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Regulatory T cells attenuate Th17 cell-mediated nigrostriatal dopaminergic neurodegeneration in a model of Parkinson's disease

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Abstract

Nitrated alpha synuclein (N-α-syn) immunization elicits adaptive immune responses to novel antigenic epitopes that exacerbate neuroinflammation and nigrostriatal degeneration in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) model of Parkinson's disease (PD). We show that such neuroimmune degenerative activities, in significant measure, are Th17 cell-mediated with $CD4^{+}CD25^{+}$ regulatory T cell (Treg) dysfunction seen amongst populations of N-α-syn induced T cells. In contrast, purified vasoactive intestinal peptide (VIP)-induced and natural Treg reversed N-α-syn T cell nigrostriatal degeneration. Combinations of adoptively transferred N-α-syn and VIP immunocytes or natural Treg administered to MPTP mice attenuated microglial inflammatory responses and led to robust nigrostriatal protection. Taken together, these results demonstrate a putative mechanism for Treg control of N-α-syn-induced neurodestructive immunity and as such provide a sound rationale for future PD immunization strategies.

Keywords

Parkinson's disease; alpha synuclein; regulatory T cells; neuroprotection; immunization

An inciting event that underlies the pathogenesis of Parkinson's disease (PD) is the accumulation of aggregated proteins within neuronal cell bodies and microglia is associated with microglial activation and neuronal death. Deposition of misfolded and nitrated alpha-synuclein (N-α-syn) into Lewy bodies (LB) within nigral dopaminergic neurons of the substantia nigra pars compacta (SNpc) (1,2), with subsequent release into extracellular spaces

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Supplemental data. PCR arrays were performed to compare cytokine gene expressions from naïve $CD4^{+}$ T cells with cytokine genes of $CD4^{+}$ T cells from N-4YSyn-immunized mice (Supplemental data 1), VIP-treated mice (Supplemental data 2), and pooled $CD4^{+}$ T cells from both groups (Supplemental data 3). Combination analyses are also included.

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and draining cervical lymph nodes affects neuronal loss by engaging innate and adaptive immune responses (3–7). This leads to oxidative stress, microglial and APC activation, and neuronal degeneration (3,6,7). Interestingly, α-syn immunization was shown to generate humoral responses for clearing protein aggregates (8). However, using nitrated forms of α-syn (N-4YSyn) as an immunogen, induces profound effector T cell (Teff) responses shown to exacerbate neuroinflammation and neurodegeneration (3) analogous to the untoward T cell-mediated meningoencephalitic responses observed after Aβ immunization (9). Others have reported that T cell responses elicited during the course of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) intoxication lead to accelerated neurodegeneration (5). The molecular and antigenic bases for these T cell-mediated neurodegenerative activities are unclear. However our own previous results demonstrate that nitrated forms of α-synuclein (N-α-syn) are recognized by the adaptive immune arm to yield antigen specific effector T cells that exacerbate neuroinflammation and accelerate nigrostriatal degeneration (3).

In contrast, regulatory components of adaptive immunity affect neural repair and protection. Indeed, regulatory T cells (Treg) protect against MPTP-induced dopaminergic degeneration (10). This raised the specter of opposing effects for $CD4^+$ T cell subsets on brain disease where auto-aggressive Teff responses speed the tempo of disease, while Treg attenuate neurodegeneration (4,10–12). This is in keeping with known anti-inflammatory and neurotrophic functions of Treg, their essential role in control of immune-mediated inflammation and regulation of the mononuclear phagocyte phenotype (10,12–15). We thus investigated whether an adjuvant to promote specific regulatory adaptive immune responses could be used with N-α-syn as a vaccine for PD. Using vasoactive intestinal peptide (VIP), a neuropeptide known to induce Treg responses (16,17), we now show that functional Treg within the N-α-syn splenocyte mixture results in neuroprotection and is modulated through a Th17-mediated mechanism.

Materials and Methods

Animals, immunizations, and MPTP intoxication

Recombinant C-terminal tail of α-synuclein (4YSyn) was purified, nitrated (N-4YSyn), and tested for endotoxin as described (3). Male C57BL/6J mice and FoxP3-GFP knock in C57BL/6J mice (5 weeks old, The Jackson Laboratory) were primed s.c. with N-4YSyn emulsified in complete Freund's adjuvant (CFA) (Sigma-Aldrich), boosted s.c. after two weeks with N-4YSyn in incomplete Freund's adjuvant (IFA) (Sigma-Aldrich), and cells harvested 5 days after boost as described (3). Each ml of CFA consisted of 1 mg dry weight of heat-killed *Mycobacterium tuberculosis* (H37Ra, ATCC 25177), 0.85 mL paraffin oil and 0.15 mL mannide monooleate. IFA contained no mycobacteria. Donor mice that did not receive immunizations were injected i.p. with 15 mg of VIP (Sigma-Aldrich) in PBS. Recipient mice received four i.p. injections of either vehicle (PBS, 10 mL/kg bodyweight) or MPTP-HCl (16 mg MPTP, free base in PBS/kg bodyweight in PBS; Sigma-Aldrich); each injection was given at 2 h intervals. Twelve hours after the last injection, SPC or Treg were adoptively transferred to MPTP-intoxicated recipient mice (n = 5–7 mice per group per time point). On days 2 and 7 post-MPTP, mice were sacrificed and brains were processed for analysis. All animal procedures were in accordance with National Institutes of Health guidelines and were approved by the Institutional Animal Care and Use Committee of the University of Nebraska Medical Center. MPTP safety measures were in accordance with published guidelines (18).

Isolation and adoptive transfer of SPC and $CD4^+CD25^+$ T cells

Five days following boost, mice were sacrificed and single cell suspensions were prepared from inguinal lymph nodes and spleens. $CD4^+$ T cell populations from spleens and lymph nodes were enriched by negative selection with CD4-enrichment columns (R & D Systems)

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followed by CD25-PE positive selection with AutoMACS (Miltenyi Biotec). As determined by flow cytometric analysis, populations of Treg and Teff were consistently greater than 95% pure using this method (12). T cells were cultured in complete RPMI 1640 [RPMI medium 1640 (Invitrogen) supplemented with 10% FBS, 2 mM L-glutamine, 25 mM HEPES, 1 mM sodium pyruvate, 1 × nonessential amino acids, 55 nM 2-mercaptoethanol, 100 U/ml penicillin, and 100 μg/ml streptomycin (Mediatech)] in the presence of anti-CD3 (145-2C11; BD Pharmingen), 4YSyn or N-4YSyn. Proliferation and inhibition assays were performed as described (3,10). MPTP-intoxicated mice received an i.v. tail injection of 5×10^7 freshly-isolated SPC or 1×10^6 freshly-enriched Treg in 0.25 ml of HBSS.

In vitro polarization of CD4$^+$ T cells

CD4$^+$ T cells were isolated from N-4YSyn immunized donors and cultured at 1×10^6/ml with 2×10^6/ml irradiated SPC and 10 μg/ml N-4YSyn in 20 ml of complete RPMI 1640. For polarization, CD4$^+$ T cells were cultured for 5 days with 10 ng/ml IL-2 for control Th cells (Thc); 10 ng/ml IL-12 and 2 μg/ml anti IL-4 for Th1; 10 ng/ml IL-4 and 2 μg/ml anti-IL-12 for Th2; and 3 ng/ml TGF-β, 10 ng/ml IL-6, 5 ng/ml IL-1β, 10 ng/ml IL-23, 2 μg/ml anti-IL-4, 2 μg/ml anti-IL-12, 2 μg/ml anti-IFN-γ, and 2 μg/ml anti-IL-2 (19) for Th17 cells. Each Th subset was harvested and 10×10^6 T cells from each subset were transferred to separate recipient groups. For stimulation of cytokine production, Th subsets were stimulated with 20 ng/ml PMA and 1 μM ionomycin (Sigma-Aldrich) for 5 h, cells washed, media replaced, and supernatants collected 24 h later for analysis.

