The MHC class II transactivator affects local and systemic immune responses in an α-synuclein seeded rat model for Parkinson's disease

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The MHC class II transactivator affects local and systemic immune responses in an α-synuclein seeded rat model for Parkinson's disease

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ABSTRACT

Parkinson's disease (PD) is characterized by intraneuronal inclusions of alpha-synuclein (α-Syn), neurodegeneration and a strong neuroinflammatory component. Studies have shown that genetic variants affecting quantity and quality of major histocompatibility complex II (MHCII) have implications in PD susceptibility and that PD patients have α-Syn specific T lymphocytes in circulation. The class II transactivator (CIITA) is the major regulator of MHCII expression and reduced CIITA expression has been shown to significantly increase α-Syn induced neurodegeneration and pathology in an α-Syn overexpression rat model combined with α-Syn pre-formed fibrils (PFF). In this study, we characterized immune profiles associated with the enhanced PD-like pathology observed in congenic rats with *Ciita* allelic variants causing lower CIITA levels compared to the background strain. Flow cytometry showed that rats with lower CIITA levels had an increased proportion of MHCII+ microglia and circulating myeloid cells, yet lower levels of MHCII on individual cells. Additionally, lower CIITA levels were associated to higher TNF levels in serum, trends of higher CD86 levels in circulating myeloid cells and a lower CD4/CD8 T lymphocyte ratio in blood. Taken together, these results indicate that CIITA regulates susceptibility to PD-like pathology through baseline immune populations and serum TNF levels.

INTRODUCTION

Parkinson's disease (PD) is a progressive and incurable neurodegenerative disorder estimated to affect 2-3% of the population above the age of 65[1]. PD is very heterogenous and approximately 95% of all cases have a multifactorial etiology where genetics, lifestyle and environment are contributing factors[2]. A characteristic feature of PD is the degeneration of dopaminergic neurons in the substantia nigra pars compacta (SN), intraneuronal inclusions of alpha-synuclein (α-Syn) and neuroinflammation[3]. The neuroinflammatory process in PD includes microglial activation, local upregulation of major histocompatibility complex II (MHCII), altered levels of pro-inflammatory cytokines in cerebrospinal fluid (CSF), as well as systemic changes in blood cytokine levels and lymphocyte populations[3]. Genetic association studies have identified single nucleotide polymorphisms in the human leukocyte antigen (*HLA*) locus that regulate the expression of MHCII to be associated with an increased risk of developing PD[4,5]. Recently, coding polymorphisms causing amino-acid changes in *HLA-D* haplotypes (*HLA-DRB1*4*) were also shown to be associated to PD with a protective effect[6]. Collectively, this indicates that both the quantity and quality of MHCII affect the risk of developing PD. Since MHCII molecules present antigens to T lymphocytes and induce antigen-specific responses they serve as a link between the innate and adaptive immune systems[7].

A role of the adaptive immune system in PD etiology is supported by the presence of lymphocytes in post-mortem brain tissue from PD patients[8] and findings of α-Syn reactive CD4+ lymphocytes[9,10] early in the disease process[11]. However, it is not clear if and how antigen presentation contributes to or protect from PD pathology. The level of MHCII on antigen-presenting cells is controlled by the class II transactivator (CIITA, also known as MHC2TA) and silencing of *Ciita in vivo* using shRNA has been shown to prevent neurodegeneration in a nigral α-Syn overexpression model of PD in mice[12]. In contrast, we have previously found that rats with naturally occurring variants in the promotor of *Ciita* and lower MHCII levels have more widespread α-Syn pathology and more activated microglia after nigral overexpression of α-Syn alone[13] and combined with striatal seeding with α-Syn pre-formed fibrils (PFF)[14]. Of note, genetic variants mediating lower CIITA expression are also found in humans and are associated with increased susceptibility to multiple sclerosis,

rheumatoid arthritis and myocardial infarction, further adding to the interest of studying CIITA in relation to PD[15].

The aim of this study was to investigate the effect of CIITA and MHCII expression on peripheral and local immune responses during α-Syn seeded PD-like pathology. To do so, we used a recombinant adeno-associated viral vector (rAAV) nigral α-Syn overexpression rat model combined with striatal seeding of human PFF in two congenic rat strains with different transcriptional activity of the *Ciita* gene. Using a flow cytometric approach, we investigated both resident and peripheral immune populations and confirmed previous results that rats with lower levels of CIITA have less MHCII expression in microglial cells compared to wild-type (wt) rats. Importantly, rats with lower CIITA expression also had lower MHCII levels and trends of higher levels of the co-stimulatory marker CD86 in circulating myeloid cells as well as higher levels of tumor necrosis factor (TNF) in serum. Collectively these results suggest that the levels of CIITA alter immune populations, immune responses and cytokine levels that in turn could affect the susceptibility to PD.

RESULTS

The rAAV-α-Syn+PFF PD model results in robust α-Syn expression, α-Syn inclusions and dopaminergic neurodegeneration

To investigate the effects of differential expression of CIITA on PD like-pathology we used wt DA rats and a congenic DA.VRA4 rat strain with lower expression levels of CIITA and MHCII[13]. Rats were injected with a rAAV6-α-Syn vector[16] into the SN followed by an injection of sonicated human α-Syn PFF two weeks later in the striatum (rAAV-α-Syn+PFF, α-Syn group) (Fig. 1a, b). Control animals were injected with an empty rAAV6 vector into the SN and vehicle (Dulbecco's phosphate buffered saline (DPBS)) into the striatum (rAAV-(-)+DPBS, control group). Rats were sacrificed at baseline (naïve), 4- or 8-weeks post nigral injection for collection of brain, blood and CSF samples (Fig. 1a).

Neurodegeneration and α-Syn pathology in the rAAV-α-Syn+PFF model used have been thoroughly characterized in a previous study[14]. Qualitative histological assessment confirmed robust positive signal of human α-Syn in the SN and striatum of both DA and DA.VRA4 rats in 4- and 8-week α-Syn groups (Fig. 1c and Supplementary Fig. 1a). Controls did not show any

human α-Syn signal (Supplementary Fig. 1b, c). As expected, the rAAV-α-Syn+PFF model resulted in loss of tyrosine hydroxylase (TH) positive signal in both DA and DA.VRA4 rats at 4- and 8-weeks (Fig. 1d and Supplementary Fig. 1d) whereas the TH-positive signal remained intact in the control groups (Supplementary Fig. 1e, f). The unilateral rAAV-α-Syn+PFF model also resulted in pathological forms of α-Syn aggregates, represented by phosphorylated α-Syn at Serine residue 129 (pS129) in the cell soma and in neurites (Fig. 1e) as well as by proteinase K-resistant α-Syn aggregates mainly observed as puncta along neurites (Fig. 1f) in ipsilateral, but not contralateral hemispheres (Supplementary Fig. 1g-h). Additionally, rAAV-α-Syn+PFF lead to upregulation of MHCII molecules in the ipsilateral but not contralateral midbrain of both the DA and DA.VRA4 rats (Fig. 1g and Supplementary Fig. 1i).

The rAAV-α-Syn+PFF PD model induces microglial MHCII+ expression and CSF cytokine levels in DA and DA.VRA4 rats

We have previously revealed that DA.VRA4 rats with lower levels of CIITA have more activated microglia compared to DA in α-Syn based PD-models[13,14]. However, this data was based on immunohistochemistry (IHC) that has limited capacity to identify cell populations. By applying flow cytometric analysis of brain cells, we found a similar percentage of microglia ($CD45^{dim}CD11b+$) in control and α-Syn groups (~93-97%) (Fig. 2a and Supplementary Fig. 2a, b). There was, however, an increased percentage of MHCII+ microglial cells in the ipsilateral compared to contralateral hemisphere in both control and α-Syn groups at 4 weeks (DA control 6.8 ± 2.0% vs 4.2 ± 0.99%, p=0.0080, 95% CI [1.0, 4.1]; DA α-Syn 14 ± 4.0% vs 4.8 ± 0.81%, p=0.0010, 95% CI [5.5, 13]; DA.VRA4 control 7.0 ± 1.3% vs 4.8 ± 0.72%, p=0.0070, 95% CI [0.90, 3.5]; DA.VRA4 α-Syn 12 ± 3.2% vs 5.0 ± 1.7%, p=0.0010, 95% CI [4.0, 9.2]) and 8 weeks (DA control 7.0 ± 1.3% vs 5.3 ± 1.5%, p=0.026, 95% CI [0.31, 3.1]; DA α-Syn 8.5 ± 1.8% vs 4.4 ± 0.87%, p=0.0020, 95% CI [2.6, 5.8]; DA.VRA4 control 6.7 ± 0.73% vs 5.3 ± 0.90%, p<0.0010, 95% CI [0.88, 1.9]; DA.VRA4 α-Syn 8.4 ± 1.3% vs 4.7 ± 0.98%, p<0.0010, 95% CI [2.9, 4.3]) (Supplementary Fig. 2c). By comparing normalized values (ipsilateral/contralateral), the increase in MHCII+ microglial cells was larger in the α-Syn groups compared to controls in both strains at 4 weeks (DA 2.9 ± 0.61 vs 1.6 ± 0.35, p=0.0012, 95% CI [0.65, 1.9]; DA.VRA4 2.4 ± 0.71 vs 1.5 ± 0.28, p=0.012, 95% CI [0.26, 1.6]) and at 8 weeks (DA 2.0 ± 0.30 vs 1.4 ± 0.28, p=0.0069, 95% CI [0.21, 1.0]; DA.VRA4 1.8 ± 0.17 vs 1.3 ± 0.12, p=00030, 95% CI [0.31,

0.71]) (Fig. 2b). The levels of MHCII (determined by median fluorescence intensity, MFI) on microglia were also elevated in the ipsilateral compared to contralateral hemisphere in response to α-Syn at 4 weeks in both strains (DA 2,600 ± 456 MFI vs 1,895 ± 131 MFI, p=0.0040, 95% CI [344, 1,065]; DA.VRA4 2,075 ± 267 MFI vs 1,684 ± 163 MFI, p=0.038, 95% CI [33, 749]) whereas it returned to control levels at 8 weeks in both strains (Supplementary Fig. 2d). After normalizing MHCII+ MFI values (ipsilateral/contralateral) the relative levels was higher in the α-Syn group compared to control at 4 weeks (DA 1.4 ± 0.17 vs 0.98 ± 0.19, p=0.0039, 95% CI [0.15, 0.61]; DA.VRA4 1.2 ± 0.22 vs 0.99 ± 0.097, p=0.028, 95% CI [0.034, 0.47]) but not at 8 weeks (Fig. 2c). We did not observe any changes in infiltrating macrophages/monocytes ($CD45^{high}$CD11b+) populations in brain (Fig. 2a) in terms of overall percentage or percentage of MHCII+ macrophages (Supplementary Fig. 2e-f). The MHCII+ MFI level of infiltrating macrophages/monocytes was higher in the ipsilateral compared to contralateral hemisphere at 4 weeks in the α-Syn group of DA.VRA4 rats (15,210 ± 1,896 MFI vs 13,870 ± 1,729 MFI, p=0.019, 95% CI [330, 2,338]) (Supplementary Fig. 2g, right).

