# Therapeutic immunization protects dopaminergic neurons in a mouse model of Parkinson's disease

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**Degeneration of the nigrostriatal dopaminergic pathway, the hallmark of Parkinson's disease, can be recapitulated in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-intoxicated mice. Herein, we demonstrate that adoptive transfer of copolymer-1 immune cells to MPTP recipient mice leads to T cell accumulation within the substantia nigra pars compacta, suppression of microglial activation, and increased local expression of astrocyte-associated glial cell line-derived neurotrophic factor. This immunization strategy resulted in significant protection of nigrostriatal neurons against MPTP-induced neurodegeneration that was abrogated by depletion of donor T cells. Such vaccine treatment strategies may provide benefit for Parkinson's disease.**

Parkinson's disease (PD) is a common neurodegenerative disease characterized clinically by resting tremor, rigidity, slowness of voluntary movement, and postural instability (1). Loss of dopaminergic neurons within the substantia nigra pars compacta (SNpc), intraneuronal cytoplasmic inclusions or "Lewy bodies," gliosis, and striatal dopamine depletion are principal neuropathological findings. With the exception of inherited cases linked to specific gene defects that account for <10% of cases, PD is a sporadic condition of unknown cause (2).

Inflammation increases the risk of PD (3). Experimental disease models show that innate immunity, especially glial inflammatory factors such as proinflammatory cytokines and reactive oxygen and nitrogen species contribute to the degeneration of the nigrostriatal dopaminergic pathway (4). Although less studied than innate immunity, T lymphocytes present in brain tissue may also affect disease progression (5, 6). For example, T cells perform surveillance functions in the nervous system (7, 8), and T cell-deficient mice show enhanced neuronal loss after CNS damage (9, 10). Adaptive immunity, after vaccination with CNS antigens expressed at the lesion site, can attenuate neuronal death. For instance, in optic nerve and spinal cord injuries, encephalitic T lymphocytes directed against myelin-associated antigens positively affect neurodegenerative processes (11–14). Such self-antigen-stimulated T cells may retard neuronal injury by producing neurotrophins (15, 16) or by influencing their production by local glial cells (17).

Based on these prior studies, we theorized that immunization strategies could induce T cells to enter inflamed nigrostriatal tissue, attenuate innate glial immunity, and increase local neurotrophic factor production. To investigate this notion, copolymer-1 (Cop-1; Copaxone, glatiramer acetate), a random amino acid polymer that generates nonencephalitic T cells, which cross-react with myelin basic protein (MBP) in humans (18) and mice (19), was tested in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-intoxicated mice. Cop-1 immunization protects against secondary CNS injury without the encephalitis associated with MBP immunization (20, 21). Moreover, s.c. Cop-1 immunization preferentially incites T cells with a $T_H2$ phenotype, which secrete antiinflammatory cytokines such as IL-4, IL-10, and transforming growth factor-$\beta$ (22). We now demonstrate that Cop-1 immune cells administered to MPTP-intoxicated mice by adoptive transfer enter inflamed brain regions, suppress microglial responses, and increase expression of glial cell line-derived neurotrophic factor (GDNF).[‡‡] The process was T cell-dependent and led to significant dopaminergic neuronal protection. Because no currently clinically approved therapy prevents progressive degeneration of dopaminergic neurons in PD, we suggest that such a vaccination strategy could be of therapeutic benefit.

## Materials and Methods

**Animals and MPTP Treatment.** Male SJL mice (6–10 weeks old, The Jackson Laboratory) received four i.p. injections at 2-h intervals of either vehicle (PBS, 10 ml/kg) or MPTP-HCl (18 mg/kg of free base in PBS; Sigma). Twelve hours after the last MPTP injection, random mice received adoptive transfers of splenocytes from Cop-1- or ovalbumin (OVA)-immunized mice or no splenocytes ($n$ = 5–9 mice per group per time point). On days 2 and 7 after MPTP intoxication, mice were killed and brains were processed for subsequent analyses. 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 handling and safety measures were in accordance with published guidelines (23).

**Immunization and Adoptive Transfers.** Mice were immunized with a total dose of 200 $\mu$g of either Cop-1 or OVA emulsified in complete Freund's adjuvant containing 1 mg/ml *Mycobacterium tuberculosis* (Sigma). Five days after immunization, mice were killed and single-cell suspensions were prepared from the draining inguinal lymph nodes and spleen. MPTP-intoxicated mice received an i.v. injection of $5 \times 10^7$ splenocytes in 0.25 ml of Hanks' balanced salt solution. In all adoptive transfer experiments, pooled immunized donor cells were tested for proliferation by [$^3$H]thymidine uptake and/or cytokine expression by ELISA after exposure to immunizing or nonrelevant antigen.

**Cytokine Measurements.** Donor splenocytes were plated at a density of $1 \times 10^6$ cells per ml of tissue culture media [RPMI medium 1640 supplemented with 10% FBS/2 mM L-glutamine/25 mM Hepes/1 mM sodium pyruvate/1× nonessential amino acids/55 $\mu$M 2-mercaptoethanol/100 units/ml penicillin/100 $\mu$g/ml streptomycin (Mediatech, Herndon, VA)] and stimulated with immunizing antigens. After incubation (37°C at 48 h), supernatants were assayed for IL-10 by ELISA (R&D Systems).

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Abbreviations: PD, Parkinson's disease; SNpc, substantia nigra pars compacta; Cop-1, copolymer-1; MBP, myelin basic protein; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; GDNF, glial cell line-derived neurotrophic factor; OVA, ovalbumin; TH, tyrosine hydroxylase; GFAP, glial fibrillary acidic protein.

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**CD90 T Cell Depletion and Flow Cytometry.** Donor splenocyte cell suspensions from Cop-1-immunized donors were depleted of T cells using anti-CD90 magnetic beads and magnetic LD columns (Miltenyi Biotec, Auburn, CA). Negatively selected cells ($CD90^-$) were pooled ahead of time and were analyzed for cell purity with a FACSCalibur flow cytometer interfaced with CELLQUEST software (BD Biosciences, Immunocytometry Systems, San Jose, CA) before adoptive transfers. Unfractionated and T cell-depleted populations were stained for T cells using FITC-conjugated anti-CD3 (clone 145-2C11, BD Biosciences, Pharmingen, San Diego) and B cells with phycoerythrin-conjugated anti-B220 (clone RA3-6B2, BD Biosciences, Pharmingen).

