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CD8 T cell nigral infiltration precedes synucleinopathy in early stages of Parkinson's disease

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There is no consensus on the exact role of the adaptive immune system in Parkinson's disease pathogenesis, although there is increasing evidence that it is somehow involved. Moreover, T cell infiltration in the brain has not been thoroughly studied in Parkinson's disease and no study has assessed the infiltration in incidental Lewy body diseases cases that are considered to be early presymptomatic stages of the disease. In this study, we performed an immunohistochemistry/immunofluorescence quantitative and phenotypic assessment of T cell infiltration in human substantia nigra pars compacta and analysed the correlations with neuronal death and synucleinopathy throughout different stages of the disease. We included two groups of incidental Lewy disease in the study. One of the groups, which is believed to be the earliest stage of the disease, showed α-synuclein aggregates only in the olfactory bulb. The second group also presented α-synuclein aggregates in the substantia nigra. We also assessed the formation of different α-synuclein aggregates throughout the different stages of the unified staging system for Lewy body disorders (I to IV). We found that CD8 T cells were increased in diagnosed Parkinson's disease cases compared to the control group and their density positively correlated with neuronal death. Some of the infiltrating CD8 T cells were indeed contacting dopaminergic neurons. No differences were found regarding CD4 T cells. In the earliest stage of the disease, when substantia nigra α-synuclein aggregation is absent, we found a robust CD8 T cell infiltration and no dopaminergic neuronal death yet. Conversely, in the next stage we found neuronal loss and a milder CD8 T cell infiltration. CD8 T cell infiltration paralleled that of α-synuclein accumulation and neuronal death throughout stages II to IV. We also confirmed that CD8 T cells in charge of immune surveillance and involved in the aetiopathogenesis of the disease are equipped with cytolytic enzymes (granzyme A, B and K) and/or proinflammatory cytokines (interferon gamma), and that phenotypic differences were observed between early and late stages of the disease. We also demonstrate that a high proportion of nigral CD8 T cells are tissue resident memory T cells. Our results show that nigral cytotoxic CD8 T cell infiltration is an earlier pathogenic event than α-synuclein aggregation and neuronal death and that it parallels the progression of neuronal death and synucleinopathy in Parkinson's disease. Overall, our study suggests that CD8 T cell cytotoxic attack may initiate and propagate neuronal death and synucleinopathy in Parkinson's disease.

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Abbreviations: CTL = cytotoxic T lymphocyte; iLBD = incidental Lewy body disease; MHC = major histocompatibility complex; SNpc = substantia nigra pars compacta; TRM = tissue resident memory T cell

Introduction

Parkinson's disease is the second most frequent form of neurodegenerative disease. It is caused mainly by the loss of neuromelanin-containing neurons in the substantia nigra pars compacta (SNpc) and locus coeruleus regions of the human brain. One of the neuropathological hallmarks of Parkinson's disease is the presence of Lewy bodies and Lewy neurites in neuromelanin-containing neurons and in other brain areas as well as in the autonomic peripheral nervous system. Several neuron autonomous (Michel *et al.*, 2016) and non-neuron autonomous mechanisms (Caggiu *et al.*, 2019) have been suggested to contribute to neuronal death, but until now there has been no definite evidence to support that one of these is the initiator of the deleterious cascade of events leading to neuronal death. Some of the proposed mechanisms may interact synergistically to trigger cell death, while others may be general indicators of neuronal stress or dysfunction. α-Synuclein is one of the candidates suggested to have a relevant role in this deleterious cascade, but controversy remains over its actual effects. While some authors believe that α-synuclein aggregation participates in neuronal death by disrupting cellular homeostasis (Dehay *et al.*, 2015), others consider that this aggregation is the result of upstream or compensatory mechanisms to cellular stress in order to prevent cell death (Espay *et al.*, 2019). Increasing evidence suggests that both the innate and the adaptive immune systems also contribute to the pathogenesis of Parkinson's disease (Tansey and Romero-Ramos, 2019). To this end, α-synuclein has been proposed as one of the possible triggers of the immune response (Stone *et al.*, 2009; Allen Reish and Standaert, 2015). Regarding the adaptive immune system, both CD4 and CD8 T cell SNpc infiltration have been observed in different Parkinson's disease animal models (Theodore *et al.*, 2008; Brochard *et al.*, 2009; Sanchez-Guajardo *et al.*, 2010; Chandra *et al.*, 2016; Harms *et al.*, 2017). Several studies have confirmed the relevance of T cells, particularly CD4 T cells, in dopaminergic cell loss in these models. In the MPTP mouse model, for example, neuronal loss is attenuated in mice that lack both T and B cells (Lira *et al.*, 2011), in mice exhibiting a global loss of T cells, and in mice lacking CD4 T cells, but not in mice without functional CD8 T cells (Brochard *et al.*, 2009). Moreover, whereas T helper 17 cells exacerbate nigrostriatal dopaminergic cell loss (Benner *et al.*, 2008), adoptive transfer of regulatory T cells has been shown to attenuate such neurodegeneration (Reynolds *et al.*, 2007) in this model. In the Parkinson's disease mouse model based on the overexpression of human α-synuclein, neurodegeneration and T cell infiltration are prevented in a global major histocompatibility complex (MHC) II knockout mouse that has an arrested CD4 T cell population (Harms *et al.*, 2013). A growing body of clinical data suggests that the adaptive immune system is indeed somehow involved in the pathogenesis of Parkinson's disease (Mosley *et al.*, 2012). However, it is not so clear in humans if CD4 T cells are more relevant than CD8 T cells as seen in animal models. For instance, it was reported that although the proportion of activated CD4 T cells remains unchanged in Parkinson's disease CSF, the activated CD8 T cell fraction does increase (Schröder *et al.*, 2018). It was also shown that the CD8 T cell fraction increases in peripheral blood while regulatory T cells are decreased, suggesting a switch from tolerance to immunity (Baba *et al.*, 2005). In fact, dopaminergic neurons express MHC-I under basal/control conditions and are therefore able to present antigens and suffer a cytotoxic attack (Cebrián *et al.*, 2014). In this sense, genome-wide association studies have suggested common genetic pathways between Parkinson's disease and autoimmune diseases (Witoelar *et al.*, 2017). In addition, a higher risk for Parkinson's disease has been associated with structural and regulatory variants in the HLA region (Hamza *et al.*, 2010; Ahmed *et al.*, 2012; Wissemann *et al.*, 2013). In light of this evidence, it is surprising that only one published study has assessed CD4 and CD8 T cell densities in the Parkinson's disease SNpc to address the relevance of each T cell population in the target organ. That study reported a 10-fold increase of both CD4 and CD8 T cells (Brochard *et al.*, 2009), but the results have not been confirmed by an independent laboratory.

The goal of the current neuropathological study was to characterize the T cell infiltration in the SNpc to shed light on their potential role in dopaminergic cell loss and α-synuclein aggregation. We included post-mortem tissue from Parkinson's disease subjects and incidental Lewy body disease (iLBD) cases, with or without synucleinopathy in the SNpc, that represent two early presymptomatic stages of Parkinson's disease. We performed a phenotypic characterization of those CD8 T cells in charge of immune surveillance and those involved in Parkinson's disease aetiopathogenesis. Our results suggest that CD8 T cells may not only propagate neurodegeneration, but initiate neuronal loss and precede α-synuclein aggregation in Parkinson's disease.

Materials and methods

Human post-mortem brain tissue

Paraffin-embedded midbrain sections (15 sections; thickness 6 μm) from deceased Parkinson's disease patients ($n = 15$), iLBD

cases ($n = 9$) and age-matched healthy control individuals ($n = 7$) were provided by Banner Sun Health Research Institute, Sun City, Arizona. The Brain and Body Donation Program operates with the approval of Western Institutional Review Board (Puyallup, WA). Two cases were obtained from the Neurological Tissue BioBank at IDIBAPS-Hospital Clinic (Barcelona). Detailed information about these 31 cases can be found in Supplementary Tables 1 and 2. Supplementary Table 2 contains the available clinical history and autopsy information with all the comorbidities of the subjects included in the study. The selection of these cases was based on neuropathological diagnosis with reference to clinical information provided by the tissue bank.

Immunohistochemistry and immunofluorescence

Before commencing with the immunolabelling protocol, a photobleaching procedure was carried out using a HYG05-MINI-100*w-b LED light source (input voltage 100–240 V AC and frequency of 50–60 Hz, TOPLANET) for 48 h at 4°C for those sections directed to immunofluorescence.

Briefly, deparaffinization was performed at 60°C for 30 min. Afterwards, sections were rehydrated using increasing ethanol concentrations and xylenes. An antigen retrieval protocol was performed using sodium citrate (10 mM; pH 6) in a waterbath at 95°C for 20 min to unmask the antigen. After cooling, sections for immunohistochemistry were quenched in 10% methanol (vol/vol) and 3% hydrogen peroxide (vol/vol) for 5 min. Sections were rinsed three times in 0.1 M Tris buffered saline (TBS) between each incubation period. Species-specific serum and 0.1% Triton^{TM} X-100 were used to block and permeabilize for 1 h at room temperature. Sections were incubated overnight with primary antibodies at 4°C: rabbit anti-human CD4 (1:100; #HPA004252; Sigma-Aldrich), rabbit anti-human CD8α (1:200; #HPA037756; Sigma-Aldrich), rat anti-human CD8α (Supernatant; #NOR132; CNIO), mouse anti-human CD69 (1:25; #310902; BioLegend), rabbit anti-human CD103 (1:100; #ab129202; Abcam), rabbit anti-human FasL (1:500; #HPA054959; Sigma-Aldrich), rabbit anti-human Granzyme A (1:100; #PA5-30054; Thermo Fisher Scientific), mouse anti-human Granzyme B (1:100; #MAB3070; Merck Millipore), rabbit anti-human Granzyme K (1:100; HPA063181; Sigma-Aldrich), rabbit anti-human IFNϒ (1:100; #ab9657; Abcam), mouse anti-human PD-1 (Supernatant; #760-4895; Roche), rabbit anti-human PD-L1 (1:200; #13684; Cell Signaling Technology®), rabbit anti-human Perforin1 (1:100; #ab180773; Abcam), rabbit anti-human pS129 α-synuclein (1:125; #ab51253; Abcam), and rabbit anti-human TNFα (1:500; #ab9635; Abcam). The following day, appropriate secondary antibodies were added for a 1-h incubation at room temperature. For immunohistochemistry, ABC kit (#32050; Thermo Fischer Scientific) and Vector SG (#SK-4700; Vector) were used to reveal antigen-antibody reaction. Afterwards, sections were dehydrated and coverslipped with DPX (Sigma-Aldrich). For the immunofluorescence procedure, nuclei were stained with Hoechst 33342 (1:2000; Thermo Fischer Scientific). Finally, sections were coverslipped using the DakoCytomation Fluorescent Mounting Medium (Dako). All antibodies were tested in positive control tissue to optimize immunohistochemistry conditions. Most of the antibodies were first tested in human tonsil and some in human lymph node (Supplementary Fig. 1).

