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 a heterogeneous disorder characterized by intraneuronal inclusions of alpha-synuclein (α-Syn), a strong neuroinflammatory component and neurodegeneration. Human genetic association studies have shown that variants affecting quantity and quality of major histocompatibility complex II (MHCII) have implications in PD susceptibility and it was recently shown 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 increase α-Syn induced neurodegeneration and pathology *in vivo*. Here we show, using flow cytometry in an α-Syn overexpression model combined with α-Syn pre-formed fibrils (PFF), that congenic rats with naturally occurring differences in Ciita expression have altered local and peripheral immune populations. Lower Ciita levels are associated with increased percentages of microglia and circulating myeloid cells being MHCII+ but with lower levels of MHCII on individual cells. Additionally, lower Ciita levels was associated to higher TNF levels in serum, trends of higher CD86 levels in circulating myeloid population and a lower CD4/CD8 T lymphocyte ratio. Taken together, these results indicate that Ciita regulates serum TNF levels and baseline immune populations which could mediate an increased susceptibility to PD-like pathology.

INTRODUCTION

Parkinson's disease (PD) is a progressive and incurable neurodegenerative disorder that is estimated to affect 2-3% of the population above the age of 65[1]. PD is a very heterogenous disorder and approximately 95% of all cases have a multifactorial etiology where the genetic makeup, lifestyle and environment determine the risk of developing PD[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 activation and upregulation of major histocompatibility complex II (MHCII) on microglia as well as altered levels of pro-inflammatory cytokines in cerebrospinal fluid (CSF) and blood[3]. Genetic association studies have identified single nucleotide polymorphisms in the noncoding region of the human leukocyte antigen (*HLA*), influencing 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 overall risk of developing PD.

Previous studies in mice using viral overexpression of α-Syn in SN have shown that knocking out MHCII protects against neurodegeneration and microglial activation[7]. Additionally, nigral overexpression of α-Syn in athymic nude rats was protective against microglial activation and dopaminergic neurodegeneration[8] whereas striatal seeding of α-Syn pre-formed fibrils (PFFs) in immunocompromised mice resulted in more α-Syn pathology in the SN[9].

It was recently shown that α-Syn peptides presented on MHCII can activate CD4+ lymphocytes and that it occurs early in PD[10,11]. Infiltrating lymphocytes have also been observed in post mortem tissue from PD patients, suggesting a role of the adaptive immune system in disease progression[12]. The MHCII molecules are important in linking the innate and adaptive immune system as antigens presented on MHCII activate antigen-specific T-lymphocyte responses by interacting with CD4 and co-stimulatory molecules such as CD86.

MHCII expression is controlled by the class II transactivator (Ciita, also known as Mhc2ta) and silencing of *Ciita in vivo* using shRNA has shown to prevent neurodegeneration in a mouse α-Syn SN overexpression model of PD[13]. In contrast, we have previously shown using congenic rats with naturally occurring variants in the promotor of *Ciita*, that DA.VRA4 rats with lower MHCII levels have more phenotypically activated microglia after α-Syn overexpression in the SN compared to wildtype (wt) DA rats[14]. Additionally, DA.VRA4 rats had more widespread α-Syn pathology after nigral overexpression of α-Syn combined with striatal seeding with PFFs[15]. The involvement of MHCII in PD is clear, however, current experimental studies show contradictory results making it difficult to state if the involvement is protective or detrimental. Additionally, studies on the effects of altered MHCII levels on peripheral immune populations and the contribution to PD are lacking.

In the current study we aim to investigate the effect of naturally occurring variation in MHCII expression on the peripheral and local immune response to a recombinant adeno-associated viral vector (rAAV) nigral α-Syn overexpression combined with striatal seeding of human PFFs. To do so, we use two congenic rats with different transcriptional activity of the *Ciita* gene and therefore high vs low levels of MHCII molecules. Using a flow cytometric approach investigating both local and peripheral immune populations we confirm previous results that DA.VRA4 rats with lower levels of Ciita have less MHCII expression in microglial cells compared to wt DA rats results. Importantly, we also found lower MHCII levels in circulating cells of myeloid lineage and that rats with less Ciita had higher levels of the co-stimulatory marker CD86 in circulating myeloid cells and higher levels of tumor necrosis factor (TNF)-α in serum. Collectively these results suggest that altered levels of MHCII through Ciita results in altered immune populations and expression of molecules involved in immune responses that could affect the susceptibility for PD.

RESULTS

Nigral injection of rAAV6-α-Syn combined with striatal seeding of PFFs results in robust α-Syn expression, α-Syn inclusions and dopaminergic neurodegeneration

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[14]. PD like-pathology was induced by human α-Syn overexpression combined with PFF seeding, adapted from Thakur

et al[15,16]. Rats were injected with a rAAV6-α-Syn vector[17] into the SN followed by an injection of sonicated human α-Syn PFFs two weeks later in the striatum[15] (Fig. 1a and b). Control animals were injected with an empty rAAV6 vector into the SN and vehicle into the striatum. Rats were sacrificed at baseline (naïve), 4- or 8-weeks post nigral injection for collection of brain, blood and CSF samples (Fig. 1a).

The combined model resulted in robust positive signal of human α-Syn in the SN and striatum of both DA and DA.VRA4 rats 4- and 8-weeks post nigral injection (Fig. 1c and Supplementary Fig. 1a), similar to what we have previously shown[15]. Controls receiving nigral injection of empty vector combined with striatal injection of Dulbecco's phosphate buffered saline (DPBS) did not show any human α-Syn (Supplementary Fig. 1b, c). As expected, the seeded α-Syn model resulted in loss of TH-positive signal in both DA and DA.VRA4 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).

rAAV-α-Syn overexpression combined with PFF seeding increase microglial MHCII+ expression and CSF cytokine levels in DA and DA.VRA4 rats

We have previously shown using an immunohistochemical (IHC) approach in an rAAV-α-Syn based PD-model that DA.VRA4 rats have lower levels of Ciita and more activated microglia compared to DA rats[14]. Flow cytometric analysis of brain cells revealed no difference in the overall percentage of CD45dimCD11b+ cells (microglia) after Injection of control vector+vehicle or rAAV-α-Syn+PFF (Fig. 2a and Supplementary Fig. 2a, b), but an increase in the percentage of MHCII+ microglial cells in the ipsilateral hemisphere at 4- and 8 weeks with a larger increase in the α-Syn groups compared to controls in both strains (Fig. 2b, Supplementary Fig. 2c). The intensity of MHCII signal (determined by median fluorescence intensity, MFI) on microglia cells was also elevated in the α-Syn group at 4 weeks whereas it remained unaltered at 8 weeks in both strains (Fig. 2c and Supplementary Fig. 2d). We did not observe any changes in CD45HighCD11b+ (macrophage) populations (Fig. 2a) in terms of overall percentage, percentage of MHCII+ macrophages, or MHCII+ MFI levels (Supplementary Fig. 2e-g).