**Immunohistochemistry and Quantitative Morphology.** Seven days after MPTP intoxication, mice were killed and their brains were processed for tyrosine hydroxylase (TH) and thionin staining (24). Total numbers of TH- and Nissl-stained neurons in SNpc were counted stereologically with STEREO INVESTIGATOR software (MicroBrightfield, Williston, VT) by using an optical fractionator (25). Quantitation of striatal TH immunostaining was performed as described (24). Optical density measurements were obtained by digital image analysis (Scion, Frederick, MD). Striatal TH optical density reflected dopaminergic fiber innervation.

Additional primary antibodies used in these studies included rat Mac-1 (1:1,000; Serotec), rabbit glial fibrillary acidic protein (GFAP; 1:1000, DAKO), and rat CD3 (1:800; Pharmingen). Immunostaining was visualized by using diaminobenzidine as the chromogen. For immunofluorescence staining on fresh frozen sections, rabbit anti-CD3 (1:200, DAKO) was used with rat-anti-Mac-1 and goat anti-GDNF (1:100, R & D Systems). Confocal images were obtained with a Zeiss confocal LSM410 microscope.

**Cell Tracking.** Splenocytes from Cop-1-immunized donors were labeled with carboxyfluorescein diacetate, succinimidyl ester (CFDA SE) by using the Vybrant CFDA SE cell tracer kit (Molecular Probes). Splenocytes ($5 \times 10^7$) were adoptively transferred into PBS- or MPTP-treated mice. At 2, 8, and 20 h ($n = 3$ mice per time point) after adoptive transfers, mice were killed, their brains were fixed (4% paraformadehyde), and cryostat-cut sections were analyzed by fluorescence microscopy.

**RNA Isolation and Real-Time RT-PCR.** Total RNA from ventral midbrain and cerebellum ($n = 5$–7 mice per group) was extracted with TRIzol (Invitrogen). RNA was reverse-transcribed with random hexamers and real-time quantitative PCR was performed on cDNA by using the Applied Biosystems PRISM 7000 sequence detector with SYBR green I as the detection system. The murine primer sequences included: Mac-1, 5′-GCCAATGCAACAGGTGCATAT-3′ (forward) and 5′-CACACATCGGTGGCTGGTAG-3′ (reverse); GDNF, 5′-TGTTCTGCCTGGGTGTTGCT-3′ (forward) and 5′-TTGGAGTCACTGGTCAGCG-3′ (reverse). Primers for GAPDH were purchased from Applied Biosystems. Data are presented as a ratio of mean threshold ($C_t$) target gene expression and GAPDH. Differences between means were analyzed by using one-way ANOVA followed by the least significant difference posthoc test for pairwise comparisons.

**$Mac\text{-}1^+$ Immunohistochemistry.** Midbrain sections (30 $\mu$m) from two mice per treatment group (four to six sections per animal) were immunostained for Mac-1. Cell counts were obtained of amoeboid $Mac\text{-}1^+$ cells within the SN by using criteria reported (26) and cells per $mm^2$ was calculated. Numbers of Mac-1-positive cells were averaged for each animal and the mean cells per $mm^2$ per animal was estimated. The average countable area between treatment groups ranged from 1.92 $mm^2$ to 2.22 $mm^3$, and no significant differences in the size of countable areas were observed by ANOVA ($P = 0.063$, $n = 84$ countable areas).

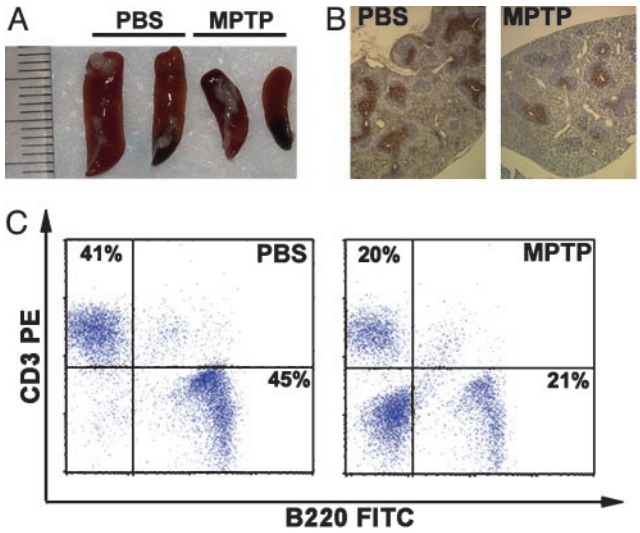


**Fig. 1.** MPTP-induced immunotoxicity. (*A* and *B*) Seven days after MPTP intoxication, spleen size (*A*) and $CD3^+$ T lymphocyte numbers (*B*) were reduced in spleens of MPTP-treated mice. (*C*) Flow cytometric analysis of splenocytes from PBS (*Left*) and MPTP (*Right*) 2 days after intoxication.

**Western Blot Assays.** Ventral midbrain protein extracts (25 $\mu$g per lane) were fractionated on SDS/4–20% PAGE (Invitrogen), and were then transferred onto PVDF membranes. Membranes were probed with horseradish peroxidase-conjugated anti-mouse IgG or rabbit anti-GFAP (1:15,000; DAKO). Secondary anti-rabbit antibodies conjugated with horseradish peroxidase were visualized by using SuperSignal West Pico chemiluminescent substrate and CCL-XPosure film (Pierce). Immunoblots were stripped and reprobed with antibodies to $\alpha$-actin (Chemicon) as an internal control.

**Measurement of Striatal Catecholamines.** Striatal dopamine and its metabolites, dihydroxyphenylacetic acid, and homovanillic acid, were analyzed 7 days after MPTP treatment by reverse-phase HPLC with electrochemical detection (25).

**Statistical Analysis.** All values are expressed as mean ± SEM. Differences among means were analyzed by one-way ANOVA followed by Bonferroni post hoc testing for pairwise comparison unless otherwise stated. The null hypothesis was rejected at the level of 0.05.