Flow cytometric analysis

Samples from cell fractions were labeled with fluorescently labeled antibodies (eBiosciences) and analyzed by flow cytometry with a FACSCalibur flow cytometer (BD Biosciences).

RNA Isolation and Real-Time PCR

RNA was purified using TRIzol reagent (Invitrogen Corp) and the RNeasy Mini Kit (QIAGEN Sciences), prior to cDNA synthesis. Real-time PCR analysis using pathway-focused gene expression profiling arrays (SA Biosciences) was performed according to manufacturer's protocol (Supplemental data).

Cytokine Analyses

A multi-analyte cytokine ELISArray (SA Biosciences) was used for cytokine analysis within cell culture supernatants according to manufacturer's protocol, Absorbance values were read at 450 nm after stopping the reaction. A cytometric bead array for Th1/Th2 cytokines (BD Biosciences) and an IL-17A Flex set (BD Biosciences) were used to quantitate cytokine concentrations within culture supernatants. The bead arrays were performed according to manufacturer's protocol and the data acquired on a BD FACSArray bioanalyzer and analyzed using the FCAP Array Software (BD biosciences). The limits of detection are 0.1 pg/ml for IL-2; 0.03 pg/ml for IL-4; 5.0 pg/ml for IL-5; 1.4 pg/ml for IL-6; 0.5 pg/ml for IFN-γ; 0.9 pg/ml for TNF–α; and 0.8 pg/ml for IL-17A. For *in vitro* cytokine analyses, CD4+ T cells were stimulated with N-4YSyn (10 μg/ml) with or without polarizing culture conditions for 5 days and cytokine levels assessed using the mouse intracellular cytokine staining kit (BD Pharmingen). Stimulated CD4$^+$ T cells were cultured an additional 4 hours in the presence of PMA, ionomycin, and brefeldin A (Leukocyte Activation Cocktail, BD Pharmingen), permeabilized and fixed in Cytofix/Cytoperm buffer, and immunostained with PE-conjugated anti-TNF-α, -IFN-γ, -IL-17, or -IL-10. Cells were analyzed with an LSRII flow cytometer (BD Biosciences).

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Immunohistochemistry

Mice were transcardially-perfused with PBS followed by 4% paraformaldehyde (PFA, Sigma). Frozen midbrain sections (30 μm) were immunostained for Mac-1 (CD11b, 1:1000; Serotec). Fluorojade C staining (Millipore) was performed on adjacent sections according to manufacturer's protocol to assess degenerating neurons and quantified using ImageJ. Overall dopaminergic neuron survival was assessed seven days following MPTP intoxication and resolution of cell death processes with polyclonal antibody to mouse tyrosine hydroxylase (TH), 1:1000 (Calbiochem) and counterstained for Nissl substance by thionin staining (20) as previously described (21). Total numbers of Mac-1$^+$ cells, CD4$^+$ T cells, TH- and Nissl-stained neurons in the SN were estimated by stereological analysis with Stereo Investigator software (MicroBrightfield) using the optical fractionator module (22). Quantitation of striatal TH (1:500; Calbiochem) was performed by densitometric analysis as described (21). Adjacent midbrain sections were immunostained for CD4 (clone RM4-5, 1:200, BD Pharmingen). Sections were incubated in streptavidin-horseradish peroxidase (HRP) solution (ABC Elite vector kit, Vector Laboratories), and color developed using a generation system consisting of diaminobenzidine (DAB) chromogen (Sigma-Aldrich) as described (21).

Statistical analyses

All values are expressed as means ± SEM. Differences between group means were analyzed by one-way ANOVA followed by Fisher's least significant *post-hoc* test for multiple comparisons (SPSS, Inc.). All effects of treatment were tested at the 95% confidence level.

Results

N-4YSyn immunity exacerbates the MPTP-induced nigrostriatal lesion

To confirm and extend our previous findings that N-α-Syn-induced immunocytes exacerbate MPTP-induced inflammation and dopaminergic neurodegeneration; splenocytes (SPC) from donors immunized with N-4YSyn were adoptively transferred to MPTP-recipients and the extent of inflammation and neurodegeneration was determined. Thus, we characterized putative T cell phenotypes and regulatory capacities of CD4$^+$ T cells from mice immunized with the nitrated form of the carboxy-terminus of α-synuclein (N-4YSyn), as this portion contains 4/5 of nitratable tyrosine residues. Stereological analysis of Mac-1$^+$ cells within the SN 2 days post-MPTP showed greater than 16-fold increase in numbers of Mac-1$^+$ cells compared with PBS controls (Fig. 1A), while adoptive transfer of N-4YSyn SPC to MPTP-recipients exacerbated Mac-1$^+$ cell numbers/mm^2 by 35% of those observed from MPTP treatment alone. Fluorojade C (FJ-C) staining of dead or dying neurons revealed that adoptive transfer of N-4YSyn SPC accelerated MPTP-induced neuronal death by 7.2-fold (Fig. 1B). Analysis of surviving nigral dopaminergic neurons [(tyrosine hydroxylase (TH)-immunoreactive (TH$^+$) and Nissl-stained (Nissl$^+$) neurons] 7 days after MPTP intoxication indicated a 45% overall neuronal loss compared with PBS controls, whereas MPTP-treated recipients receiving N-4YSyn SPC exhibited a 63% reduction of TH$^+$ neurons (Fig. 1C). PBS-treated mice that received N-4YSyn SPC showed no change in TH$^+$ neuron numbers compared with control mice that received PBS alone (data not shown). MPTP mice that received SPC from PBS/adjuvant- or non-nitrated α-syn (4YSyn)/adjuvant-immunized donors showed no significant additive or protective effect on microglial activation or neuronal survival compared with MPTP alone (data not shown) as previously described (3). No significant effects of any treatment were observed among numbers of non-dopaminergic neurons (TH$^-$Nissl$^+$). Analysis of CD4$^+$ T cell infiltration into the SN revealed that MPTP-intoxicated mice and MPTP-treated recipients of N-4YSyn SPC had increased infiltration of CD4$^+$ cells (8.5- and 10.3-fold, respectively) within the SN at 7 days-post-intoxication compared with PBS-treated controls (Fig. 1D). These data demonstrate that adaptive immune responses against N-α-syn exacerbate

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MPTP-induced neuroinflammation and nigrostriatal degeneration and support our previous works (3).

T cells isolated from N-4YSyn donors stimulated *in vitro* with anti-CD3 for 24 h produced greater concentrations of IL-17A and TNF-α relative to naïve T cells (Fig. 1E). N-4YSyn antigenic stimulation of $CD4^+$ effector T cells isolated from immunized mice induced IL-17A, TNF-α, IFN-γ and IL-2, but not IL-4 or IL-5 (Fig. 1F) suggesting that immunization partially polarized $CD4^+$ T cells *in vivo* towards either a Th1 or Th17 phenotype. Functional characterization of Treg isolated from immunized forkhead box P3 (FoxP3)/GFP transgenic mice revealed that Treg were functionally deficient in the capacity to inhibit effector T cell proliferation to anti-CD3 stimulation following immunization with N-4YSyn (20%) as compared with Treg isolated from naïve donors (80%) at a ratio of 1:1 (Fig. 1G). Intracellular cytokine tests demonstrated that N-4YSyn-expanded $CD4^+$ T cells from N-4YSyn-immunized mice were comprised of 38.2% TNF-α-, 9.0% IFN-γ-, and 11.2% IL-17A-expressing effector T cells (data not shown). Taken together, these results indicate that immunization with N-4YSyn induces Th1 and/or Th17 T cells, as well as producing a deficiency in Treg function, and suggests putative roles for Th1, Th17, and Tregs in exacerbation of MPTP-induced inflammation and dopaminergic neuronal death.