CD86 (also known as B7-2) is a co-stimulatory signal expressed by antigen presenting cells (APCs) necessary for activation of T lymphocytes[18]. The α-Syn seeded model and control did not result in elevated CD86 MFI levels in microglia or macrophages in the ipsilateral hemisphere (Supplementary Fig. 2h, i). After normalization to the contralateral hemisphere, the CD86 MFI levels were reduced in the DA 4-week α-Syn group only (Fig. 2d). Additionally, there was a slight increase in CD3+ T lymphocytes at 4 weeks in the DA α-Syn group as well as in DA.VRA4 control and α-Syn groups (Fig. 2e). These results indicates that there is no major infiltration nor activation of T lymphocytes in the seeded α-Syn rat model for PD.

To investigate the effect of α-Syn+PFF on CSF cytokine levels, we performed multiplexed ELISA. At 4-weeks there were no differences in cytokine levels (Fig. 2f) whereas at 8-weeks, TNF levels were increased in DA rats and IL-6 levels were increased in both DA and DA.VRA4 rats compared to their controls (Fig. 2g).

rAAV-α-Syn+PFF model-induced changes in blood myeloid- and T lymphocytic populations

We next investigated changes in blood immune cell populations induced by the α-Syn+PFF model by flow cytometry (Supplementary Fig. 3a) and observed a reduction of CD45+CD11b+ circulating myeloid cells in DA rats at 4 weeks compared to controls (Fig. 3a, b). However, the percentage of these cells that were MHCII+ was increased (Fig. 3c). No change in CD4+ T lymphocytes was observed (Fig. 3d, e) whereas there was an increase in the percentage of CD8+ T lymphocytes in DA rats at 8 weeks compared to controls (Fig. 3f). No difference in CD4/CD8 ratios was detected (Supplementary Fig. 3b).

To investigate the effect of α-Syn+PFF on blood cytokine levels, we performed multiplexed ELISA. Levels of IL-1β and IL-5 were elevated at 4 weeks in DA.VRA4 rats only, with no changes at 8 weeks (Fig. 3g, h).

Differential expression of Ciita regulates MHCII levels on brain macrophages and microglia during seeded α-Syn pathology

DA and congenic DA.VRA4 rats responded similarly to the α-Syn+PFF seeded model in terms of microglial population size, proportion of MHCII+ microglia (Fig. 4a, b) and infiltration of

CD3+ T lymphocytes (Supplementary Fig. 4a). Similar to previous findings, the intensity of MHCII+ signal (ipsilateral MFI normalized to contralateral DA values) on microglia was lower in DA.VRA4 rats with decreased Ciita levels compared to DA (Fig 4c). This Ciita -dependent difference was observed between strains in naïve, control and α-Syn+PFF groups (Fig. 4c). However, microglial CD86 levels (MFI) did not differ between strains (Fig. 4d).

There were no differences in percentages of infiltrating macrophages or percentage of MHCII+ macrophages between DA and DA.VRA4 rats (Fig. 4e-f). Similar to the results for microglia, DA.VRA4 rats with reduced Ciita levels had lower levels of MHCII in the macrophage population compared to DA (Fig. 4g). 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 (Fig. 4h). No differences in CSF cytokine levels was observed between the two strains apart from higher IL-10 levels in the DA.VRA4 strain in the α-Syn+PFF group at 8 weeks (Supplementary Fig. 4b-f).

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

We used flow cytometric analyses of CD45+CD11b+ myeloid cells and CD4+/CD8+ T lymphocytes (CD45+CD3+) to determine if differential expression of Ciita affects circulating immune cells in blood in naïve, control and α-Syn+PFF injected DA and DA.VRA4 rats. There was no difference between strains in overall percentage of circulating myeloid cells (Fig. 5a). Similar to results from brain, naïve DA.VRA4 rats with lower Ciita levels had a higher percentage of MHCII+ myeloid cells in blood compared to DA (Fig. 5b). Also similar to the results from brain, myeloid cells in blood from DA.VRA4 showed a clear trend of lower levels of MHCII, determined by MFI values, independent of intervention (Fig. 5c). Interestingly, the opposite trend was observed for CD86, with higher MFI levels in DA.VRA4 compared to DA rats (Fig. 5d). The percentage of CD4+ T lymphocytes was decreased and CD8+ T lymphocytes increased in naïve and control DA.VRA4 rats compared to DA (Fig. 5e, f). This trend was also reflected in a reduced CD4/CD8 ratio in DA.VRA4 compared to DA rats (Fig. 5g). The differences in T lymphocyte populations were, however, not depending on the α-Syn+PFF model but observed in naïve rats and control groups.

Naïve DA.VRA4 rats with lower levels of Ciita had higher levels of TNF in serum compared to DA, and this difference remained after control- or α-Syn+PFF injections (Fig. 5h-l). Additionally, DA.VRA4 rats had higher levels of IL-1β when naïve (Fig. 5h) and higher IL-5 levels 4 weeks after α-Syn+PFF injections (Fig. 5j).