## Results

**Cop-1 Immunity Confers Dopaminergic Neuroprotection.** To test whether Cop-1 immunity confers dopaminergic neuroprotection, MPTP-intoxicated SJL mice received, by adoptive transfer, 12 h after MPTP treatment, $5 \times 10^7$ donor splenocytes from nonintoxicated mice previously immunized with either Cop-1 or chicken egg OVA. Replicate MPTP- and PBS-treated mice that did not receive splenocytes served as controls. Adoptive transfer of Cop-1 immune cells to MPTP-treated recipients was used because immunotoxicity precluded active immunization studies. Indeed, MPTP induced significant changes in spleen size with diminished numbers of $CD3^+$ T cells 7 days after MPTP intoxication (Fig. 1 *A* and *B*). Flow cytometric analysis of splenocyte populations revealed a 51% and 53% decrease in $CD3^+$ T cell and $B220^+$ B cell numbers, respectively (Fig. 1*C*). Because MPTP intoxication occurs rapidly and its metabolism into the active toxin, 1-methyl-4-phenylpyridinium ($MPP^+$), is complete within minutes (27) and is undetectable after 8 h (28), the timing of splenocyte adoptive transfers was designed to avoid confounding effects of MPTP metabolism and its induced hematopoietic toxicity (29). Seven days after MPTP treatment, after which no further dopaminergic neurodegeneration is detected

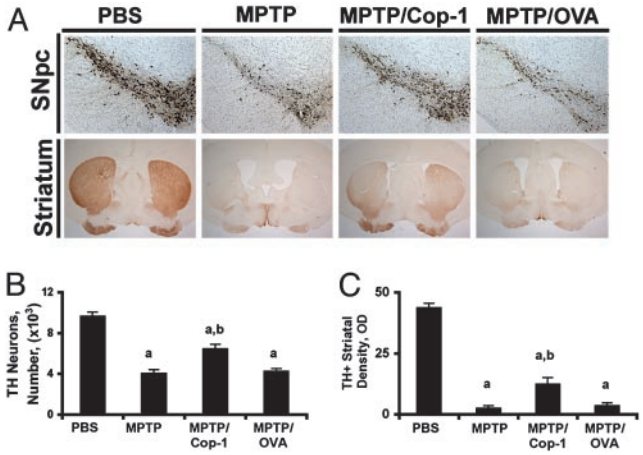


**Fig. 2.** Cop-1 immunization protects against MPTP-induced dopaminergic neuronal loss. (*A*) Photomicrographs of SNpc and striatum TH immunostaining from PBS, MPTP, MPTP/Cop-1, or MPTP/OVA groups. (*B*) SNpc $TH^+$ neuronal counts of SNpc $TH^+$ neurons. (*C*) Optical densities of striatal $TH^+$ fibers. Values represent means ± SEM for five to nine mice per group. $P < 0.05$ compared with PBS (a), MPTP (b), and MPTP/OVA (b).

(30), mice were transcardially perfused with saline followed by 4% paraformaldehyde, their brains were removed, were cryosectioned, and immunostained for expression of TH, the rate-limiting enzyme in dopamine synthesis (Fig. 2*A*). Stereological counts revealed that MPTP caused a 58% loss of SNpc TH-positive neurons compared with PBS controls (Fig. 2*B*). Similar results were observed in MPTP-injected mice that received splenocytes from OVA-immune donors (MPTP/OVA; Fig. 2 *A* and *B*). In contrast, MPTP-injected mice that received Cop-1 splenocytes (MPTP/Cop-1) exhibited a much smaller reduction in the number of SNpc dopaminergic neurons compared with MPTP or MPTP/OVA animals (Fig. 2 *A* and *B*). Counts of SNpc neurons after Nissl staining with thionin correlated with TH-positive neuron counts ($r = 0.993, P < 0.0001$). This finding confirmed that differences in TH-positive neuron counts were due to numbers of structurally intact neurons and eliminated the possibility that differences resulted from the down regulation of TH itself (Table 2, which is published as supporting information on the PNAS web site, and ref. 30).

Sparing of SNpc dopaminergic cell bodies does not always correlate with protection of their corresponding striatal nerve fibers (25), which is essential for maintaining dopaminergic neurotransmission. To determine whether adoptive transfer of Cop-1 splenocytes affected the integrity of striatal dopaminergic fibers, the density of TH-immunoreactivity in striata (Fig. 2 *A* and *C*) was assessed. MPTP reduced striatal TH density by 94% (MPTP) and 92% (MPTP/OVA) compared with PBS controls (Fig. 2*C*). In contrast, loss of striatal TH density in MPTP/Cop-1 mice (72% loss) was significantly less compared with what was observed in MPTP and MPTP/OVA animals (Fig. 2*C*). The dopaminergic nerve terminals are consistently more affected than the cell bodies in both PD and its MPTP model and are often less amenable to neuroprotection (25, 31). Thus, given the severity of damage at level of the nerve terminals, any significant protection is deemed relevant. Taken together, these findings indicate that Cop-1 immune cells mitigate the deleterious action of MPTP on dopaminergic nerve fibers in the striatum and cell bodies in the SNpc. The ability of splenocytes from Cop-1-immunized mice to confer neuroprotection to myelinated axons is consistent with prior studies where Cop-1 immunization protected against traumatic nerve injury (20).

To determine whether adoptive transfer of Cop-1 immune cells also protects against biochemical deficits caused by MPTP, we assessed levels of dopamine and two of its metabolites, dihydroxyphenylacetic acid and homovanillic acid, in striata 7 days after MPTP treatment. Characteristic diminution in striatal dopamine levels by 51% for MPTP-treated mice and 41% for the MPTP/OVA group was observed compared with levels in striata of PBS controls. In contrast, animals that received Cop-1 splenocytes showed only a 4% decrease in striatal dopamine (Table 1). Together, these results indicate that spleen cells from Cop-1-immunized mice protect neuronal dopamine metabolism as well as structural neuronal elements and its projections.

**Table 1. Striatal neurotransmitter levels from mice 7 days after MPTP treatment**

| | Neurotransmitter levels, ng/mg tissue | | |
|---|---|---|---|
| Treatment | Dopamine | DOPAC | HVA |
| PBS ($n = 4$) | 10.0 ± 0.1 | 0.9 ± 0.2 | 8.8 ± 0.8 |
| MPTP ($n = 6$) | 4.9 ± 0.05* | 1.4 ± 0.2 | 5.4 ± 1.1* |
| COP-1/MPTP ($n = 5$) | 9.6 ± 1.1 | 1.3 ± 0.2 | 7.7 ± 1.0 |
| OVA/MPTP ($n = 6$) | 6.0 ± 0.07* | 2.3 ± 0.2† | 5.0 ± 0.2* |

Values in parentheses are the mean ± SEM for no. of mice per treatment group. DOPAC, dihydroxyphenylacetic acid; HVA, homovanillic acid.
*$P < 0.01$ compared with PBS and COP-1.
†$P < 0.02$ compared with all groups.

