NEUROSCIENCE

Therapeutics potentiating microglial p21-Nrf2 axis can rescue neurodegeneration caused by neuroinflammation

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Neurodegenerative disorders are caused by progressive neuronal loss, and there is no complete treatment available yet. Neuroinflammation is a common feature across neurodegenerative disorders and implicated in the progression of neurodegeneration. Dysregulated activation of microglia causes neuroinflammation and has been highlighted as a treatment target in therapeutic strategies. Here, we identified novel therapeutic candidate ALGERNON2 (altered generation of neurons 2) and demonstrate that ALGERNON2 suppressed the production of proinflammatory cytokines and rescued neurodegeneration in a 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)–induced Parkinson's disease model. ALGERNON2 stabilized cyclinD1/p21 complex, leading to up-regulation of nuclear factor erythroid 2–related factor 2 (Nrf2), which contributes to antioxidative and anti-inflammatory responses. Notably, ALGERNON2 enhanced neuronal survival in other neuroinflammatory conditions such as the transplantation of induced pluripotent stem cell–derived dopaminergic neurons into murine brains. In conclusion, we present that the microglial potentiation of the p21-Nrf2 pathway can contribute to neuronal survival and provide novel therapeutic potential for neuroinflammation-triggered neurodegeneration.

INTRODUCTION

Neuroinflammation is a hallmark in the progression of neurodegenerative conditions such as Alzheimer's disease (AD) and Parkinson's disease (PD) (*1*, *2*). The brains of patients with these conditions are characterized by elevated levels of proinflammatory cytokines and marked activation of microglia (*3*), the resident macrophages of the central nervous system (CNS). Microglia contribute to brain immune homeostasis by surveying brain tissue for pathogens, removing debris, and providing neurotrophic factors, while overactivated microglia also release harmful reactive oxygen species (ROS) and proinflammatory cytokines (*4*). Many lines of evidence indicate that dysregulation of microglial functions leads to the pathogenesis of neurodegenerative diseases, including AD and PD (*5*). Given the role of microglia in neuroinflammation, concerted efforts have been made to investigate the potential to target and modify dysregulated microglial functions for therapeutic purposes.

Nuclear factor erythroid 2–related factor 2 (Nrf2) is a transcription factor that induces the expression of genes involved in antioxidant pathways, which reduce ROS levels (*6*). Under normal conditions, Nrf2 expression is maintained at low levels by Kelch-like ECH-associated protein 1 (Keap1), which binds to Nrf2 and mediates ubiquitin-proteasome degradation (*7*). Upon exposure to oxidative stress or agents that modulate cysteine residues in Keap1, Nrf2 is released from continual degradation by dissociating from Keap1 and translocates into the nucleus where it activates downstream gene expression (*8*). In addition to activating antioxidant response genes, Nrf2 contributes to anti-inflammatory pathways (*9*–*12*), suppressing inflammatory responses by binding in proximity to the promoters of genes that encode proinflammatory cytokines in macrophages (*13*). Extensive research has focused on identifying the agents/factors that regulate the association between Nrf2 and Keap1 (*14*). Recently, it has been reported that p21, an inhibitor of cyclin-dependent kinase (CDK), interacts with Nrf2 through the DLG motif, which mediates weak binding to form the Nrf2-Keap1 complex, resulting in the inhibition of Nrf2 degradation (*15*). Thus, p21 plays a physiological role in regulating Nrf2 expression (*16*).

Harmine, a potent inhibitor of dual-specificity tyrosine phosphorylation–regulated kinase 1A (Dyrk1A) from natural plants, is known to exhibit anti-inflammatory activity (*17*–*19*); however, its hallucinogenic properties due to inhibition of monoamine oxidase A (MAO-A) prevent its clinical application (*20*). With the aim of regulating neuroinflammation using chemical compounds that specifically target Dyrk1A, we investigated a novel Dyrk1A inhibitor, ALGERNON2 (altered generation of neurons 2), with better availability in brain tissue, which can be used to ameliorate neurodegeneration caused by neuroinflammation. In this study, we showed that targeting Dyrk1A can potentiate the neuroprotective p21-Nrf2 pathway and contribute to neuronal survival by suppressing proinflammatory cytokine production caused by neuroinflammation.

RESULTS

ALGERNON2 rescues neurodegeneration following MPTP administration

Our previous study showed that the Dyrk1A inhibitor ALGERNON normalizes the impaired neurogenesis observed in a Down syndrome model by enhancing the proliferation of neural stem cells (NSCs) (*21*). To explore potential therapies for neuroinflammation-related neurodegenerative disorders, we used data from our previous screening

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to search for a potent Dyrk1A inhibitor with pronounced brain tissue distribution and retention in vivo (fig. S1A). The selected compound displayed comparable levels of brain distribution within the first 30 min to that of ALGERNON, but greater retention was observed in brain tissue at 4 hours after oral administration (fig. S1B) compared to ALGERNON when administered through subcutaneous injection. This previously unidentified compound exhibited inhibitory activity on the Dyrk/Clk kinase family (fig. S1C), with a half-maximal inhibitory concentration (IC_{50}) of 32.9 nM against Dyrk1A (fig. S1A), which was slightly better than ALGERNON (IC_{50} = 76.9 nM). The previously unidentified compound induced the stabilization of cyclin D1 protein (fig. S1D) and enhanced the proliferation in NSCs in vitro (fig. S1E). The previously unidentified compound was orally bioavailable, which was confirmed by the in vivo dose-dependent up-regulation of NSC proliferation in the dentate gyrus of the hippocampus (fig. S1F), and the up-regulated expression of doublecortin, a marker of immature neurons, indicating an increase in newborn NSCs differentiated into neurons (fig. S1G). We named this previously unidentified compound ALGERNON2 on the basis of its similar neurogenic activity to that of the pioneer neurogenesis inducer ALGERNON (*21*). Notably, ALGERNON2 lacked the inhibitory activity of MAO-A and did not induce obvious behavioral alterations or changes in body weight after 4 weeks of administration (fig. S2).

To examine the potential application of ALGERNON2 in neurodegenerative disorders, we used the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) injection model where dopaminergic neurodegeneration occurs by the 1-methyl-4-phenyl-pyridium ion (MPP^+) generated from blood-brain barrier–penetrable MPTP. Four injections of MPTP (20 mg/ml; intraperitoneal) at 2-hour intervals [day 0 (D0)] were administered, followed by the commencement of treatment the subsequent day (D1) for 11 days (Fig. $1A_1$). After drug administration, we assessed the dopaminergic degeneration by analyzing the expression level of tyrosine hydroxylase (TH) and dopamine (DA) transporter (DAT) as dopaminergic neuronal markers in the striatal tissue (Fig. $1A_2$). Treatment with ALGERNON2 substantially rescued MPTP-induced neurodegeneration (Fig. $1A_3$), suggesting therapeutic potential by either neuronal supplementation or suppression of neuronal loss.