DISCUSSION

Studies investigating human PD cohorts or experimental models of PD has revealed important findings on how local and peripheral immune responses might contribute to or protect against different aspects of PD. However, contradictory outcomes are often reported. A contributing factor is likely the multiple different murine models used to study PD-related changes in the immune system, including toxin-induced models (e.g., MPTP)[12], transgenic models[19-21] or models with overexpression of α-Syn using viral vectors[7,13,14,22], injection of PFFs[9,23], or a combination of the two[15,16]. Additionally, knockout models are often used to study the role of immune-related proteins or molecules[9,12,13,21,24,25], although these often have a dysfunctional immune system. In the current study we instead use rats with naturally occurring variants in the *Ciita* gene, leading to differential expression of MHCII. Similar variations in the human orthologue of Ciita also regulate MHCII expression and are associated with susceptibility of rheumatoid arthritis, multiple sclerosis and myocardial infarction[26]. We argue that the use of these congenic rats is a physiologically highly relevant model to study the effects of antigen presentation on immune populations and PD-like pathology. To our knowledge this is the first study to investigate the effects of Ciita/MHCII on immune populations in a model with genetic and pathophysiologic similarities with PD.

In the current study we show that differential expression of the master regulator of MHCII, Ciita, have implications in both the local and peripheral immune populations. Here, we use a PD model of nigral overexpression of human α-Syn combined with striatal seeding of human PFFs to model PD. We confirm previous findings of lower Ciita levels affecting MHCII levels in microglial cells in response to α-Syn[13-15]. Additionally, we show that Ciita levels also affect MHCII expression in circulating cells of myeloid lineage, and DA.VRA4 rats with less Ciita also show tendencies of increased levels of CD86. Interestingly, naïve DA.VRA4 rats had a higher percentage of MHCII+ circulating cells of myeloid lineage, and relatively more MHCII+

microglia in the brain. The observed differences for untreated rats suggest that the differential expression of *Ciita* affects the baseline levels of MHCII+ microglia as well as circulating myeloid cells, and not only observed after initiation of PD like pathology[13-15]. Additionally, lower Ciita levels were associated with higher levels of TNF in circulation, independent of intervention. Thus, lower levels of Ciita affect baseline/naïve immune conditions that could mediate an increased susceptibility to pathological aggregation, spread and toxicity of α-Syn [15].

MHCII molecules are essential in linking the innate and adaptive immune system. Based on the presence of MHCII+ cells in the SN of PD patients[27] and the observed infiltration of T-lymphocytes into the PD brain[12] it has been proposed that PD shares features of autoimmune disorders. In such a scenario, α-Syn is processed by microglia/macrophages and presented to CD4+ lymphocytes which results in an antigen-specific immune response. In 2017, a key study was published with the first evidence of autoreactive CD4+ lymphocytes upon antigen presentation of α-Syn peptides[10]. A follow-up study showed that the α-Syn reactive CD4+ lymphocytes were mainly observed close to PD diagnosis[11]. In our current study, we show that DA.VRA4 rats with lower levels of Ciita have lower MHCII levels on both microglia and circulating myeloid cells. Additionally, the lower levels of Ciita affected levels of CD86, a co-stimulatory marker necessary for CD4+ lymphocyte activation. In the circulating myeloid population there was a clear trend of increased CD86 levels compared to the DA background strain. To determine if the altered CD86 levels have implications on PD-like pathology and progression, further functional studies of both antigen presentation and lymphocyte activation are necessary.

Our previous findings demonstrate that, based on morphology, microglia of DA.VRA4 rats with lower Ciita levels are more activated but have lower levels of MHCII and this was associated with a more widespread α-Syn pathology[14,15]. One hypothesis is that the altered levels of CD86 associated with lower Ciita levels has an impact on immune responses to α-Syn pathology and exacerbates the pathological spread in part mediated by CD4+ lymphocyte responses.

Previous studies in a nigral α-Syn overexpression model in mice found that PD-like pathology was mainly driven by infiltrating monocytes[25] and that silencing of *Ciita* greatly reduces both monocyte and lymphocyte infiltration[13]. In the current study, we did not detect any differences in lymphocyte or macrophage/monocyte infiltration between the wt DA and congenic DA.VRA4 strain with the exception of reduced MHCII macrophage/monocyte levels in the DA.VRA4 strain. However, it is important to highlight that very limited numbers of infiltrating lymphocytes and macrophages/monocytes are observed in our model (<1,000 events/hemisphere) compared to studies using α-Syn nigral overexpression in mice.

In our current study we show that rats with less Ciita have higher levels of TNF in serum independent of intervention compared to DA rats and additionally we observe increased levels of the pro-inflammatory cytokine IL-1β in CSF in response to α-Syn in DA.VRA4 compared to controls. Increased TNF and IL-1β levels in blood have been seen in PD patients from multiple studies[28]. Additionally, IL-1β levels have been shown to influence the NLRP3 inflammasome and contribute to neurodegeneration in a 6-OHDA mouse model of PD[29] and correlate to disease progression in PD patients[30]. 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 upregulation of IL-1β exacerbates α-Syn pathological spread and neurodegeneration.

We observed a lower CD4/CD8 ratio in naïve DA.VRA4 rats with lower Ciita levels, driven by a decrease in CD4+ T lymphocytes and increase in CD8+ T lymphoctes, although this difference was not seen between rats receiving α-Syn+PFF injections. Lower CD4/CD8 ratio, due to decreased numbers of CD4+ lymphocytes, has been reported in PD patients[31]. A recent study reported protective effects of lymphocytes in a striatal PFF seeding model of PD in mice, where adoptive transfer of CD4+ lymphocytes to immunocompromised mice reduced α-Syn pathology and additionally increased the microglial activation based on morphology compared to immunocompromised mice without striatal PFF seeding[9]. Of note, another recent study, using a nigral AAV overexpression model of α-Syn in mice, reported that CD4 knockout protected against neurodegeneration in the SN and inhibited myeloid activation, based on MHCII levels[24]. Studies on lymphocyte populations in PD patients have also shown

differing outcomes; some have reported an overall decrease in circulating CD4+ lymphocyte subpopulations due to decreased levels of the T-helper (Th) 2 and 17 subsets as well as reduced numbers of regulatory lymphocytes[32]. Others have reported overall decrease in circulating lymphocytes with increased Th1 and 17 subsets and decreased Th2 and regulatory T cells[33] whereas a third study did not observe any changes in Th1 and Th2 subsets but only an increase in the Th17 lymphocyte population[34]. Collectively, the contradictory findings on the contribution of T lymphocytes on PD susceptibility and progression might be due to the different methodological approaches used and more studies are necessary to get a better understanding on the role of lymphocytes in PD.