**Cop-1 Immune Cells Reduce Microglial Reactions.** Based on studies that demonstrate antiinflammatory cytokine profiles by Cop-1-reactive T cells (19, 22), we theorized that the protective effects of Cop-1 immune cells resulted from the modulation of glial inflammatory responses. In line with previously reported results, our immunization strategy generated T cells that proliferate (data not shown) and secrete IL-10 and IL-4 in response to MBP and/or Cop-1 (Fig. 3 *A* and *B*). Because the active phase of neuronal death and neuroinflammatory activities peak at ≈2 days after MPTP injection (25, 30), we assessed lymphocyte infiltration and IgG in the nigrostriatal region at this time point. $CD3^+$ T cells were detected within nigrostriatal tissue (Fig. 3 *C* and *D*) in all mice after MPTP intoxication and adoptive transfer. However, differences were not observed in the ventral midbrain IgG by either Western blot (Fig. 3*E*) or immunohistochemical tests (data not shown). These findings suggested that T cells, not IgG, play the principal roles in the neuroprotective activities observed in these studies. To confirm whether infiltrating T cells in immunized mice were donor-derived, splenocytes were labeled *ex vivo* with the succinimidyl ester of carboxyfluorescein diacetate (Molecular Probes) and transferred intravenously to MPTP mice. As early as 2 h after adoptive transfer and for 20 h thereafter, carboxyfluorescein diacetate-labeled lymphocytes were readily observed both in ventral midbrains and striata of MPTP mice. No labeled cells were found in the cerebellum, a region not afflicted by MPTP. These data demonstrate that donor-derived T cells rapidly enter affected regions of the brain during active inflammation and neuronal loss.

Based on observations that peripheral lymphocytes enter and accumulate in areas of tissue damage early after cell transfer and at times of peak inflammation, we assessed the potential of Cop-1 immune cells to regulate MPTP-induced microglial reactions. In MPTP-treated animals, $CD3^+$ T cells were readily seen in close association with activated microglial cells (Fig. 3*F*); the latter evidenced by increased expression of Mac-1 (CD11b), a cell-surface receptor for complement that is up-regulated by activated microglia in both PD and the MPTP model (31). The evidence that Cop-1 immune spleen cells secreted IL-10 and IL-4 upon *in vitro* stimulation with Cop-1 or MBP (Fig. 3 *A* and *B*), suggested that T cell cytokines may affect glial cell function. Because MPTP-induced neurodegeneration may be attenuated by microglia deactivation (25, 31, 32), we analyzed the ventral midbrain for Mac-1 gene expression by real-time RT-PCR 24 h after adoptive transfer of

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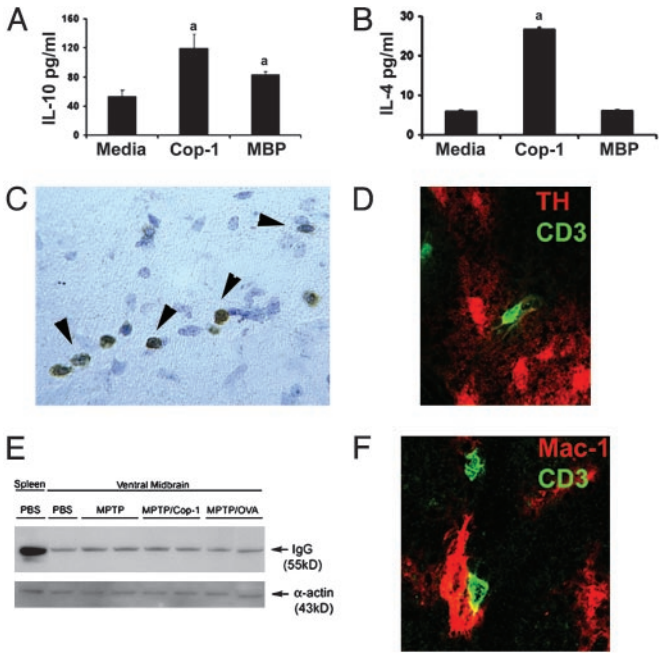


**Fig. 3.** Cytokine secretion by Cop-1 donor immune cells and T cell infiltration of the SNpc. (*A* and *B*) IL-10 (*A*) and IL-4 (*B*) secretion by Cop-1-immunized splenocytes cultured in media or stimulated with Cop-1 or MBP (30 $\mu$g/ml). Values are means of IL-10 or IL-4 concentrations ± SEM for three to four mice. a, $P < 0.05$ compared with PBS treatment group. (*C*) $CD3^+$ T cells in the SNpc of MPTP-intoxicated mice 2 days after adoptive transfer of Cop-1 splenocytes (arrows). (*D*) $CD3^+$ T cells in proximity (green) to $TH^+$ neurons (red) within the SNpc of an MPTP mouse. (*E*) Western blot analysis for IgG in ventral midbrains after adoptive transfer of splenocytes. (*F*) Ventral midbrain $CD3^+$ T cells (green) in direct contact with Mac-$1^+$ cells (red; magnification: ×2,000).