N-4YSyn immunity neurodegeneration is mediated by $CD4^+$ T cell subsets

To test the abilities of Th1 and Th17 cells to enhance dopaminergic neurodegeneration, $CD4^+$ T cells were isolated from N-4YSyn immunized donors, polarized *in vitro* for 5 days in culture conditions to favor a Th1, Th2, or Th17 phenotype, and then each polarized T cell subset was adoptively transferred to separate groups of MPTP-intoxicated recipients (Fig. 2A). Cytometric bead array analysis confirmed that culture conditions indeed polarized N-4YSyn $CD4^+$ T cells to each designated phenotype as characterized by production of IFN-γ by Th1 cells, IL-4 by Th2 cells, and IL-17A by Th17 cells (Fig. 2B). Moreover, flow cytometric analysis of intracellular cytokine expression indicated that polarization of N-4YSyn $CD4^+$ T cells under Th1 conditions yielded 4.1% IFN-γ- and 59.5% TNF-α-producing T cells. Th17 polarizing conditions yielded 11.8% IL-17A- and 86.8% TNF-α-producing T cells, while Th2 culture conditions yielded 69.1% IL-10 producing T cells (data not shown). Compared with non-polarized $CD4^+$ T cell controls (Thc), quantitative PCR analyses of transcription factors and interleukin genes indicated that polarized Th1 cells showed increased expression of *Stat4* (2-fold), *Ifng* (31-fold), *Tnf* (2-fold) and *Il6* (15-fold); polarized Th2 cells showed increased expression of *Gata3* (5-fold), *Stat6* (13-fold) *Il4* (3-fold), *Il10* (5-fold) and *Il13* (5-fold); and polarized Th17 cells showed 351-fold increase in transcription activator RAR-related orphan receptor c (*Rorc*) as well as increased expression of *Il22* (4-fold), *Il23* (8-fold), *Il17a* (51-fold), and *Tnf* (76-fold) (data not shown).

Analysis of surviving dopaminergic neurons in TH-immunostained ventral midbrain and striatum 7 days after MPTP treatment and adoptive transfer revealed that adoptive transfer of either N-4YSyn Th1 or Th17 cells resulted in decreased numbers of surviving TH^+ neurons within the SN; whereas, only N-4YSyn Th17 cells induced remarkably diminished TH termini densities within the striatum (Fig. 2C). To validate those observations, unbiased stereological analysis of ventral midbrain sections indicated that while PBS-treated controls averaged 7994 ± 212 total TH^+ neurons within the SN, MPTP-intoxication induced a 25% loss of TH^+ neurons to 5971 ± 250 (Fig. 2D). Adoptive transfer of N-4YSyn Th1 cells increased the MPTP-lesion to 46% neuronal loss resulting in 4320 ± 252 total TH^+ neurons; whereas, adoptive transfer of N-4YSyn Th2 cells had no significant protective or exacerbative effect on the total TH^+ neurons in response to MPTP-intoxication. In contrast, adoptive transfer of N-4YSyn Th17 cells induced a 65% loss in the number of surviving TH^+ neurons (2800 ± 243), a 2.6-fold increased lesion relative to MPTP-intoxication alone. Analysis of the surviving TH^+ dopaminergic

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neuronal termini showed that the TH density within the striatum of MPTP-intoxicated mice was 26% relative to PBS-treated controls. Adoptive transfer of neither N-4YSyn Th1 nor Th2 cells significantly affected the striatal densities relative to MPTP-intoxication alone. In contrast, adoptive transfer of N-4YSyn Th17 cells exacerbated the MPTP-induced loss of striatal TH density to 5% of PBS-treated controls (Fig. 2E).

Co-transfer of VIP SPC with N-4YSyn SPC attenuates N-4YSyn mediated microglial responses

VIP increases Treg numbers or suppressive function (16,17) or through abrogation of Th17 differentiation (23). To determine whether VIP modulation of N-α-syn-directed immune responses regulate neurodegenerative activities, SPC populations from VIP-treated or N-4YSyn-immunized mice were adoptively transferred either separately or together to MPTP recipients and the neuroinflammatory and neurodegenerative responses were evaluated at 2 days post MPTP intoxication; a time of peak inflammation and rate of neuronal death (24). MPTP mice that received N-4YSyn SPC exhibited an exacerbated inflammatory response as demonstrated by increased nigral Mac-1 expression, which was diminished in mice treated with VIP SPC (Fig. 3A). Similarly, neuronal death as demonstrated by FJ-C staining was demonstrably increased in N-4YSyn SPC-treated MPTP mice compared with MPTP treatment alone; whereas, FJ-C staining was diminished in MPTP mice treated with VIP SPC and more acutely diminished in mice treated with pooled VIP and N-4YSyn SPC. Quantitative validation of these observations indicated that in MPTP-intoxicated mice, transfer of VIP SPC reduced the numbers of activated microglia by 33%, whereas transfer of N-4YSyn SPC numbers increased Mac-1^+ cell densities by 55% (Fig. 3B). More importantly, co-transfer of VIP SPC with N-4YSyn SPC not only attenuated the exacerbative effects mediated by N-4YSyn SPC by 75%, but diminished numbers of Mac-1^+ microglia by 61% less than MPTP alone. Analysis of nigral Mac-1^+ microglia 7 days post-MPTP demonstrated sustained microglial activation in both mice that received N-4YSyn SPC alone or in combination with naïve SPC; whereas, significant numbers of Mac-1^+ cells were not observed within any other treatment group (data not shown). Analysis of total nigral FJ-C^+ cells revealed a 7-fold increase in the number of dead or dying neurons in MPTP-recipients that received N-4YSyn SPC compared with MPTP alone; while transfer of pooled VIP-and N-4YSyn SPC resulted in 64% fewer injured neurons (Fig. 3C). Adoptive transfer of naïve SPCs alone or together with N-4YSyn SPC showed no significant detrimental or protective effect on microglial activation, and numbers of FJ-C^+ neurons were not significantly different compared with MPTP alone (data not shown). These data suggested that a cell population within SPC from VIP-treated mice, but not naïve SPC was able to inhibit or suppress N-4YSyn-mediated effector cells and ameliorate the exacerbated microglial response and neuropathology.

VIP SPC modulate N-4YSyn immunity to confer neuroprotection

To validate that neuroprotection was not a transient effect, observed only at day 2, we assessed TH immunostained ventral midbrain and striatal sections 7 days after MPTP treatment and adoptive transfers; a time after which MPTP-induced neuroinflammation and neurodegeneration has subsided (3,21). MPTP mice that received VIP SPC showed a modest increase in TH^+ neuronal density (Fig. 4A). In contrast, TH^+ neurons within the SN of MPTP recipients that received N-4YSyn SPC were markedly diminished compared with MPTP treatment alone; whereas, those that received pooled VIP and N-4YSyn SPC exhibited TH^+ neuronal densities reminiscent of PBS controls. Although lesions of the dopaminergic striatal termini are typically more severe, similar patterns of dopaminergic loss were observed in mice treated with MPTP alone and with separate SPC populations, while those treated with pooled N-4YSyn SPC and VIP SPC exhibited discernable increases in the density of TH^+ striatal termini. To confirm those findings, stereological analysis and comparison with PBS controls showed that MPTP reduced SN TH^+ neurons by 48%, while MPTP and N-4YSyn SPC reduced

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those neurons by 64% (Fig. 4B). MPTP recipients of VIP SPC showed a modest, yet significant 18% increase in TH^+ neuron number compared with MPTP alone. In comparison, 91% of TH^+ neurons survived in MPTP-mice receiving SPC from N-4YSyn-immunized and VIP-treated donors. Striatal TH^+ density in MPTP-intoxicated mice was 16% of PBS controls, whereas transfer of N-4YSyn SPC to MPTP recipients reduced striatal TH densities to 7% of PBS controls (Fig. 4C). Although transfer of VIP SPC to MPTP-mice showed no significant additive or protective effect, co-transfer of VIP and N-4YSyn SPC increased striatal termini survival to 39% of PBS controls. Adoptive transfer of naïve SPC alone showed no significant detrimental or protective effect on dopaminergic neuronal survival (data not shown).