Quantitative analyses

All SNpc sections were obtained from the level of the third cranial nerve. CD4 and CD8-positive T cell densities were determined using three sections per case and the rest of the parameters were determined using one section. The whole left SNpc was analysed.

T cell density

CD4 and CD8-positive cell densities were quantified using three sections per case for each T cell type. Quantification was performed using a Zeiss AX10 microscope with a motorized stage and Stereo Investigator software. SNpc was delineated with a contour with a 4× magnification lens. Then, all the fields included in this contour were counted with a 20× magnification lens and a 450 × 300 µm counting frame. The average fields counted per section were 140. Infiltrating and perivascular T cells were counted separately with different markers. To obtain the density of T cells, the number of T cells was divided by the area delineated by the contour. Representative images were acquired using an Olympus BX61 microscope fitted with an Olympus DP72 camera.

Dopaminergic neuron density

Dopaminergic neurons containing neuromelanin were counted using one section of SNpc for each case; this was done with a Stemi 2000-C microscope fitted with an AxioCam ERc55 camera.

Density of synucleinopathy-related structures

To detect α-synuclein aggregates, we used pS129 α-synuclein antibody. Lewy bodies, neurons with diffuse synuclein staining, and Lewy neurites were counted using one section of SNpc for each case. A similar methodology to assess T cell density was followed. Representative images were acquired using an Olympus BX61 microscope fitted with an Olympus DP72 camera.

Characterization of CD8-positive T cells

Double staining for CD8 and various markers was performed and reported as the percentage of CD8-positive T cells expressing the specific marker in one section of SNpc for each case. Infiltrating and perivascular T cells were counted separately. Quantification was performed with an Olympus FSX100 microscope and images were acquired using standard filter sets with an Olympus FluoViewTM FV1000 confocal microscope and FV10-ASW 4.2 visualization software.

Statistical analysis

All statistical analyses were performed using GraphPad Prism 6 software (version 6, GraphPad Software, USA). Comparisons between two groups were performed by two-tailed Student's *t*-test or Mann-Whitney U-test for groups with unequal variances. Comparisons between more than two groups of only one variable were done by one-way ANOVA. The Tukey test for *post hoc* multiple comparisons was used as recommended by the software itself. Correlations between two numerical variables

were performed using the Pearson correlation test. Results were considered to be significant for values of $P < 0.05$.

Data availability

The data that support the findings of this study are available from the corresponding author, upon reasonable request.

Results

The density of CD8, not CD4, T cells increased in Parkinson's disease SNpc

T cell traffic into the CNS is thought to occur when activated T cells cross the blood–brain barrier from the perivascular space to the parenchyma where they can interact with target cells. To this end, we counted CD4 and CD8 T cells in age-matched control and Parkinson's disease SNpc tissue to determine the density of non-infiltrating T cells and of cells that are actively infiltrating the brain parenchyma. T cells in the perivascular space and the parenchyma can be easily distinguished by a trained observer even when the perivascular space is collapsed and not perceptible as T cells in this compartment are attached to blood vessels (Fig. 1A and B). Our analysis revealed that CD4 T cells are not abundant in both the parenchyma (0.267 cells/mm^2) and perivascular (0.25 cells/mm^2) spaces of control SNpc tissue. Moreover, the presence of a small number of T helper cells in control SNpc parenchyma was not different from that in SNpc tissue from patients diagnosed with Parkinson's disease (0.295 cells/mm^2) (Fig. 1A), suggesting that there is no infiltration of CD4 T cells in Parkinson's disease. No statistically significant differences were found between groups regarding perivascular CD4 T cells density, although much more dispersion of values were found in the Parkinson's disease group (Fig. 1A). We cannot rule out that perivascular CD4 T cells are playing a role in the aetiopathogenesis of the disease, but no correlation between their density and neuronal loss was found (data not shown). CD8 T cell numbers in the parenchyma of control SNpc (0.86 cells/mm^2) were higher than that of CD4 T cells, but they were much more abundant in the perivascular space (4.5 cells/mm^2) (Fig. 1B). All the parenchymal densities of control cases clustered around 0–1 cells/mm^2 with the exemption of one case that had a higher density. This case was reported to have mild senile intention tremor (Supplementary Table 2, Control-5). Although most of the intention tremors are of cerebellar origin, this higher density may be explained by the presence of this movement disorder. In Parkinson's disease brains, CD8 T cell densities increased 2.5 times (2.1 cells/mm^2) compared to controls in the SNpc parenchyma, demonstrating a nigral infiltration of cytotoxic T lymphocytes (CTLs) in Parkinson disease ($P = 0.0225$). Nevertheless, a considerable variability in cell numbers was observed, which resulted in a non-statistically significant increase of CD8 T cells in the perivascular space (7.3 cells/mm^2) even though some patients showed higher cell densities in this compartment (Fig. 1B).

CD8 T cells density correlates positively with neuronal death in Parkinson's disease

We wanted to explore the possible link of CD8 T cell infiltration to neuronal cell death. CTL-mediated killing requires the migration of CTLs and interaction between their TCR (T cell receptor) and cognate peptide-MHC-I molecules presented by the target cell. Once TCR-triggered immunological synapses with their target are established, the content of their cytotoxic granules is secreted and the cell-death machinery in the target cell is triggered (Kabanova *et al.*, 2018). Dopaminergic neurons can be targeted by CTLs because they express MHC-I (Cebrián *et al.*, 2014). In this sense, we observed CTLs surrounding or contacting dopaminergic neurons; they were also present in regions where extracellular neuromelanin was present as part of the remains of dead neurons in SNpc tissue from Parkinson's disease patients (Fig. 2A). As shown in Fig. 2B, more than one CTL contacted one neuron in some cases, suggesting that CTLs exhibit co-operativity in killing targets as has been demonstrated *in vivo*. The accumulation of sublethal pro-death signals delivered by different single CTLs might lead to faster apoptosis of the target (Halle *et al.*, 2016, 2017). In this regard, we found CTLs surrounding what seemed to be degenerating neurons (Fig. 2B). Halle and collaborators elegantly demonstrated that the average *in vivo* CTL killing capacity is somewhat limited and that CTL densities need to be much higher than in an *in vitro* setting. Along with CTL density, the ratio of CTLs to target cells is a critical parameter for CTL-mediated killing. We found a large increase of this ratio in Parkinson's disease patients ($P = 0.0005$; U = 7) (Fig. 2C), signifying a higher probability of contact with target cells. To examine this concept, we next assessed the relationship between the density of CTLs and the density of dopaminergic neurons among all subjects. As shown in the scatter plot (Fig. 2D), a higher density of CTLs corresponded to a lower density of dopaminergic neurons ($P = 0.0021$; $r = -0.492$). This negative linear correlation between CTL and dopaminergic neuron densities strengthens the idea that CTLs are key elements in neuronal cell death in Parkinson's disease.

CD8 T cell infiltration occurs in absence of neuronal death and synucleinopathy in incidental Lewy body disease SNpc

To assess whether CD8 T cell infiltration may have a relevant role in early stages of Parkinson's disease and to determine its relationship with synucleinopathy, we included two