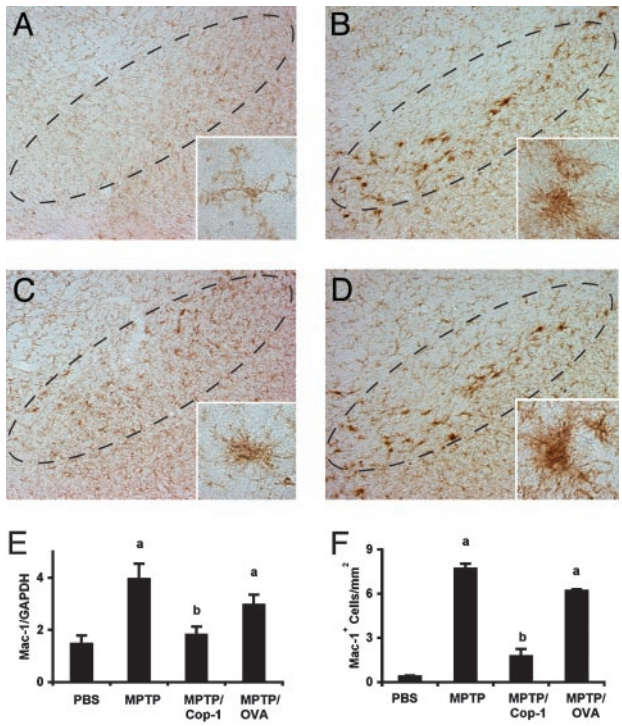


**Fig. 4.** Cop-1 immunization reduces MPTP-induced microglial reaction in the SNpc. (*A–D*) Mac-1 immunostaining within the SNpc (area circumscribed by dashed line and *Insets* at ×100 magnification) from PBS (*A*), MPTP (*B*), MPTP/Cop-1 (*C*), or MPTP/OVA (*D*) groups. (*E*) Real-time RT-PCR assessment of Mac-1/GAPDH mRNA from ventral midbrain. (*F*) Counts of Mac-$1^+$-reactive microglia from SNpc. $P < 0.05$ compared with PBS (a), MPTP (b), and MPTP/OVA (b) groups.

Cop-1 splenocytes (48 h after last MPTP injection). In agreement with prior studies (31), brains from MPTP-treated animals showed significant increases in Mac-1 mRNA. In contrast, MPTP/Cop-1 mice showed lower Mac-1 expression compared with both MPTP and MPTP/OVA animal groups (Fig. 4*E*). Immunohistochemical staining for cell-surface expression of Mac-1 in the ventral midbrain 48 h after adoptive transfer reflects levels of Mac-1 mRNA (Fig. 4 *A–D*). In PBS control mice, Mac-1 expression was associated with small microglial cells having thin ramifications (Fig. 4*A*). MPTP-injected and MPTP/OVA mice showed intense Mac-1 immunoreactivity, which revealed larger microglial cells with thicker short ramifications (Fig. 4 *B* and *D*). In MPTP/Cop-1 mice, Mac-$1^+$ cells were smaller, with finer processes approximating those in PBS controls (Fig. 4*C*). Enumeration within the SN of Mac-$1^+$ microglia with an activated phenotype showed a significant reduction in reactive microglia in the MPTP/Cop-1 group compared with MPTP- or MPTP/OVA-treated mice (Fig. 4*F*). Correlation analysis of Mac-1 mRNA expression and Mac-$1^+$ microglia counts from PBS-, MPTP-, MPTP/Cop-1-, and MPTP/OVA-treated groups indicated a strong correlation ($r = 0.76$, $P = 0.03$). Taken together, these data indicate that Cop-1 splenocytes are capable of attenuating MPTP-induced microglial reactions.

Although Cop-1 immune transfer significantly diminished the microglial reaction, astrocyte morphology was not affected. Expression of the astrocyte-specific antigen, GFAP, was comparable among all MPTP treatment groups as revealed by Western blot analysis of ventral midbrain 2 days after MPTP administration (data not shown). Astrocytosis by day 7 after MPTP treatment, shown by enhanced GFAP immunostaining and astrocyte morphology was similar among MPTP-treated groups, irrespective of passive immunization strategies (data not shown).

**Neuroprotection Is T Cell-Dependent.** As stated, T cells entered the damaged nigrostriatal tissue after MPTP intoxication in the absence of any noticeable alterations in nigrostriatal IgG levels. This finding suggested that the cellular arm of the immune system was responsible for neuroprotection. To test this hypothesis, T cell-depleted splenocytes from Cop-1-immunized mice were prepared by anti-CD90-conjugated magnetic beads. This action resulted in the removal of >90% of $CD3^+$ T lymphocytes without affecting B cell ($B220^+$) populations (Fig. 5*A*). In the experiments, MPTP-treated mice received unfractionated or T cell-depleted splenocytes from Cop-1-immunized donors, unfractionated splenocytes from OVA-immunized donors, or no splenocytes. On day 7, mice were killed and brain tissue was immunostained for TH content in the SNpc and striatum (Fig. 7, which is published as supporting information on the PNAS web site). A significant reduction in the number of TH-positive neurons within the SNpc was observed in MPTP-treated mice that received no splenocytes or splenocytes from OVA-immunized donors (Fig. 5*B*). Significant neuroprotection was afforded to MPTP-treated recipients of splenocytes from Cop-1-immunized mice (Fig. 5*B*). However, neuroprotection was ablated in mice that received T cell-depleted Cop-1 splenocytes (Fig. 5*B*). Parallel changes in striatal dopaminergic nerve fibers was also demonstrated. The diminution of TH optical density in striatal sections was significantly less in MPTP-treated recipients of unfractionated Cop-1 splenocytes compared with MPTP-treated control groups; however, this neuroprotection was ablated in recipients of T cell-depleted Cop-1 splenocytes (Fig. 5*C*). These results indicate that T cells from Cop-1-immunized donors are required for the observed neuroprotective activities.

**Cop-1 Immunization Increases Expression of GDNF in Ventral Midbrain.** Finally, we investigated whether Cop-1 immunization affects neurotrophin production at the site of disease. GDNF mitigates neu-