We chose to exclude a control vector carrying a fluorescent protein which is commonly used to control for protein overexpression, mainly to avoid any fluorescent protein interfering with our flow cytometric readout. Additionally, we have previously observed that using a control vector carrying GFP additionally leads to a neuroinflammatory response[15,25], therefore an empty vector was a more appropriate control to study immune responses in the current study. However, based on our results it seems like the stereotactic injections per se cause a neuroinflammatory response.

We have previously investigated the effects of differential Ciita expression on PD-like α-Syn pathology and neurodegeneration at 8-weeks post nigral injection of α-Syn-rAAV6 combined with striatal seeding of human PFFs[15] and microglia profile and neurodegeneration 12-weeks post nigral injection using a α-Syn-rAAV6 vector only[14]. Studies have shown that there is an inflammatory response ongoing prior to neurodegeneration in animal models[23,35] and in PD patients[10], therefore we chose to include an earlier timepoint in our current study; 4 weeks post nigral injection of human α-Syn-rAAV6. However, a limitation of models using intracranial injections is the physical damage and blood-brain barrier disruption that are caused from the injection itself. It is possible that changes observed in immune populations at the 4-week timepoint are due to inflammatory responses to the stereotactic surgeries rather than the vector and transgene. Another limitation in our current study design is the inclusion of cortex in brain samples used for flow cytometric analyses, as this might dilute striatal and midbrain immune populations.

In the current study we show that naturally occurring variation in Ciita levels alter molecules linking the innate and adaptive immune system in both local and peripheral immune populations, possibly affecting the susceptibility to α-Syn-induced neurodegeneration and pathological protein spread[15]. We observed continuously elevated levels of TNF in congenic DA.VRA4 rats with lower Ciita levels. To answer if these elevated TNF levels are causally related to the increased susceptibility to α-Syn-induced PD-like pathology requires further experiments. Collectively, our work together with other experimental and human studies highlight the importance of understanding the link between the innate and adaptive immune systems and PD.

MATERIALS AND METHODS

Experimental design

To investigate the effects of differential expression of Ciita (also known as Mhc2ta) we used wt DA rats and a congenic DA.VRA4 rat strain with lower expression levels of Ciita and MHCII[14]. Male rats entered the study at around 12 weeks of age and a total of 77 rats were included, 6-9 rats/group. We used a combination of viral overexpression of human α-Syn combined with seeding of human PFFs, adapted from Thakur et al[16]. In our current study, rats were injected with a rAAV6 vector overexpressing human α-Syn[17] into the SN followed by an injection of human α-Syn PFFs two weeks later in the striatum to induce a robust PD-like α-Syn pathology (Fig. 1)[15]. Animals were sacrificed at 4- and 8-weeks post nigral injection for collection of brain, serum and CSF samples. 6 animals per strain and timepoint were used for flow cytometric analysis of brain and blood samples and 2-3 animals were used for qualitative IHC validation of α-Syn expression and TH loss. 6 naïve rats per strain were sacrificed at around 12 weeks of age and used as baseline levels for cytokine levels and flow cytometric analyses of blood and brain. One naïve DA and one DA.VRA4 rat from the 8-week α-Syn+PFF group was 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+PFF 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[36]. 2-3 rats were housed 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. A total of 77 male rats were included in the study. 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[17] and injected at a concentration of 1.3-1.7E+10 gc/µl. The same vector but without the human α-Syn gene was used as a control. Concentration was determined by ITR-qPCR.

Pre-formed fibrils

Human α-Syn PFFs were produced in Dr. K. Luk's laboratory as previously described[37] and stored at -80°C. PFFs 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.).

Electron microscopy of PFFs

The gross structure of PFFs before or after sonication were imaged using transmission electron microscopy. PFFs 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 T12 Tecnai Spirit BioTWIN electron microscope.

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 analgesic. Burr holes was created using a dental drill. For nigral injections of 3 µl empty rAAV6 vector or vector carrying human α-Syn the following coordinates taken from bregma was used; 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 PFFs (2.5 µg/µl) or DPBS as control was injected using the following coordinates relative to bregma; 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. 1 mg/kg of Metacam (Boehringer Ingelheim Animal Health, Germany) was injected s.c. for post-operative analgesics. The rats were left to recover in new 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 sampling

CSF samples were collected at baseline, 4- and 8-weeks post nigral injection from all 77 rats by attaching the rats to 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 samples were collected into protein LoBind tubes (Eppendorf, Germany) and 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 timepoints 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 for flow cytometric analysis 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

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) 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 without calcium, magnesium or phenol red (HBSS) (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 two-layer density gradient separation of myelin and brain mononuclear cells.

Brain mononuclear cell isolation by gradient separation

Brain mononuclear cells were isolated and myelin removed using a two-layer density gradient adapted from[38,39]. 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.). 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 and 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

200 µl of whole blood collected in EDTA coated tubes were prepared 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)).

Antibody staining for flow cytometric analysis

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

Table 1. List of 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)

Samples were analyzed using an LSR Fortessa (BD, U.S.), configuration specified in Table 2. Compensation was performed using BD CompBeads (BD, U.S.) and prepared according to manufacturer's instructions. Fluorescence minus one (FMO), unstained and unstained cells with viability dye were included for each recording session and for each sample type (blood or brain). Gating strategy for brain and blood samples can be seen in Supplementary Fig. 2a and 3a. Microglial cells were gated as CD45dimCD11b+ in brain samples. Infiltrating macrophages/monocytes (CD45HighCD11b+) 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+. T-helper cells were gated as CD4+ and cytotoxic T lymphocytes as CD8+. Data was analyzed using FlowJo software version 10.8.1 (BD, U.S.).

Table 2. Configuration of the LSR Fortessa used for flow cytometric analysis and indication of what filters were 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. Sections were rinsed with PBS or 0.1% PBS with Triton-X 100 (v/v) (PBST) between all incubation steps. 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 h at RT. All antibodies used for IHC can be 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 and dehydrated and coverslipped using Pertex (Histolab, Sweden).