To elucidate the neuroprotective cell populations within the SPC pools, we enriched $CD4^+CD25^+$ Treg from naïve and VIP-treated donors and adoptively transferred each population with N-4YSyn SPC into MPTP mice. Co-transfer of N-4YSyn and VIP SPC to MPTP recipients provided 100% protection of TH^+ nigral dopaminergic neurons; whereas, neuroprotection was not observed in MPTP mice that received SPC from N-4YSyn-immunized and naïve donors (45% TH^+ neuron survival) compared with percentages of surviving neurons after treatment with MPTP alone (49%) or in combination with N-4YSyn SPC (34%) (Fig. 4D). In comparison, co-adoptive transfer of Treg from either naïve or VIP-treated donors with N-4YSyn SPC afforded significant neuroprotection with VIP Treg being more effective (96% survival) than naïve Treg (80% survival). Analysis of striatal dopaminergic termini density was comparable, showing that N-4YSyn SPC exacerbated the MPTP-induced lesion to 13% of PBS controls (Fig. 4E). Co-transfer of naïve with N-4YSyn SPC was not effective in perturbing N-4YSyn exacerbative effects; whereas co-transfer of VIP and N-4Syn SPC increased dopaminergic termini survival to 47% of PBS controls, as did co-transfer of naïve Treg and N-4YSyn SPC (50% of PBS controls). Co-transfer of Treg from VIP-treated mice with N-4YSyn SPC was the most efficacious, increasing the mean terminal density to 62% of PBS controls. These results demonstrate that Treg from VIP-treated mice are most efficacious for neuroprotection of both dopaminergic nigral neurons and striatal termini against the neurodegenerative activities of N-4YSyn adaptive immune responses.

VIP Treg abrogate development of a Th17 response

Immunization with adjuvant, N-4YSyn in adjuvant or treatment with VIP afforded little change in the frequencies of splenic $CD3^+$, $CD19^+$, $CD4^+$, and $CD4^+CD25^+$ cells (Fig. 5A). Flow cytometric analysis for $CD4^+CD25^+$ T cells within immune SPC populations revealed minimal increases in frequencies of $CD4^+CD25^+$ T cells in SPC of mice immunized with adjuvant, N-4YSyn in adjuvant, or treated with VIP, while the majority of the $CD4^+CD25^+$ T cells also expressed $FoxP3^+$. Analysis of antigen-induced proliferative responses showed that while N-4YSyn T cells proliferated in response to N-4YSyn, naïve or VIP-treated donors did not (Benner et al., 2008 and data not shown). In contrast, anti-CD3 stimulation of T cells from all donor groups induced proliferative responses in excess of 10-fold. Such responses, however, were not observed against non-nitrated α-syn in any of the experimental or control groups.

To assess whether VIP SPC suppress effector T cell proliferative responses, we evaluated SPC co-cultures from N-4YSyn-immunized and VIP-treated donors for their proliferative capacity in the presence of either anti-CD3 or N-4YSyn. At a 1:1 ratio of N-4YSyn SPC to VIP SPC, proliferation to both anti-CD3 stimulation and N-4YSyn were suppressed by 67% and 81%, respectively and diminished in a dose dependent fashion with the diminution of VIP SPC number (data not shown). Given the dichotomy between Treg and Th17 differentiation, we hypothesized that Treg function or development may be inhibited by immunization with N-4YSyn. To test this hypothesis, we evaluated $CD4^+CD25^+CD62L^{low}$ Treg isolated from naïve, N-4YSyn-immunized, and VIP-treated mice for their capacity to inhibit CD3-mediated proliferation of $CD4^+CD25^-$ naïve T cells (Fig. 5B). VIP Treg showed increased functional

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capacity to suppress T cell proliferation compared with naïve Treg showing a consistent 5% or greater inhibition of proliferation. Importantly, N-4YSyn Treg were functionally deficient in their suppressive function of Teff proliferation, showing 3-fold less percent inhibition of Teff proliferation compared with naïve Treg. In contrast, pooled VIP and N-4YSyn Treg showed enhanced suppressive capacity compared with all other Treg populations, with 10% greater inhibition versus naïve Treg at a 1:1 Treg to responder ratio. These data suggested that while N-4YSyn immunization abrogates Treg function, VIP Treg in the presence of N-4YSyn T cells additively restores regulatory function.

To characterize effector and regulatory T cell subset cytokine profiles, isolated $CD4^+$ T cells from N-4YSyn-immunized or VIP-treated mice were stimulated with anti-CD3 separately or pooled at a 1:1 ratio to induce cytokine expression. Quantitative RT-PCR revealed that N-4YSyn $CD4^+$ T cells showed increased expression of Th17 and Th17-associated genes relative to naïve T cells (Fig. 5C). This included *Il21*, *Il17a* and *Rorc*, whereas genes linked to Th1 (*Stat4, Ifng, IL-12, and IL-2*), Th2 (*Gata3, Stat6, Il4, Il10, and Il13*), and Treg (*Foxp3* and *Il10*) (25) were decreased. The increased expression of *Il17* and *Rorc* with concomitant decreases in Th1, Th2, and Treg associated genes, suggested that N-4YSyn immunization polarized $CD4^+$ T cells toward a Th17 phenotype. Moreover, genes encoding cytokines known to inhibit Th17 differentiation including IL-2, IL-4, IFN-γ, and IL-15 (−2.85-fold, not shown) were decreased in N-4YSyn T cells compared with naïve T cells. Interestingly, VIP T cells showed few changes in gene expression relative to naïve T cells, with genes predominately associated with a Th1 phenotype decreased in expression, whereas expression of Th2, Treg, or Th17 related genes were generally not affected. Importantly and in contrast, T cells pooled from N-4YSyn-immune and VIP-treated mice showed decreased expression of Th1 and Th17 related genes; whereas, genes for Treg were increased (Fig. 5C and supplemental data 1, 2 and 3). Qualitative analysis of cytokine production in response to anti-CD3 stimulation showed increased production of IL-17A and IL-6 from N-4YSyn T cells relative to naïve T cells; while, production of IL-2, IFN-γ (Fig. 5D), and IL-4 (data not shown) were decreased. TNF-α production was also increased greater than 2.5-fold relative to naïve T cells (data not shown). Analysis of cytokine production by VIP T cells revealed that the cytokine production was not significantly different to that of naïve T cells, although production of Th2-related cytokines IL-10 (Fig. 5D), IL-4, and IL-13 (data not shown) were marginally increased. In comparison, co-culture of N-4YSyn and VIP T cells resulted in increased production of regulatory cytokines including IL-10, IFN-γ (Fig. 5D), and IL-13 (data not shown), with concomitant decreased production of IL-17A and IL-6 compared with N-4YSyn T cells. TGF-β1 production was increased 2-fold relative to naïve T cells in supernatants of N-4YSyn-immunized, VIP-treated, and pooled T cell populations (data not shown). Cytokine concentrations within culture supernatants assessed by cytometric bead array were congruent with these results and revealed that while T cells from N-4YSyn immunized donors cultured alone produced nearly a 2-fold greater amount of IL-17A relative to naïve T cells (87.7 ± 3.3 pg/ml vs. 52.2 ± 0.8 pg/ml), co-culture with T cells from VIP treated donors resulted in reduced production of IL-17A equal to that produced by naïve T cells. Production of IFN-γ and TNF-α were also reduced in cultures of pooled N-4YSyn and VIP T cells relative to N-4YSyn T cells cultured alone (data not shown).