To clarify when ALGERNON2 rescues neurodegeneration, we assessed the survival of DA neurons at D3, during the acute phase of injury when oxidative stress was most robust and neurogenesis was expected to be impossible to accomplish (Fig. $1B_1$). We compared the preadministration and pre- and postadministration of ALGERNON2 (Fig. $1B_1$). Mice received ALGERNON2 1 hour before MPTP injection and were left 3 days without any further treatment (before) or treated daily for the following 3 days (before and after). We found that dopaminergic neuronal loss was rescued in both conditions (Fig. $1B_2$ and B_3), indicating that only a single pretreatment was sufficient to induce neuronal survival upon MPTP-induced oxidative stress. These results also suggested that ALGERNON2 exerted neuroprotective effects rather than enhanced neurogenesis, which requires several weeks to occur. To further confirm this hypothesis, we administered ALGERNON2 once and left animals untreated for the following 3 days (Fig. $1C_1$). We analyzed TH-positive neurons in the substantia nigra (SN) and striatal tissues (Cpu) (Fig. $1C_2$). Dopaminergic neurodegeneration was rescued by ALGERNON2 in this condition (Fig. 1D). We measured the amount of DA in striatal tissues and observed a recovery of DA quantity upon drug administration (Fig. 1E). These data indicated that a single pregavage treatment could protect dopaminergic neurons from oxidative stress. We also examined, using pole test, whether ALGERNON2 administration improves behavioral deficit. MPTP-treated mice required a longer period to make a turn on the pole (T-turn), while ALGERNON2-treated mice displayed an improved performance, indicating that ALGERNON2 ameliorated the impaired locomotion caused by MPTP injection by reducing dopaminergic neuronal loss (Fig. 1F).

ALGERNONs mediate neuroprotection in the presence of glial cells

To identify the neuroprotective mechanism of ALGERNON2, we isolated hippocampal neurons from murine brains and tested whether the compound could suppress neuronal death triggered by oxidative stress in vitro. In contrast to the results of the MPTP model in vivo, ALGERNON2 failed to rescue neurodegeneration upon MPP^+ treatment in vitro (fig. S3A). This result suggested that the neuroprotective effects of ALGERNON2 in an MPTP injection model required the presence of glial cells. To test this hypothesis, we evaluated the survival of primary dopaminergic neurons in the presence or absence of glial cells in vitro. We either depleted the proliferative glial cells by treatment with cytosine arabinoside (araC) or allowed glial survival and proliferation by addition of fetal bovine serum in primary cultures (Fig. 2A). We measured the survival of neurons following MPP^+ treatment and observed that ALGERNON treatment rescued MPP^+-induced DA neuronal loss in mixed culture conditions (no araC in Fig. 2B). In contrast, no beneficial effect of drug administration on neuronal survival was observed in neuronal cultures treated with araC, indicating that ALGERNONs only protected neurons in conditions containing glia. To confirm the glial contribution to neuronal survival with drug treatment, we established a coculture system containing DA neurons differentiated from human induced pluripotent stem cells (iPSCs) and glial cultures isolated from the murine brain (Fig. 2C and fig. S3B). The glial cultures contained glial fibrillary acidic protein (GFAP)–positive and ionized calcium-binding adapter molecule 1 (Iba1)–positive cells, but not TH-positive cells (fig. S3B). Oxidative stress was induced by H_2O_2 treatment, and neuronal survival was assessed with immunostaining based on the number of human nuclei (hNuclei) (Fig. 2, C and D) and Nurr1 and TH dopaminergic neuronal markers by quantitative polymerase chain reaction (qPCR; Fig. 2D). ALGERNON2 treatment enhanced the survival of DA neurons in the cocultured conditions, implying that ALGERNONs exert their neuroprotective effects in a glial cell–dependent manner.

Nrf2 and p21 enhancement by ALGERNONs

Increased cyclin D1 levels induce costabilization of p21 by forming the cyclin D1/p21 complex, which escapes the proteasomal degradation pathway (*22*). We previously reported that ALGERNON enhanced the proliferation of NSCs by stabilizing cyclin D1 (*21*). Therefore, we first examined the possibility that these cell cycle regulators are affected by ALGERNON treatment. We verified the expression of cyclin D1 and p21 in mixed cultures of astrocytes and microglia (fig. S4A). Strong p21 and cyclin D1 signals were observed in all three cell types: Cd11b-positive, GFAP-positive, or GFAP/Cd11b-negative cells (fig. S4A). In particular, higher expression of p21 was observed in Cd11b-positive microglial cells than in GFAP-positive astrocytes (Fig. 3A, arrowheads). We tested the effect of ALGERNONs on the expression of these molecules.

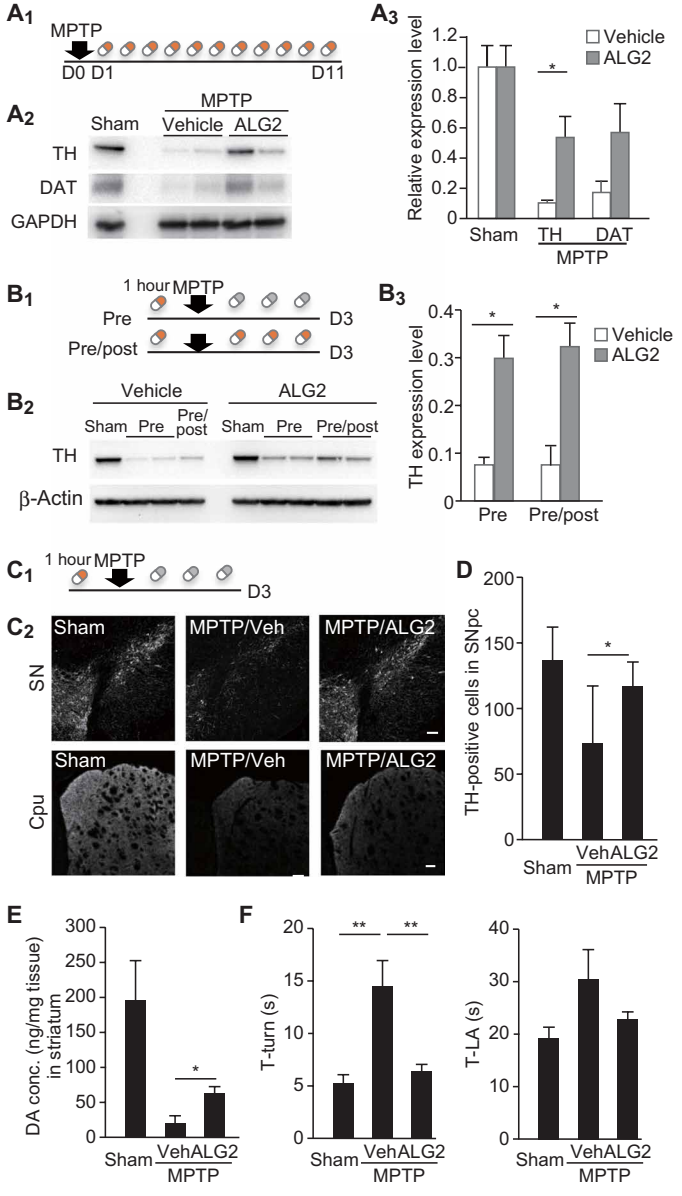

Fig. 1. ALGERNON2 rescues DA neuronal loss upon MPTP injection. (**A$_1$**) Experimental scheme. (**A$_2$**) Western blotting of the striatal tissue of animals treated as indicated. TH, tyrosine hydroxylase; DAT, dopamine transporter; GAPDH, glyceraldehyde-3-phosphate dehydrogenase. (**A$_3$**) Quantification of Western blotting analysis. *$P < 0.05$. (**B$_1$**) Experimental scheme. (**B$_2$**) Western blotting analyses from the striatal tissue of animals with indicated treatment. (**B$_3$**) Quantification of Western blotting analysis. The expression level of TH was normalized to that of sham control. *$P < 0.05$. (**C$_1$**) Experimental scheme. (**C$_2$**) Representative images of the substantia nigra (SN) and striatum (Cpu) of sham control or MPTP-treated animals administered with vehicle (Veh) or ALGERNON2. Tissue was stained with anti-TH antibody. Scale bars, 200 μm (SN) and 100 μm (Cpu). (**D**) Quantification of the number of TH-positive cells in SN. *$P < 0.05$. $n = 3$ each. (**E**) Measurement of DA amount in striatal tissues of animals with indicated treatment. *$P < 0.05$. $n = 6$ each. (**F**) Average time taken to make a turn (T-turn) and of locomotor activity (T-LA) of animals with indicated treatment during a pole test. **$P < 0.01$. $n = 6$, 7, and 6 for each group.