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)
Biotinylated anti-rabbit	Goat	1:200	Vector Laboratories (BA-1000)

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. Briefly the plates were blocked for 1 h at RT on shake using "blocker H". Serum samples were diluted 4-fold and CSF samples 2-fold in "diluent 42", samples and calibrators were incubated at 4°C on shake O/N. Detection antibodies were diluted in "diluent 40" and added to the plate for 2 h on shake. "Read buffer T" was added to the plate immediately prior to reading the plates 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 can differ 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 one duplicate was undetected for a group the non-detected value was replaced with the lowest quantifiable value for the specific cytokine. If more than one duplicate was undetected for a group results are presented as non-detected "ND".

Statistical analyses

Statistical analyses were conducted using the GraphPad Prism software version 9.3.1 (San Diego, CA, U.S.). QQ-plot of residuals was used to determine the use of parametric or non-parametric tests. Data 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. A significance level of $\alpha \leq 0.05$ 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. Manuscript was written by F.B., L.B. and M.S.

COMPETING INTEREST

The authors declare no competing interest.

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FIGURES AND FIGURE LEGENDS

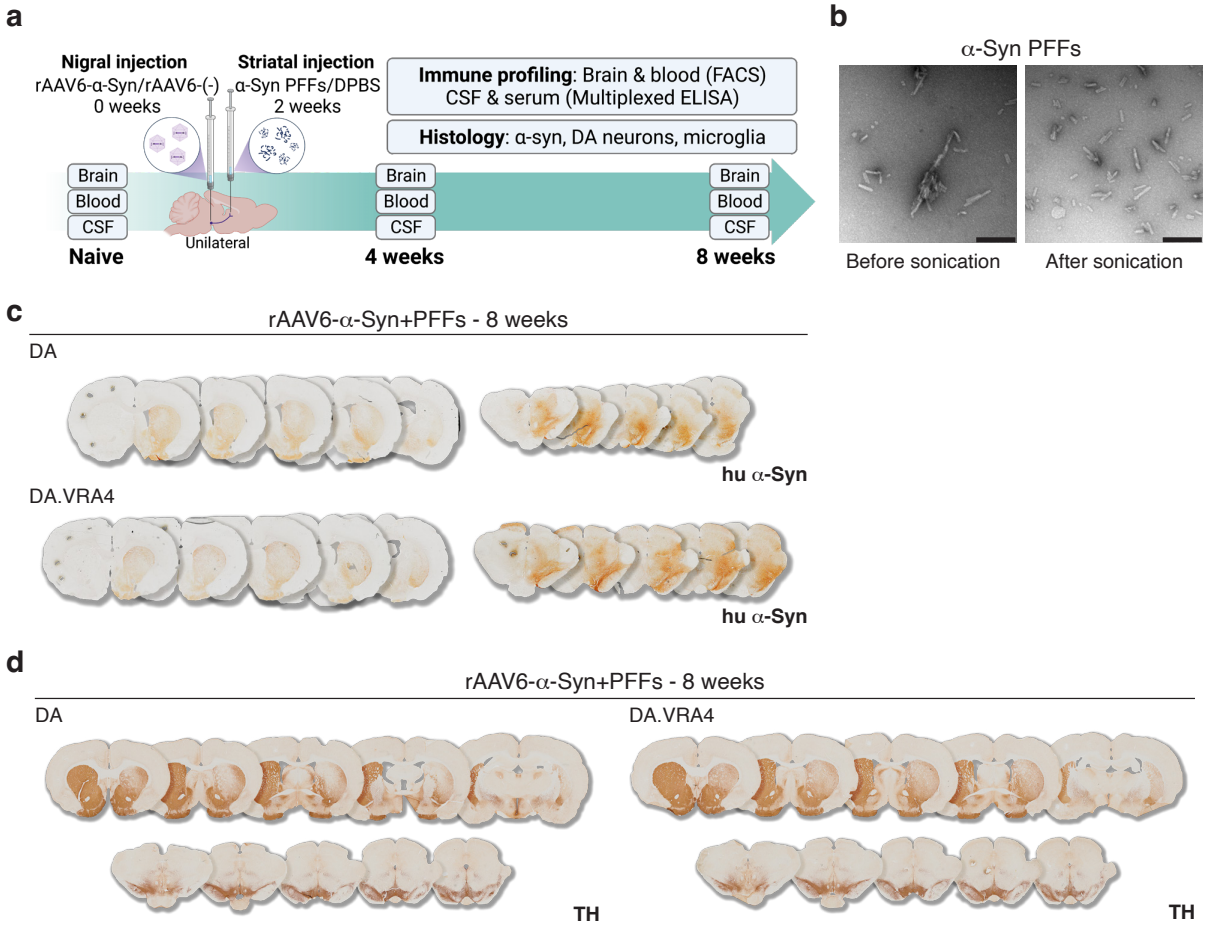

Fig. 1. α-Syn overexpression combined with striatal seeding of α-Syn pre-formed fibrils (PFFs) leads to TH loss. a Experimental outline. **b** TEM images of α-Syn PFFs before (left) and after (right) sonication; sonicated PFFs were used for striatal seeding. Scale bar = 200 nm. **c** Unilateral nigral overexpression of human rAAV6-α-Syn combined with striatal seeding of human PFFs results in robust human α-Syn signal in substantia nigra (SN) and striatum. **d** α-Syn+PFF injection leads to loss of TH-signal in both striatum and SN.