CD86 (also known as B7-2) is a co-stimulatory signal expressed by antigen-presenting cells necessary for activation of T lymphocytes[7]. The rAAV-α-Syn+PFF model or control did not change CD86 MFI levels in macrophages in the ipsilateral hemisphere (Supplementary Fig. 2h). Microglial CD86 MFI values were lower in the ipsilateral compared to contralateral hemisphere at 4 weeks in the α-Syn group in both strains (DA 714 ± 30 MFI vs 784 ± 53 MFI, p=0.0070, 95% CI [-111, -29]; DA.VRA4 713 ± 114 MFI vs 774 ± 93 MFI, p=0.0050, 95% CI [-93, -29]), at 8 weeks in the control groups (DA 1,115 ± 169 MFI vs 1,182 ± 159 MFI, p=0.021, 95% CI [-117, -15]; DA.VRA4 1,053 ± 128 MFI vs 1,085 ± 143 MFI, p=0.029, 95% CI [-57, -4.9] and at 8 weeks in the DA α-Syn group (924 ± 64 MFI vs 1,018 ± 43 MFI, p=0.0050, 95% CI [-140, -48]) (Supplementary Fig. 2i). After normalization to the contralateral hemisphere, the relative CD86 MFI levels were reduced in the DA α-Syn group compared to control at 4 weeks only (0.91 ± 0.0046 vs 0.99 ± 0.073, p=0.042, 95% CI [-0.16, -0.0038) (Fig. 2d). Additionally, there was a slight increase in infiltrating T lymphocytes (CD45+CD3+) in the ipsilateral compared to contralateral hemisphere at 4 weeks in the DA α-Syn group (0.96 ± 0.36% vs 0.65 ± 0.25%, p=0.032, 95% CI [0.039, 0.57]), DA.VRA4 control (1.0 ± 0.19% vs 0.77 ± 0.32%, p=0.034, 95% CI [0.030, 0.53]) and DA.VRA4 α-Syn (1.2 ± 0.40% vs 0.73 ± 0.24%, p=0.0040, 95% CI [0.26,

0.77]) (Fig. 2e). These results indicate that there is early infiltration of T lymphocytes but no upregulation of the co-stimulatory marker CD86 necessary for T lymphocyte activation in the rAAV-α-Syn+PFF rat model for PD.

Altered levels of cytokines in the CSF are reported in PD patients[3]. To investigate the effect of rAAV-α-Syn+PFF on CSF cytokine levels, we performed multiplexed ELISA. Compared to control, CSF cytokine levels were unaffected in α-Syn groups at 4 weeks (Fig. 2f), but increased at 8 weeks; TNF in DA rats (0.41 ± 0.18 pg/ml vs 0.20 ± 0.19 pg/ml, p=0.049, 95% CI [0.00060, 0.42]) and IL-6 in both DA (44 ± 21 pg/ml vs 12 ± 12 pg/ml, p=0.0029, 95% CI [13, 52]) and DA.VRA4 (31 ± 11 pg/ml vs 6.6 ± 7.6 pg/ml, p=0.00020, 95% CI [14, 35]) (Fig. 2g).

The rAAV-α-Syn+PFF PD model induces changes in blood myeloid- and T lymphocyte populations

To investigate changes in peripheral immune cell populations, we performed flow cytometry of blood collected 4- and 8-weeks post nigral injection (Supplementary Fig. 3a). At 4 weeks, we observed lower levels of circulating myeloid cells (CD45+CD11b+) in DA rats injected with rAAV-α-Syn+PFF compared to controls (20 ± 4.4% vs 27 ± 3.4%, p=0.012, 95% CI [-12, -2.0]) (Fig. 3a, b), but a higher percentage were MHCII+ (9.0 ± 1.4% vs 7.0 ± 1.4%, p=0.036, 95% CI [0.16, 3.7]) (Fig. 3c). The overall percentage of T lymphocytes, CD4+ T lymphocytes or CD4/CD8 ratio did not change in response to α-Syn in DA or DA.VRA4 rats (Supplementary Fig. 3b, c and Fig. 3e). There was, however, a higher percentage of CD8+ T lymphocytes in DA -α-Syn rats compared to controls at 8 weeks (37 ± 7.3% vs 28 ± 4.9%, p=0.047, 95% CI [0.16, 18]) (Fig. 3f).

To investigate blood cytokine levels, we performed multiplexed ELISA on serum. The rAAV-α-Syn+PFF model lead to higher levels of IL-1β (23 ± 6.7 pg/ml vs 14 ± 5.8 pg/ml, p=0.022, 95% CI [1.5, 16]) and IL-5 (37 ± 5.8 pg/ml vs 29 ± 4.2 pg/ml, p=0.0077, 95% CI [2.7, 14]) in DA.VRA4 rats compared to controls at 4 weeks, but no changes at 8 weeks or in DA rats (Fig. 3g, h).

Differential expression of CIITA regulates MHCII levels on brain macrophages and microglia during rAAV-α-Syn+PFF induced pathology

Since DA.VRA4 rats with lower CIITA levels are more susceptible to α-Syn pathology and dopaminergic neurodegeneration than DA rats[14], we compared immune cell populations by flow cytometry of brain tissue between the strains. DA and DA.VRA4 rats injected with rAAV-α-Syn+PFF did not differ in terms of microglial population size, proportion of MHCII+ microglia (Fig. 4a, b) or infiltration of T lymphocytes (Supplementary Fig. 4a). However, naïve DA.VRA4 rats had, compared to DA, a lower percentage of microglia (93 ± 1.9% vs 96 ± 1.1%, p=0.017, 95% CI [-5.1, -0.65]) but increased percentage of MHCII+ microglia (5.7 ± 0.60% vs 4.9 ± 0.35%, p=0.019, 95% CI [0.18, 1.6]) (Fig. 4a, b). In line with previous IHC-based data, the intensity of the MHCII+ signal (ipsilateral MFI normalized to contralateral MFI for DA) on microglia was lower in DA.VRA4 rats compared to DA (Fig 4c). This CIITA-dependent difference was observed between DA.VRA4 and DA naïve (0.88 ± 0.032 vs 0.97 ± 0.077, p=0.029, 95% CI [-0.17, -0.011]), 8-week control (0.95 ± 0.038 vs 1.0 ± 0.030, p=0.0014, 95% CI [-0.13, -0.042]), α-Syn 4 weeks (1.1 ± 0.097 vs 1.4 ± 0.19, p=0.011, 95% CI [-0.47, -0.079]) and α-Syn 8 weeks (0.91 ± 0.072 vs 1.0 ± 0.063, p=0.016, 95% CI [-0.23, -0.031]) (Fig. 4c). There were no differences between DA and DA.VRA4 rats in percentages of brain macrophages or MHCII+ brain macrophages (Fig. 4d, e). However, brain macrophages from DA.VRA4 rats with reduced CIITA levels had lower levels of MHCII compared to DA naïve (0.76 ± 0.15 vs 1.0 ± 0.094, p=0.010, 95% CI [-0.42, -0.073]), α-Syn 4 weeks (0.76 ± 0.061 vs 1.0 ± 0.046, p<0.00010, 95% CI [-0.34, -0.21]) and α-Syn 8 weeks (0.92 ± 0.047 vs 1.1 ± 0.081, p=0.0080, 95% CI [-0.24, -0.050]) (Fig. 4f).

Microglial CD86 levels (ipsilateral MFI values normalized to contralateral MFI values for DA) did not differ between strains (Fig. 4g). Similarly, brain macrophage CD86 MFI levels did not differ between DA and DA.VRA4 rats except for the 4 week control groups, where DA.VRA4 had lower CD86 MFI compared to DA (1.0 ± 0.13 vs 1.2 ± 0.076, p=0.032, 95% CI [-0.28, -0.016]) (Fig. 4h).

No differences in CSF cytokine levels were observed between the two strains apart from higher IL-10 levels in DA.VRA4 compared to DA rats in the α-Syn 8 week group (3.2 ± 1.1 pg/ml vs 1.7 ± 0.74 pg/ml, p=0.0084, 95% CI [0.42, 2.4]) (Supplementary Fig. 4b-d).

Lower CIITA expression is associated with increased percentage of blood MHCII+ myeloid cells, decreased CD4/CD8 ratio and elevated TNF levels in serum

We used flow cytometric analyses of myeloid cells and CD4+/CD8+ T lymphocytes to determine if differential expression of CIITA affects circulating immune cells in naïve, control and α-Syn groups of DA and DA.VRA4 rats. There was no difference between rat strains in overall percentage of circulating myeloid cells (Fig. 5a). Similar to the results from brain, naïve DA.VRA4 rats with lower CIITA levels had a higher percentage of MHCII+ myeloid cells in blood compared to DA (12 ± 2.6% vs 7.0 ± 2.9%, p=0.0071, 95% CI [1.8, 8.9]) (Fig. 5b). Also similar to the results from brain, myeloid cells in blood from DA.VRA4 showed a trend of lower MHCII levels, determined by normalized MFI values for DA, and this was independent of intervention (Fig. 5c). Opposite to infiltrating myeloid cells in brain, blood myeloid cell CD86 levels (MFI values normalized for DA) were higher in DA.VRA4 compared to DA rats, although only significantly higher in the control group at 4 weeks (1.6 ± 0.48 vs 1.0 ± 0.084, p=0.014, 95% CI [0.15, 1.0]) (Fig. 5d).

Even though we did not observe a difference in overall percentage of T lymphocytes in blood in response to α-Syn, the percentage was lower in DA.VRA4 rats compared to DA at 4 weeks in the α-Syn group (49 ± 6.8% vs 59 ± 5.6%, p=0.021, 95% CI [-18, -1.9]) (Fig. 5e). Investigating T lymphocyte subpopulations, the percentage of CD4+ cells was lower in DA.VRA4 rats compared to DA in both naïve (70 ± 2.5% vs 74 ± 1.4%, p=0.0037, 95% CI [-7.0, -1.8]) and the 8 week control group (59 ± 4.9% vs 66 ± 3.2%, p=0.024, 95% CI [-13, -1.2]) whereas CD8+ T lymphocytes were increased in naïve DA.VRA4 rats compared to DA (27 ± 1.5% vs 23 ± 1.4%, p=0.0023, 95% CI [1.5, 5.2]) (Fig. 5f-g). Consequently, a reduced CD4/CD8 ratio was observed in DA.VRA4 compared to DA rats in both naïve (2.6 ± 0.21 vs 3.2 ± 0.26, p=0.0019, 95% CI [-0.87, -0.27]) and the 8 week control group (1.8 ± 0.37 vs 2.4 ± 0.50, p=0.036, 95% CI [-1.2, -0.055]) (Fig. 5h). The differences in T lymphocyte subpopulations in rats with differing CIITA levels were, thus, not depending on α-Syn.