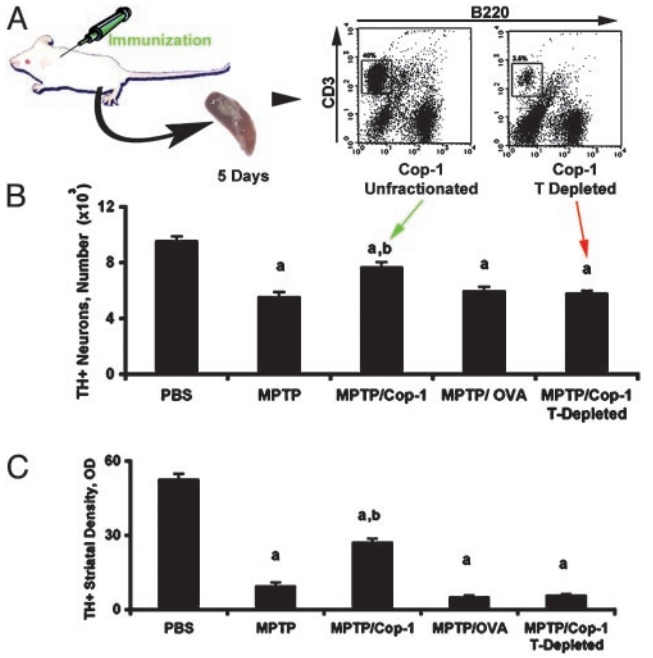


**Fig. 5.** T cell depletion ablates Cop-1-mediated dopaminergic neuroprotection. (*A*) Flow cytometric analysis of Cop-1 immune splenocytes before (unfractionated) and after T cell depletion. (*B*) Counts of SNpc $TH^+$ neurons for PBS ($n = 5$), MPTP ($n = 7$), MPTP/Cop-1 ($n = 8$), MPTP/OVA ($n = 8$), and MPTP/Cop-1/T cell-depleted groups ($n = 6$). (*C*) Densities of striatal $TH^+$ fibers. Values are means ± SEM. $P < 0.01$ compared with PBS (a), MPTP (b), and MPTP/OVA (b), MPTP/Cop-1/T (b)-depleted groups.

rodegenerative processes in MPTP animals and leads to symptomatic recovery after dopaminergic injury (33), This finding formed the basis for PD clinical trials that so far have yielded promising results (34). In our study, we quantitated by real-time RT-PCR analysis, GDNF mRNA levels in ventral midbrains from PBS, MPTP, MPTP/Cop-1, and MPTP/OVA mice 20 h after adoptive transfer. MPTP/Cop-1 mice showed significantly greater levels of ventral midbrain GDNF mRNA compared with all other groups (Fig. 6*A*). To identify the cellular source of GDNF within the SN

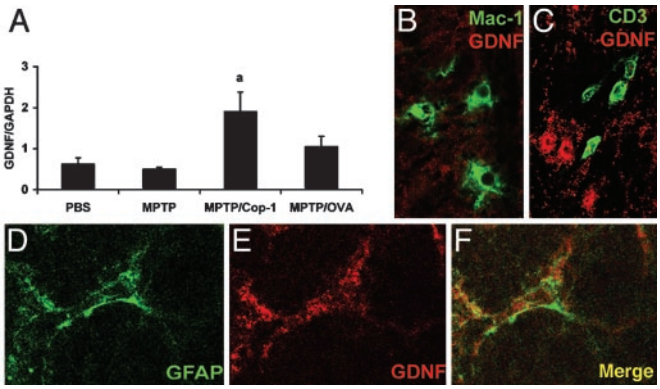


**Fig. 6.** GDNF expression in MPTP-intoxicated mice after adoptive transfer of Cop-1 splenocytes. (*A*) Real-time RT-PCR of GDNF mRNA expression from ventral midbrains of PBS, MPTP, MPTP/Cop-1, or MPTP/OVA groups. Values represent ratios of GDNF mRNA normalized to GAPDH and are means ± SEM for five to six mice per group. $P < 0.05$ compared with PBS (a), MPTP, and MPTP/OVA groups. (*B–F*) Confocal microscopy of SNpc from MPTP-treated recipients of splenocytes from Cop-1-immunized mice showing GDNF immunostaining (red) (*B–D* and *F*) and $Mac\text{-}1^+$ (green) microglia (*B*), $CD3^+$ (green) T cells (*C*), and $GFAP^+$ (green) astrocytes (*D* and *F*). Magnification: ×2000.

of MPTP/Cop-1 mice, sections were double immunostained for GDNF and cell markers. Analysis by confocal microscopy demonstrated that GDNF expression colocalized with cells expressing GFAP, but not with CD3 or Mac-1 (Fig. 6 *B–F*). These data suggest that astrocytes, not T cells or microglia, are the primary source of GDNF production in this model.

## Discussion

Epidemiological, immunopathological, and animal model studies support the notion that innate immunity affects nigrostriatal dopaminergic neurodegeneration in PD (2, 35). Many of the pathogenic processes operative in PD are recapitulated in MPTP-intoxicated animals. For example, animals injected with MPTP exhibit early microglial-associated neuroinflammatory events and subsequent nigrostriatal degeneration. Based on a number of prior studies linking neuroinflammation to neurodegenerative processes, we hypothesized that negatively regulating innate immunity in the CNS through $T_H2$-polarized adaptive immune responses through vaccination could lead to positive disease outcomes. Consistent with this idea, we demonstrate that passive immunization with Cop-1 immune cells into acutely MPTP-intoxicated mice protects the nigrostriatal dopaminergic system. This finding was evidenced by higher numbers of surviving SNpc $TH^+$ neuronal bodies and striatal fibers, in addition to elevated striatal dopamine levels in MPTP mice receiving Cop-1 immune cells. Taken together, the data indicate that Cop-1 immune cells accumulate specifically in affected brain areas during the most active phase of MPTP-induced neurodegeneration (30), and by so doing, trigger a T cell-dependent neuroprotective response.

The neuroprotection seen in our studies could result as a consequence of $T_H1$ (proinflammatory, IFN-$\gamma$) or a $T_H2$ or $T_H3$ (antiinflammatory, IL-10, IL-4, and TGF-$\beta$) immune response. However, Cop-1 immunization, in particular, is well known to generate $T_H2$ or $T_H3$ T cells (19, 22), which secrete cytokines known to suppress innate immunity (36–38). Cop-1 immunization, in the MPTP model, could exploit immunoregulatory activities of $T_H2$ or $T_H3$ T cells and thus provide a vehicle to attenuate microglial neurotoxic responses. Several of our observations support the notion that this scenario may underlie, at least in part, Cop-1 neuroprotective effects in the MPTP model. First, infiltration of the nigrostriatal pathway with donor-derived T cells was seen in close proximity to or in direct cell–cell contact with activated microglia. Second, a marked decrease in MPTP-associated microglial responses was observed after transfer of Cop-1 immune cells. This finding was supported by a profound reduction of ventral midbrain Mac-1 mRNA content and SNpc Mac-1 immunostaining. Third, MPTP-associated astrocytosis, a putative neuroprotective response, remained unchanged by passive immunization with Cop-1 cells. Fourth, IL-10 and IL-4, but not IFN-$\gamma$, was secreted by the Cop-1 cells in laboratory assays, providing evidence for the induction of an antiinflammatory $T_H2$ phenotype in affected brain tissue. Taken together, these results suggest that Cop-1 immune cells in MPTP mice can attenuate the microglial inflammatory responses that contribute to nigrostriatal dopaminergic neurodegeneration.