We next theorized that VIP could induce antigenic tolerance when given with N-4YSyn immunization. To test this idea, mice treated with or without VIP were immunized with N-4YSyn, and T cells were assessed for their proliferation capacity to N-4YSyn antigen. T cell proliferation to N-4YSyn was suppressed 2.5-fold in T cells isolated from N-4YSyn-immunized and VIP-treated donors compared with T cells from mice immunized with N-4YSyn alone (Fig. 5E and F). Notably, T cells from N-4YSyn immunized mice did not proliferate in response to non-cognate, non-nitrated 4YSyn antigen, while demonstrating significant proliferative capacity to cognate N-4YSyn (Fig. 5E). VIP treatment of N-4YSyn-

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immunized mice reduced 4YSyn-stimulated as well as background T cell proliferation (0–10 μg/ml) compared with non-VIP treated mice ($P < 0.035$). VIP treatment of immunized mice also inhibited the capacity to generate any significant N-4YSyn-induced proliferative responses at any antigen concentration compared to media controls ($P > 0.05$) (Fig. 5F). These data suggest that VIP treatment at the time of N-4YSyn immunization induces antigen-specific tolerance to N-4YSyn. Moreover, T cells from VIP-treated and N-4YSyn-immunized mice showed 1.7- and 1.9-fold increases in expression of genes encoding Foxp3 and IL-10, respectively, while IL-17A gene expression was diminished 2.4 fold compared with T cells from N-4YSyn-immunized mice (Fig. 5G). This demonstrated that VIP treatment favors the induction of regulatory responses that suppress Th17 function.

Discussion

We present a unique hypothesis for the pathogenesis of PD in that opposing adaptive immune responses mediated by Teff or Treg could lead to divergent outcomes in the tempo and progression of disease. As such, the current study was designed to generate a platform for the development of a vaccine strategy that can be applied to intervene against the ongoing inflammatory cascade that occurs in PD. Importantly, the neuroprotective responses seen were distinct from dendritic cells (DC) activities. Simply stated, VIP-induced DC were not required for the effects observed. Indeed, adoptive transfer of purified populations of Tregs (natural and VIP-induced), cells devoid of DC, attenuated both neuroinflammatory and neurodegenerative activities in MPTP intoxicated and N-α-syn-T cell administered mice. Additional works in models of chronic disease may serve to better translate the findings to human investigations.

Treg modulate inflammation, attenuate microglial activation, and promote neuronal survival after MPTP intoxication (10,12,26,27). The observation that Treg responses are neuroprotective is contradictory to the hypothesis of protective autoimmunity as proposed by others (28–30). Moreover, the T cell repertoire in the elderly is skewed toward a Th2 and regulatory T cell phenotype, and an increased suppressive function of $CD4^+CD25^+$ Treg has been linked to neurodegenerative conditions (31). Possible explanations for this discrepancy are that Treg have decreased migratory capabilities to the brain in the elderly or increased inflammation within the brain perturbs Treg function. Another possibility is that the repertoire of naturally occurring Treg is diminished with age due to thymic involution. Our observation that immunization with N-4YSyn induces a Th17 response lends the possibility that increased Treg may perpetuate Th17 differentiation in the presence of copious amounts of IL-6 during inflammation (32), as Treg are a primary source of TGF-β. Whereas, IFN-γ producing Th1 cells would inhibit Th17 differentiation, lending to the concept of protective autoimmunity as observed by others following acute injury to the CNS (33). Alternatively, Treg may be deficient in response to neoantigens such as N-α-syn. Indeed, we show that Treg from N-4YSyn immunized mice are functionally deficient in suppression of effector T cell proliferation.

Most notable for the current study is the ability of VIP-induced Treg to overcome the toxicities of Th17 amplified N-α-syn-mediated nigrostriatal degeneration. Transfer of splenocytes from VIP-treated donors alone was less effective than transfer of pooled splenocytes from N-4YSyn-immunized donors and VIP-treated donors, suggesting that Treg need to be activated or antigen-specific to confer neuroprotection. In this study, the systemic inflammation induced by N-4YSyn immunity may have activated Treg within the VIP population. Alternatively, the phenotype of N-4YSyn $CD4^+$ T cells may have been modulated away from a Th17 phenotype in favor of a Treg phenotype mediated by VIP. Our in vitro data lend support for the later, as co-culture of T cells from N-4YSyn immunized mice with T cells from VIP-injected mice attenuated the development of a Th17 phenotype in preference of a regulatory T cell phenotype. Possible mechanisms could include divergent transcriptional regulation either in favor of RORγt/RORc or FoxP3 pathways, as induction of one activator of transcription may inhibit

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the induction of the other (32,34). Alternatively, VIP-induced Treg could induce greater expression of IL-10 amongst the Th17 cell population. Increased IL-10 expression has been noted in approximately half of TGF-β/IL-6-synergized Th17 cells (35,36) and shown to limit Th17-driven inflammation in a mouse model of multiple sclerosis (35). Whether these mechanisms are exclusive or synergistic in our model is still under investigation.