Microglial BV-2 cells were treated with ALGERNON, and we subsequently assessed protein levels by Western blotting. Cyclin D1 expression gradually increased following ALGERNON treatment in a dose- and time-dependent manner, as we reported previously (fig. S4B) (*21*). Notably, we observed co–up-regulation of p21 alongside cyclin D1 stabilization. To better understand the corelationship between p21 and cyclin D1, we visualized and measured the respective levels of the two proteins in each cell of primary microglial cultures using an automated image scanning and analysis system (Fig. 3B). Scatter plots from each single-cell analysis revealed a correlation between the expression of p21 and cyclin D1 as the population shifted to the top-right quadrant upon ALGERNON incubation (fig. S4C). The average intensity of nuclear cyclin D1 gradually increased after ALGERNON treatment, which was followed by an increase in that of p21 in Cd11b-positive cells (fig. S4, D and E).

p21 was originally described as a universal inhibitor of CDKs, but it has also been reported to bind to Nrf2 and suppress Nrf2 degradation (*15*). On the basis of previous reports indicating that the up-regulation of p21 potentiates the Nrf2 pathway (*16*), we assessed whether ALGERNONs could affect the expression level of Nrf2. We found that Nrf2 was up-regulated following ALGERNON treatment in BV-2 microglial cells (fig. S4B). We next examined whether the expression of Nrf2 could be induced in Cd11b-positive microglial cells by ALGERNON treatment. We observed a gradual increase in the intensity of nuclear Nrf2 signal with ALGERNON treatment in Cd11b-positive cells (Fig. 3C and fig. S4F). Nrf2 up-regulation peaked at 6 hours and returned to baseline after overnight treatment, which is consistent with the observation of transient up-regulation of cyclin D1 and p21 following ALGERNON treatment, which decreased after 6 hours of treatment (fig. S4D).

To examine whether p21 influences Nrf2 up-regulation, we transfected a destabilizing domain–fused p21 into microglial BV-2 cells and rapidly induced its expression by adding Shield-1 (*23*). Shield-1 induced p21 expression, which in turn up-regulated Nrf2 expression. The up-regulation of Nrf2 observed was further enhanced in the presence of ALGERNON (fig. S4G). We also confirmed that p21 interacted with Nrf2 in the microglial BV-2 cell line (fig. S4H). To further clarify whether the up-regulation of Nrf2 following ALGERNON treatment was mediated via cyclin D1/p21 complex stabilization, we monitored Nrf2 expression in the presence or absence of p21. When microglial cells were stimulated by exposure to lipopolysaccharide (LPS), Nrf2 expression was up-regulated as indicated by the right-shifted histogram (shown in orange in Fig. 3D$_1$). Under this condition, further Nrf2 enhancement was achieved following ALGERNON2 treatment (yellow histogram in Fig. 3D$_1$, left). However, this additional induction of Nrf2 by ALGERNON2 treatment was not observed in the absence of p21 (Fig. 3D$_1$, right histogram), indicating that the induction of Nrf2 by ALGERNON treatment required p21. Furthermore, the dot plot showing the nuclear Nrf2 level quantified from each cell revealed that Nrf2 expression was increased following ALGERNON2 treatment in cells treated with control small interfering RNA (siRNA), but not in cells treated with *p21* siRNA (Fig. 3D$_2$). Thus, we concluded that ALGERNON treatment mediated Nrf2 up-regulation by stabilizing the cyclin D1/p21 complex.

We also examined the expression level of Nrf2 in striatal tissue from animals injected with MPTP. We observed an elevated expression of Nrf2 following ALGERNON treatment (Fig. 3E). Higher expression of NAD(P)H dehydrogenase-1 (NQO-1) and heme oxygenase-1

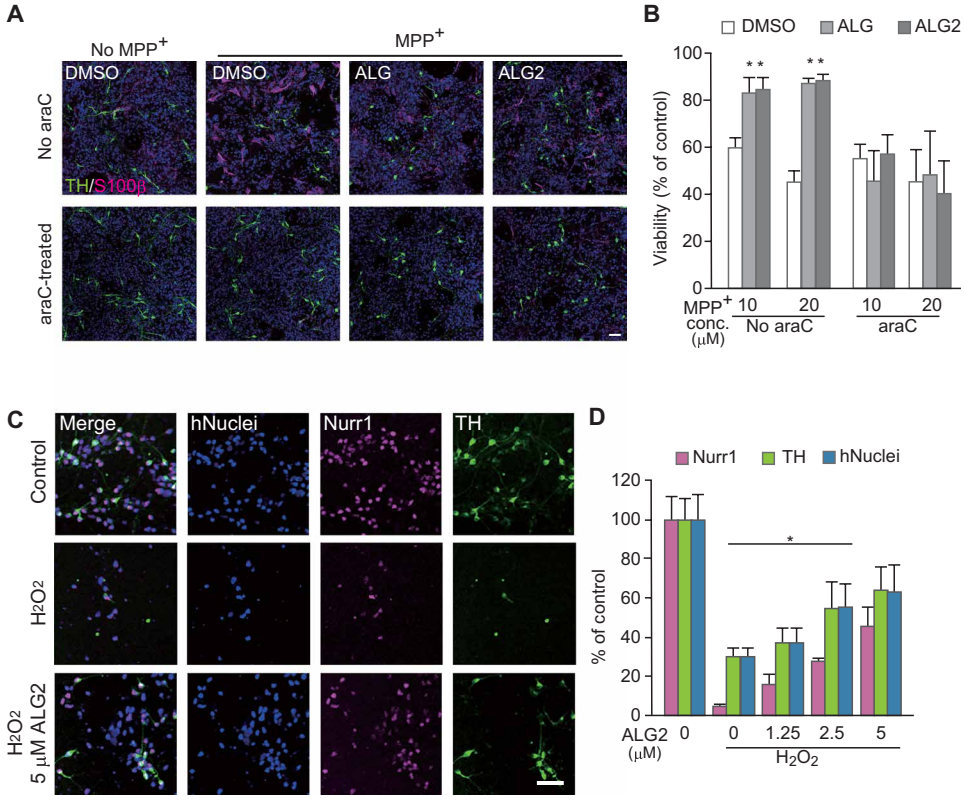

Fig. 2. ALGERNONs mediate neuroprotection in the presence of glial cells. (**A**) Representative images of ventral midbrain–derived neurons at 7 days in vitro. DA neurons and glial cells were visualized with TH (green) and S100β (magenta), respectively. Nuclei were stained with Hoechst 33342 (blue). Scale bar, 50 μm. (**B**) Viability was assessed by the number of TH-positive neurons and normalized to that of control conditions at 0 μM MPP^+. Treatment with ALGERNONs improved neuronal survival upon oxidative stress in the presence of glial cells. *$P < 0.05$. DMSO, dimethyl sulfoxide. (**C**) Representative images of human induced pluripotent stem cell (iPSC)–derived DA progenitors cultured with murine glial cells. Human iPSC-derived DA neurons were visualized with anti–human nuclei (hNuclei; blue), Nurr1 (magenta), and TH (green) antibodies. Scale bar, 50 μm. (**D**) Quantitative analyses of the number of hNuclei, and the level of Nurr1 and TH mRNA. Nurr1 and TH were used as markers of DA neurons in early differentiation and maturation, respectively. ALGERNON2 rescued neuronal loss in the presence of glial cells. Data were normalized to the control condition without H_2O_2 treatment. *$P < 0.05$.