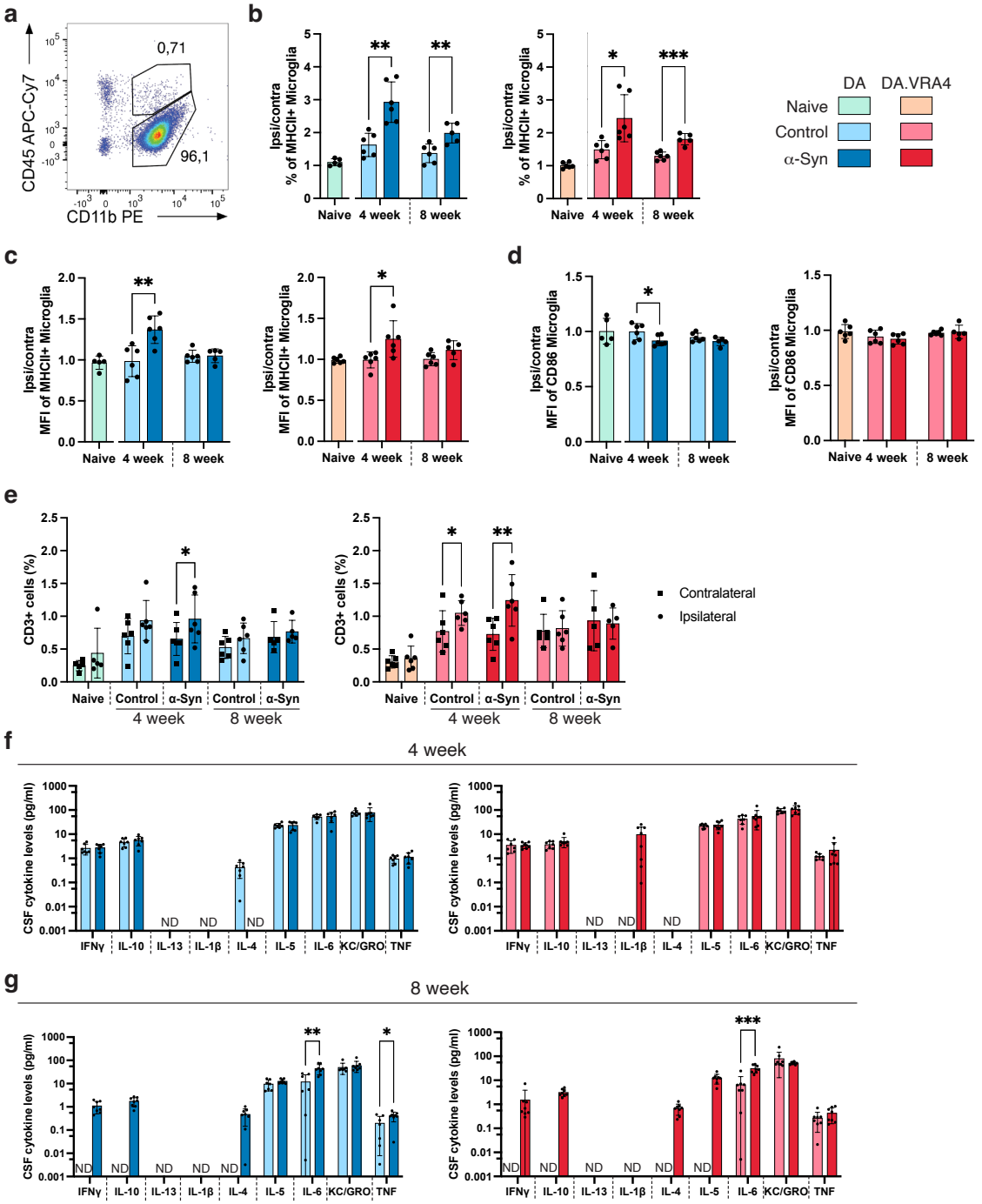

Fig. 2 α-Syn+PFF injection leads to robust upregulation of MHCII on microglial cells in the brain. **a** Gating of CD45dimCD11b+ (microglia) and CD45highCD11b+ (infiltrating macrophages/monocytes) cells in brain samples. **b** Normalized (ipsilateral/contralateral hemisphere) percentage of MHCII+ microglia is higher in α-Syn injected animals compared to control in both DA (left) and congenic DA.VRA4 (right) rats. **c** MHCII levels determined by normalized median fluorescence intensity (MFI) values in DA (left) and DA.VRA4 (right) rats are higher after 4- but not 8-weeks post nigral α-Syn overexpression. **d** Normalized MFI values

of CD86 in DA (left) and DA.VRA4 (right) rats. **e** Stereotactic injection leads to increased percentage of CD3+ cells (T lymphocytes) in DA (left) and congenic DA.VRA4 (right) rats. **b-e** Naïve (DA n=5, 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=6, DA.VRA4 n=6) and α-Syn (DA n=5, DA.VRA4 n=5). **f-g** Cytokine levels in cerebrospinal fluid (CSF) 4- and 8-weeks post nigral injection, respectively, in DA (left) and DA.VRA4 (right) rats. α-Syn injection results in elevated IL-6 levels in both DA and DA.VRA4 and TNF in DA.VRA4 at 8 weeks. Non-detected values are indicated by "ND". 4-week; control (DA n=7, DA.VRA4 n=7) and α-Syn (DA n=7, DA.VRA4 n=8), 8-week; control (DA n=7), DA.VRA4 n=8) and α-Syn (DA n=8, DA.VRA4 n=8). **b-d, f-g** Unpaired Student's t-test. **e** Paired Student's t-test. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$. Data presented as median ± SD with individual values.