Naïve DA.VRA4 rats with lower levels of CIITA had higher levels of TNF in serum compared to DA (4.2 ± 0.57 pg/ml vs 3.3 ± 0.56 pg/ml, p=0.022, 95% CI [0.15, 1.6]), and this difference remained in control- (4-week 2.7 ± 0.37 pg/ml vs 2.0 ± 0.16 pg/ml, p=0.0012, 95% CI [0.31, 0.98], 8-week 4.3 ± 0.58 pg/ml vs 3.1 ± 0.36 pg/ml, p=0.00060, 95% CI [0.64, 1.8]) and α-Syn groups (4-week 2.4 ± 0.56 pg/ml vs 1.9 ± 0.22 pg/ml, p=0.039, 95% CI [0.030, 1.0], 8-week 4.3 ± 0.40 pg/ml vs 3.1 ± 0.43 pg/ml, p=0.00020, 95% CI [0.71, 1.7]) (Fig. 5i-k). Additionally, DA.VRA4 rats had higher levels of IL-1β compared to DA for naïve (29 ± 14 pg/ml vs 11 ± 8.3 pg/ml, p=0.025, 95% CI: [2.8, 33]) (Fig. 5i) and higher IL-5 levels for α-Syn 4 weeks (37 ± 5.8 pg/ml vs 25 ± 11 pg/ml, p=0.019, 95% CI [2.5, 23]) (Fig. 5j, right).

DISCUSSION

Studies investigating human cohorts and experimental models support a role for antigen presentation and adaptive immune responses in PD etiology. However, there are contradictory findings on how local and peripheral immune responses contribute to or protect against different aspects of PD. Contributing factors to these discrepancies likely include difficulties in determining causality versus consequence in an ongoing pathological process, as well as the multiple different murine models used to study PD-related changes in the immune system. In a recent study, we showed that lower CIITA levels are associated with increased susceptibility to α-Syn pathology and dopaminergic neurodegeneration in the rAAV-α-Syn+PFF PD model[14]. This strongly supports CIITA, MHCII and the process of antigen presentation to have causal impact on PD risk and outcome. The relative contribution of resident/local (brain) and peripheral (systemic) immune cells and cytokines in this process is, however, not known. Therefore, in the current study we have characterized the effects of CIITA expression levels on local and peripheral immune populations in the rAAV-α-Syn+PFF model.

To assess the role of antigen presentation in PD-like pathology, we have used rats with naturally occurring variants in the *Ciita* gene, leading to differential expression of MHCII. Genetic variants regulating *Ciita* expression are found in multiple rat strains and in humans, where the orthologue regulates MHCII expression and are associated with susceptibility to rheumatoid arthritis, multiple sclerosis and myocardial infarction[15]. We argue that these

congenic rats provide a physiologically highly relevant model to study the effects of antigen presentation on immune populations and PD-like pathology. This is in sharp contrast to the use of knockout (KO) models with dysfunctional immune systems to study the role of immune-related proteins or molecules[8,12,17-21].

Even though there is substantial evidence of the involvement of *HLA*/MHCII in PD indicated by genetic association studies and elevated MHCII levels at the site of neurodegeneration[3-6,22,23], the knowledge on the role of MHCII in disease etiology is lacking. Moreover, the results from animal models are somewhat contradictory, probably due to the different methodologies used to study the impact of MHCII and CIITA on PD-like pathology. The various approaches include different species (rats[13,14,24] or mice[12,21,25]), different models of PD/synucleinopathies (transgenic[21], rAAV-α-Syn[12,13,24,25], or rAAV-α-Syn+PFF[14]) and the approach on how to manipulate CIITA/MHCII levels or the adaptive immune system (KO models[12,21,25], nude rats[24], silencing through shRNA[12], or the use of congenic strains[13,14]). The model employed in the current study has high construct validity (common *CIITA* genetic variants regulate MHCII levels both in rats and humans) and high face validity (the rAAV-α-Syn+PFF rat model displays seeded α-Syn pathology, dopaminergic neurodegeneration, motor impairment and neuroinflammation). Together, these characteristics allow for a good predictive validity of the model.

Our results are partly contradictory to other *in vivo* studies of CIITA impact on PD-like pathology, but differences in the respective *in vivo* models are important to consider. In mice, silencing of *Ciita* through shRNA reduced T lymphocyte and monocyte infiltration and protected against SN dopaminergic loss upon nigral rAAV-α-Syn overexpression[12]. However, the silencing of *Ciita* was also associated with a significant reduction of MHCII+ microglia cells ($CD45^{dim}CD11b+$)[12]. Other studies found that complete KO of MHCII or CIITA fully protected against dopaminergic cell loss[12,25] and microglial activation[25] in response to α-Syn overexpression. In one study KO of MHCII resulted in accelerated pathology in the brain and an overall reduction of T lymphocytes in the CNS of transgenic mice expressing human α-Syn with the A53T mutation (M83+/0) combined with injection of PFF into the hindlimb[21]. The KO of MHCII or CIITA creates a dysfunctional immune system and lost interplay between CD4+ T

lymphocytes and antigen presenting cells that limits the physiological relevance of these models. In rats, T lymphocyte deficient (homozygous nude) rats did not upregulate MHCII levels in response to nigral rAAV-α-Syn injection and were partially protected against dopaminergic cell loss compared to heterozygous nude rats[24]. Interestingly, there was no difference in α-Syn pathological load in SN between homozygous and heterozygous nude rats, which may be influenced by the fact that heterozygous nude rats have significantly fewer T lymphocytes compared to wt rats.

Using flow cytometry, this study confirms previous semi-quantitative findings from brain immunostaining and RT-qPCR regarding microglial MHCII expression in response to α-Syn; lower CIITA levels are associated to a larger proportion of microglia expressing MHCII but with lower levels of MHCII per cell[13,14]. Additionally, by analyzing blood, we show that lower CIITA levels in naïve DA.VRA4 rats affect MHCII expression in circulating cells of the myeloid lineage in a similar way as in microglia; a higher percentage is MHCII+ but the MHCII level per cell is lower. Thus, differential expression/levels of *Ciita* affects the baseline levels of MHCII+ microglia and MHCII+ circulating myeloid cells, and not only after initiation of PD like pathology[12-14]. We hypothesize that increased numbers of MHCII+ microglia could accelerate dopaminergic neurodegeneration through pathological spread of α-Syn, as we have previously reported an increased aggregation and propagation of α-Syn in DA.VRA4 rats, along with pathological α-Syn (pS129) co-localized within MHCII+ microglial cells in the rAAV-α-Syn+PFF model[14] (Fig. 6).

Compared to studies using α-Syn nigral overexpression or striatal PFF injection in mice[12,26,27], we found very limited numbers of brain infiltrating macrophages/monocytes and lymphocytes in the rAAV-α-Syn+PFF rat model. Among live cells analyzed from brain tissue, 93-97% were $CD45^{dim}$CD11b+ microglia and only 0.5-1.5% $CD45^{high}$CD11b+ macrophages/monocytes. However, as much as 70-85% of the macrophages/monocytes but only 5-15% of the microglia were MHCII+, indicating that infiltrating macrophages/monocytes might still play an active role in CNS antigen presentation. The low number of infiltrating macrophages/monocytes and lack of differences between α-Syn and control groups in this model is contradictory to a previous study reporting that PD-like pathology was mainly driven

by infiltrating monocytes in a nigral α-Syn overexpression model in mice[20]. In addition, CIITA levels did not affect the number of infiltrating macrophages/monocytes or lymphocytes in our model, while KO and silencing of *Ciita* have been reported to greatly reduce both monocyte and lymphocyte infiltration in mice overexpressing α-Syn in SN[12].

As for T lymphocytes (CD45+CD3+) infiltrating the brain, we recorded few events from brain tissue, and while infiltration of T lymphocytes increased after rAAV-α-Syn+PFF injection at 4 weeks, this response was not dependent on CIITA levels. This finding suggests that T lymphocyte infiltration mainly occurs at early stages, prior to any major neurodegeneration, which has been reported in other murine PFF models[27,28]. In blood, DA.VRA4 rats had fewer T lymphocytes in circulation compared to DA at 4 weeks in response to α-Syn. We also observed a reduced CD4/CD8 ratio in blood from naïve DA.VRA4 rats with lower CIITA levels, driven by a decrease in CD4+ and increase in CD8+ T lymphocytes, although this difference was not seen between rats receiving rAAV-α-Syn+PFF injections.

Studies in α-Syn-based PD models indicate both detrimental and protective roles of lymphocytes. Neurodegeneration-promoting effects are supported by findings that mice lacking lymphocytes (*Rag1* KO) were protected against dopaminergic cell loss in SN, that lymphocyte reconstitution resulted in dopaminergic cell loss comparable to wt mice[29] and that CD4 KO protected against neurodegeneration in the SN and inhibited myeloid activation[19]. In contrast, protective effects of lymphocytes have been reported in a striatal α-Syn PFF model, where adoptive transfer of CD4+ lymphocytes to immunocompromised mice reduced α-Syn pathology[18]. Studies on lymphocyte populations in PD patients are also inconclusive. A recent study reported no difference in T lymphocytes overall or in subpopulations (CD4+ or CD8+), but a reduction in effector and regulatory T lymphocytes in PD[30]. Others report a decrease in T lymphocytes overall, and in both CD4+ and CD8+ subpopulations[31]. Additionally, there are reports on a lower CD4/CD8 ratio in PD patients due to decreased numbers of CD4+ lymphocytes[32] and on an overall decrease in circulating CD4+ lymphocyte subpopulations due to decreased levels of T-helper (Th) 2, Th17 and regulatory lymphocytes[33]. Other research suggests an overall decrease in circulating lymphocytes with increased Th1 and Th17 but decreased Th2 and regulatory T lymphocytes[34] or no changes in

Th1 and Th2 subsets but an increase in the Th17 lymphocyte population[35]. In addition to differences in population sizes and ratios, functional studies indicate altered functions of lymphocyte populations in PD. One study found deficits in migratory capacity of CD4+ T lymphocytes from PD patients[36]. Another study reported impaired suppressor functions of T regulatory cells in PD, which could be resorted by *ex vivo* expansion[30]. A third study reported that higher level of activation of T lymphocytes in response to phytohemagglutinin stimulation was associated with PD disease severity[31]. To decipher the contribution of T lymphocytes on PD susceptibility and progression more studies on the role of T lymphocytes and MHC-dependent immune responses in PD are required.