(HO-1), downstream transcripts of Nrf2, was also observed in ALGERNON-treated striatal tissue (Fig. 3E). This led to the hypothesis that ALGERNON administration up-regulates Nrf2 expression under oxidative conditions in vivo.

ALGERNONs up-regulate Nrf2 protein but do not induce ARE-driven down stream transcripts in the basal condition

To examine whether ALGERNONs induced antioxidative responses and promoted neuronal survival, we examined the transcriptional activity of Nrf2 using a luciferase reporter expressed under the antioxidant responsive element (ARE) (ARE-*luc2P*). ALGERNON up-regulated the expression of the ARE-driven luciferase; however, the degree of induction was smaller than that with the Nrf2 stabilizer sulforaphane (SLF) treatment or by cotransfection with an Nrf2-expressing plasmid (fig. S5A).

We next compared ALGERNON with Nrf2 inducers, diethyl maleate (DEM) and 15-deoxy-Δ12,14-prostaglandin J2 (Δ15). ALGERNON treatment up-regulated Nrf2 in a dose-dependent manner; however, no HO-1 induction was observed (fig. S5B). In contrast, both DEM and Δ15, direct modulators of cysteine residues in Keap1, independently showed robust induction of HO-1 despite no obvious Nrf2 up-regulation (fig. S5B). These results suggest that ALGERNON treatment halts Nrf2 degradation but does not induce nuclear translocation of Nrf2 to exert its transcriptional functions. Cotreatment with ALGERNON and SLF showed additive effects on the induction of Nrf2 downstream transcripts, NQO-1, UDP-glucuronosyltransferase 1-6 (UGT1a6), and catalase (Cat) (fig. S5C), indicating that ALGERNON potentiates Nrf2 in the presence of Nrf2 inducers. Note that ALGERNON treatment alone did not induce any of these transcripts in the basal condition (fig. S5C). To further examine whether ALGERNONs were involved in the Nrf2 signaling pathway, we performed a chromatin immunoprecipitation (ChIP) assay. Astrocytes were treated with ALGERNON, SLF, or both for 3 hours and subjected to a ChIP assay using an anti-Nrf2 antibody. The treatment with ALGERNON barely increased the binding of Nrf2 to the NQO-1 promoter region when compared to SLF (fig. S5D). In accordance with the results shown in fig. S5C, cotreatment with ALGERNON and SLF additively increased the binding of Nrf2 to the NQO-1 promoter, indicating that while ALGERNON itself did not up-regulate the transcriptional activity of Nrf2, it enhanced the effect of the Nrf2 inducer SLF. We assessed the expression of Nrf2 in cytosolic and nuclear fractions extracted from astrocytes after 3 hours of treatment with ALGERNON. We observed that a robust nuclear translocation of Nrf2 was induced by SLF, but not by ALGERNON (fig. S5E). The induction of HO-1 was