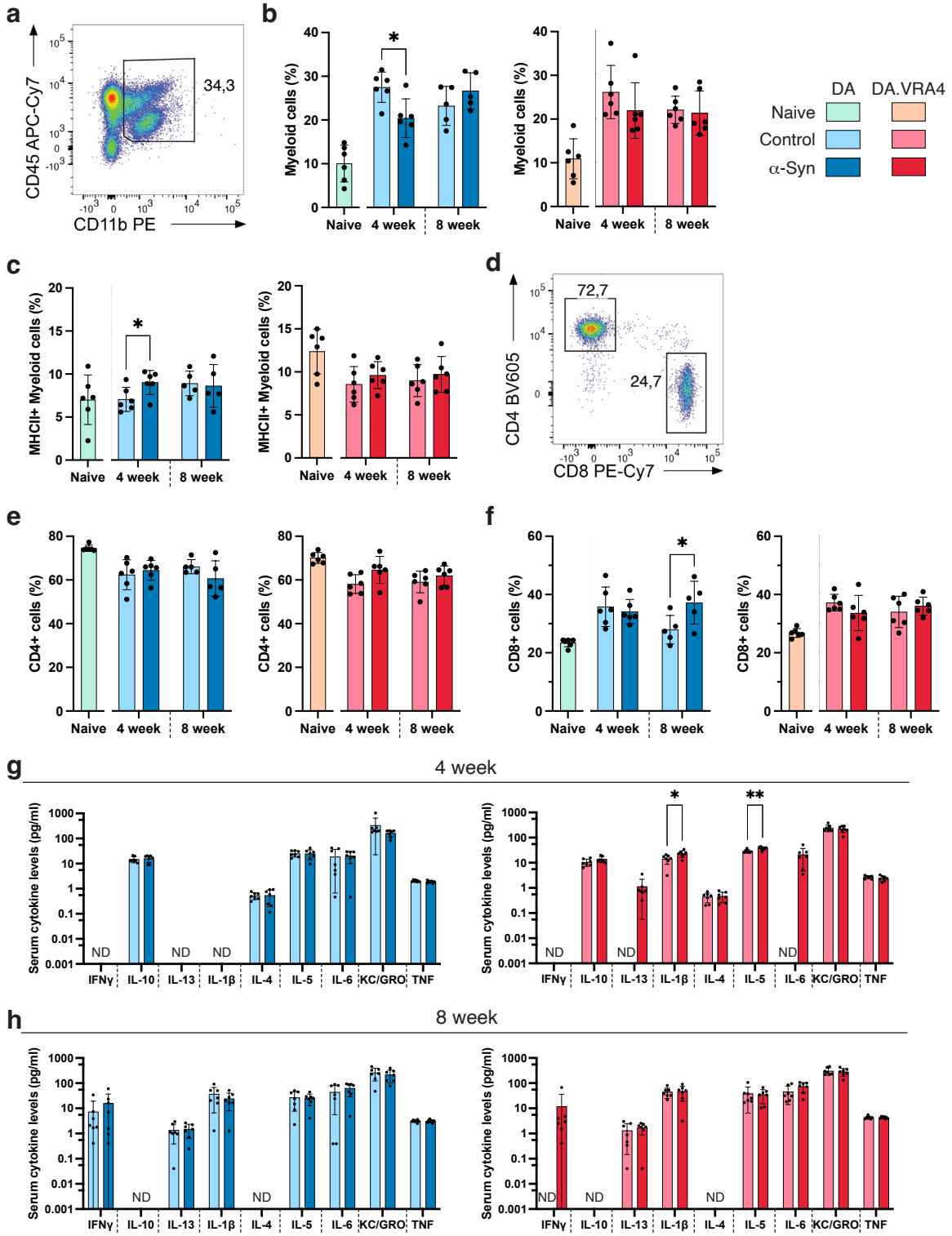

Fig. 3 α-Syn+PFF injection leads to minor changes in myeloid and lymphocyte populations in blood of DA but not congenic DA.VRA4 rats. a Gating strategy for CD45+CD11b+ cells (myeloid) in blood. **b** Overall percentage of myeloid cells in DA (left) and DA.VRA4 (right) at 4- and 8-weeks post α-Syn+PFF injection. **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 strategy for CD4+ or CD8+ T lymphocytes (CD45+CD3+ cells). **e** Overall percentage of CD4+ T lymphocytes in blood does not change after α-Syn 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** α-Syn-expression in the brain 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 rAAV6-α-Syn injection in DA (left) or DA.VRA4 (right) rats, n=7/group. **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 median ± SD with individual values.

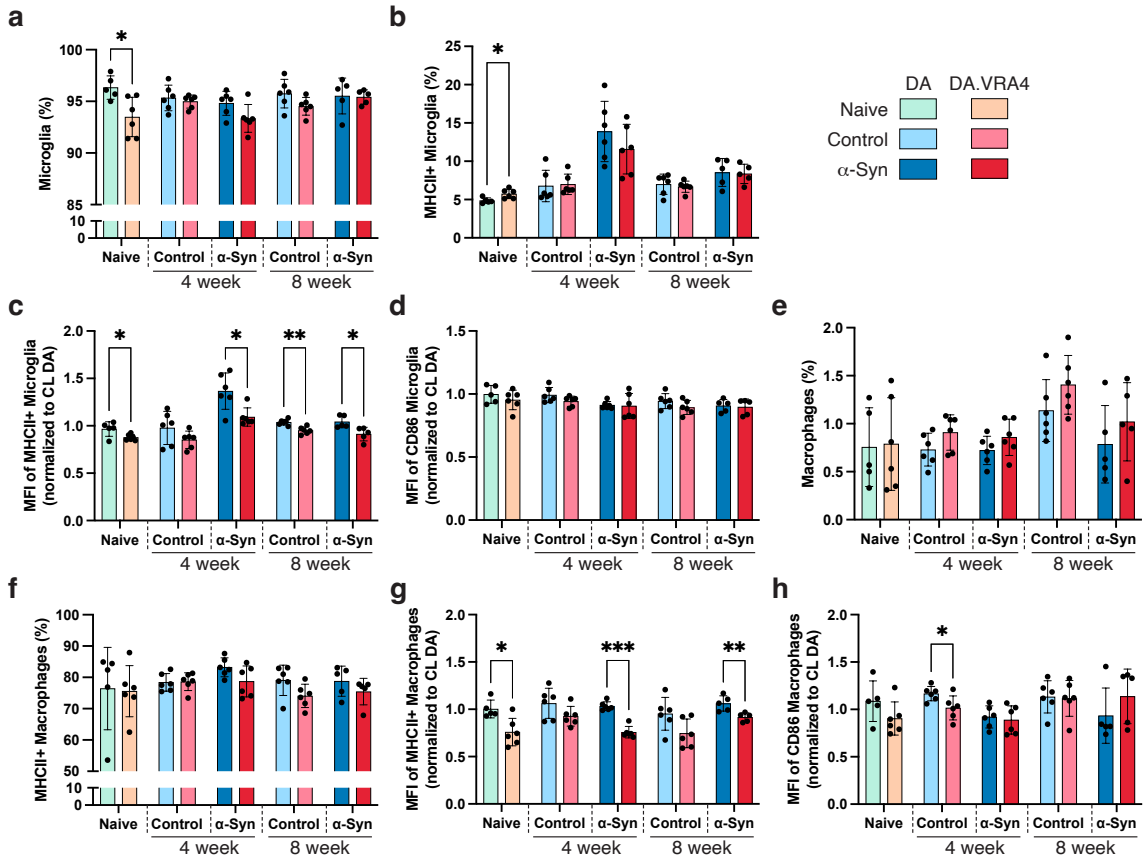

Fig. 4 Ciita regulates MHCII levels on microglia and infiltrating macrophages in response to α-Syn in the brain. a Total percentage of CD45dimCD11b+ cells (microglia) is reduced in naïve DA rats compared to DA.VRA4 rats with lower Ciita levels. **b** Percentage of MHCII+ microglia is higher in naïve DA compared to DA.VRA4. **c** Congenic DA.VRA4 rats with lower Ciita have reduced MHCII MFI levels on microglia independent of α-Syn, normalized to contralateral (CL)

