, Tokyo, Japan. [3]Neurodegenerative Disorders Collaboration Laboratory, RIKEN Center for Brain Science, Wako, Saitama, Japan.

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