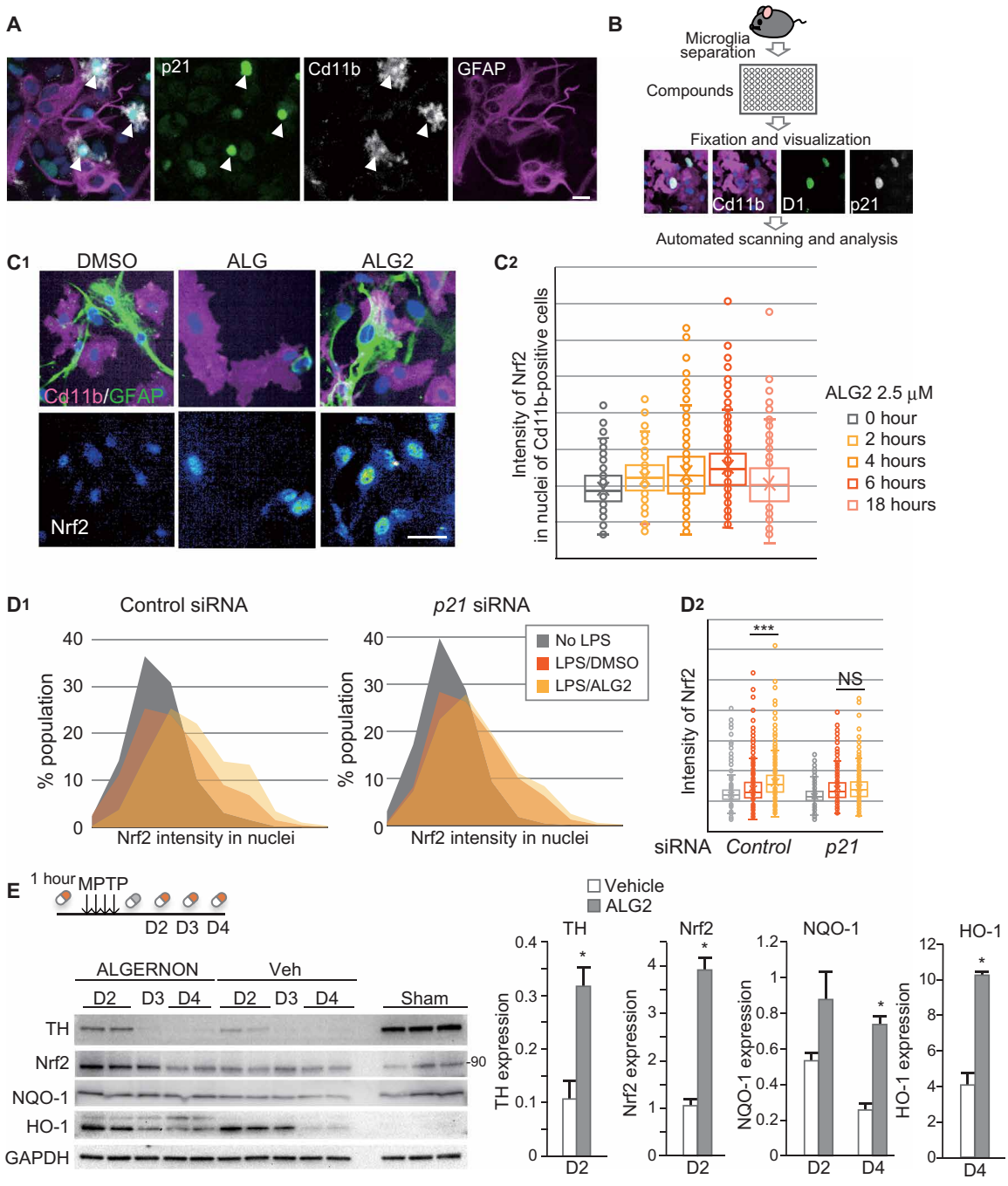

Fig. 3. Nrf2 and p21 enhancement by ALGERNONs. (**A**) Representative images of glial cultures isolated from the murine brain. Scale bar, 10 μm. (**B**). Experimental scheme for the automated evaluation of the expression of cyclin D1 and p21. The image acquisition and analyses were performed in an automated manner. (**C_1**) Representative images of glial cells treated with indicated compounds. Scale bar, 25 μm. (**C_2**) An example of quantification of the signal intensity of Nrf2 in nuclei of Cd11b-positive cells. Glial cells were treated with ALGERNON2 (2.5 μM) for indicated periods. The full set of experimental data is available in fig. S3F. (**D_1**) Representative examples of the histogram of Nrf2 intensity in nuclei of Cd11b-positive cells treated as indicated. ALGERNON2 treatment shifted the histogram rightward; this shift was abolished in the presence of *p21* small interfering RNA (siRNA). (**D_2**) Dot plots from the same experiments shown in (D_1). ***$P < 0.001$. Note that the increase in nuclear Nrf2 signal upon lipopolysaccharide (LPS) and LPS/ALGERNON2 treatment was not observed in cells treated with *p21* siRNA compared with those that were treated with control siRNA. (**E**) Experimental scheme (top) and Western blotting of striatal tissue from animals treated as indicated (bottom). Quantitative analyses of Western blotting (right). *$P < 0.05$.

accompanied by nuclear translocation of Nrf2 upon SLF treatment, but not following ALGERNON exposure, indicating that ALGERNON could not potentiate Nrf2 nuclear translocation in the basal condition. Moreover, ALGERNON and SLF cotreatment increased the levels of nuclear Nrf2 and induced HO-1 (fig. S5E). These results indicated that ALGERNONs could potentiate Nrf2 expression but were insufficient to activate Nrf2 transcriptional activity to mediate the antioxidant pathway in the basal condition.

Nrf2 is controlled by ubiquitination, and Keap1 is primarily a ubiquitin ligase adaptor and substrate receptor to regulate Nrf2 degradation by sensing redox conditions. Since p21 inhibits Nrf2 degradation by disrupting the weak binding motif between Keap1 and Nrf2, the effect of p21 on Nrf2 stabilization could be affected in the absence of Keap1. To elucidate the relationship between p21-mediated Nrf2 regulation and Keap1 functions, we simultaneously knocked down Keap1 and p21 and quantified the Nrf2 nuclear level (fig. S6A). We found that p21 knockdown had no effects on the basal level of nuclear Nrf2, while a treatment with SLF or DEM increased the nuclear level of Nrf2 in the p21-depleted condition. Conversely, Keap1 knockdown up-regulated the basal nuclear Nrf2 level and abolished the effect of SLF/DEM to further stabilize Nrf2. The double knockdown experiment showed similar results as that of Keap1 knockdown alone (fig. S6A). This indicates that Keap1 is a primary influencer in Nrf2 expression regulation.

ALGERNONs target Dyrk1A kinase, which is a priming kinase for subsequent phosphorylation of targets by glycogen synthase kinase (GSK3) (*24*). GSK3 phosphorylates Nrf2, creating a phosphodegron within its Neh6 domain that targets Nrf2 for β-TrCP/Cul1–mediated ubiquitination and proteasomal degradation (*25*). It is thus possible that the inhibition of Dyrk1A by ALGERNONs prevents priming and, consequently, phosphorylation by GSK3, leading to Nrf2 stabilization. To examine whether Dyrk1A is involved in the priming of phosphorylation events on Nrf2 and contributes to the down-regulation of Nrf2 at the protein level, we performed an in vitro kinase assay on Nrf2 recombinant protein and three Dyrk1A-target motives that exist within Nrf2 (S408, S433, and S577). We did not observe any effect of Dyrk1A on Nrf2 phosphorylation (fig. S7). We thus eliminated the possibility that Dyrk1A phosphorylates to regulate the level of Nrf2 protein.

ALGERNONs suppress the cytokine production upon LPS stimulation through Nrf2 stabilization

How did Nrf2 up-regulation by ALGERNONs contribute to neuronal survival? We next focused our attention on microglia, as we observed clear p21 expression in microglial cells rather than in astrocytes (Fig. 3A). We examined the glial activation in a MPTP model at 3 days following MPTP injection. In the SN of animals treated with MPTP, some microglia were stained with strong Iba1 signal surrounding TH-positive neurons (Fig. 4A). These microglia presented distinct morphology, which indicates activation (Fig. 4A) (*26*). Note that these amoeboid-shaped microglia were not observed in mice treated with ALGERNON2, making microglia candidate targets of ALGERNONs.

It has been reported that Nrf2 suppresses cytokine production by binding in proximity to the promoters of genes encoding cytokines in macrophages (*13*). We examined whether our compounds could suppress microglial cytokine production. LPS stimulation drastically induced proinflammatory cytokine gene expression. Notably, this up-regulation was effectively suppressed by treatment with ALGERNONs (Fig. 4, B and C). In addition, the induction of several chemokines and the expression of inducible nitric oxide synthase (*iNOS*) mRNA were also suppressed (Fig. 4D). To determine whether the effect of the drugs was mediated through Nrf2, we depleted Nrf2 with targeted siRNA and tested whether drug treatment could still suppress cytokine production. In the control siRNA condition, ALGERNON suppressed proinflammatory cytokine production upon LPS stimulation, but not in conditions without Nrf2 (Fig. 4E). This indicated that the suppression of cytokine production by ALGERNONs was mediated through Nrf2 stabilization. We also examined whether the suppression of cytokine production by ALGERNON was enhanced through Nrf2 up-regulation by cotreatment experiment. The single treatment of ALGERNON or SLF suppressed the expression of cytokine and chemokine genes (Fig. 4F), while their cotreatment further suppressed the production of these genes (Fig. 4F), proving that Nrf2 was involved in the effects of ALGERNONs observed to suppress the production of proinflammatory cytokine genes. These ALGERNON effects were abolished in the p21-depleted condition (fig. S6B), proving that ALGERNONs suppressed cytokine production in conditions where Nrf2 and p21 were both present.

Next, to evaluate cytokine production in the MPTP-injected model, we collected striatal tissues at 24 hours after MPTP injections, a time point where more robust glial activation and cytokine production are typical (Fig. 4G, scheme in the upper panel). We observed that the expression of *CD68* mRNA, which encodes a lysosomal protein highly expressed in activated microglia but minimally expressed in resting microglia, was up-regulated upon MPTP injection and that it was suppressed by ALGERNON treatment (Fig. 4G, left graph). ALGERNON treatment also suppressed cytokine production induced by MPTP (Fig. 4G, right graph).