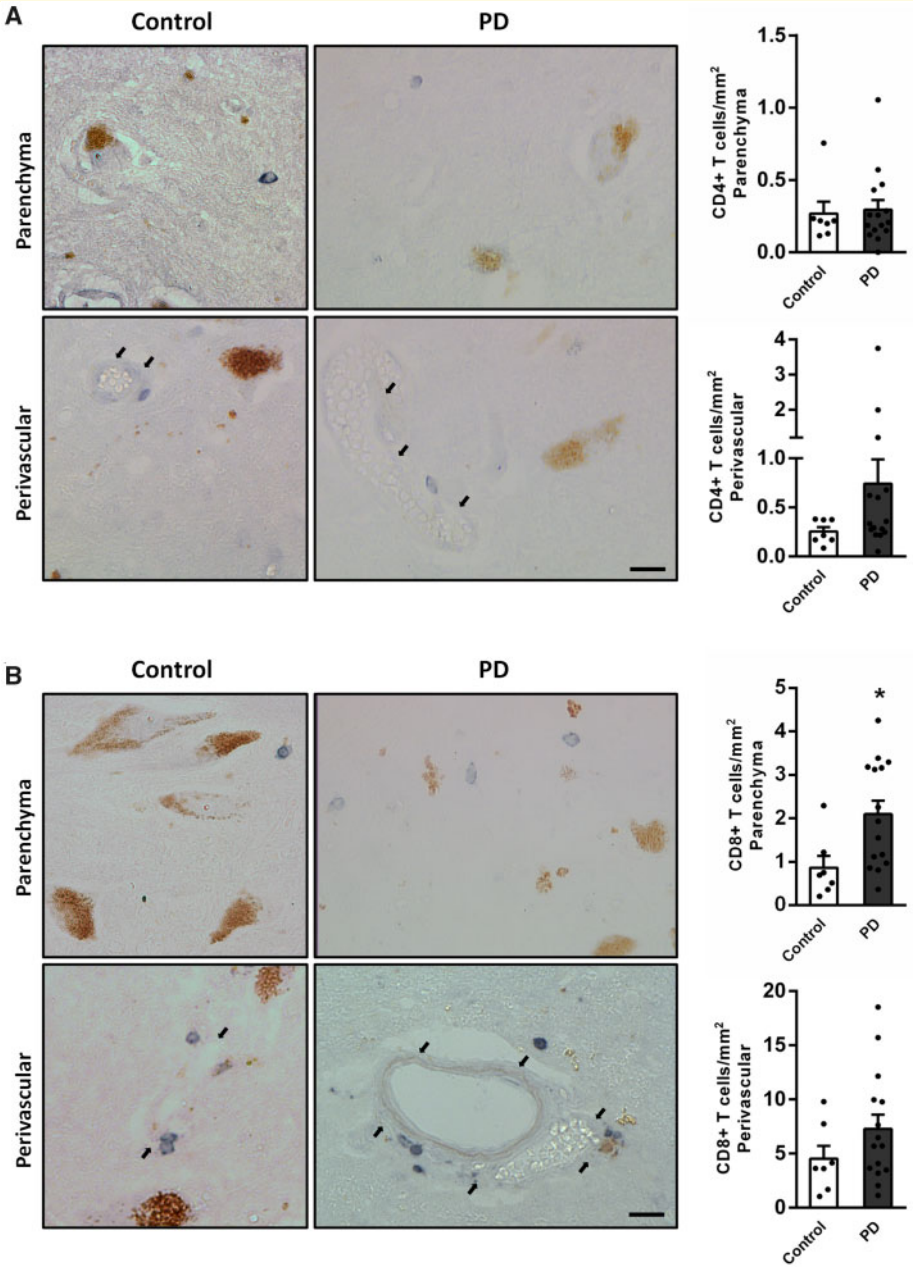

Figure 1 CD4 and CD8 T cell densities in control and Parkinson's disease SNpc. (**A**) *Left*: Representative photomicrographs of parenchymal and perivascular CD4 T cells (blue). *Right*: Density of CD4 T cells in the parenchyma and in the perivascular space (**B**) *Left*: Representative photomicrographs of parenchymal and perivascular CD8 T cells (blue). *Right*: Density of CD8 T cells in the parenchyma and in the perivascular space. Unpaired *t*-test **P*-value < 0.05 compared to control. In all four graphs, bars represent the mean number of cells per mm^2 ± standard error of the mean (SEM). Control ($n = 7$), Parkinson's disease (PD, $n = 15$). Scale bar = 15 μm.

iLBD groups in the study. iLBD cases consist of autopsied control individuals who had Lewy bodies or Lewy neurites without clinical findings of Parkinson's disease and are considered to represent presymptomatic early stages of the disease (Dickson *et al.*, 2008; Iacono *et al.*, 2015). One of the groups showed α-synuclein aggregates in the olfactory bulb but not in the SNpc, while the second group also presented α-synuclein aggregates in the SNpc. For the sake of simplicity, we will call these groups iLBD1 and iLBD2, respectively, because the first group is assumed to represent an earlier stage of the disease (Beach *et al.*, 2009*a*, *b*). We first determined the density of dopaminergic neurons in both groups (Fig. 3A), with the average being 147.9 neurons/mm^2 in iLBD1, which was similar to the mean density in control subjects (144.9 neurons/mm^2). The mean density of dopaminergic neurons in iLBD2 (113.9 neurons/mm^2) was

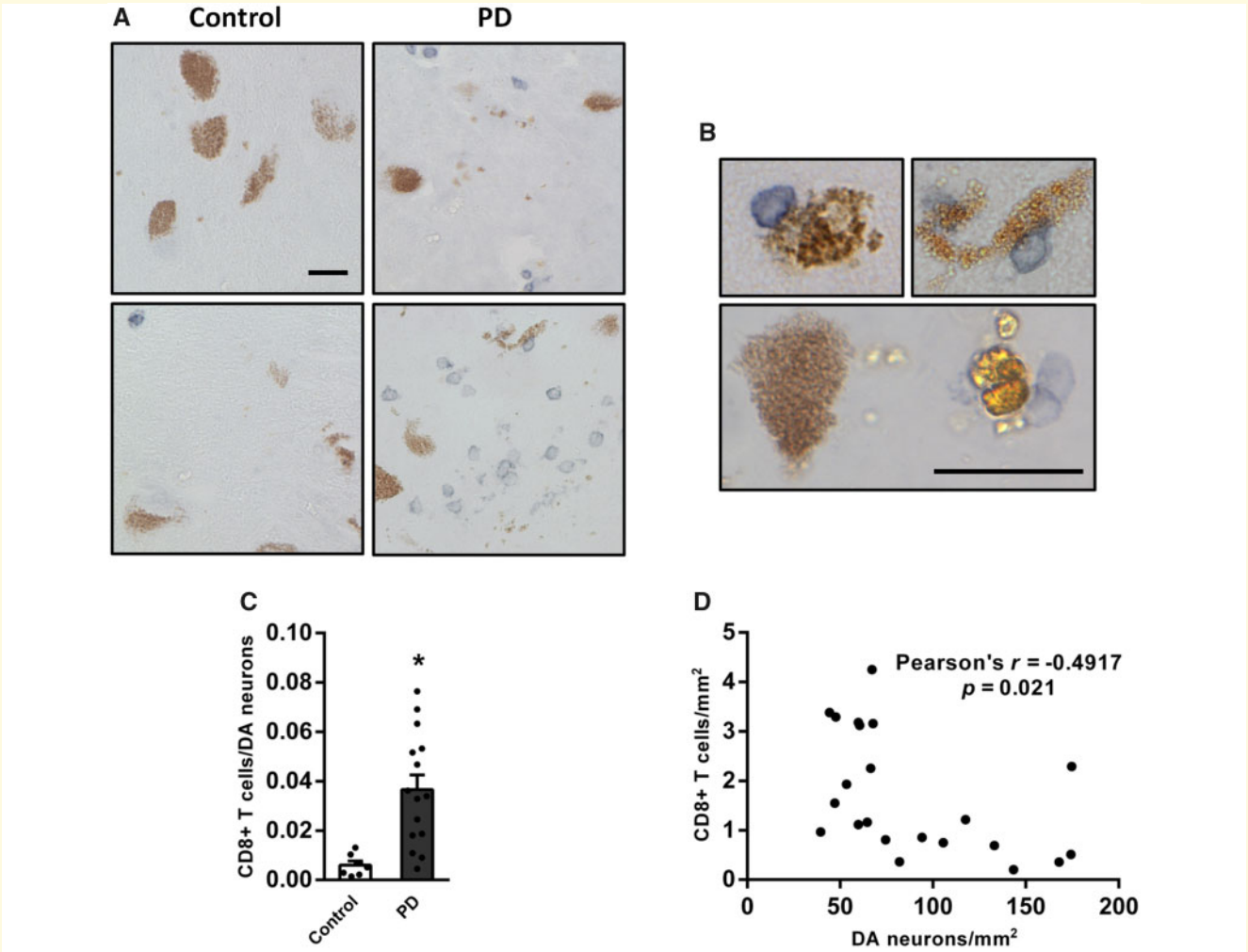

Figure 2 CD8 T cell neuronal contacts/appositions and correlation of CD8 T cell density with dopaminergic cell loss. (**A**) Photomicrographs of CD8 T cells in the SNpc of two controls and two Parkinson's disease (PD) cases. *Top left*: Control photomicrograph shows no parenchymal CD8 T cells, and in the *bottom left* image only one CD8 T cell can be seen. Parkinson's disease cases show much more variability, from just a few CD8 T cells (*top right*) to many (*bottom right*), particularly in regions with extracellular neuromelanin. (**B**) Photomicrographs showing CD8 T cells in close contact with dopaminergic neurons. Neurons in contact with one to three T cells are shown. The neuron contacted by three CTLs (*bottom*) is clearly dying if not already dead. (**C**) CD8 T cell/dopaminergic (DA) neuron ratio in control ($n = 7$) and Parkinson's disease ($n = 15$) groups. Graph bars represent mean value ± SEM. Mann-Whitney test *P-value = 0.0005 compared to control. (**D**) Scatter diagram of dopaminergic neuron density versus CD8 T cell density in all cases. Pearson correlation $r = -0.4917$; P-value < 0.05. Scale bar = 15 μm.

between that seen in control subjects and Parkinson's disease patients (61.9 neurons/mm^2), suggesting that neuronal cell loss occurs concomitantly with the appearance of α-synuclein aggregates ($P < 0.0001$; F = 32.69; $df = 31$). We subsequently assessed CD8 T cell densities in both groups. iLBD1 cases had higher densities of CD8 T cells than iLBD2 cases (Fig. 3B). The average parenchymal density in the iLBD1 cases was even higher than in Parkinson's disease cases (4.6 versus 2.1 CTLs/mm^2) (Fig. 3C), with one of the iLBD1 cases exhibiting the highest CD8 T cell density (7.9 CTLs/mm^2) of all the iLBD and Parkinson's disease cases examined. As seen for Parkinson's disease patients, some CD8 T cells were in contact with dopaminergic neurons (Fig. 3D). In iLBD2, where cell loss and synucleinopathy in the SNpc starts, the parenchymal density of CD8 T cells (1.1 CTLs/mm^2) is more than four times lower than in the iLBD group, indicating a certain degree of inhibition of the immune response. Both groups were statistically different ($P = 0.0317$; U = 1). The same pattern was seen for the perivascular space ($P = 0.0232$; $t = 2.893$; $df = 7$). CTL perivascular densities were also high in the iLBD1 group (7.8 cells/mm^2) as in Parkinson's disease cases, while iLBD2 levels (2.6 cells/mm^2) were even lower than that seen in control subjects (Fig. 3C). These findings suggest that CD8 T cell infiltration precedes both α-synuclein aggregation and neuronal cell death. The fact that we did not observe clear neuronal loss in the earliest stage (iLBD1) even though CD8 T cells were contacting neurons (Fig. 3D), reinforces the idea that the lethality of