In addition to altered immune cell profiles, we found alterations in CSF and serum levels of several cytokines with possible links to PD. The rAAV-α-syn+PFF model resulted in increased CSF IL-6 levels in both DA and DA.VRA4 rats. These results are in line with clinical findings in which elevated CSF IL-6 levels were observed in PD patients[37]. We also found higher levels of the anti-inflammatory cytokine IL-10 in CSF from DA.VRA4 rats compared to DA (8-week α-syn), which has previously been shown to be neuroprotective and reduce microglial activation in toxin models of PD[38]. Higher serum levels of IL-5, reported to be elevated in CD4+ Th2 lymphocytes from PD patients stimulated with α-Syn peptides *ex vivo*[10], were also found in the DA.VRA4 α-Syn group compared to control. Increased IL-1β and TNF levels in blood have been seen in PD patients from multiple studies[39], and we found higher levels of TNF in serum in DA.VRA4 compared to DA and higher levels of IL-1β in DA.VRA4 α-Syn compared to control. IL-1β levels have also been shown to influence the NLRP3 inflammasome and contribute to neurodegeneration in a 6-OHDA mouse model of PD[40] and correlate to disease progression in PD patients[41]. Together with our previous findings, it is possible that elevated levels of TNF in DA.VRA4 rats affect the susceptibility to PD-like pathology and together with IL-1β and IL-5 exacerbates α-Syn pathological spread and neurodegeneration. In fact, inhibition of soluble TNF has been shown to attenuate microglia and astrocyte activation and protect against dopaminergic neurodegeneration in a rat 6-OHDA model of PD[42]. Further investigation would be necessary to assess if TNF inhibition could modulate neuroinflammation, neurodegeneration and α-Syn pathology in the rAAV-α-syn+PFF model.

As all models, the rAAV-α-Syn+PFF PD rat model has both strengths, as highlighted earlier, and limitations. A limitation of models using intracranial injections is the physical damage and blood-brain barrier disruption possibly causing changes in immune populations, independent of what is injected. In order to control for immune responses not related to α-Syn, injections of an empty vector in SN and vehicle in striatum was used for the control groups. We chose an empty vector since we and others have observed that the commonly used rAAV-GFP control vector elicits a neuroinflammatory response[14,20], and a fluorescent control protein would also interfere with flow cytometry. As control for PFF, we chose to use vehicle, since we have found that bovine serum albumin elicits a neuroinflammatory response[14] and other studies report that α-Syn monomers and saline are comparable controls for the PFF model in rats[28]. We have previously investigated the effects of differential CIITA expression on PD-like α-Syn pathology and neurodegeneration at 8-weeks post SN injection of rAAV6-α-Syn in the rAAV-α-Syn+PFF model[14] and microglia profile and neurodegeneration 12-weeks post SN injection using a rAAV-α-Syn vector only[13]. Studies have shown that there is an inflammatory response ongoing prior to neurodegeneration in animal models[27,28,43] and in PD patients[10]. Therefore, we chose to include an earlier time point in our current study; 4 weeks post nigral injection of rAAV-α-Syn. Since α-Syn pathology and MHCII+ microglial cells are widespread in the brain in the rAAV-α-Syn+PFF model[14] we included entire hemispheres for flow cytometric analyses in the current study. Consequently, it is possible that region-specific differences affected by CIITA levels or responses to α-Syn are missed.

In conclusion, our results show that CIITA levels alter molecules linking the innate and adaptive immune system in both local and peripheral immune populations that could explain the increased susceptibility to α-Syn-induced neurodegeneration and pathological protein spread observed in DA.VRA4 rats with naturally occurring lower CIITA levels[13,14] (Fig. 6). We also observed continuously elevated levels of serum TNF in DA.VRA4 rats. To assess if these elevated TNF levels are causally related to the increased susceptibility to α-Syn-induced PD-like pathology requires further studies. Collectively, our work together with other experimental and human studies highlight the complexity and importance of understanding the link between innate and adaptive immune responses in PD.

MATERIALS AND METHODS

Experimental design

To investigate the effects of differential expression of CIITA we used wt DA rats and a congenic DA.VRA4 rat strain with lower expression levels of CIITA and MHCII[13]. Male rats entered the study at 12±1 weeks of age and a total of 77 rats were included with 6-9 rats/group. We used a combination of viral overexpression of human α-Syn combined with seeding of human PFF, adapted from Thakur et al[44]. Rats were injected with a rAAV6 vector carrying human α-Syn[16] into the SN followed two weeks later by an injection of human α-Syn PFF in the striatum (Fig. 1)[14]. Animals were sacrificed at 4- and 8-weeks post nigral injection for collection of brain, serum and CSF samples. Six animals per strain and time point were used for flow cytometric analysis of brain and blood samples and 2-3 animals per strain and time point were used for qualitative IHC validation of α-Syn expression, TH loss, α-Syn pathology and MHCII upregulation. Naïve rats (n=6 per strain) were sacrificed at 12±1 weeks of age and used as baseline for cytokine levels and flow cytometric analyses of blood and brain. Two rats (1 naïve DA and 1 DA.VRA4 α-Syn 8 week) were excluded from flow cytometry analysis of brain due to unsatisfactory perfusion and blood-filled ventricles, respectively. One DA rat from the 8-week α-Syn group was excluded from flow cytometry analysis for both blood and brain due to a clogged capillary during stereotactic surgery. One DA rat from the 8-week control group was excluded for flow cytometry analysis of blood due to inadequate number of events.

Animals

The DA.VRA4 strain was generated by transfer of the VRA4 locus from the PVG strain to a DA background[45]. Rats were housed 2-3 per cage in “type III high” individually ventilated cages with free access to standard rodent chow and water and kept in a pathogen-free and climate-controlled environment with a 12-hour light/dark cycle at the Biomedical Center in Lund. All procedures were approved by the local ethics committee in the Malmö-Lund region and specified in permit 18037-19.

Viral vectors

rAAV6 carrying human α-Syn under transcriptional regulation by the Synapsin-1 promotor and the woodchuck hepatitis virus posttranscriptional regulatory element (WPRE) was

generated as previously described[16] and injected at a concentration of 1.3E+10 gc/µl. The same vector but without the human α-Syn gene was used as a control and injected at a concentration of 1.7E+10 gc/µl. Concentration was determined by ITR-qPCR.

Pre-formed fibrils

Human α-Syn PFF were produced as previously described[46] and stored at -80°C until use. PFF were diluted to a concentration of 2.5 µg/µl in sterile DPBS and sonicated for 6 min with 1 s ON/1 s OFF pulses at 70% power using a Q125 sonicator and cup horn (Qsonica, U.S.). The gross structure of PFF before and after sonication were imaged using transmission electron microscopy. PFF were diluted to a concentration of 0.025 µg/µl and transferred to a hexagonal pattern 400 mesh cupper grid with a pioloform film reinforced with a carbon coat, for 20 min at room temperature (RT). Samples were stabilized with uranyl acetate for 1 min. Excess uranyl acetate was removed and the grids were left to dry for at least 5 min prior to imaging using a FEI Tecnai Spirit BioTWIN transmission electron microscope (FEI, U.S.).

Surgical procedure

Rats were anaesthetized with 5% and maintained with 1-3% isoflurane (Isoflo vet, Orion Pharma) with a 2:1 mixture of O_2: NO_2 during the surgical procedure. Rats were attached to a stereotactic frame with a flat-skull position and 0.2 ml. Marcain (2.5 mg/ml, Aspen Nordic, Denmark) was subcutaneously (s.c.) injected under the scalp for local analgesia. Burr holes were created using a dental drill. For nigral injections, 3 µl rAAV6-(-) or rAAV6-α-Syn was injected in the following coordinates taken from bregma[47]; Anterior/posterior (A/P) -5.3 mm, medial/lateral (M/L) ±1.7 mm and dorsal/ventral (D/V) -7.2 mm. For striatal injections, 3 µl PFF (2.5 µg/µl) or DPBS as control was injected using the following coordinates relative to bregma[47]; A/P -0.4 mm, M/L ±3.0 mm and D/V -4.5 mm. Injections were made unilaterally in the right hemisphere using a 10 µl Hamilton syringe (Hamilton, U.S.) fitted with a glass capillary. Injections were made with a flow rate of 0.5 µl/2 min and the capillary was left for 2 min after the injection before it was slowly retracted. The wound was sutured using surgical staples. Metacam (1 mg/kg) (Boehringer Ingelheim Animal Health, Germany) was injected s.c. for post-operative analgesia. The rats were left to recover in clean cages and monitored for 48 h post-surgery.

Tissue collection

Rats were euthanized by intraperitoneal injection of 200-300 mg/kg sodium pentobarbital (APL, Sweden).

Cerebrospinal fluid (CSF) sampling

CSF samples were collected at baseline, 4- and 8-weeks post nigral injection from all 77 rats in a stereotactic frame with an approximate 50-60° downward flex of the head. A midline incision was made over the neck and muscles covering the cisterna magna were severed using a scalpel. CSF samples were aspirated using a 27G scalp vein set (Vygon, France) by inserting the bevel of the needle perpendicular to the cisterna magna. CSF was collected into protein LoBind tubes (Eppendorf, Germany), immediately put on dry ice and stored at -80°C until analysis. CSF samples contaminated with blood were excluded from analysis.

Serum and whole blood collection

Blood from naïve, 4- and 8-week time points from all 77 rats included in the study was collected by cardiac puncture. For cytokine analysis, serum was prepared by leaving whole blood undisturbed at RT for 30-60 min followed by centrifugation for 10 min at 4°C and 2,000xg. Serum was aliquoted into protein LoBind tubes (Eppendorf, Germany) and stored at -80°C until analysis. Whole blood was collected into K3E EDTA coated tubes (BD, U.S.) and stored at 4°C for 3-4 h until preparation for flow cytometric analysis.

Brain processing for immunohistochemistry and flow cytometry

After CSF and blood sampling, rats were transcardially perfused with 0.9% saline (w/v) with the descending aorta clamped using hemostatic forceps for at least 5 min or until no blood was visible. For IHC analysis, rats were subsequently perfused with ice-cold 4% paraformaldehyde (PFA) for 5 min and the brains post-fixed in 4% PFA at 4°C overnight (O/N) followed by cryopreservation in PBS containing 30% sucrose (w/v) and 0.01% sodium azide (w/v), pH 7.2 until sectioning. For flow cytometric analysis, brains were collected into ice-cold Roswell Park Memorial Institute 1640 medium without phenol red (Gibco/Thermo Fischer Scientific, U.S.) and stored at 4°C for a maximum of 3 h until processing.