ALGERNON2 rescues neurodegeneration caused by neuroinflammation

To examine whether ALGERNONs could suppress neuroinflammation in other deleterious conditions where glial activation is critical, we used a systemic inflammation model induced by LPS injection, which results in DA neuronal loss caused by robust brain inflammation (*27*, *28*). After repeated LPS injections (Fig. 5A), robust glial activation was observed on D1, followed by a gradual decrease on D7 (Fig. 5, B and C). On D7, a loss of DA neurons was observed in the SN (Fig. 5C). Using this model, we examined whether ALGERNONs could rescue neurodegeneration caused by neuroinflammation. We administered ALGERNON2 1 hour before LPS injection. We evaluated glial activation and cytokine production on D1 and dopaminergic neurodegeneration on D7 and D14 (Fig. 5, D and E). ALGERNON2 administration suppressed both glial activation (Fig. 5F) and cytokine production (Fig. 5, G and H). Notably, dopaminergic neuronal loss was rescued following ALGERNON2 administration (Fig. 5E). These results suggested that ALGERNONs suppressed neuroinflammation not only caused by MPTP injection in the CNS but also induced peripherally by LPS injection.

ALGERNON2 enhances the efficacy of iPSC-DA neuron transplantation

Last, we tested the effect of ALGERNONs on the survival of transplanted iPSC-derived DA neurons. We produced DA neurons from human iPSCs and transplanted them into the murine brains to determine the efficacy of the integration of iPSC-derived DA neurons in the presence of ALGERNON2. ALGERNON2 was administered 1 hour before transplantation and for the four subsequent days when robust glial activation was expected (experimental scheme shown in Fig. 6A). The transplanted DA neurons were identified by hNuclei staining, and surviving DA-iPSCs were quantified by costaining with the dopaminergic markers TH and Nurr1 (Fig. 6B). We observed that treatment with ALGERNON2 improved the survival rate of iPSC-DA cells 4 weeks after transplantation (Fig. 6C).

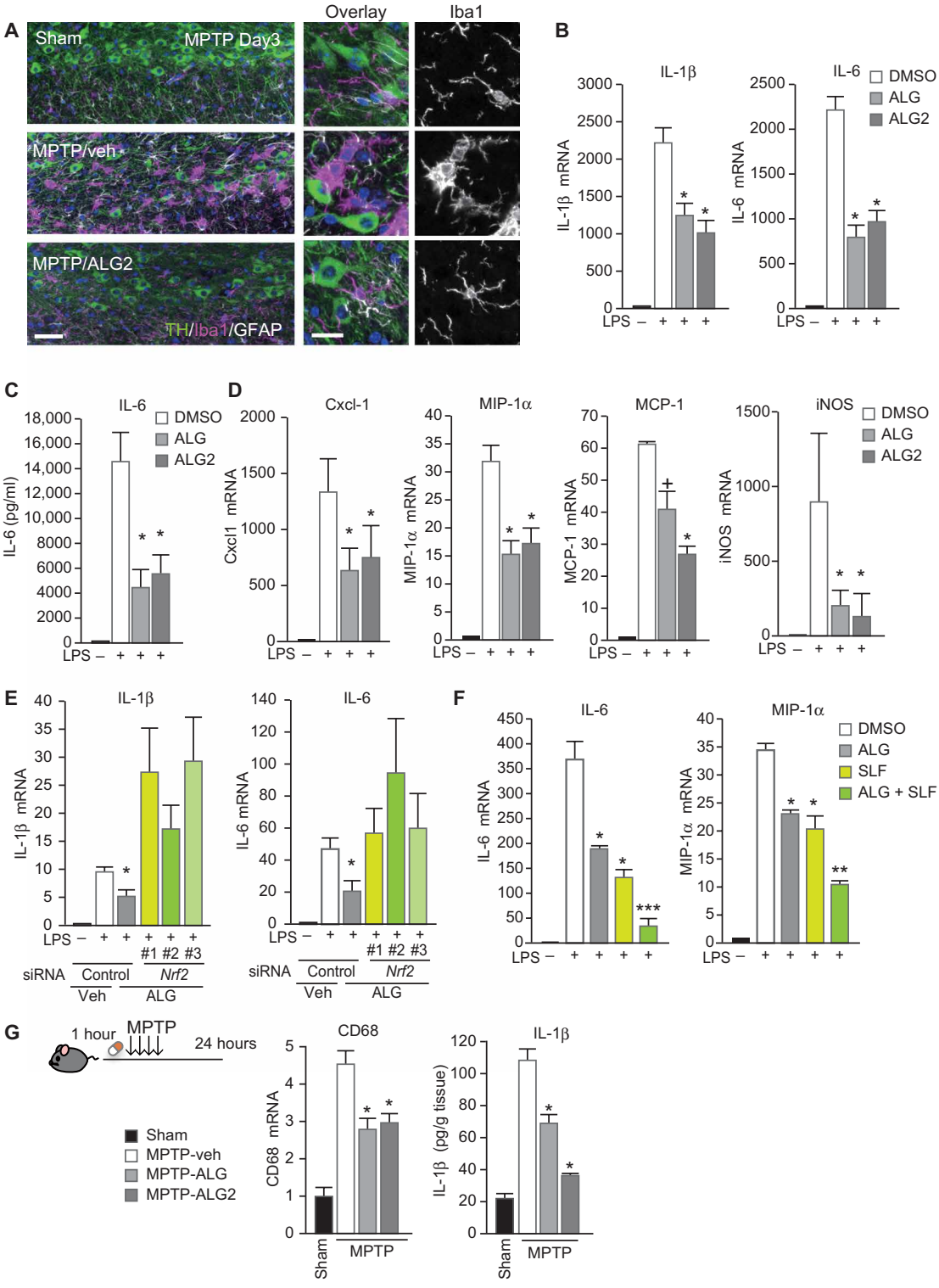

Fig. 4. ALGERNONs suppress cytokine production following LPS stimulation through Nrf2 stabilization in microglia. (**A**) Representative images of SN tissues of sham control or MPTP-treated animals administered with vehicle or ALGERNON2 at day 3. Tissues were stained with anti-TH (green), Iba1 (magenta), and GFAP (gray scale) antibodies. Scale bar, 50 μm (left panels). Amoeboid-shaped activated microglia were observed in MPTP-treated animals with vehicle administration. Scale bar, 20 μm (right panels). (**B** and **D**) The production of cytokines (B), chemokines, and *iNOS* mRNA (D) upon LPS treatment was assessed by qPCR. The values were normalized to those of nontreated conditions. *$P < 0.05$. (**C**) Cytokine production upon LPS stimulation was quantified with enzyme-linked immunosorbent assay (ELISA). *$P < 0.05$. IL, interleukin. (**E** and **F**) Real-time qPCR analyses of the production of indicated genes in the presence or absence of Nrf2 (E) or upon treatment of ALG, SLF, or combined (F). *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$. (**G**) Experimental scheme (top). Animals were administered with ALGERNONs 1 hour before MPTP injection. Tissues were collected 24 hours after the last MPTP injection. Quantitative analyses of striatal tissue by qPCR (left) and ELISA (right) (lower graphs). *$P < 0.05$. $n = 6$ each.