In addition to targeting the innate immune system, this therapeutic vaccine strategy was shown to augment GDNF within brain regions of active disease. It is likely that this effect is also implicated in the neuroprotective activities of Cop-1 because GDNF delivered to MPTP-intoxicated animals shows significant benefit (33). This observation may also be relevant to human disease given the therapeutic benefits of surgically implanted pumps which directly infuse GDNF into affected dopaminergic structures of PD patients in early human clinical trials (34). Activated T cells express both neurotrophins (39) and the neurotrophic factor receptors, trkB and trkC (40), thus providing sufficient mechanistic means to establish T cell–neuron communications. Consistent with this view, T cells can increase local CNS neurotrophic factor production *in vivo* (11). Our data demonstrate a dramatic increase in ventral midbrain

GDNF expression in Cop-1-immunized MPTP-injected mice. Interestingly, confocal microscopy revealed GDNF in astrocytes, but not in microglia or infiltrating T cells. Thus, these findings suggest that Cop-1 immune cells stimulate the local production of GDNF by astrocytes. In keeping with this idea, T cell cytokines are well known to affect the regulation of neurotrophins (17, 41) that in turn, could actively participate in the observed Cop-1-induced neuroprotective effects.

To our knowledge, this is the first time that a vaccine strategy has been used to confer neuroprotection for dopaminergic neurons. We posit that Cop-1-specific $T_H2$ cells, which recognize MBP, simultaneously suppress cytotoxic inflammatory responses and increase local neurotrophic factor production. It is possible that both mechanisms converge to ultimately abate the dopaminergic neurodegenerative process that occurs in the MPTP model of PD. In this regard, Cop-1 vaccination reflects the anticipated outcomes of gene therapy. Indeed, both approaches attempt to deliver factors that would attenuate disease to damaged microenvironments. This method is implemented to enhance the therapeutic index by delivering maximal levels of factors to specific diseased areas, thus minimizing system toxicity. Still, immunization avoids the inherent limitation of gene delivery and, by directing immune cells to areas of injury and producing a spectrum of disease mitigating factors, positively alters the neurodegenerative process. Additional studies performed in the MPTP model wherein animals analyzed over time and $TH^+$ neuronal counts substantiated with other tests, including behavioral and spectroscopic assays, may serve to further validate our experimental observations. In keeping with this concept, preliminary reports from our laboratories based on measures of *N*-acetyl aspartate (a biochemical neuronal marker) by using magnetic resonance spectroscopy and immunopathological coregistration and reverse-phase HPLC in the SNpc confirmed the neuroprotective effects of Cop-1 immune cells in MPTP-injected mice.[§§]

We found unexpectedly that MPTP induces a profound toxicity on cellular components of the peripheral immune system. Adoptive transfer of Cop-1 immune cells to MPTP recipient animals was used as immunotoxicity precluded active immunization. Moreover, our initial work used adoptive transfers with whole splenocyte populations to determine whether collective immune responses elicited against Cop-1 and reflecting active immunization could elicit neuroprotective activities in an animal model of PD. This step is critical in preclinical studies because both T and B cells can affect outcomes in nerve injury models (9) and may work in concert in doing so (42). Indeed, the pharmacokinetics of MPTP are well studied and demonstrate that the toxin is rapidly metabolized in mice and no longer detectable 8 h after the final dose (28). Although passive transfer is commonly performed in humans, there are no contraindications for PD patients to receive direct vaccination with Cop-1 or other related antigens that might elicit similar neuroprotective responses.

We conclude that this report opens a field of investigation toward the development of neuroprotective therapeutic modalities for PD. The reported Cop-1-specific immune-mediated neuroprotection has direct implications for the treatment of PD. As a Food and Drug Administration-approved and well tolerated drug, Cop-1 has been used effectively in patients with chronic neuroinflammatory disease such as relapsing remitting multiple sclerosis for more than a decade. Given the safety record of Cop-1 and that current treatments for PD remain palliative, such a vaccination strategy represents a promising therapeutic avenue that can readily be tested in human clinical trials.