Sample preparation for Flow cytometry

Brain sample collection and homogenization

Hemispheres of freshly collected brains were separated and put into a 7 ml glass dounce tissue grinder (DWK, Germany) with 3-5 ml ice-cold 1x Hank's Balanced Salt Solution (HBSS) without calcium, magnesium or phenol red (Gibco/Thermo Fischer Scientific, U.S.), pH 7.0-7.4. Each hemisphere was homogenized on ice using the large clearance pestle followed by the small clearance pestle until complete homogenization. The glass dounce tissue grinder set was washed with detergent and dried between samples. Homogenized samples were passed through a 100 µm nylon cell strainer (Falcon, U.S.) into a 50 ml conical tube to remove any remaining large debris. 1x HBSS (pH 7.0-7.4) was added until a total volume of 12 ml was reached and samples were kept on ice until separation of myelin and brain mononuclear cells.

Brain mononuclear cell isolation by gradient separation

Brain mononuclear cells were isolated and myelin removed using an adapted two-layer density gradient protocol[48,49]. A 100% stock isotonic Percoll (SIP) was prepared by diluting Percoll (GE Healthcare, U.S.) 9:1 in 10x HBSS (Gibco/Thermo Fischer Scientific, U.S.) and 35% SIP was prepared by diluting 100% SIP 0.35:1 in 1x HBSS pH 7.0-7.4. Homogenized brain samples were centrifuged for 5 min at 4°C and 400xg, the supernatant was discarded and the pellet was thoroughly resuspended in 16 ml of 35% SIP. The cell suspension was carefully layered with 5 ml of 1x HBSS pH 7.0-7.4 and centrifuged for 30 min at 4°C and 800xg without brake. The HBSS layer (top), myelin layer (between HBSS and 35% SIP) and 35% SIP was aspirated and the pelleted isolated brain mononuclear cells were washed in 10 ml of 1x HBSS pH 7.0-7.4 and resuspended in ice-cold fluorescence-activated cell sorting (FACS) buffer.

Blood sample preparation

Whole blood (200 µl) samples collected in EDTA coated tubes was used for flow cytometric analysis. Red blood cells (RBCs) were lysed by adding 1.8 ml of 1x Pharm Lyse (BD, U.S.) to whole blood cell samples and incubated at RT for 15-20 min. Cells were washed in sterile-filtered PBS (pH 7.2) and resuspended in sterile-filtered ice-cold FACS buffer (2% (w/v) bovine serum albumin fraction V (Roche, Switzerland) and 0.01% sodium azide (w/v) in PBS (pH 7.2)).

554

555 Antibody staining for flow cytometric analysis

556 FcγII receptors on blood and brain samples were blocked by adding anti-rat CD32 diluted
557 1:200 and incubated for 5 min at 4°C. 50 µl of cell suspension was stained using an antibody
558 cocktail (Table 1) diluted in Brilliant Stain Buffer (BD, U.S.). Cells were incubated with
559 antibodies for 30 min at 4°C in dark followed by washing in sterile PBS (pH 7.2). Cells were
560 resuspended in 250 µl of sterile FACS buffer containing DRAQ7 diluted 1:1,000 prior to
561 analysis.

562

563 **Table 1. Antibodies, viability marker and compensation beads used for flow cytometry**

Antigen/ Target	Species specificity	Fluorochrome/ Conjugation	Clone	Isotype/ Host	Dilution	Company
CD45	Rat	APC-eFluor 780	OX1	Mouse IgG1, κ	1:100	Invitrogen (47-0461-82)
CD3	Rat	BV421	1F4	Mouse IgM, κ	1:200	BD Horizon (563948)
CD4	Rat	BV605	OX-35	Mouse IgG2a, κ	1:200	BD OptiBuild (740369)
CD8a	Rat	PE-Cy7	OX8	Mouse IgG1, κ	1:200	Invitrogen (25-0084-82)
CD11b	Rat	PE	WT.5	Mouse IgA, κ	1:200	BD Pharmingen (562105)
MHCII RT1B	Rat	Alexa Fluor 647	OX-6	Mouse IgG1, κ	1:400	Bio-Rad (MCA46A647)
CD86	Rat	BV711	24F	Mouse IgG1, κ	1:100	BD OptiBuild (743215)
FcγRII	Rat	-	D34-485	Mouse IgG1, κ	1:200	BD Pharmingen (550270)
Compensation	Mouse, κ	-	-	-	-	BD CompBeads (552843)
Viability/ dsDNA	-	DRAQ7	-	-	1:1,000	Invitrogen (D15106)

564

565 Samples were analyzed using an LSR Fortessa (BD, U.S.), configuration specified in Table 2.
566 Compensation was performed using BD CompBeads (BD, U.S.) and prepared according to
567 manufacturer's instructions. Fluorescence minus one, unstained and unstained cells with
568 viability dye were included for each recording session and for each sample type (blood or
569 brain) and used to set gates. Gating strategy for brain and blood samples can be seen in
570 Supplementary Fig. 2a and 3a. Microglial cells were gated as $CD45^{dim}$CD11b+ in brain samples.

Infiltrating macrophages/monocytes ($CD45^{high}CD11b+$) and T lymphocytes (CD45+CD3+) in brain samples were rare with <1,000 events/hemisphere. Myeloid population in blood was gated as CD45+CD11b+ and T lymphocytes as CD45+CD3+. Th cells were gated as CD4+ and cytotoxic T lymphocytes as CD8+. Data was analyzed using FlowJo software version 10.8.1 (BD, U.S.). All analyses were done on freshly isolated tissue and recorded during multiple sessions. 4-6 rats were used at each recording session (equal number of DA and DA.VRA4 rats per session) from the same experimental group (naïve/control/α-Syn) and time point (4- or 8- weeks). To minimize variation introduced by the instrument or sample preparation from each session all comparisons from full groups are made from percentages or normalized values.

Table 2. Configuration of the LSR Fortessa used for flow cytometric analysis and filters used for recording of isolated blood and brain cells.

Laser	Filter	Fluorochrome
Blue – 488 nm	780/60	PE-Cy7
	695/40	-
	610/20	-
	575/26	PE
	530/30	-
	488/10	SSC
Red – 640 nm	780/60	APC-eFluor 780
	730/45	DRAQ7
	670/30	Alexa Fluor 647
Violet – 405 nm	780/60	-
	710/50	BV711
	660/20	-
	610/20	BV605
	525/50	-
	442/46	BV421

Immunohistochemistry

Fixed brains were coronally sectioned on a Microm HM450 freezing microtome (Thermo Scientific, U.S.) with 35 µm thickness in series of 12 and stored in Walter's antifreeze solution at 4°C until IHC staining. All stainings were done on free floating sections except for proteinase K treated α-Syn staining which was done on mounted sections on gelatin-coated glass slides. Sections were rinsed with PBS or 0.1% PBS with Triton-X 100 (v/v) (PBST) between all incubation steps. For proteinase K resistant α-Syn aggregates, sections were incubated with

5 µg/ml Proteinase K diluted in TBS (Thermo Fischer Scientific, U.S.) for 1 h at RT prior to quenching. For 3,3´-diaminobenzidine (DAB) stainings sections were quenched with 3% H_2O_2 (v/v) and 10% MetOH (v/v) in PBS. Sections were blocked with 10% serum (same species as secondary antibody) in 0.3% PBST. Primary antibody was diluted in 0.3% PBST with 5% serum (same species as secondary antibody) and incubated at 4°C O/N. On the following day sections were incubated with biotinylated secondary antibody and incubated for 1 or 2 h at RT (DAB or Fluorescence, respectively). All antibodies used for IHC are found in Table 3. For DAB stainings, horseradish peroxidase conjugated avidin/biotin-complex (Vector laboratories, U.S.) was prepared according to manufacturer's instructions and added to the sections for 30 min at RT. A DAB substrate kit (Vector laboratories, U.S.) was prepared according to manufacturer's instructions and used as a chromogen for visualization. DAB sections were mounted on gelatin-coated glass slides, dehydrated and coverslipped using Pertex (Histolab, Sweden). Fluorescently stained sections were coverslipped using PVA/DABCO and stored at 4°C in dark. Brightfield overview images of TH and human α-Syn were acquired using an Olympus VS-120 virtual slide scanner (Olympus, Japan). Brightfield images of pS129 α-Syn and proteinase K treated human α-Syn in SN was acquired using an Olympus BX53 (Olympus, Japan). MHCII+ microglia cells were imaged using a Leica SP8 scanning confocal microscope (Leica, Germany).

Table 3. List of antibodies used for immunohistochemistry

Antigen/Secondary antibody	Host	Dilution	Company
Human α-Syn	Mouse	1:1,000	Santa Cruz (sc-12767)
Biotinylated anti-mouse	Horse	1:200	Vector Laboratories (BA-2001)
TH	Rabbit	1:1,000	EMD Millipore (AB152)
pS129 α-Syn	Rabbit	1:2,000	Abcam (ab51253)
Biotinylated anti-rabbit	Goat	1:200	Vector Laboratories (BA-1000)
MHCII	Mouse	1:500	Abcam (ab23990)
Alexa Fluor 488 anti-mouse	Donkey	1:200	Abcam (ab150105)

Cytokine analysis

Cytokine analysis in serum and CSF was performed using the V-PLEX Proinflammatory panel 2 Rat Kit from Mesoscale diagnostics (MSD, U.S.) according to manufacturer's instructions. The plates were washed using PBS with 0.05% Tween-20 between incubation steps. Serum samples were diluted 4-fold and CSF samples 2-fold. Plates were read on a MESO QuickPlex SQ 120 analyzer (MSD, U.S.). Results were analyzed using the Discovery Workbench software

version 4.0.13 (MSD, U.S.). The number of samples used for cytokine analysis differs as a consequence for available wells on the MSD plate. All samples were run in duplicates and the mean value was used for analysis. If only one replicate was detected it was included in the analysis. If both replicates were undetected for a sample the non-detected (ND) value was replaced with the lowest quantifiable value for the specific cytokine. If duplicates for more than one sample was undetected for a group no statistical comparisons were made due to uncertainty of the results, however, all detected values are presented. If all samples were undetected for a group it is indicated by "ND".

Statistical analyses

Statistical analyses were conducted using the GraphPad Prism software version 9.3.1 (San Diego, CA, U.S.). Quantile-quantile plot of residuals was used to determine the use of parametric or non-parametric tests. Data in figures is presented as mean ± SD and individual values. Comparisons between contralateral and ipsilateral hemispheres was done by paired Student's t-test. Unpaired Student's t-test was used to compare control and α-Syn+PFF groups within strain or naïve/control/α-Syn+PFF between strains. Data in text is presented as (mean1 ± SD1 vs mean2 ± SD2, p-value, 95% CI of difference [lower limit, upper limit]). A significance level of $\alpha<0.05$ was used for all analyses.