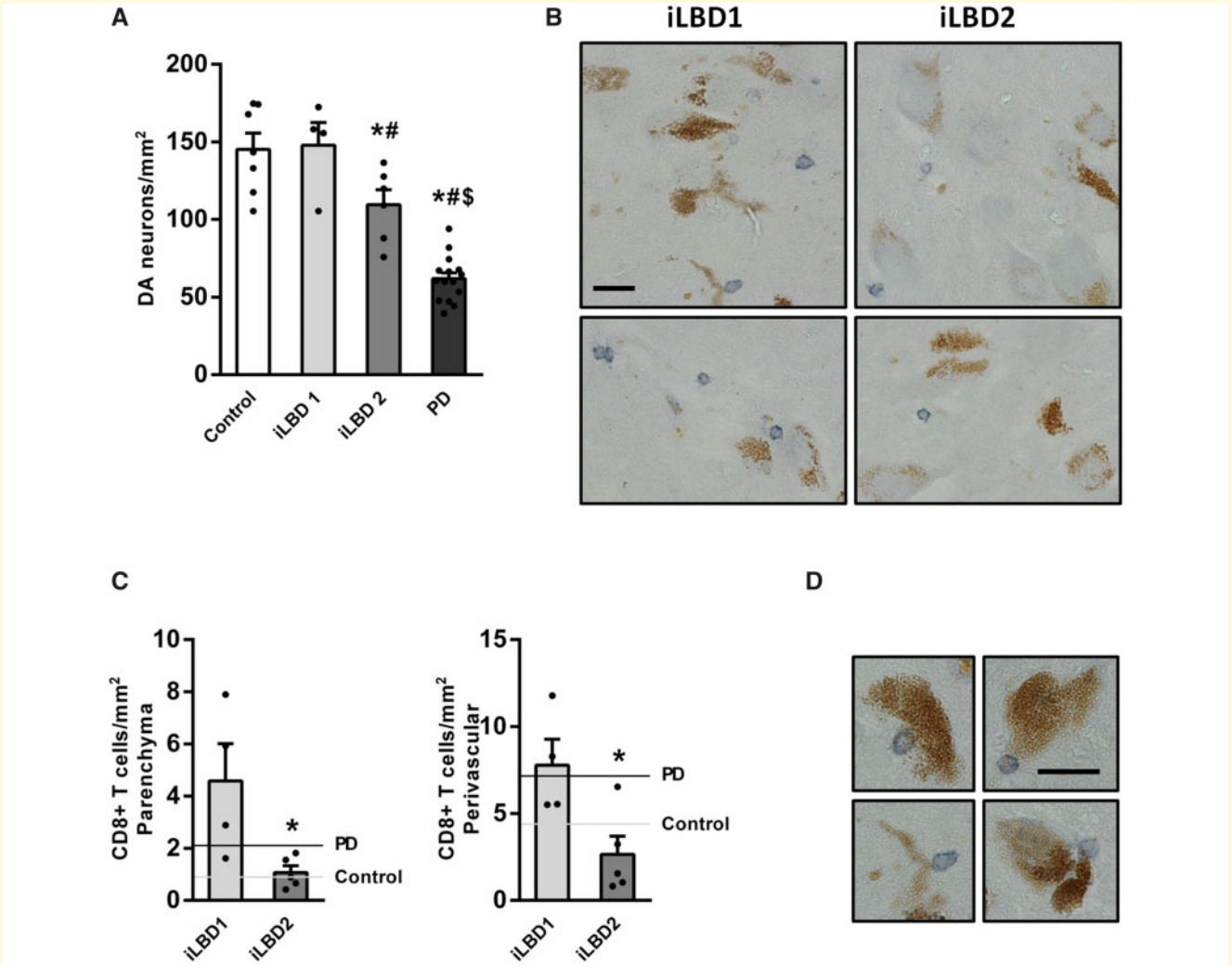

Figure 3 Dopaminergic cell loss, CD8 T cell infiltration and neuronal contacts in iLBD with or without synucleinopathy in the SNpc. Cases of iLBD without α-synuclein aggregates in the SNpc are considered to represent the earliest stage of Parkinson's disease (iLBD1) whereas iLBD cases with α-synuclein in the SNpc represent the next stage (iLBD2). (**A**) Dopaminergic (DA) neuron density in control ($n = 7$), iLBD1 ($n = 4$), iLBD2 ($n = 5$) and Parkinson's disease (PD, $n = 15$) cases. One-way ANOVA; *post hoc* Tukey's test. *P-value < 0.05 compared to controls; $^{\#}P$-value < 0.05 compared to iLBD1; $^{\$}P$-value < 0.05 compared to iLBD2. (**B**) Representative SNpc CD8 T cell (*blue*) photomicrographs of two iLBD1 and two iLBD2 cases. Cases with iLBD1 show much more infiltration of CD8 T cells than iLBD2. (**C**) CD8 T cell SNpc density in the parenchyma (*left*) and perivascular space (*right*). White and black lines show the mean densities of control and Parkinson's disease, respectively, to assist in comparing values. Mann-Whitney test for parenchyma and unpaired *t*-test for perivascular (*P-value < 0.05 compared to iLBD1). Bars in both graphs represent means of neurons or T cells per mm^2 ± SEM. (**D**) Photomicrographs showing CD8 T cells (blue) contacting neuromelanin-containing dopaminergic neurons. Scale bar = 15 μm.

these cytotoxic cells is low, as discussed above. No changes in CD4 T cell density were observed in iLBD subjects (data not shown). To explore the relationship between CTL infiltration and synucleinopathy, we reanalysed CTL densities and neuronal loss according to the unified staging system for Lewy body disorders (stages I to IV) (Beach *et al.*, 2009*a*); iLBD1 and iLBD2 belonged to stages I and II, respectively. Stage III affects the brainstem and limbic structures, while in stage IV synucleinopathy is also found in the cerebral cortex. We assessed the formation of different phospho-α-synuclein aggregates in the SNpc to understand the temporal dynamics of their formation and to determine whether synucleinopathy progressively increases in the SNpc throughout the various stages. We assessed the density of Lewy neurites, the percentage of dopaminergic neurons with α-synuclein diffusely accumulated in the cytoplasm, and Lewy body density regardless of whether the Lewy bodies were located intracellularly or extracellularly. All three parameters progressively increased in each stage, suggesting that α-synuclein aggregates are indeed continuously forming in the SNpc (Fig. 4A). Lewy neurites seemed to form before Lewy bodies because several stage II cases harboured Lewy

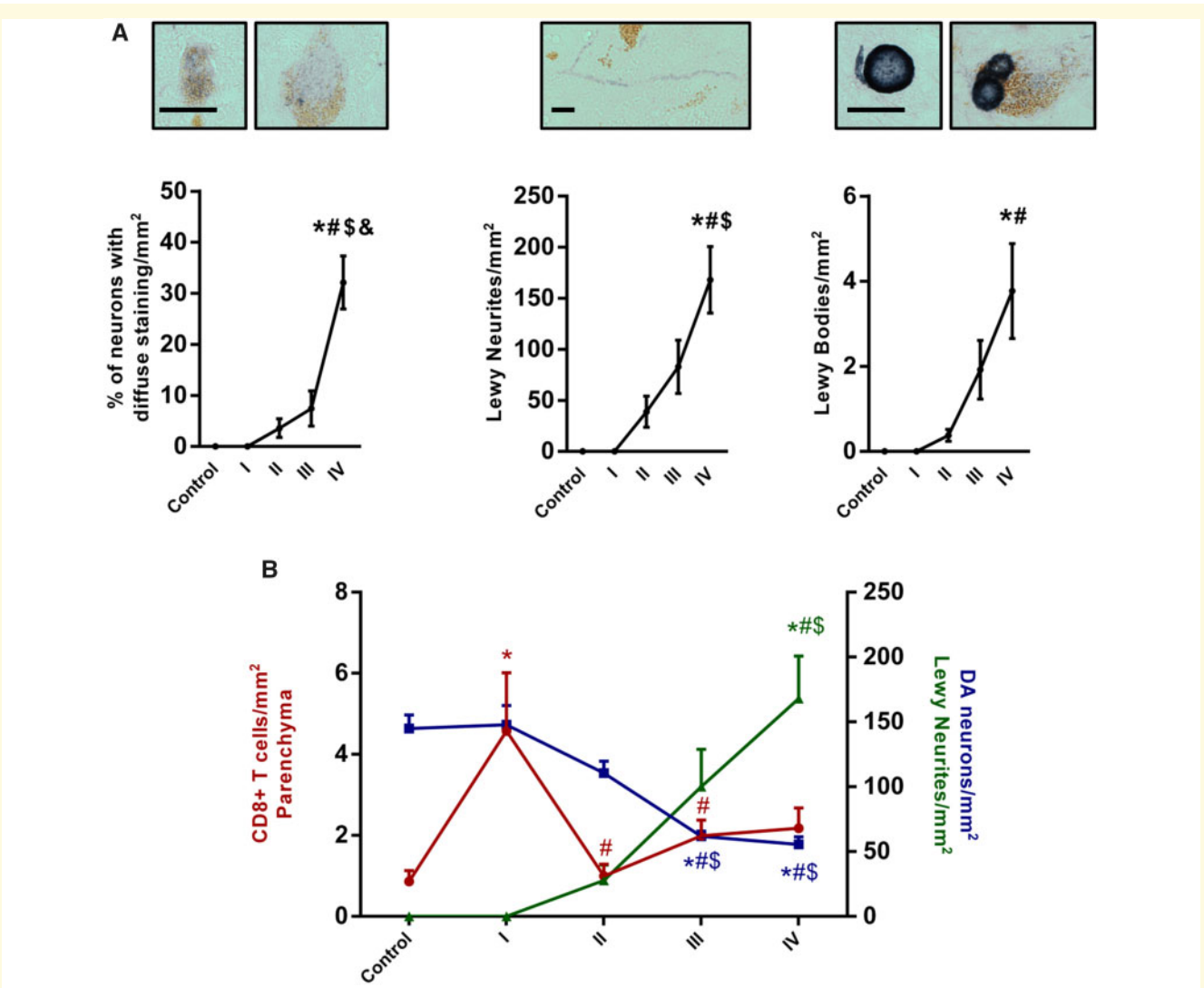

Figure 4 Density of distinct phospho-α-synuclein aggregates at different stages of the unified scale for Lewy body disorders and their relationship with neuronal loss and CD8 T cell infiltration. (**A**) From *left* to *right*: Density of neurons with diffuse aggregates ($P < 0.0001$; F = 12.83; *df* = 30), density of Lewy neurites ($P = 0.0006$; F = 6.957; *df* = 30) and density of intracellular and extracellular Lewy bodies ($P = 0.0050$; F = 4.786; *df* = 30) from stages I to IV of the Lewy body disorders scale. Values are shown as mean ± SEM. One-way ANOVA; *post hoc* Tukey's test. *P-value < 0.05 compared to controls; $^{\#}P$-value < 0.05 compared to stage I; $^{\$}P$-value < 0.05 compared to stage II; $^{\&}P$-value < 0.05 compared to stage III. Representative images are presented above each graph (scale bar = 15 μm). (**B**) Density of dopaminergic (DA) neurons (blue), density of Lewy neurites (green) and CD8 T cell density (red) in the parenchyma for the different stages. One-way ANOVA; *post hoc* Tukey's test. *P-value < 0.05 compared to controls; $^{\#}P$-value < 0.05 compared to stage I; $^{\$}P$-value < 0.05 compared to stage II.