Immunotherapeutic approaches should be directed towards a Th2/Treg response, which promotes antibody production and downregulates pro-inflammatory Th1/Th17 responses. This is important with the observation that T cells in the Phase IIa trial of the Aβ vaccination study were Th1-based underscores the importance of mounting a Th2/Treg- response (37). Methods of generating Treg are likely to have an important role in the development of therapies for PD, AD, stroke, amyotrophic lateral sclerosis, and HIV-1 associated neurocognitive disorders (4, 10–12). Tolerogenic molecules can promote production of Treg, including neuropeptides such as VIP which may be useful as a therapeutic tool to control immunity. Moreover, this work is congruent with our previous findings that immune cells from mice immunized with Copolymer-1 (Cop-1; Copaxone, glatiramer acetate) protect against MPTP toxicities (21). The commonality of results between reports reflects the fact that Cop-1 can induce Th2 and Th3 Treg, and through IL-4, IL-10, and TGF-β promote $CD4^{+}CD25^{-}$ T cells to $CD4^{+}CD25^{+}$ Treg conversions. The interaction of Treg with Teff by direct cell contact or through soluble factors may modulate adaptive immune responses, boost attenuation of microglial function, and thus provide increased neuroprotection. The data, taken together, provide a platform for a PD immunization strategy. Nonetheless, we realize the limitations seen in the current study. Indeed, acute MPTP intoxication does not exactly reflect human disease in course and pathobiology. The chronicity of immune responses also seen in PD is distinct from that observed following acute MPTP exposure. All together, the abilities to induce a regulatory T cell response rather than autoreactive immunity towards CNS antigens may in fact generate protective T cell responses without secondarily exacerbating neuronal damage due to inflammation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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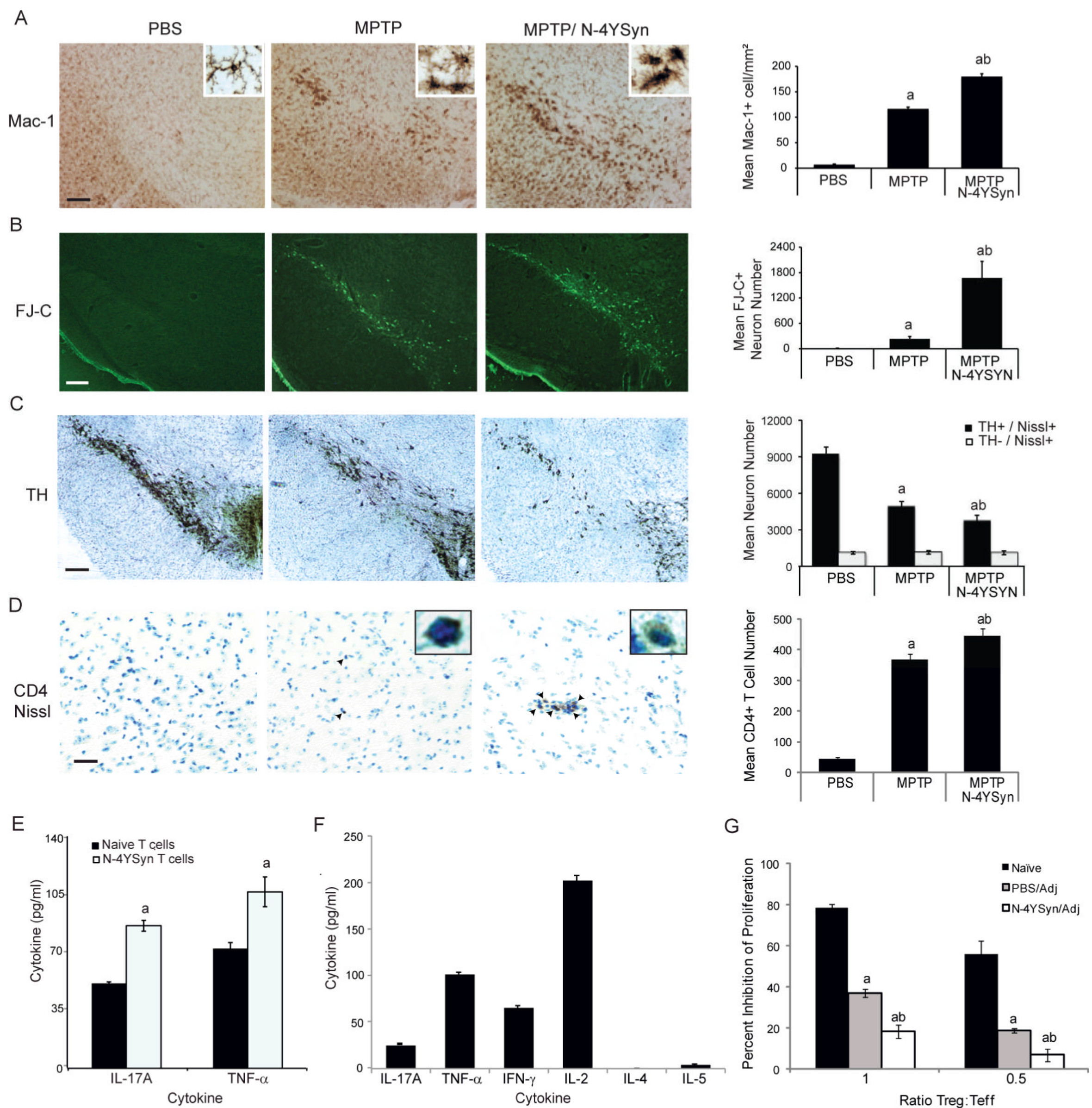

FIGURE 1. N-α-syn adaptive immunity accelerates MPTP-nigrostriatal degeneration

Photomicrographs and enumeration of Mac-1$^+$, FJ-C$^+$, TH$^+$ neurons and CD4$^+$ T cells in the SN of mice treated with PBS, MPTP, or MPTP and N-4YSyn SPCs. Shown are midbrains from mice 2 days after MPTP treatment and immunostained for (A) Mac-1$^+$ reactive microglia (scale bar, 200 μm) with estimated numbers of Mac-1$^+$ microglia per mm^2; and (B) stained for FJ-C$^+$ neurons (scale bar, 200 μm) with estimated total numbers of FJ-C$^+$ neurons. (C) From the midbrains of mice 7 days post MPTP treatment, dopaminergic neurons in the SN were identified as TH$^+$Nissl$^+$ neurons, while non-dopaminergic neurons were identified as TH$^-$Nissl$^+$ neurons (scale bar, 200 μm). Total numbers of nigral neurons were determined and represented as mean number of TH$^+$Nissl$^+$ neurons (black bars and TH$^-$Nissl$^+$ neurons (gray bars). (D) Infiltration of CD4$^+$ T cells (arrow heads) within the SN of mice 7 days following MPTP-intoxication (scale bar, 100 μm) and total numbers of CD4$^+$ T cells within the SN were estimated. (A, B, C, and D) Differences in means (± SEM, n=7 mice per group) were determined, where $P<0.05$ compared with groups treated with [a]PBS or [b]MPTP. Concentration

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of cytokines in culture supernatants of (E) anti-CD3 stimulated naïve (black bars) and N-4YSyn T cells (white bars) and (F) N-4YSyn-stimulated N-4YSyn effector $CD4^+$ T cells. (G) Assessment of Treg-mediated inhibition of effector T cell proliferation by Treg sorted from naïve (black bars), PBS/adjuvant- (PBS/Adj, gray bars), or N-4YSyn/adjuvant- (N-4YSyn/Adj, white bars) immunized FoxP3-GFP transgenic mice. (E and G) Differences in means (± SEM) were determined where $P < 0.05$ compared with Treg from [a]naïve mice or [b]PBS/Adj immune mice, (n=4).

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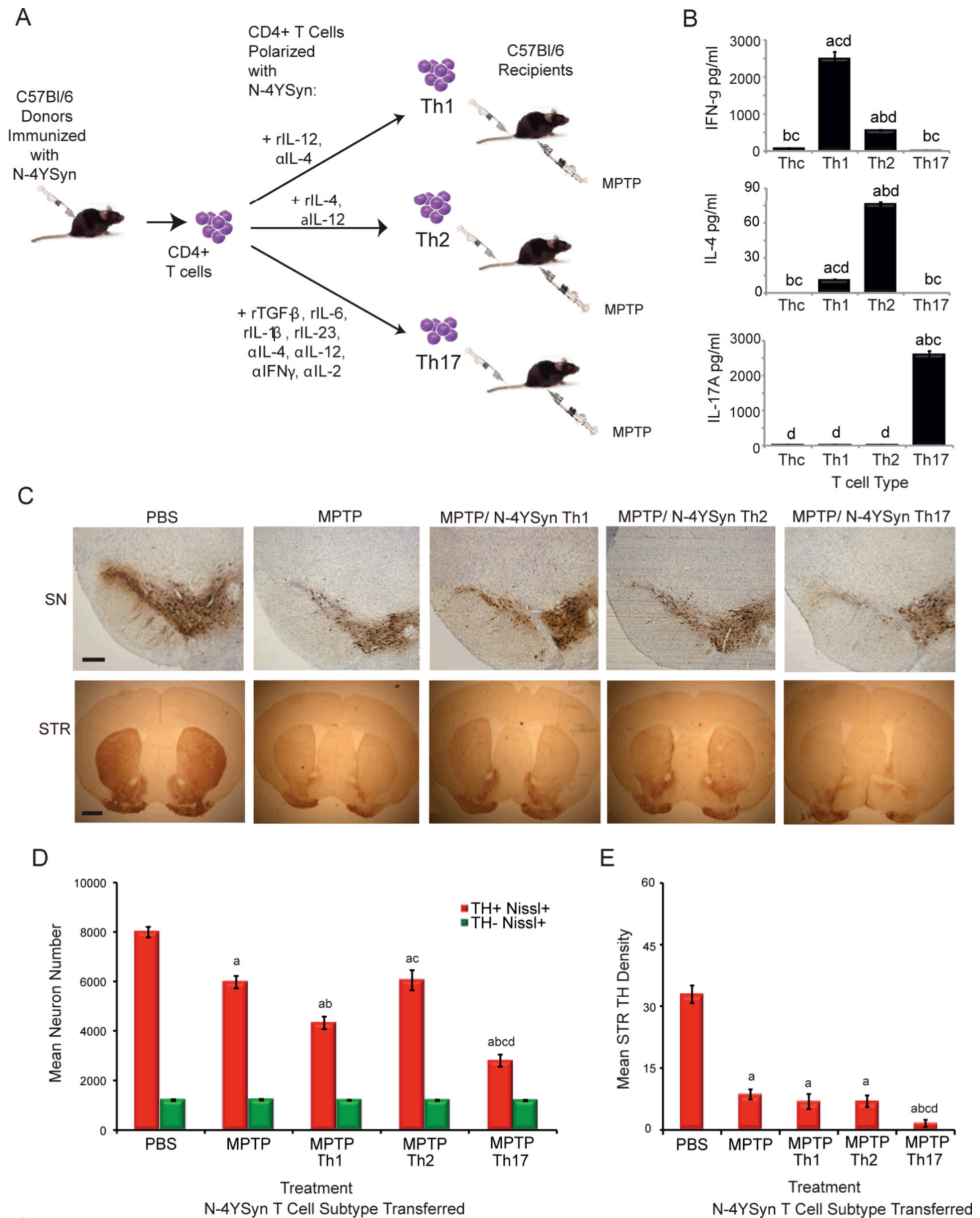