DATA AVAILABILITY

All original data is available from the corresponding author upon reasonable request.

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ATHOR CONTRIBUTION

F.B., I.J.F. and M.S. designed the study. K.C.L. produced α-Syn pre-formed fibrils. F.B. and I.J.F. performed stereotactic injections, sample collection and flow cytometric recordings. F.B. performed enzyme-linked immunosorbent assays. Data was analyzed by F.B., K.G., M.S. and L.B. All authors contributed to the manuscript.

COMPETING INTEREST

The authors declare no competing interest.

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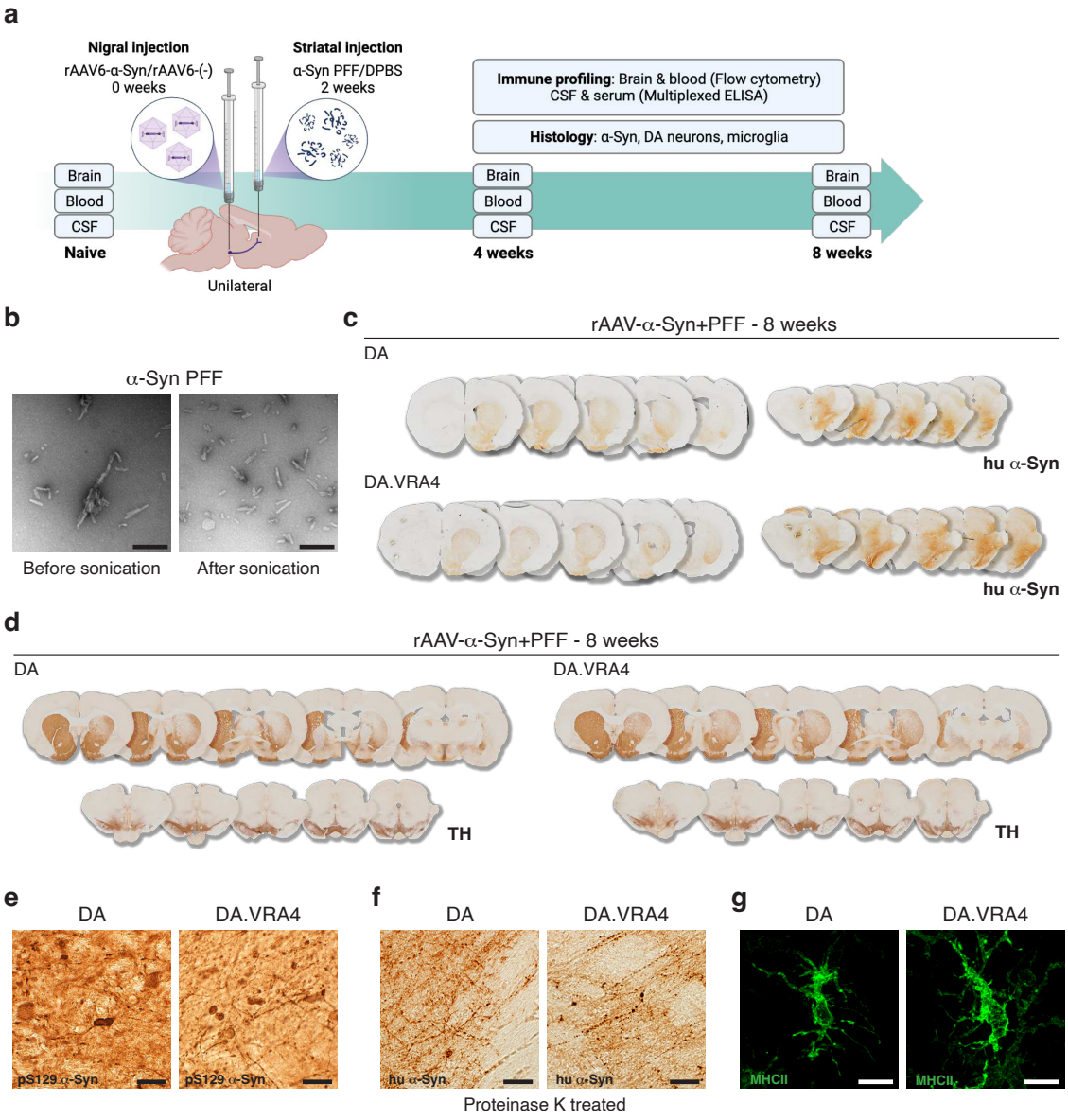
804 FIGURES AND FIGURE LEGENDS



805

806 **Fig. 1. α-Syn overexpression combined with striatal seeding of α-Syn pre-formed fibrils**
807 **(PFF) leads to TH loss, α-Syn pathology and MHCII upregulation. a** Experimental outline
808 (created with BioRender.com). **b** TEM images of α-Syn PFF before (left) and after (right)
809 sonication; sonicated PFF were used for striatal seeding. Scale bar = 200 nm. **c** Unilateral nigral
810 overexpression of human α-Syn combined with striatal seeding of human PFF results in robust
811 human α-Syn signal in substantia nigra (SN) and striatum. rAAV-α-Syn+PFF injection leads to
812 **d** loss of TH-signal in both striatum and SN, **e** positive signal for phosphorylated α-Syn on
813 serine residue 129 (pS129 α-Syn) and **f** proteinase K resistant α-Syn aggregates. **e-f**
814 Representative images from 8 weeks ipsilateral SN, scale bar = 20 µm. **g** rAAV-α-Syn+PFF

injection leads to upregulation of MHCII on microglia, representative image from 8 weeks ipsilateral midbrain, scale bar = 10 µm.

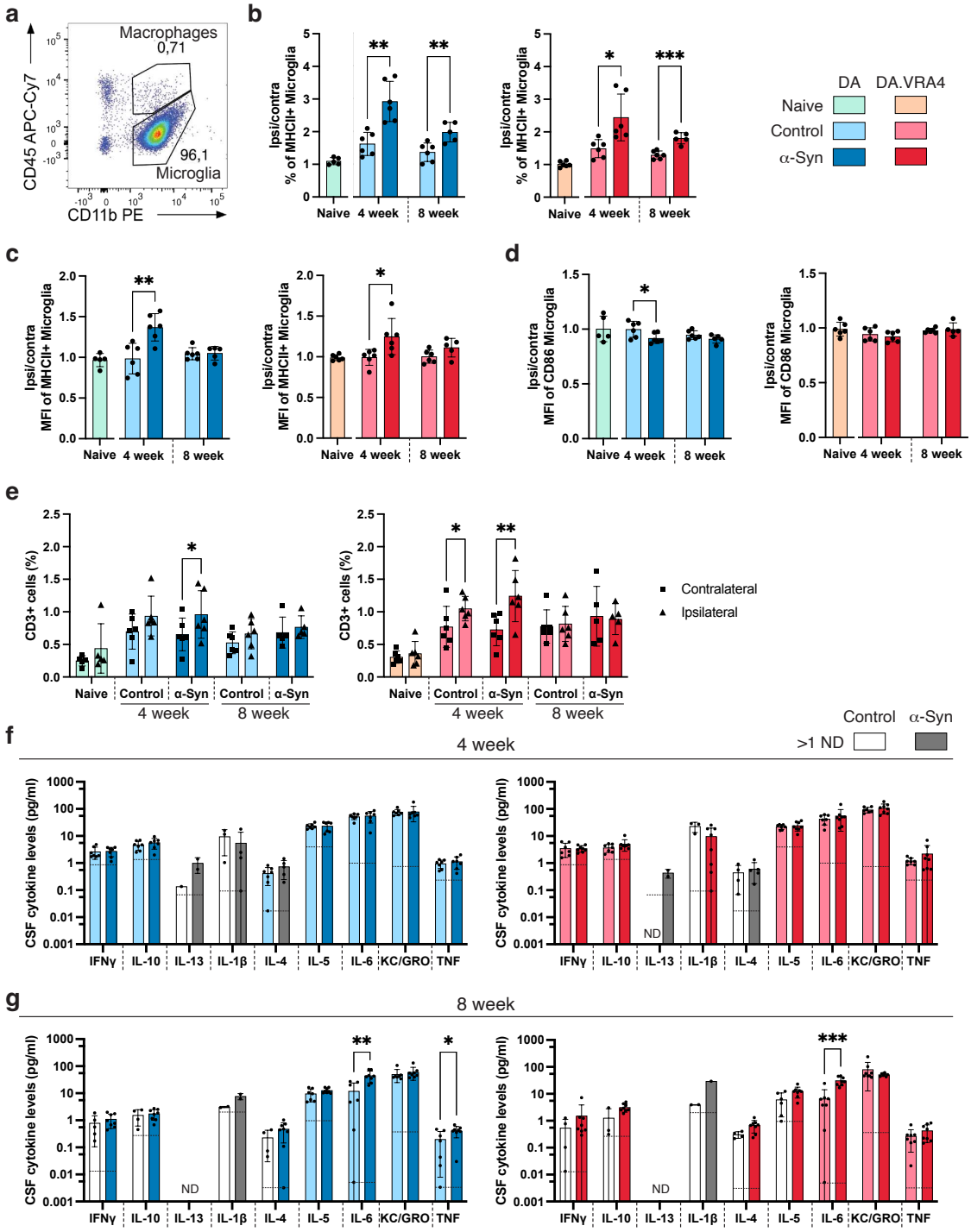


Fig. 2. Local effects of rAAV-α-Syn+PFF on microglial MHCII expression, infiltrating lymphocytes and CSF cytokine profiles. a Gating of microglia ($CD45^{dim}CD11b+$) and infiltrating macrophages/monocytes ($CD45^{high}CD11b+$) in brain samples. **b** Normalized (ipsilateral/contralateral hemisphere) percentage of MHCII+ microglia is higher in rAAV-α-

822 Syn+PFF injected animals compared to control in both DA (left) and congenic DA.VRA4 (right)
823 rats. **c** MHCII levels determined by normalized median fluorescence intensity (MFI) values in
824 DA (left) and DA.VRA4 (right) rats are higher after 4- but not 8-weeks post nigral α-Syn
825 overexpression. **d** Normalized MFI values of CD86 in DA (left) and DA.VRA4 (right) rats. **e**
826 Stereotactic injection leads to increased percentage of T lymphocytes (CD45+CD3+) in DA
827 (left) and congenic DA.VRA4 (right) rats. **b-e** Naïve (DA n=5, DA.VRA4 n=6), 4-week; control
828 (DA n=6, DA.VRA4 n=6) and α-Syn (DA n=6, DA.VRA4 n=6), 8-week; control (DA n=6, DA.VRA4
829 n=6) and α-Syn (DA n=5, DA.VRA4 n=5). **f-g** Cytokine levels in cerebrospinal fluid (CSF) 4- and
830 8-weeks post nigral injection, respectively, in DA (left) and DA.VRA4 (right) rats. α-Syn
831 injection results in elevated IL-6 levels in both DA and DA.VRA4 and TNF in DA.VRA4 at 8
832 weeks. No statistical analysis was done if >1 value/group was non-detected (ND). The limit
833 for lowest quantifiable value for each cytokine is indicated by a horizontal dashed line. Groups
834 with all values non-detected are indicated by "ND". 4-week; control (DA n=7, DA.VRA4 n=7)
835 and α-Syn (DA n=7, DA.VRA4 n=8), 8-week; control (DA n=7), DA.VRA4 n=8) and α-Syn (DA
836 n=8, DA.VRA4 n=8). **b-d, f-g** Unpaired Student's t-test. **e** Paired Student's t-test. *p < 0.05, **p
837 < 0.01 and ***p < 0.001. Data presented as mean ± SD with individual values.