neurites but not Lewy bodies. Once this framework had been established, we compared the densities of CTLs and dopaminergic neurons in the different stages. As shown in Fig. 4B, dopaminergic cell loss progressed continuously from stage II (115 neurons/mm^2), when α-synuclein aggregates appear, to stage III (69.1 neurons/mm^2) and stage IV (55.5 neurons/mm^2). Stage III and IV were statistically different from the rest of the groups ($P < 0.0001$; F = 26.54; *df* = 30). Regarding CTL infiltration, two phases could be delineated. In the first (from stage I to II), which is the most intense, CTLs might have eventually triggered synucleinopathy and neuronal loss, but this was somehow counteracted to limit the immune pathology. In the second phase (from stage II to IV), CTL infiltration paralleled that of α-synuclein accumulation and neuronal death, suggesting that synucleinopathy is linked to the adaptive immune response. Stage I was statistically different from controls and stages I and II, but not from stage IV ($P = 0.0033$; F = 5.19; *df* = 30). Some authors have hypothesized that the adaptive immune response is triggered by an antigen derived from α-synuclein (Benner *et al.*, 2008; Sulzer *et al.*, 2017), but our results suggest that in the early stage of Parkinson's disease

the recruitment of CD8 T cells may be independent of nigral synucleinopathy. Nevertheless, it is not possible to rule out the fact that in later stages of the disease, when α-synuclein further accumulates, α-synuclein-derived antigens contribute to the immune response in a new wave of CD8 T cell infiltration. Overall, our results suggest that CD8 T cells may be relevant in both the initiation and the progression of disease as they precede neuronal death and synucleinopathy.

CD8 T cells in the SNpc express the CD103 tissue-resident memory T cell marker

It is accepted that there is a permanent need for immune surveillance of the CNS to defend it against pathogens (Loeffler *et al.*, 2011). In this respect, our study confirms the presence of CD8 T cells in the parenchyma and more abundantly in the perivascular spaces in all human SNpc tissue, including that of healthy control subjects. Few studies have assessed the phenotype of CD8 T cells in human brain tissue, both under healthy and disease conditions (Stone *et al.*, 2009; Loeffler *et al.*, 2011; Mosley *et al.*, 2012; Steinbach *et al.*, 2016), and none have assessed this in Parkinson's disease. Therefore, it was necessary to not only characterize the phenotype of these cells in Parkinson's disease and iLBD cases, but also under physiological conditions. This should make it possible to better understand the surveillance role of the adaptive immune system in the brain and to elucidate what cell phenotypes are responsible for the immune response involved in Parkinson's disease. Unlike flow cytometry analysis, immunofluorescence studies with tissue sections allow the topography of CTLs to be examined. In other words, such studies enable elucidation of the cell phenotypes that are infiltrating the brain parenchyma and contacting neurons compared to those that remain in the perivascular space. When a CD8 T cell recognizes its antigen and becomes activated, it can invoke any of three principal mechanisms to kill infected or tumour cells (Chávez-Galán *et al.*, 2009). The first of these mechanisms is by secreting cytokines like interferon γ (IFNγ) and tumour necrosis factor α (TNFα). The second is via Fas/FasL interactions, and the third is by releasing cytotoxic granules containing granzyme and perforin. To determine the killing machinery relevant to Parkinson's disease, we assessed all three of these mechanisms in our samples. We also assessed PD-1 and PD-L1 as the most relevant immune checkpoint system that regulates effector T cell activity within tissues to maintain tolerance and to prevent host tissue damage (Pey *et al.*, 2014).

It has recently been demonstrated that CD8 T cells in the white matter of the human corpus callosum harbour tissue-resident memory (TRM) cell features (Smolders *et al.*, 2018). TRM cells are generated in and persist at the site of primary infection and during resolution. As there is phenotypic heterogeneity in the TRM cell subset, the identification of TRM cells on the basis of phenotypic markers like CD69, CD103, CD49a and CD44 may therefore not reliably identify all resident T cells (Topham and Reilly, 2018). However, since it has been demonstrated that CD103 may be restricted to TRM cells in mouse neuronal tissue (Wakim *et al.*, 2010) and a relevant proportion of TRM cells in the human corpus callosum express CD103 (Smolders *et al.*, 2018), we decided to assess the expression of this marker in our samples and determine the percentage of CD8 T cells that express it. We performed double immunofluorescence for CD8 and CD103, and found CD103 plasma membrane staining in a relevant proportion of CTLs (Fig. 5A). About half of the CD8 T cells in the parenchyma of all control subjects expressed CD103, but only ∼25% of these cells in the perivascular space expressed this marker (Fig. 5B). These results demonstrate that TRM cells exist under healthy conditions in human grey matter. The percentage of CD103-positive CD8 T cells in the perivascular space in iLBD (31.7%) and Parkinson's disease (31.8%) was similar to that of the controls. Regarding percentages in the parenchyma, no statistically significant differences were seen among the three groups (Fig. 5B). It was previously reported that half of the CD8/CD69-positive TRM cells in the human corpus callosum do not express CD103. If this is also true for the SNpc, then the majority of CTLs detected could be TRM cells. To determine if CD8 T cells in the SNpc were CD69 positive, we carried out a double immunofluorescence and found out that almost all the perivascular and parenchymal CD8 T cells were positive for CD69 (Supplementary Fig. 3). This is particularly relevant for the aetiology of Parkinson's disease because TRM cells are those CD8 T cells that have been previously activated by their cognate antigen. We also assessed PD1 and PD-L1 staining in all three groups and found no staining at all (data not shown), suggesting that the PD1 immune checkpoint does not play a role in the SNpc or in Parkinson's disease.

Half of CD8 T cells in human SNpc express IFNγ but not TNFα or FasL

IFNγ can be released by activated macrophages and promote the recruitment of several immune cells such as activated CD8 T cells. It boosts their killing power by increasing the expression of MHC-I, but can also trigger targeted cell death directly (Castro *et al.*, 2018). More recently, it was demonstrated that the autocrine production of IFNγ by CTLs enhances their motility and promotes killing of the primary target (Bhat *et al.*, 2017). To determine whether patrolling and killing CD8 T cells were producing IFNγ we performed double immunofluorescence experiments for CD8 and IFNγ (Fig. 5C) and counted the percentage of CTLs expressing this cytokine in the parenchyma and perivascular spaces of control, iLBD and Parkinson's disease SNpc tissue. Around half of the CTLs in both compartments were positive for IFNγ regardless of the group (Fig. 5D). We then explored the expression of TNFα which, aside from a plethora of different immune functions, can activate death