FIGURE 2. Th17 immunity amplifies N-α-syn nigrostriatal degeneration

(A) Experimental design for *in vitro* polarization and adoptive transfers of $CD4^+$ T cells of a Th1, Th2 or Th17 phenotype. Donor mice were immunized with N-4YSyn in adjuvant and $CD4^+$ T cells were polarized in culture with indicated cytokine/anti-cytokine cocktails in the presence of N-4YSyn to yield N-4YSyn Th1-, Th2-, or Th17-enriched T cells. Recipient mice were intoxicated with MPTP and polarized T cell subsets were adoptively transferred to separate recipient groups at 12 hours post-MPTP treatment. Survival of nigrostriatal dopaminergic neurons and termini were evaluated 7 days post MPTP-treatment. (B) Concentration of cytokines produced by $CD4^+$ T cells isolated from donor N-4YSyn immunized mice and cultured for 5 days under nonpolarizing control conditions (Thc) or

polarized to Th1, Th2 or Th17 T cells. (C) Photomicrographs of TH^+ neurons in the SN (scale bar 500 μm) and striatal termini (scale bar 1000 μm) of mice treated with PBS, MPTP, or MPTP and polarized Th1, Th2 or Th17 CD4+ effector cells. (D) Total numbers of TH^+ $Nissl^+$ dopaminergic neurons in the SN (red bars) and non-dopaminergic neurons ($TH^−$ $Nissl^+$, green bars). (E) TH densitometry of dopaminergic neuronal termini within the striatum. (D and E) Differences in means (± SEM, n=7 mice per group) were determined where $P<0.05$ compared with groups treated with [a]PBS, [b]MPTP, [c]MPTP/N-4YSyn Th1 and [d]MPTP/N-4YSyn Th2.

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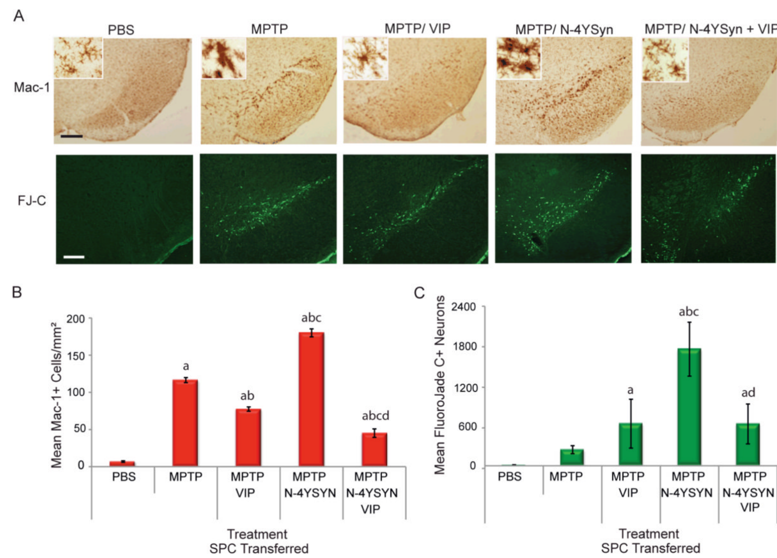

FIGURE 3. Microglial activation and nigrostriatal responses

Recipient mice were treated with MPTP. SPC from donor mice treated with VIP or immunized with N-4YSyn, or equal numbers of pooled SPC were adoptively transferred 12 hours post-MPTP treatment. Mice were sacrificed on day 2 post-MPTP treatment for evaluation of the substantia nigra for reactive Mac-1^+ microglia and neuronal cell injury by Fluorojade C (FJ-C) stain. (A) Photomicrographs (scale bars, 200 μm) of midbrain immunostained for Mac-1 (top panels) to identify reactive microglia or FJ-C to identify dead or dying neurons (bottom panels). (B) Mean numbers of Mac-1^+ microglia and (C) FJ-C^+ neurons within the SN were determined. (B and C) Differences in means (± SEM, n=5 mice per group) were determined where $P<0.05$ compared with groups treated with ^{a}PBS, ^{b}MPTP, ^{c}MPTP/VIP SPC, or ^{d}MPTP/N-4YSyn SPC.

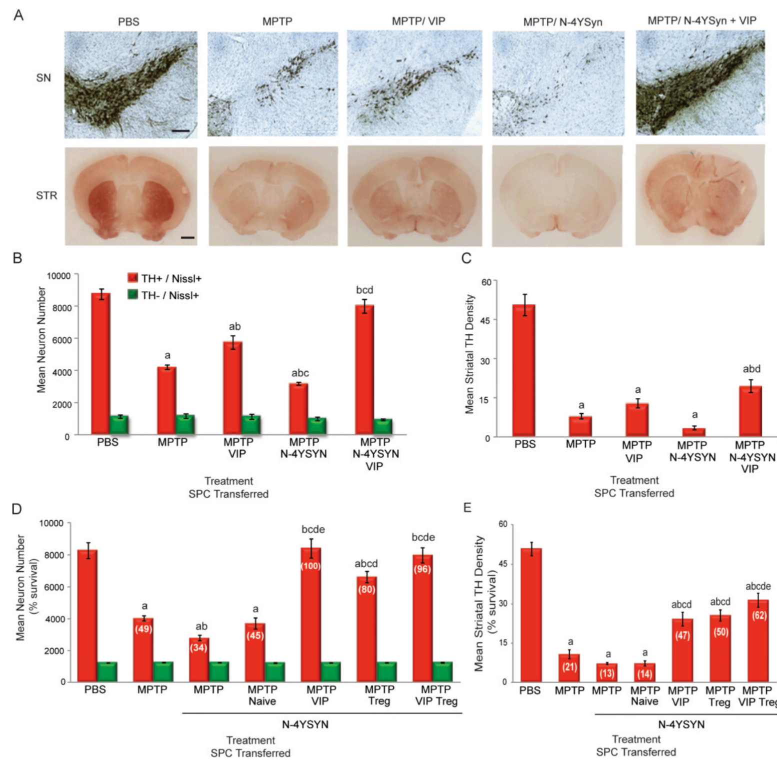

FIGURE 4. Treg mediated nigrostriatal neuroprotection in immunized MPTP-intoxicated mice