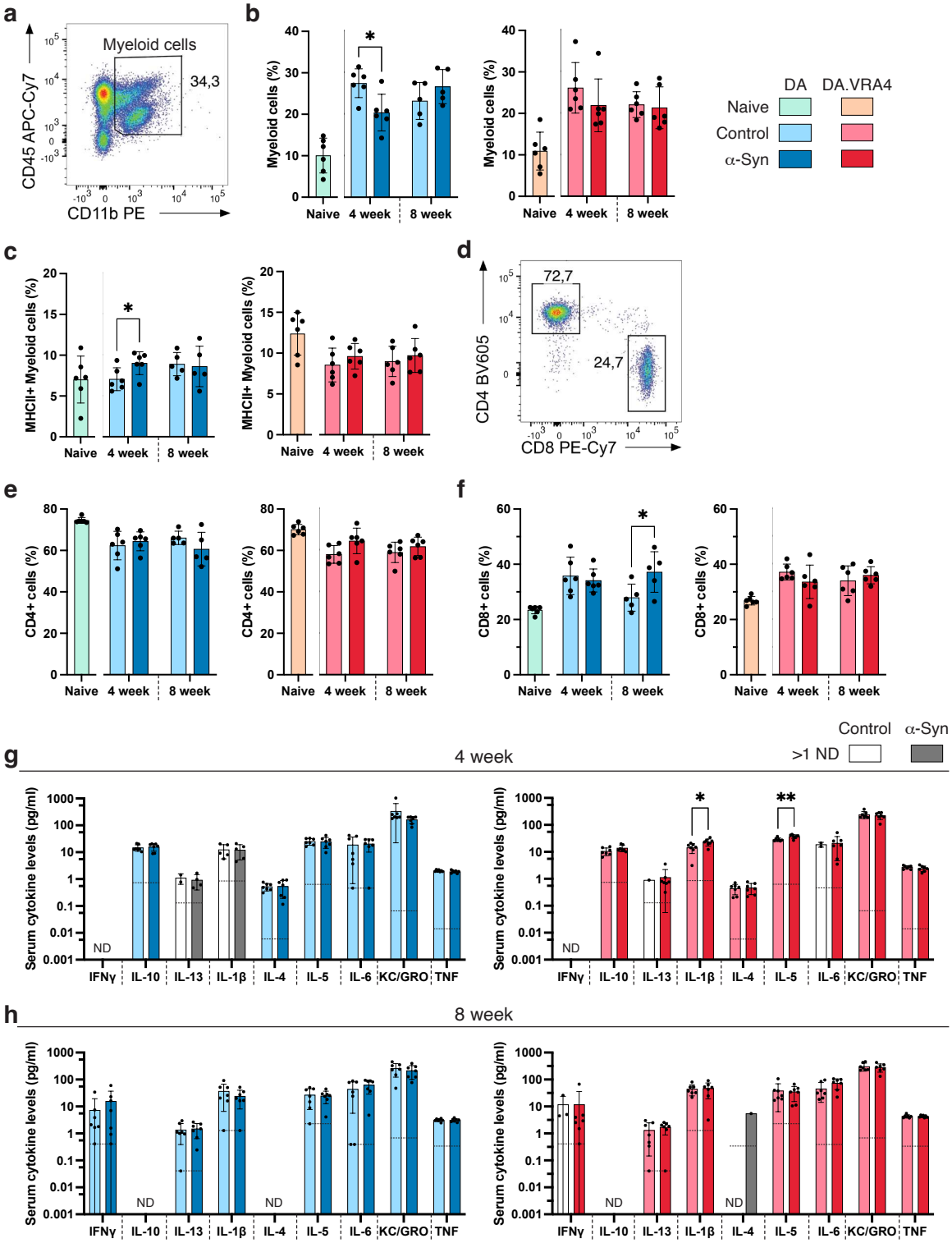
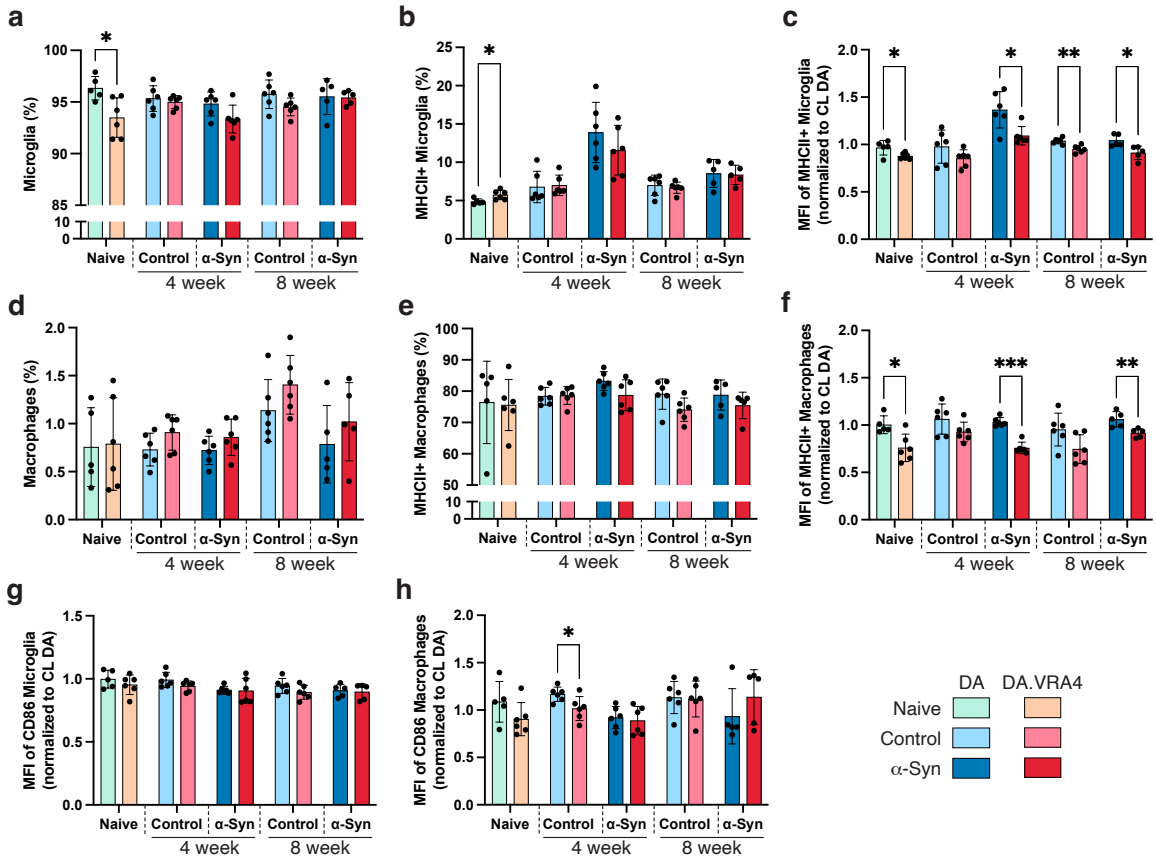


Fig. 3. Systemic effects of rAAV-α-Syn+PFF on blood myeloid cells' MHCII expression, circulating lymphocytes and serum cytokine profiles. **a** Gating of myeloid cells (CD45+CD11b+) in blood. **b** Overall percentage of myeloid cells in DA (left) and DA.VRA4 (right) at 4- and 8-weeks. **c** Percentage of MHCII+ myeloid cells is reduced in DA rats (left) α-Syn group compared to control but unaltered in congenic DA.VRA4 rats (right). **d** Gating of

CD4+ or CD8+ T lymphocytes (CD45+CD3+ cells). **e** Overall percentage of CD4+ T lymphocytes in blood does not change after rAAV-α-Syn+PFF injection in the brain of DA (left) or DA.VRA4 (right) rats. **f** Percentage of CD8+ T lymphocytes increase in α-Syn group at 8 weeks in DA (left) rats. **g-h** rAAV-α-Syn+PFF injection results in increased serum IL-1β and IL-5 levels in DA.VRA4 rats (right) after 4 weeks, n=7/group. **h** No change in serum cytokine levels is observed at 8 weeks post nigral rAAV-α-Syn injection in DA (left) or DA.VRA4 (right) rats, n=7/group. No statistical analysis was done if >1 value/group was ND. Groups with all values non-detected are indicated by "ND". The limit for lowest quantifiable value for each cytokine is indicated by a horizontal dashed line. **a-f** Naïve (DA n=6, DA.VRA4 n=6), 4 week; control (DA n=6, DA.VRA4 n=6) and α-Syn (DA n=6, DA.VRA4 n=6), 8 week; control (DA n=5, DA.VRA4 n=6) and α-Syn (DA n=5, DA.VRA4 n=6). **b,c and e-h** Unpaired Student's t-test. *$p < 0.05$, **$p < 0.01$. Data presented as mean ± SD with individual values.



857

858 **Fig. 4 CIITA regulates local MHCII levels on both microglia and infiltrating macrophages in**
859 **response to rAAV-α-Syn+PFF. a** Total percentage of microglia (CD45^{dim}CD11b+) is reduced in
860 naïve DA rats compared to DA.VRA4 rats with lower CIITA levels. **b** Percentage of MHCII+
861 microglia is higher in naïve DA compared to DA.VRA4. **c** Congenic DA.VRA4 rats with lower
862 CIITA have reduced MHCII MFI levels on microglia independent of α-Syn, normalized to
863 contralateral (CL) DA values. **d** Percentage of infiltrating macrophages/monocytes
864 (CD45^{high}CD11b+) and **e** MHCII+ macrophages are not regulated by differing CIITA levels. **f**
865 DA.VRA4 rats have reduced MHCII MFI levels on infiltrating macrophages compared to DA
866 independent of rAAV-α-Syn+PFF injections (normalized to CL DA). **g** Normalized microglial
867 CD86 MFI levels is not regulated by CIITA. **h** CD86 MFI levels (normalized to CL DA) are not
868 regulated by CIITA in response to α-Syn. Naïve (DA n=5, DA.VRA4 n=6), 4-week; control (DA
869 n=6, DA.VRA4 n=6) and α-Syn (DA n=6, DA.VRA4 n=6), 8-week; control (DA n=6, DA.VRA4 n=6)
870 and α-Syn (DA n=5, DA.VRA4 n=5). Data presented as mean ± SD with individual values.
871 Unpaired Student's t-test. *p < 0.05, **p < 0.01 and ***p < 0.001.