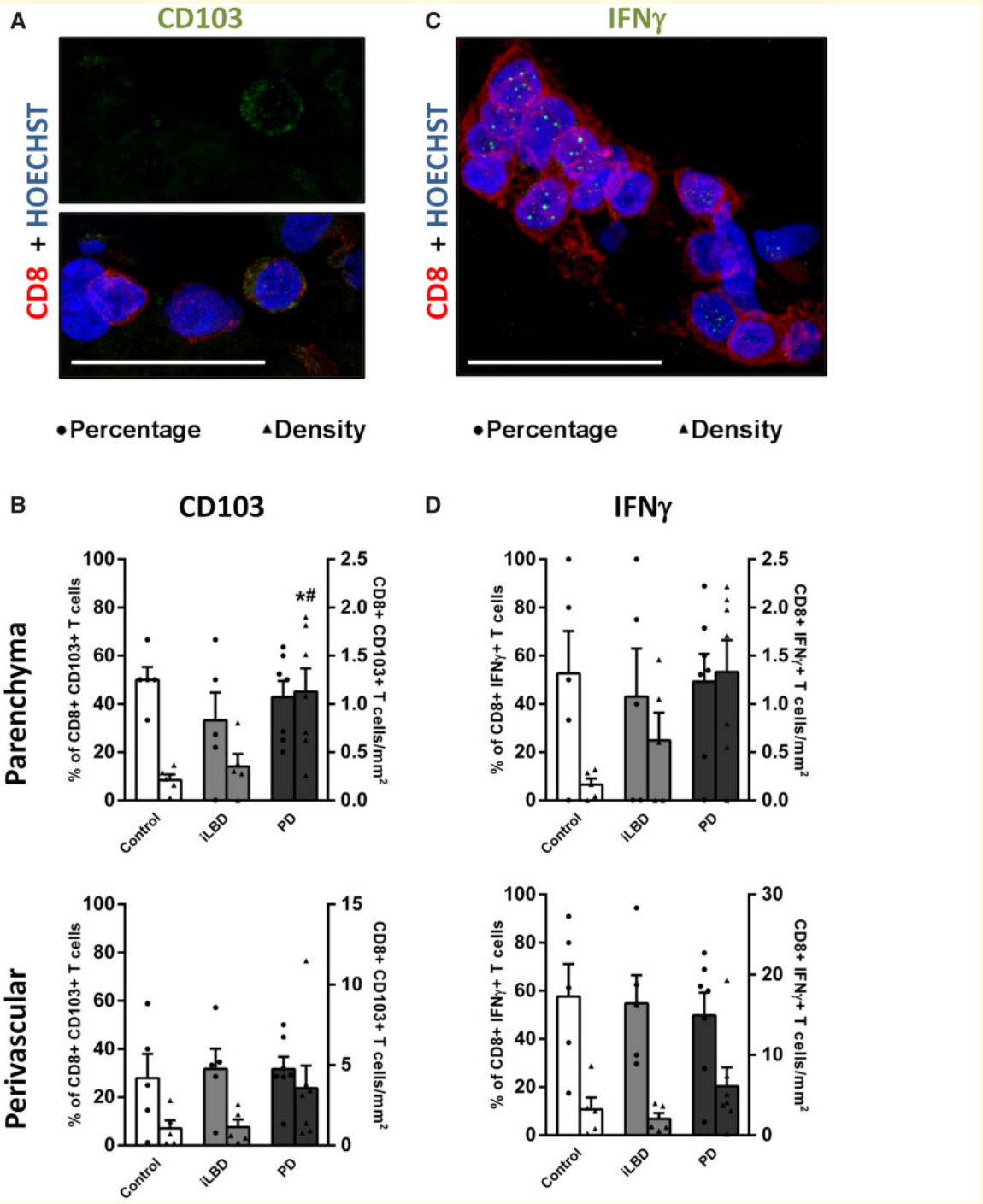

Figure 5 CD103+ and IFNγ+ CD8 T cells in control, iLBD and Parkinson's disease cases. (**A**) Immunofluorescence image showing one CD103-positive (green) and two CD103-negative CD8-positive (red) T cells. CD103 can be seen in the membrane. (**B**) Percentages (dots) and densities (triangles) of CD103-positive CD8 T cells in the parenchyma (*top*) and perivascular spaces (*bottom*) in control ($n = 5$), iLBD ($n = 5$) and Parkinson's disease (PD, $n = 7$) cases. One-way ANOVA; *post hoc* Tukey's test. *P-value < 0.05 compared to controls; #P-value < 0.05 compared to iLBD ($P = 0.0062$; $F = 7.463$; $df = 16$). (**C**) Immunofluorescence of IFNγ-positive (green) and -negative CD8 (red) T cells. (**D**) Percentages (dots) and densities (triangles) of IFNγ CD8 T cells in the parenchyma (*top*) and perivascular spaces (*bottom*) in control ($n = 5$), iLBD ($n = 5$) and Parkinson's disease ($n = 7$) cases. One-way ANOVA; *post hoc* Tukey's test. *P-value < 0.05 compared to controls. Bars represent mean value ± SEM in all graphs. Scale bar = 15 μm.

signalling (Micheau and Tschopp, 2003). No TNFα-positive CTLs were found in either of the compartments in any of the groups (data not shown). Finally, to assess the relevance of the Fas/FasL pathway, we conducted double immunofluorescence experiments for CD8 and FasL. As no FasL-positive CD8 T cells were found (data not shown), our results thus demonstrate that ~50% of CD8 T cells in the SNpc express IFNγ, and this may contribute to the killing capacity of CTLs in Parkinson's disease.

Different granzyme contents in SNpc infiltrating cytotoxic lymphocytes

Granzymes induce targeted cell death through non-redundant diverse pathways that complement each other (Voskoboinik *et al.*, 2015). Granzyme A (GrzA) and granzyme B (GrzB) are the most abundant and also the most studied granzymes. GrzB mimics caspases that cleave proteins after selected aspartate residues, and is the most powerful granzyme inducing programmed cell death via caspase-dependent and -independent pathways (Afonina *et al.*, 2010). Conversely, GrzA cleaves proteins at sites after basic amino acids and activates a slower form of cell death through different substrates. Among the other granzymes identified in humans, granzyme K has also been shown to induce cell death in mice (Harari *et al.*, 2009) and to be present in TRM cells of the human corpus callosum as GrzA and GrzB (Smolders *et al.*, 2018). For these reasons, we assessed the presence of the three granzyme types in our samples. We started with GrzB and found very few granules in most of the positive CTLs, but in some cases the granules were more abundant (Fig. 6A). Surprisingly, we found that infiltrating CTLs in five of seven iLBD cases contained GrzB (15% of CTLs), regardless of the presence of α-synuclein in the SNpc. One case had almost 40% of CTLs positive for GrzB, while none of the control or Parkinson's disease cases had a single CTL in the parenchyma with this granzyme ($P = 0.0027$; $F = 8.342$; $df = 20$) (Fig. 6B). As shown in Fig. 6C, some CTLs containing GrzB granules were in close contact with dopaminergic neurons or surrounding those neurons (Supplementary Fig. 2). We found similar percentages of CTLs with GrzB in the perivascular space (12%) of control and iLBD groups, whereas the Parkinson's disease group had the lowest percentage (4%) (Fig. 6B). In summary, CTLs containing GrzB granules participate in early stages of Parkinson's disease but not in the late stages. We also looked for the presence of GrzA in SNpc CTLs. Overall, GrzA granules were more abundant per CTL than GrzB (Fig. 7A). GrzA-positive CTLs were present in both compartments of all groups; however, iLBD SNpc tissue had a higher percentage of infiltrating CTLs containing this granzyme (64%) compared to the control (36%) and Parkinson's disease (35%) groups ($P = 0.0080$; $F = 6.391$; $df = 20$) (Fig. 7B). A similar but less pronounced trend could be found in perivascular spaces (Fig. 7C). In iLBD and Parkinson's disease SNpc some of the CTLs containing GrzA could be seen in contact with dopaminergic neurons (Fig. 7D). We also assessed GrzK expression and found CTLs containing this granzyme in all groups in both compartments even though the number of granules was not very high (Fig. 8A). In iLBD cases, half of the CTLs contained GrzK regardless of the compartment, but no statistically significant differences were found among the groups (Fig. 8B). Finally, we studied the expression of perforin in CD8 T cells, but no staining was found in the brain parenchyma or perivascular space for any of the groups (data not shown). Our findings are in agreement with those published previously showing that surveillance CTLs in the brain are devoid of perforin (Loeffler *et al.*, 2011; Smolders *et al.*, 2018). In fact, it was elegantly demonstrated that CTLs with GrzB but without perforin are responsible for neuronal damage in multiple sclerosis (Haile *et al.*, 2011). The lack of perforin may explain, in part, the moderate toxicity of these CTLs.

Overall, our results demonstrate that CD8 T cells in charge of immune surveillance contain granzyme A-, B- and K-containing granules and IFNγ, but not perforin or Fas-L. We also demonstrate that in a very early stage of Parkinson's disease, even before neuronal cell death and synucleinopathy occur, GrzB-positive CD8 T cells infiltrate the SNpc along with other CTLs that express GrzA and K, and/or IFNγ. In this early stage, the percentage of GrzA-positive CTLs is much higher than that in controls. In later stages of the disease, when Parkinson's disease has been diagnosed, only CTLs with GrzA, GrzK and IFNγ but not GrzB can be found in the parenchyma.

Discussion

So far, it is not known what 'spark' initiates the neuronal death that leads to Parkinson's disease. The results of the present post-mortem study strongly suggest that CTLs could initiate and propagate dopaminergic neuronal loss and α-synuclein pathology in Parkinson's disease. We demonstrate that CTLs with different killing machinery contact dopaminergic neurons and that CTL density in the SNpc correlates positively with neuronal loss in Parkinson's disease. Even though the role of α-synuclein in Parkinson's disease is still not completely understood, α-synuclein aggregation in different areas of the brain have been used to define a staging system for the disease (Braak *et al.*, 2003; Braak and Del Tredici, 2017) and a unified staging system for Lewy body disorders in general (Beach *et al.*, 2009). Putting aside the debate on how accurate and universal those systems are, it is broadly accepted that other areas of the brain can be affected by synucleinopathy before the SNpc is involved. There is also consensus that the olfactory bulb is one of the areas that could be initially affected because Lewy pathology can be found exclusively in this area in some iLBD cases. Moreover, a vast majority of Parkinson's disease cases show aggregates in this area. In fact, olfactory bulb synucleinopathy has a high specificity and sensitivity for Parkinson's