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1. Fahn, S. & Przedborski, S. (2000) *Merritt's Neurology* (Lippincott, Williams & Wilkins, New York).
2. Dauer, W. & Przedborski, S. (2003) *Neuron* **39,** 889–909.
3. Chen, H., Zhang, S. M., Hernan, M. A., Schwarzschild, M. A., Willett, W. C., Colditz, G. A., Speizer, F. E. & Ascherio, A. (2003) *Arch. Neurol. (Chicago)* **60,** 1059–1064.
4. Gao, H. M., Liu, B., Zhang, W. & Hong, J. S. (2003) *Trends Pharmacol. Sci.* **24,** 395–401.
5. McGeer, P. L., Itagaki, S., Boyes, B. E. & McGeer, E. G. (1988) *Neurology* **38,** 1285–1291.
6. Kurkowska-Jastrzebska, I., Wronska, A., Kohutnicka, M., Czlonkowski, A. & Czlonkowska, A. (1999) *Exp. Neurol.* **156,** 50–61.
7. Babcock, A. A., Kuziel, W. A., Rivest, S. & Owens, T. (2003) *J Neurosci* **23,** 7922–7930.
8. Raivich, G., Jones, L. L., Kloss, C. U., Werner, A., Neumann, H. & Kreutzberg, G. W. (1998) *J. Neurosci.* **18,** 5804–5816.
9. Schori, H., Lantner, F., Shachar, I. & Schwartz, M. (2002) *J. Immunol.* **169,** 2861–2865.
10. Serpe, C. J., Sanders, V. M. & Jones, K. J. (2000) *J. Neurosci. Res.* **62,** 273–278.
11. Hammarberg, H., Lidman, O., Lundberg, C., Eltayeb, S. Y., Gielen, A. W., Muhallab, S., Svenningsson, A., Linda, H., van Der Meide, P. H., Cullheim, S., *et al.* (2000) *J. Neurosci.* **20,** 5283–5291.
12. Hauben, E., Butovsky, O., Nevo, U., Yoles, E., Moalem, G., Agranov, E., Mor, F., Leibowitz-Amit, R., Pevsner, E., Akselrod, S., *et al.* (2000) *J Neurosci* **20,** 6421–6430.
13. Moalem, G., Leibowitz-Amit, R., Yoles, E., Mor, F., Cohen, I. R. & Schwartz, M. (1999) *Nat. Med.* **5,** 49–55.
14. Fisher, J., Levkovitch-Verbin, H., Schori, H., Yoles, E., Butovsky, O., Kaye, J. F., Ben-Nun, A. & Schwartz, M. (2001) *J. Neurosci.* **21,** 136–142.
15. Kerschensteiner, M., Gallmeier, E., Behrens, L., Leal, V. V., Misgeld, T., Klinkert, W. E., Kolbeck, R., Hoppe, E., Oropeza-Wekerle, R. L., Bartke, I., *et al.* (1999) *J. Exp. Med.* **189,** 865–870.
16. Chen, M., Valenzuela, R. M. & Dhib-Jalbut, S. (2003) *J. Neurol. Sci.* **215,** 37–44.
17. Awatsuji, H., Furukawa, Y., Hirota, M., Murakami, Y., Nii, S., Furukawa, S. & Hayashi, K. (1993) *J. Neurosci. Res.* **34,** 539–545.
18. Chen, M., Gran, B., Costello, K., Johnson, K., Martin, R. & Dhib-Jalbut, S. (2001) *Mult. Scler.* **7,** 209–219.
19. Aharoni, R., Teitelbaum, D., Sela, M. & Arnon, R. (1997) *Proc. Natl. Acad. Sci. USA* **94,** 10821–10826.
20. Kipnis, J., Yoles, E., Porat, Z., Cohen, A., Mor, F., Sela, M., Cohen, I. R. & Schwartz, M. (2000) *Proc. Natl. Acad. Sci. USA* **97,** 7446–7451.
21. Schori, H., Kipnis, J., Yoles, E., WoldeMussie, E., Ruiz, G., Wheeler, L. A. & Schwartz, M. (2001) *Proc. Natl. Acad. Sci. USA* **98,** 3398–3403.
22. Aharoni, R., Teitelbaum, D., Leitner, O., Meshorer, A., Sela, M. & Arnon, R. (2000) *Proc. Natl. Acad. Sci. USA* **97,** 11472–11477.
23. Przedborski, S., Jackson-Lewis, V., Naini, A. B., Jakowec, M., Petzinger, G., Miller, R. & Akram, M. (2001) *J. Neurochem.* **76,** 1265–1274.
24. Tieu, K., Perier, C., Caspersen, C., Teismann, P., Wu, D. C., Yan, S. D., Naini, A., Vila, M., Jackson-Lewis, V., Ramasamy, R. & Przedborski, S. (2003) *J. Clin. Invest.* **112,** 892–901.
25. Liberatore, G. T., Jackson-Lewis, V., Vukosavic, S., Mandir, A. S., Vila, M., McAuliffe, W. G., Dawson, V. L., Dawson, T. M. & Przedborski, S. (1999) *Nat. Med.* **5,** 1403–1409.
26. Muthane, U., Ramsay, K. A., Jiang, H., Jackson-Lewis, V., Donaldson, D., Fernando, S., Ferreira, M. & Przedborski, S. (1994) *Exp. Neurol.* **126,** 195–204.
27. Markey, S. P., Johannessen, J. N., Chiueh, C. C., Burns, R. S. & Herkenham, M. A. (1984) *Nature* **311,** 464–467.
28. Giovanni, A., Sonsalla, P. K. & Heikkila, R. E. (1994) *J. Pharmacol. Exp. Ther.* **270,** 1008–1014.
29. Ortiz, G., Zuniga-Gonzalez, G., Garcia, J., Torres-Bugarin, O., Zamora-Perez, A. & Bitzer-Quintero, O. (2003) *Environ. Mol. Mutagen.* **41,** 64–68.
30. Jackson-Lewis, V., Jakowec, M., Burke, R. E. & Przedborski, S. (1995) *Neurodegeneration* **4,** 257–269.
31. Wu, D. C., Jackson-Lewis, V., Vila, M., Tieu, K., Teismann, P., Vadseth, C., Choi, D. K., Ischiropoulos, H. & Przedborski, S. (2002) *J. Neurosci.* **22,** 1763–1771.
32. Wu, D. C., Teismann, P., Tieu, K., Vila, M., Jackson-Lewis, V., Ischiropoulos, H. & Przedborski, S. (2003) *Proc. Natl. Acad. Sci. USA* **100,** 6145–6150.
33. Kordower, J. H., Emborg, M. E., Bloch, J., Ma, S. Y., Chu, Y., Leventhal, L., McBride, J., Chen, E. Y., Palfi, S., Roitberg, B. Z., *et al.* (2000) *Science* **290,** 767–773.
34. Gill, S. S., Patel, N. K., Hotton, G. R., O'Sullivan, K., McCarter, R., Bunnage, M., Brooks, D. J., Svendsen, C. N. & Heywood, P. (2003) *Nat. Med.* **9,** 589–595.
35. Przedborski, S. & Goldman, J. E. (2004) in *Nonneuronal Cells of the Nervous System: Function and Dysfunction. Part III. Pathological Conditions*, ed. Hertz, L. Advances in Molecular and Cell Biology, Series ed. Bittar, E. E. (Elsevier, Boston), pp. 31–111.
36. Sawada, M., Suzumura, A., Hosoya, H., Marunouchi, T. & Nagatsu, T. (1999) *J. Neurochem.* **72,** 1466–1471.
37. Nguyen, V. T. & Benveniste, E. N. (2000) *J. Immunol.* **165,** 6235–6243.
38. Paglinawan, R., Malipiero, U., Schlapbach, R., Frei, K., Reith, W. & Fontana, A. (2003) *Glia* **44,** 219–231.
39. Moalem, G., Gdalyahu, A., Shani, Y., Otten, U., Lazarovici, P., Cohen, I. R. & Schwartz, M. (2000) *J. Autoimmun.* **15,** 331–345.
40. Besser, M. & Wank, R. (1999) *J. Immunol.* **162,** 6303–6306.
41. Brodie, C., Goldreich, N., Haiman, T. & Kazimirsky, G. (1998) *J. Neuroimmunol.* **81,** 20–30.
42. Barouch, R. & Schwartz, M. (2002) *FASEB J* **16,** 1304–1306.