DA values. **d** Normalized microglial CD86 MFI levels is not regulated by Ciita. **e** Percentage of CD45highCD11b+ cells (infiltrating macrophages) and MHCII+ macrophages (**f**) are not regulated by differing Ciita levels. **g** Congenic DA.VRA4 rats have reduced MHCII MFI levels on infiltrating macrophages compared to wildtype DA independent of α-Syn injections (normalized to CL DA). **h** CD86 MFI levels (normalized to CL DA) are not regulated by Ciita in response to α-Syn. Naïve (DA n=5, 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=6, DA.VRA4 n=6) and α-Syn (DA n=5, DA.VRA4 n=5). Data presented as median ± SD with individual values. Unpaired Student's t-test. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$.

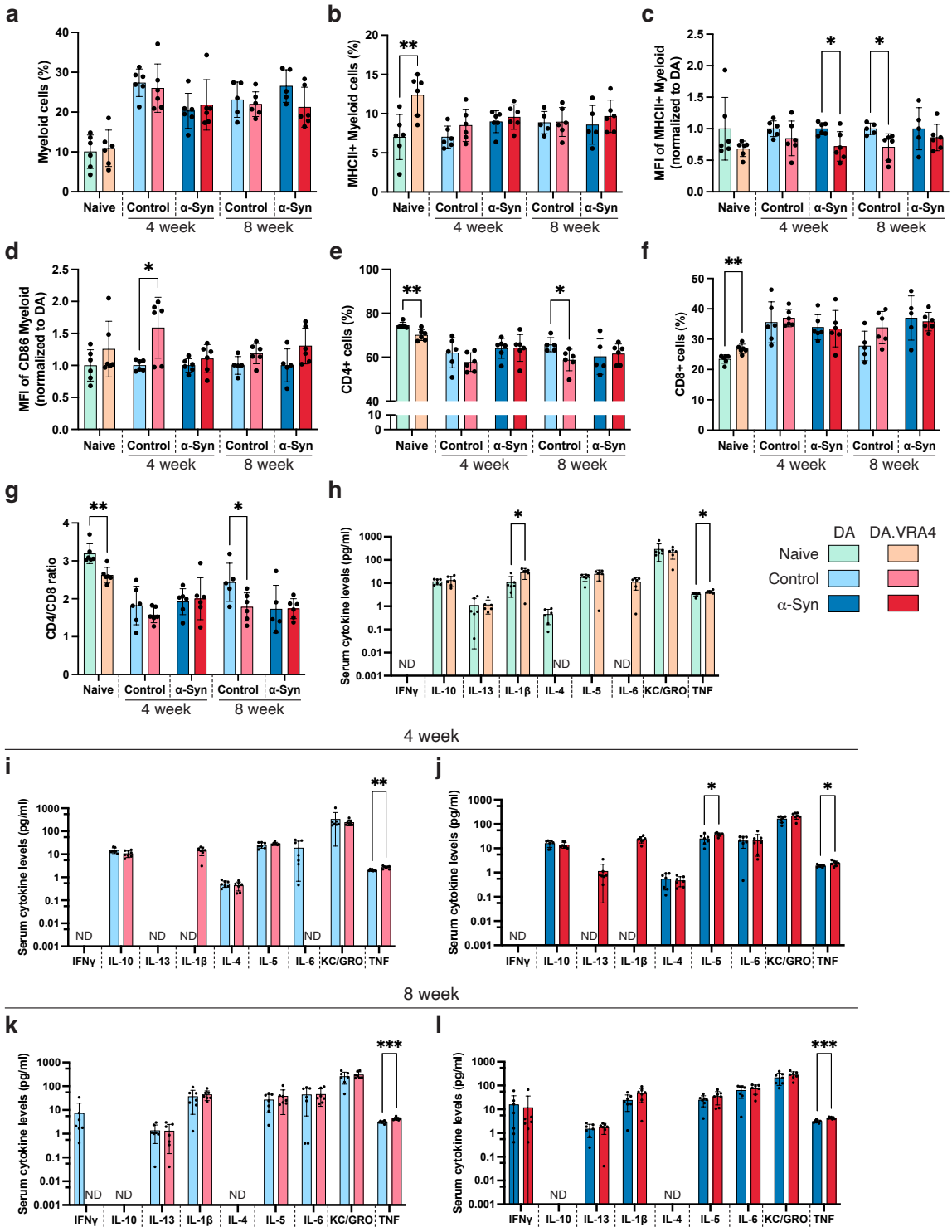

Fig. 5 Lower Ciita levels are associated with reduced MHCII levels on myeloid cells and higher TNF in serum. a The proportion of CD45+CD11b+ cells (myeloid) is not regulated by Ciita. **b** Naïve DA.VRA4 rats with less Ciita have an increased proportion of MHCII+ myeloid cells compared to wildtype DA. **c** Ciita regulates MHCII MFI levels in circulating myeloid cells. **d** CD86 MFI levels are not regulated by Ciita in response to α-Syn. **e-g** Naïve congenic DA.VRA4

rats with lower Ciita levels have less CD4+ but more CD8+ T lymphocytes (CD45+CD3+) leading to a reduced CD4/CD8 ratio. **a-g** 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). **h-l** Congenic DA.VRA4 rats with less Ciita have increased TNF levels in serum independent of α-Syn. **h** n=6/group. **i-l** n=7/group. Data presented as median ± SD with individual values. Unpaired Student's t-test. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$.