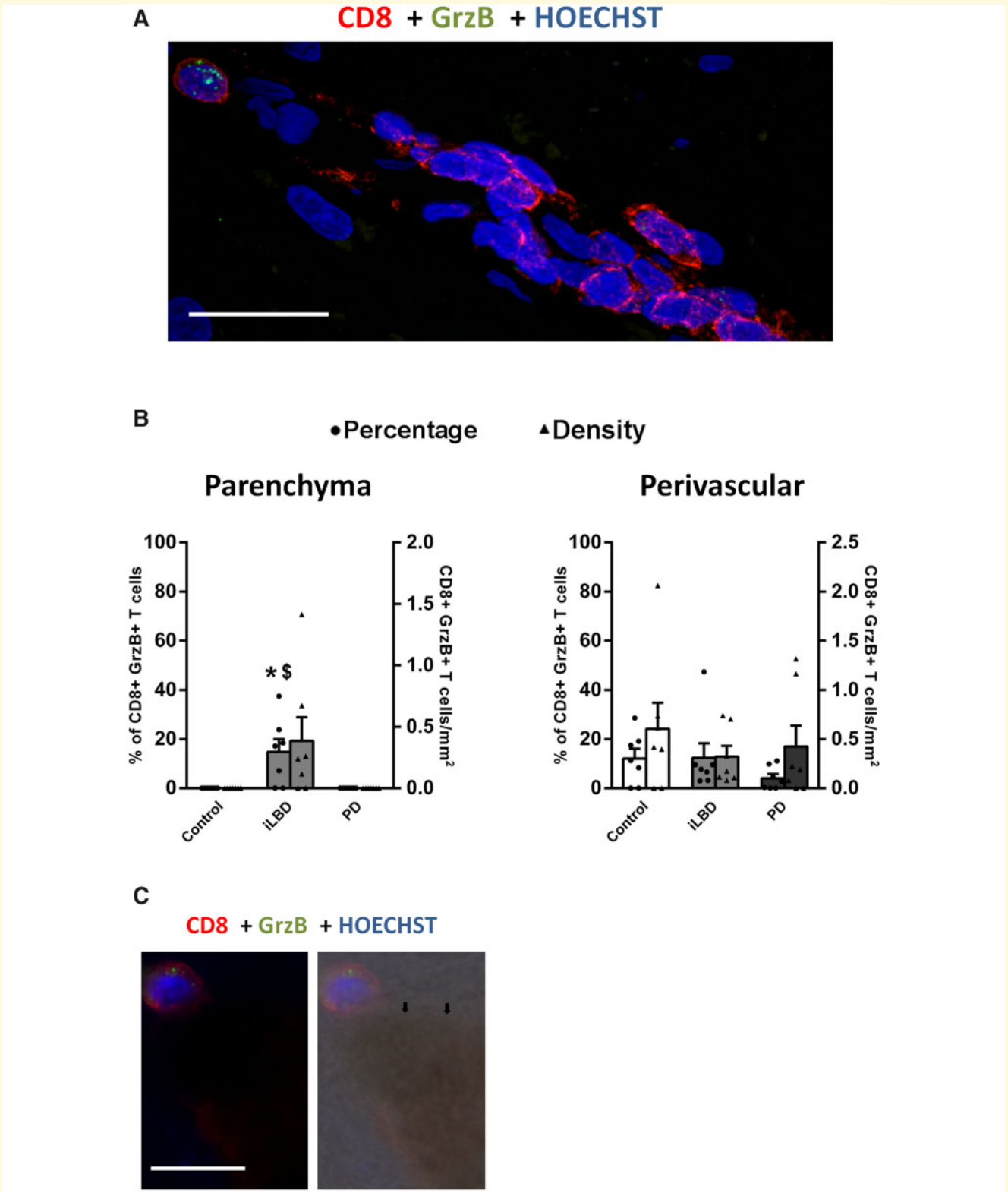

Figure 6 Granzyme B-positive CD8 T cells in control, iLBD and Parkinson's disease cases. (**A**) Immunofluorescence image showing a CD8 (red) T cell with many granzyme B granules among several granzyme B-negative CD8 T cells and some with one or two granules. (**B**) Percentages (dots) and densities (triangles) of granzyme B-positive CD8 T cells in the parenchyma (*left*) and perivascular spaces (*right*) in control ($n = 7$), iLBD ($n = 7$) and Parkinson's disease ($n = 7$) cases. One-way ANOVA; *post hoc* Tukey's test. *P-value < 0.05 compared to controls; $^{\$}P$-value < 0.05 compared to Parkinson's disease. Bars represent mean value ± SEM in all graphs. (**C**) Photomicrograph showing a CD8 T cell containing several granzyme B granules (green) and contacting a dopaminergic neuron in the SNpc of an iLBD case. Arrows point at a neuromelanin-containing neuron. All scale bars = 15 μm.

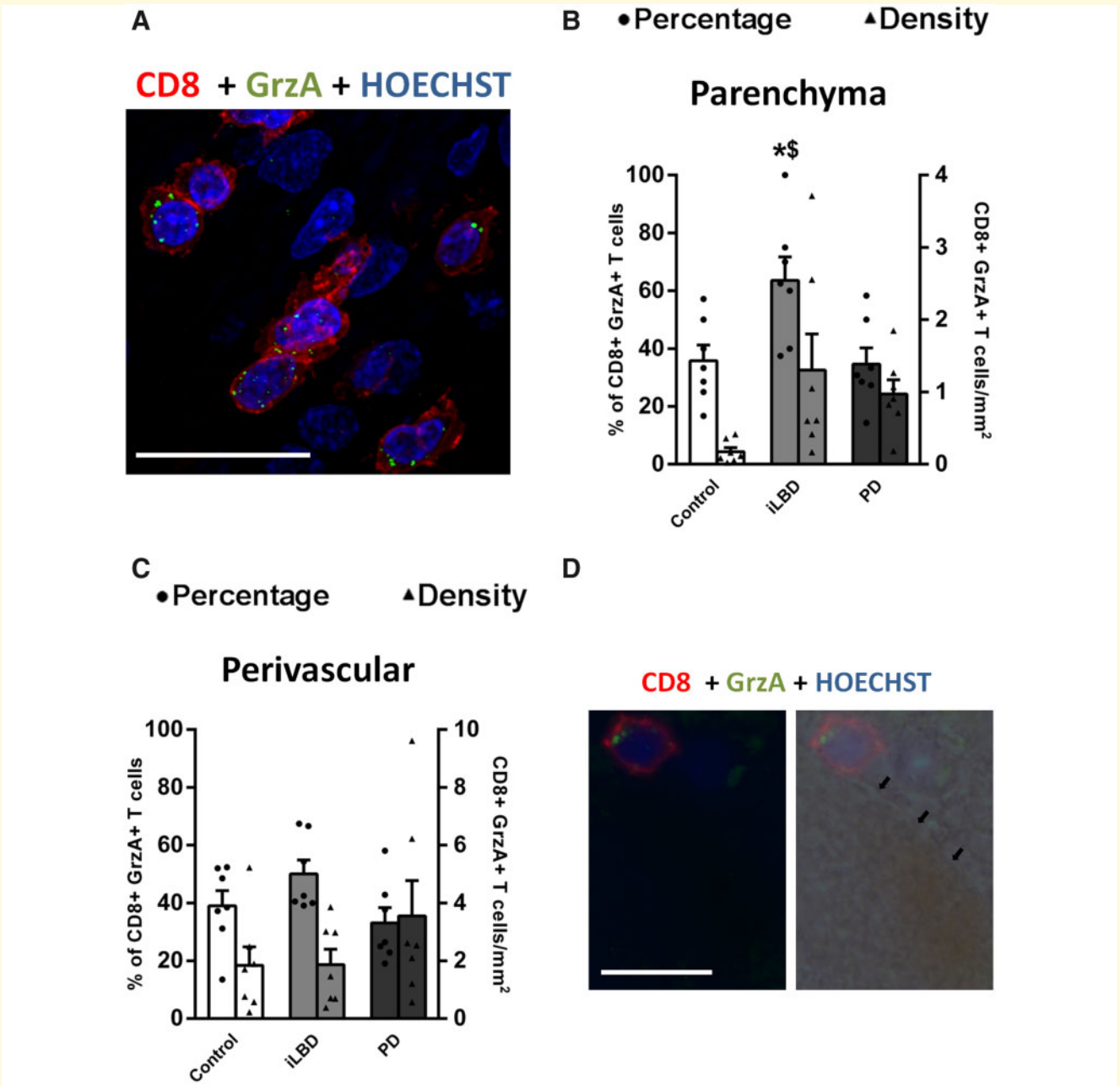

Figure 7 Granzyme A-positive CD8 T cells in control, iLBD and Parkinson's disease cases. (**A**) Representative immunofluorescence image of CD8 T cells positively and negatively stained for granzyme A (green). Scale bar = 15 μm. (**B**) Percentages (dots) and densities (triangles) of granzyme A-positive CD8 T cells in the parenchyma. One-way ANOVA; *post hoc* Tukey's test. *P-value < 0.05 compared to controls; $^{\$}P$-value < 0.05 compared to Parkinson's disease. (**C**) Percentages (dots) and densities (triangles) of granzyme A-positive CD8 T cells in the perivascular spaces. Bars represent mean ± SEM in both graphs for control ($n = 7$), iLBD ($n = 7$) and Parkinson's disease ($n = 7$) cases. (**D**) Photomicrograph of a CD8 T cell with granzyme A (green) granules contacting a neuromelanin-containing dopaminergic neuron. Arrows point at a neuromelanin-containing neuron.

disease and dementia with Lewy bodies (Beach *et al.*, 2009). We were surprised to find a strong infiltration of CTLs in the absence of α-synuclein aggregation in the SNpc since over the last decade α-synuclein-derived antigens have been proposed as candidates to trigger the adaptive immune response in Parkinson's disease (Benner *et al.*, 2008; Sulzer *et al.*, 2017). Our findings not only shed light on the aetiopathogenesis of the disease but also confirm that those iLBD cases with synucleinopathy in the olfactory bulb would have very likely developed Parkinson's disease, or another associated disease, if they had lived longer as they showed the pathological presence of CTLs in the SNpc. In those cases

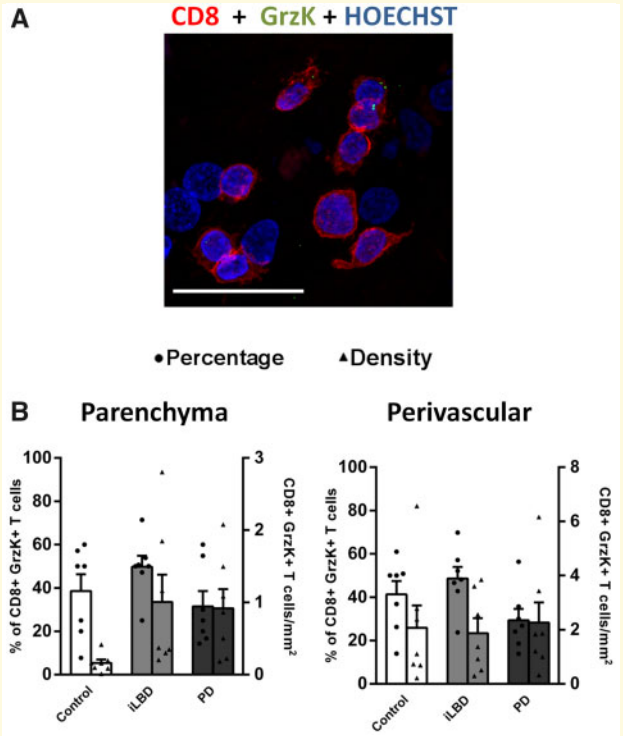

Figure 8 Granzyme K-positive CD8 T cells in control, iLBD and Parkinson's disease cases. (**A**) Immunofluorescence image showing CD8 T cells in the SNpc that stain positively and negatively for granzyme K (green). Scale bar = 15 μm. (**B**) Percentages (dots) and densities (triangles) of granzyme K-positive CD8 T cells in the parenchyma (*left*) and perivascular spaces (*right*) in control ($n = 7$), iLBD ($n = 7$) and Parkinson's disease (PD, $n = 7$) cases. Bars represent mean value ± SEM in all graphs.

with an intact SNpc, we found a robust CTL infiltration and captured CTLs in close apposition to neuromelanin-containing neurons. In other words, we were able to detect a CTL-mediated immune attack even before neuronal death started. Our results suggest two phases in the pathogenesis of Parkinson's disease that are due to the CTL-mediated immune response. The first is an early, strong CTL infiltration that initiates mild neuronal loss and α-synuclein aggregation but is partially suppressed or resolved. This is followed by a second wave of CTL infiltration that expands the α-synuclein pathology and neurodegeneration subsequently seen in Parkinson's disease diagnosed cases. This two-phase model is also supported by the fact that SNpc-infiltrating CTLs contain GrzB granules in iLBD cases but not in Parkinson's disease cases after years of progression. These changes in the number and phenotype of infiltrating CTLs suggest that different immunosuppressive mechanisms are activated at different stages of the disease. However, in our work we have ruled out the PD-1/PD-L1 checkpoint pathway as a participant in this process. Elucidating which inhibitory pathways are relevant for brain infiltrating CTLs will be helpful for the development of immunotherapies targeting these T cells.