Recipient mice were treated with MPTP, and SPC from donor mice immunized with N-4YSyn were adoptively transferred with SPC from naïve mice, VIP-treated mice, naïve Treg, Treg from donor mice treated with VIP, or pooled SPC populations were adoptively transferred 12 hours post-MPTP treatment. Mice were sacrificed on day 7 post-MPTP treatment and evaluated for surviving dopaminergic nigral neurons and striatal termini. (A) Midbrain sections (top panels, scale bar 200 μm) and striatum (bottom panel, scale bar 1000 μm) immunostained for TH. (B) Dopaminergic neurons in the SN were identified as $TH^{+}Nissl^{+}$ neurons (red bars), while non-dopaminergic neurons were identified as $TH^{-}Nissl^{+}$ neurons (green bars). (C) Mean densities of striatal dopaminergic termini were determined by digital image analysis. (B and C) Differences in means (± SEM, n=7 mice per group) were determined where $P<0.05$ compared with groups treated with ^{a}PBS, ^{b}MPTP, ^{c}MPTP/VIP SPC, ^{d}MPTP/N-4YSyn SPC (D) Dopaminergic neuronal survival in the SN and (E) density of striatal dopaminergic termini following adoptive transfer of 5×10^{7} N-4YSyn SPC without other cells or in combination with 5×10^{7} naïve SPC (Naïve), 5×10^{7} SPC from VIP-treated mice (VIP), 1×10^{6} Treg from naïve mice (Treg), or 1×10^{6} Treg from mice treated with VIP (VIP Treg). (D and E) Differences in means of neuron number and striatal TH density (± SEM, n=7 mice per group) were determined where $P<0.05$ compared with groups treated with ^{a}PBS, ^{b}MPTP, ^{c}MPTP/N-4YSyn SPC, dMPTP/N-4YSyn SPC + naive SPC, or ^{e}MPTP/N-4YSyn SPC + naïve Treg. (E) Mean percentages of survival for nigral dopaminergic neuronal bodies or striatal termini based on PBS controls are presented parenthetically in white on each bar.

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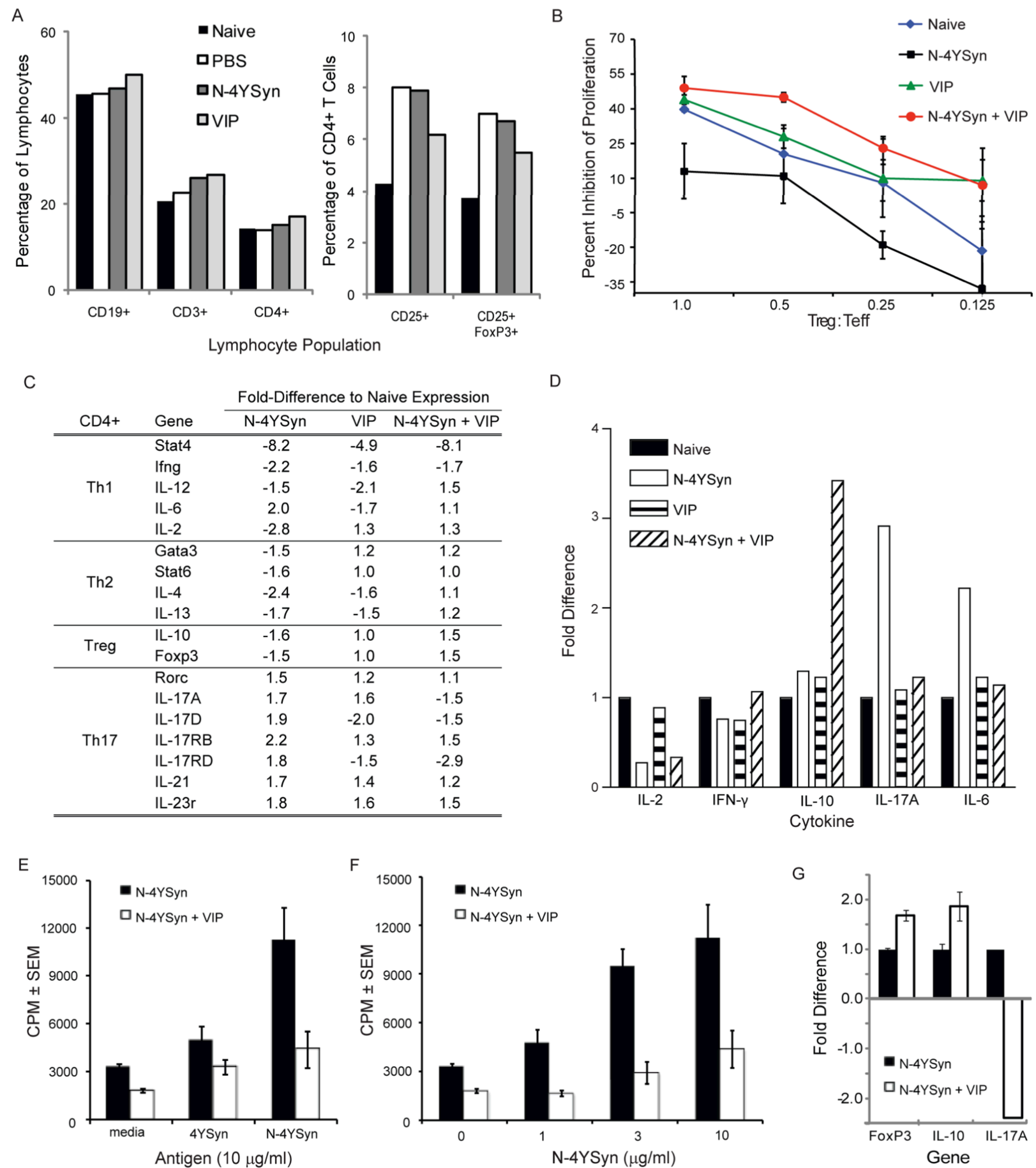

CD4+	Gene	N-4YSyn	VIP	N-4YSyn + VIP
		Fold-Difference to Naive Expression		
Th1	Stat4	-8.2	-4.9	-8.1
	Ifng	-2.2	-1.6	-1.7
	IL-12	-1.5	-2.1	1.5
	IL-6	2.0	-1.7	1.1
	IL-2	-2.8	1.3	1.3
Th2	Gata3	-1.5	1.2	1.2
	Stat6	-1.6	1.0	1.0
	IL-4	-2.4	-1.6	1.1
	IL-13	-1.7	-1.5	1.2
Treg	IL-10	-1.6	1.0	1.5
	Foxp3	-1.5	1.0	1.5
Th17	Rorc	1.5	1.2	1.1
	IL-17A	1.7	1.6	-1.5
	IL-17D	1.9	-2.0	-1.5
	IL-17RB	2.2	1.3	1.5
	IL-17RD	1.8	-1.5	-2.9
	IL-21	1.7	1.4	1.2
	IL-23r	1.8	1.6	1.5

FIGURE 5. Phenotypic and functional characterization of Treg

SPC, $CD4^+$ T cells and Treg from naïve mice, mice immunized with N-4YSyn, or mice treated with VIP were evaluated for B and T cell frequencies, ability to inhibit T cell proliferation, and expression of Teff and Treg genes. (A) Flow cytometric analysis of cell subsets within splenic lymphocytes from naïve mice (black bars), mice immunized with PBS/adjuvant (white bars) or N-4YSyn/adjuvant (dark gray bars), or treated with VIP (light gray bars). (B) Inhibition assay to assess the suppressive function of Treg isolated from each donor group on proliferation of anti-CD3 stimulated naïve $CD4^+CD25^-$ T cells. (C) Table of relative fold-differences in expression of $CD4^+$ T cell related genes from T cells isolated from N-4YSyn immunized, VIP-treated, and N-4YSyn+VIP T cells compared with T cells from naïve mice (Supplemental data

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1,2 and 3). (D) Cytokine production assayed from T cell supernatants, normalized to absorbance obtained from supernatants of naïve T cells. (E) T cell proliferation cultured in the absence of antigen (media) or presence of nonnitrated 4YSyn or nitrated N-4YSyn. (F) Dose response of N-4YSyn on T cell proliferation. (G) Fold difference of gene expression for T cells from N-4YSyn-immunized mice treated with or without VIP. Values shown are differences in means (± SEM, n=4).