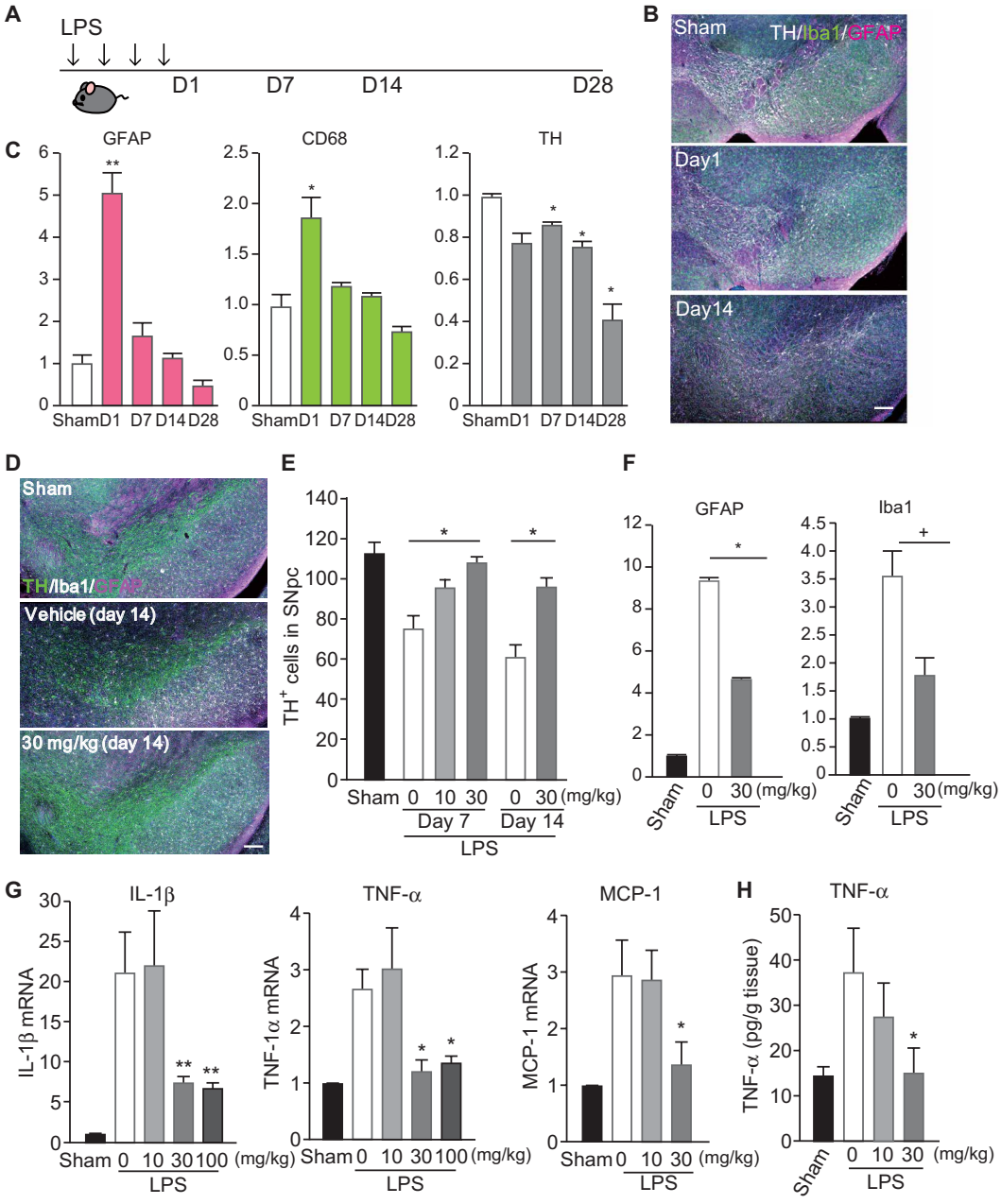

Fig. 5. ALGERNON2 rescues the neurodegeneration caused by neuroinflammation. (**A**) Experimental scheme. LPS was intraperitoneally injected at 1 mg/kg for four consecutive days. Brains were collected for analyses on indicated days. (**B**) Representative images of the SN on indicated days. Scale bar, 200 μm. (**C**) Quantitative analysis of mRNA expression in the striatum. $n = 10$ each. $^{*}P < 0.05$ and $^{**}P < 0.01$. (**D**) Representative images of the SN from animals treated as indicated. TH (green), Iba1 (gray scale), and GFAP (magenta) were used as markers of DA neurons, microglia, and astrocytes, respectively. Scale bar, 200 μm. (**E**) Quantification of the number of TH-positive cells in the SN pars compacta (SNpc). $n = 5$ to 6 animals analyzed for each condition. Error bars represent SEM. $^{*}P < 0.05$. (**F**) Quantitative analyses of glial activation by qPCR in striatal tissues at D1 from last LPS injection. Error bars represent SEM. $^{*}P < 0.05$. $+P = 0.11$. (**G** and **H**) The levels of indicated cytokines and chemokines from striatal tissues on D1 were analyzed by qPCR (G) and ELISA (H). Error bars represent SEM. $^{*}P < 0.05$. TNF-α, tumor necrosis factor–α.

DISCUSSION

In the current study, we identified the neuroprotective role of ALGERNONs, chemical Dyrk1A inhibitors, in neuroinflammatory conditions. By inhibiting Dyrk1A activity, ALGERNONs costabilized the p21/cyclin D1 complex and anti-inflammatory transcription factor Nrf2, leading to suppression of proinflammatory cytokine gene expression in microglia (Fig. 6D). ALGERNONs rescued neurodegeneration not only triggered in the CNS but also induced by peripherally activated inflammation, indicating their potential for broader applications.

We revealed that targeting Dyrk1A can regulate gene expression of proinflammatory genes in microglia (Fig. 4) via the Nrf2 pathway.