Neuronal death occurs concomitantly with synucleinopathy in the cases that we have called iLBD2; however, whether α-synuclein has a deleterious or protective role is still not clear. Further experiments should be conducted to elucidate the role of α-synuclein in CTL-mediated cytotoxicity to determine whether CTL-induced synucleinopathy is instrumental for neuronal death or if it mirrors a compensatory mechanism. Both these possibilities may be compatible. In any event, our results suggest that CTL infiltration occurs upstream of α-synuclein aggregation and may trigger the constant formation of aggregates during chronification of the disease. Even though α-synuclein-derived antigens do not seem to be what triggers the initial immune attack, it has been demonstrated that α-synuclein peptides induce a humoral immune response in Parkinson's disease patients that possibly helps to eliminate the aggregates (Sulzer *et al.*, 2017). However, it is not clear if those antigens are also inducing a CTL response (Mosley and Gendelman, 2017), thus making it necessary to determine the antigens that initiate and propagate the immunogenic attack. These antigens could be exoantigens or autoantigens. Neuromelanin is one candidate as a source of autoantigens since it appears several years after birth.

We have confirmed the presence of CD4 and CD8 T cells not only in the perivascular space but also in the SNpc parenchyma in aged controls. Our study differs from a previously published report particularly in relation to the density of CD4 T cells in the control group (Brochard *et al.*, 2009). While the mentioned study found almost no CD4 T cells, we found as many such cells as in the Parkinson's disease group. Because we did not include young controls in the study, we cannot say if T cell infiltration is linked to the ageing process or reflects a normal T cell physiological surveillance role. It is more likely that T cells infiltrate and then, since TCR engagement does not occur, they egress from the parenchyma. In fact, we demonstrated here for the first time that CD8 T cells in both compartments express the TRM markers CD103 and CD69, suggesting recognition of a previous antigen. It has been demonstrated in mice that after viral reinfection and presentation of cognate antigen on MHC-I, TRM cells rapidly acquire a cytotoxic effector function and prevent fatal brain infection, even in the absence of circulating CD8 T cells (Steinbach *et al.*, 2016). It is probable that this is also true for the human brain. One study has previously demonstrated that most of the CD8 T cells in human white matter are TRM cells (Smolders *et al.*, 2018). That same study, using flow cytometry, showed that these CTLs are equipped with the killing machinery that we have described. However, they could not determine if the CTLs were actually infiltrating. Because we used immunohistochemistry/immunofluorescence techniques we could tell that the CTLs were in the parenchyma, with the exception of those containing GrzB granules that were infiltrating only in the case of iLBD patients. Our findings are similar to what has been found, for instance, in an autoimmune disease such as multiple sclerosis, although the later shows much higher CD8 T cell densities: dominance of CD8 T cells with

cytotoxic and TRM features in all disease courses and lesion stages, while CD4 T cells were sparse (Machado-Santos *et al.*, 2018). Because of the nature of our study, we could not match the results obtained in the SNpc with a phenotypic assessment of the T lymphocyte subsets in blood samples. In line of our results, it has been demonstrated that the peripheral immune profile in Parkinson's disease patients is characterized by the lack of CD8 T cell replicative senescence which characterizes normal ageing, while no changes in CD4 T cells subsets were found (Williams-Gray *et al.*, 2018). However, several studies have reported alterations in the CD4 T cell population in Parkinson's diseases peripheral blood that suggest the involvement of this cell subsets in the pathology of the disease. For example, an abnormal preponderance of effector memory CD4 T cells over naïve CD4 T cells has been reported in several works (Fiszer *et al.*, 1994; Bas *et al.*, 2001; Stevens *et al.*, 2012) and this activated profile has been associated to motor impairment severity (Saunders *et al.*, 2012). It has also been demonstrated that the decrease of CD4 helper T cells in Parkinson's disease is due to a reduction in Th2, Th17 and regulatory T cells populations (Kustrimovic *et al.*, 2018). Moreover, *in vitro* experiments have demonstrated that naïve CD4 T cells isolated from blood of patients preferentially differentiate towards the Th1 lineage (Kustrimovic *et al.*, 2018) while the regulatory T cell suppressive activity is impaired (Saunders *et al.*, 2012). Therefore, further studies with post-mortem tissue and blood need to be performed to elucidate the role of each T cell subtype during the initiation and progression of the disease.

The study has several limitations. The first is that this is a descriptive and correlative study and the aetiopathogenic relevance of CD8 T cells should be confirmed with an appropriate model that allows temporal dynamics assessment of their infiltration, transformation into TRM and their role in neuronal cell death. The second limitation of the study is that the ratio between males and females is not the same among the different groups and we cannot tell if there is a gender effect on the parameters that we have assessed. In addition, using one to three sections depending on the parameter assessed is also a limitation of the study. More accurate values would have been obtained if more tissue sections were included in the study. Lastly, we have not confirmed the absence of α-synuclein aggregates in the striatum of the cases that the Brain Bank reported as stage I (olfactory bulb only) of the Unified Staging System for Lewy Body Disorders and that are the cases that we have called iLBD1. Therefore, we cannot completely rule out that in some cases α-synuclein aggregates in the synaptic terminals are starting dopaminergic degeneration.

One of the aspects that challenges the interpretation of any study performed with aged population is the frequent existence of multiple chronic diseases. Multimorbidity or coexistence of several diseases in the same individual may occur for several reasons, including common risk factors or mechanisms, iatrogenic complications, and random chance. As shown in the clinical and autopsy information in Supplementary Table 2, the vast majority of the cases regardless of the experimental groups, developed several diseases during their lives, some of which were chronic. Therefore, we cannot rule out that that their health status and the medication associated had influenced our results. Any disease or treatment that involves or impacts the immune system could potentially have influenced our results. For instance, administration of chemotherapeutics or the coexistence of infection or inflammatory diseases could affect CD8 T cell infiltration. However, looking at the individual values we can reach the conclusion that these conditions have not introduced a bias in the study. We have checked those patients that had received chemotherapy and overall, they are not different in terms of T cell infiltration from their respective groups. White blood cell counts, including T lymphocytes, are known to decline in most cancer patients after chemotherapy, however, this decline is transient. For example, it has been reported that patients that received four treatments with gemcitabine recovered their basal counts 28 days after the last administration (Plate *et al.*, 2005). Moreover, not all chemotherapeutic drugs have the same effect on T cells. For example, taxol mainly affects the regulatory T cell population rather than other subsets including effector T cells (Zhang *et al.*, 2008). Most of the cases included in our study that died because of cancer did not receive chemotherapy or received it several months or even more than 1 year before death. However, there is an exception. Case iLBD-2, which belonged to the iLBD group with no synulceinopathy in the substantia nigra (what we called 'iLBD1' group), received chemotherapy just 3 weeks before death. CD8 T cell density in the parenchyma of this case was 5.9 cells/mm^2, similar to that of Case iLBD-6 from the same group and died because of renal disease (7.9 cells/mm^2). In fact, the average density of this group was 4.6 cells/mm^2. Moreover, iLBD-2 CD8 T cells contained granules of GrzB like most of the iLBD cases. Therefore, it seems that chemotherapy did not influence the infiltration in this case. Cases ILBD-1 and iLBD-9 did not receive chemotherapy. Regarding the control group, Control-3 received chemotherapy and radiotherapy 4 years before and 8 months before death, respectively. CD8 T cell parenchymal density in this case was 0.51 cells/mm^2, which is similar to that of Control-7 (0.68 cells/mm^2) who died because of heart disease and have never received a chemotherapeutic drug. Control-6 also received chemotherapy and had even higher density of CD8 T cells (0.74 cells/mm^2). Control-1 received the last chemotherapy round 5 months before death and had a lower CD8 T cell parenchymal density (the lowest one). Based on a previously published study, this value is not far from the expected range and therefore we believe that it is linked to interindividual variability. Even though we have tested that excluding this case does not change the significance of the results of our statistical analysis, we believe that this case should not be excluded. Among the Parkinson's disease cases that died because of cancer some of them did not receive chemotherapy or were treated with these drugs years before death. Therefore, chemotherapy did

not seem to introduce a bias in our study. Regarding the infectious/inflammatory conditions, none of them suffered from sepsis during the last disease stage but a high proportion of the subjects were affected somehow by an infectious/inflammatory process, but all the groups (Control, iLBD and Parkinson's disease) included subjects with this condition. For example, two controls, one iLBD and five Parkinson's disease cases appear to have had bronchopneumonia, and six controls, five iLBD and eight Parkinson's disease had arthritis. Regarding the iLBD cases, infection could not be the cause of the infiltration of CD8 T cells with GrzB as Cases ILBD-2, ILBD-4 and ILBD-6 had no infection and had average levels of CD8 T cells with GrzB, while Case ILBD-5 had mild chronic hepatitis and did not show CD8 T cells with Grz in the parenchyma.

Based on our results, we propose that CD8 T cells contribute to nigral dopaminergic neuron dysfunction and death in Parkinson's disease before overt Lewy bodies appear.

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Competing interests

The authors report no competing interests.

Supplementary material

Supplementary material is available at *Brain* online.

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