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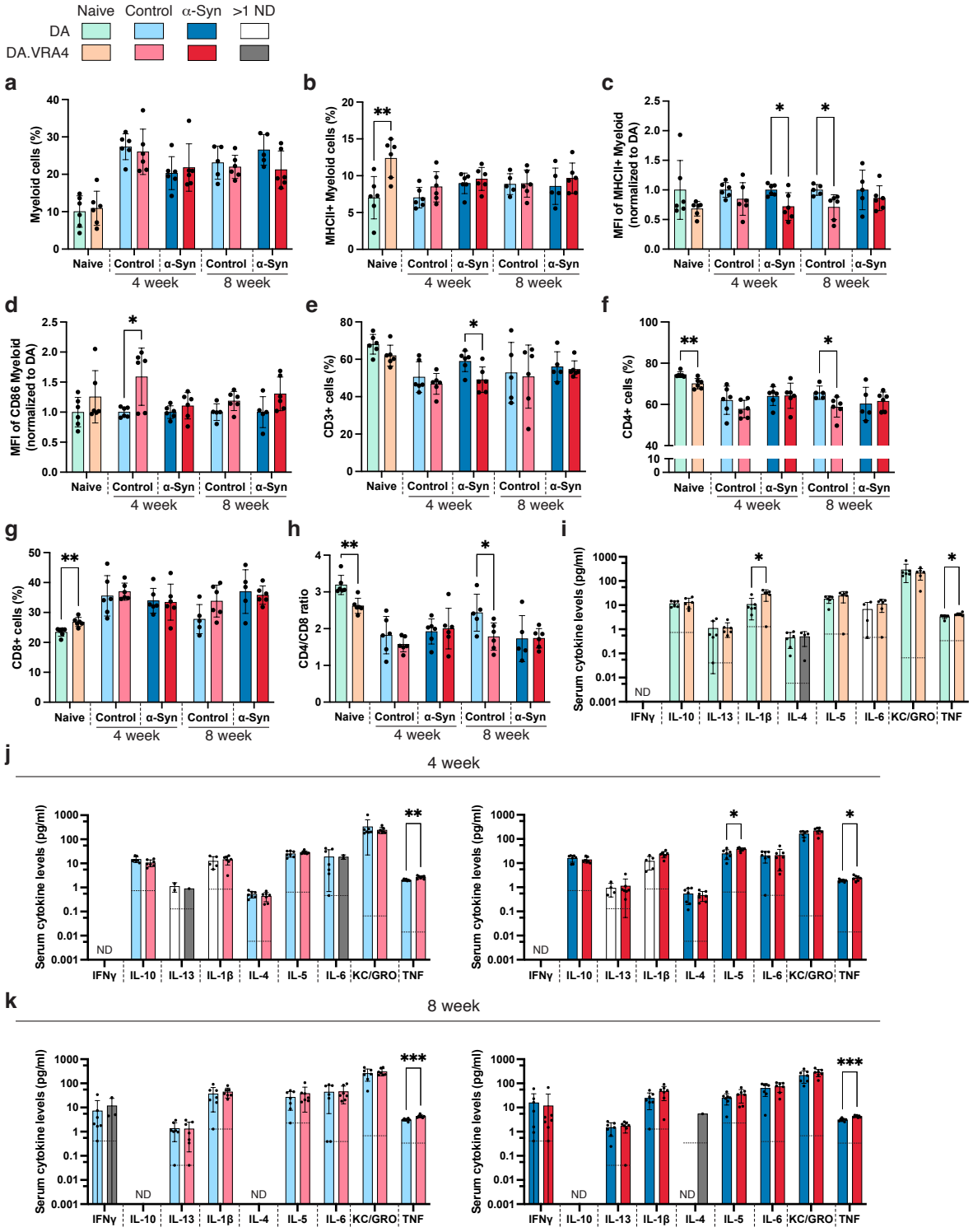

873

874 **Fig. 5. CIITA regulates MHCII levels on blood myeloid cells and TNF levels in serum. a** The
875 proportion of myeloid cells (CD45+CD11b+) in blood is not regulated by CIITA. **b** Naïve
876 DA.VRA4 rats with less CIITA have an increased proportion of MHCII+ myeloid cells compared
877 to DA. **c** CIITA regulates MHCII MFI levels in circulating myeloid cells. **d** CD86 MFI levels are
878 not regulated by CIITA in response to α-Syn. **e** Reduced percentage of T lymphocytes

879 (CD45+CD3+) in rats with lower CIITA levels 4 weeks after rAAV-α-Syn SN injection. **f-h** Naïve
880 congenic DA.VRA4 rats with lower CIITA levels have less CD4+ but more CD8+ T lymphocytes
881 (CD45+CD3+) leading to a reduced CD4/CD8 ratio. **a-h** Naïve (DA n=6, DA.VRA4 n=6), 4 week;
882 control (DA n=6, DA.VRA4 n=6) and α-Syn (DA n=6, DA.VRA4 n=6), 8 week; control (DA n=5,
883 DA.VRA4 n=6) and α-Syn (DA n=5, DA.VRA4 n=6). **i-k** Congenic DA.VRA4 rats with less CIITA
884 have increased TNF levels in serum independent of α-Syn. No statistical analysis was done if
885 >1 value/group was ND. The limit for lowest quantifiable value for each cytokine is indicated
886 by a horizontal dashed line. Groups with all values non-detected are indicated by "ND". **i**
887 n=6/group. **j-k** n=7/group. Data presented as mean ± SD with individual values. Unpaired
888 Student's t-test. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$.
889

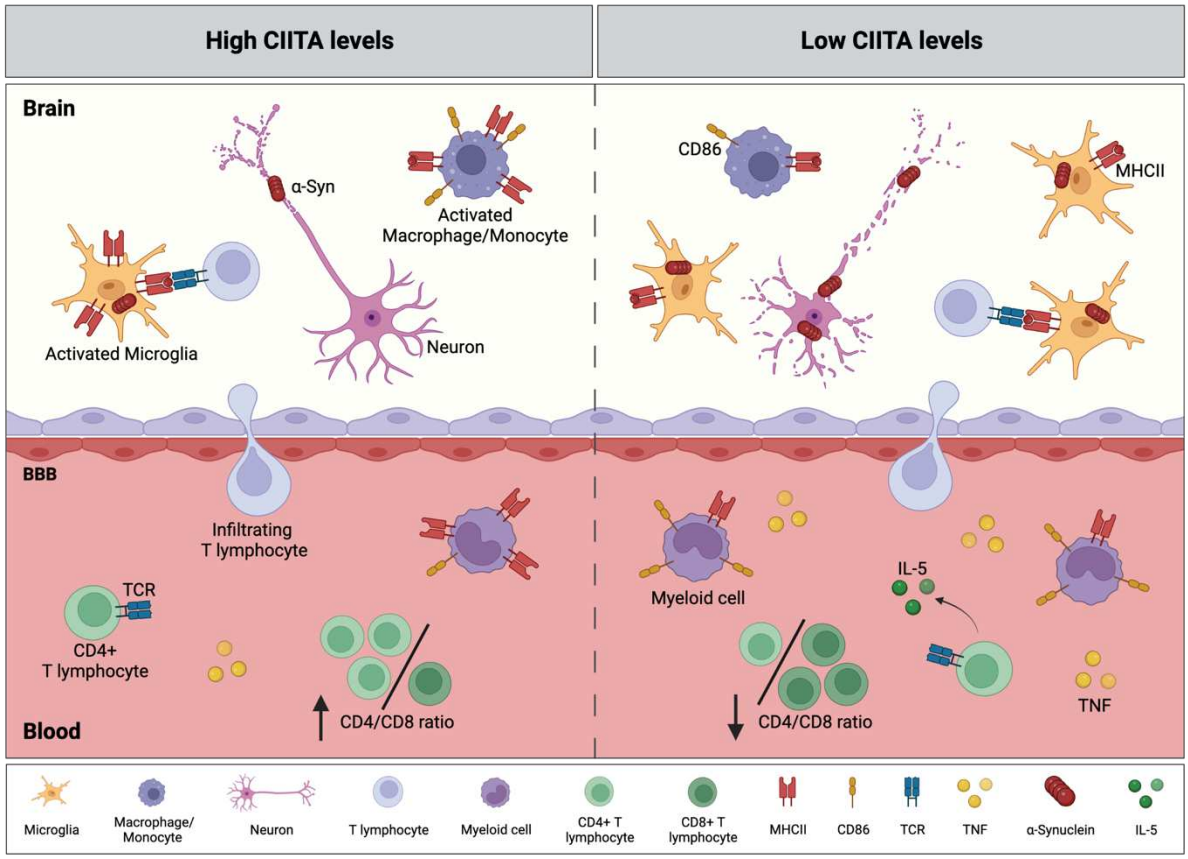

Fig. 6. CIITA regulates baseline immune populations that could affect the susceptibility to PD-like pathology and exacerbate α-Syn propagation. Lower CIITA levels are associated with motor impairments[13,14], neurodegeneration[13,14] and exacerbated α-Syn pathological spread[14] in response to α-Syn. Reduced CIITA levels are also associated with lower MHCII levels on microglia[13,14] and myeloid cells (both in brain and blood, reported in the current study). In addition to an increased number of activated microglia (determined by morphology[13] and MHCII positive cells in striatum[13,14]) in response to α-Syn, the current study also reveals that reduced CIITA levels are associated with increased numbers of MHCII+ microglia in brain and MHCII+ myeloid cells in circulation in naïve rats, which could influence the susceptibility to PD-like pathology. We believe that the increased number of activated microglia in rats with low CIITA levels are important to consider in terms of susceptibility and progression of PD-like pathology. Infiltrating T lymphocytes could be presented with processed α-Syn peptides in the brain, leading to an adaptive immune response (lower CIITA levels are associated with elevated levels of IL-5 in serum in response to α-Syn). α-Syn reactive T lymphocytes in circulation have been reported by *ex vivo* studies in PD patients[9,10]. CIITA levels regulated CD4/CD8 ratio in blood in naïve rats and TNF levels in serum. If TNF levels have an impact on

susceptibility and progression of α-Syn seeded PD-like pathology requires further investigation. The illustration was created with BioRender.com.

Supplementary Files

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