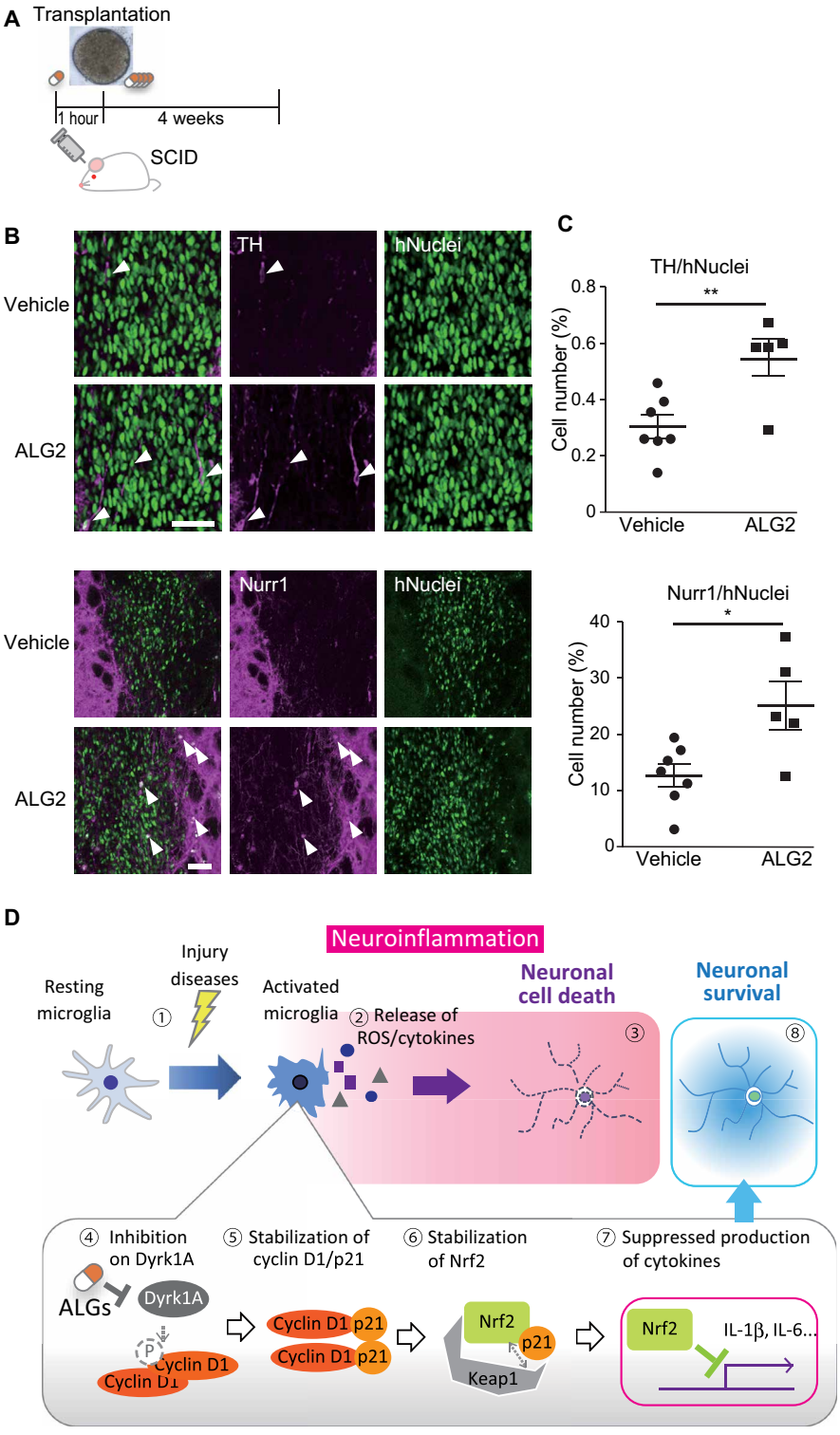

Fig. 6. ALGERNON2 enhances the efficacy of transplantation of iPSC-DA neurons. (**A**) Experimental scheme. Recipients were administered ALGERNON2 1 hour before transplantation of human iPSC-derived DA neurons. Drug administration was performed for four consecutive days after the operation. Animals were left untreated for 4 weeks. (**B**) Representative images of transplanted cells in striatal tissues. Arrowheads indicate iPSC-derived cells colabeled with TH or Nurr1. Scale bar, 50 μm. (**C**) Quantitative analyses of transplanted cells. The number of hNuclei/TH or Nurr1 double-positive cells was normalized to the number of hNuclei-positive cells. $n = 7$ and 5 for each condition, respectively. $^{*}P < 0.05$ and $^{**}P < 0.01$. (**D**) Graphic abstract: Upon injury or exposure to neuroinflammatory pathogens (1), microglia are activated and release cytokines and ROS (2), which triggers neuronal degeneration (3) ("neuroinflammation"). Following treatment with Dyrk1A inhibitors (4), the cyclin D1/p21 complex becomes stabilized (5). Stabilized p21 halts the degradation of Nrf2 by interrupting Nrf2-Keap1 binding (6). Stabilized Nrf2 suppresses the production of proinflammatory cytokine genes (7), which contributes to neuronal survival under neuroinflammatory conditions (8).

In the SN tissues of MPTP-treated mice, activated microglia with distinct morphology were observed (Fig. 4A). Notably, this type of activated microglia was not observed in those of mice treated with ALGERNON2. The level of cytokine was reduced upon ALGERNON treatment as well (Fig. 4G), suggesting that ALGERNON can regulate microglial cytokine production. To note, the administration of ALGERNONs also suppressed the up-regulation of GFAP in a systemic LPS injection model (Fig. 5F), implying that the treatment can contribute to the regulation of reactive astrogliosis through Nrf2. These observations also support their potential for the remedies of neurodegeneration since the block of toxic A1 as

(*29*). Moreover, the role of Nrf2 in astrocytes has been reported as neuroprotective (*30*). Nrf2 has become the subject of widespread interest and investigation as a drug target since its transcriptional pathway induces antioxidant and anti-inflammatory phase II enzymes to prevent chronic inflammation (*31*–*33*). Electrophiles that modulate the cysteine residues of Keap1, however, produce severe systemic side effects because of nonspecific S-alkylation of cysteine thiols and the resulting depletion of glutathione (*34*). Here, we identified that specific targeting to Dyrk1A can function as an enhancer for the p21-Nrf2 axis without these side effects. It is notable that the up-regulation of Nrf2 by ALGERNONs was transient, peaking at 2 to 6 hours and then returning to basal levels (fig. S4F), because of the sequential and transient nature of cyclin D1 and p21 stabilization (fig. S4D). This would indicate the substantial advantage of avoiding hyperactivation of the Nrf2 pathway, which is often a condition observed in cancer tissues (*35*, *36*), while allowing sufficient functional up-regulation to exert neuroprotective signaling when required during neural insults. Moreover, ALGERNON treatment alone could not robustly induce HO-1, NQO-1, and other downstream targets of Nrf2, in contrast to the Nrf2-inducing compounds, SLF, DEM, and Δ15 (fig. S5). This agrees with the fact that p21 disrupts the interaction between the weak binding motif of the Keap1-Nrf2 complex, thus inhibiting Nrf2 degradation via the proteasomal pathway (*15*), while Nrf2 remains bound to Keap1, requiring additional triggers such as oxidative stress to translocate into the nucleus (fig. S8). The cytokine production upon LPS stimulation was reduced in the p21-depleted condition (fig. S6B), suggesting another regulatory pathway of p21. The cytosolic Nrf2 level with ALGERNON treatment was low compared to the rapid accumulation of Nrf2 in the nucleus upon SLF treatment (fig. S5E), reflecting the level of p21 disruption of the Nrf2-Keap1 complex in glial cells. We speculate that ALGERNONs increase the Nrf2 pool for nuclear translocation and that this small increase in cytosolic Nrf2 would be sufficient to rescue neurodegeneration in vivo as shown in several neuroinflammatory models (Figs. 1, 5, and 6). This underlines the potentially beneficial effects of ALGERNONs, as ALGERNONs only activate the Nrf2 pathway when triggers such as neuroinflammation are encountered.

Several reports have shown that the plant alkaloid harmine, a known Dyrk1A inhibitor, can suppress inflammation by inhibiting the nuclear factor κB pathway (*17*–*19*). Harmine displays good tissue distribution but likely contributes to hallucinations because of its inhibitory activity on MAO-A (*20*). This prompted us to identify Dyrk1A inhibitors that lack MAO-A inhibitory property. ALGERNON2 showed potent Dyrk1A inhibition with equivalent neurogenic enhancement as ALGERNON and better brain tissue retention, but no MAO-A inhibitory activity (fig. S2A). Notably, using ALGERNONs, we revealed a novel mechanism of neuroprotection that targets Dyrk1A-potentiated neuronal survival by enhancing the p21-Nrf2 pathway in glial cells. ALGERNONs rescued neurodegeneration caused by both central and peripheral inflammation, which indicates that they can be broadly applied to inflammatory conditions. ALGERNON2 enhanced the transplantation of iPSC-derived DA neurons (Fig. 6), which is currently being investigated further in our clinical trial for PD. This highlights the potential therapeutic application of ALGERNONs for iPSC transplantation in PD. Furthermore, neuroinflammation has drawn considerable attention due to its implicated association with neurodegenerative disorders and neurological psychiatric diseases (*37*). In conclusion, our results shed light on the therapeutic potential of ALGERNONs for addressing neuroinflammation-related disorders and providing a novel approach to the treatment of incurable disorders including those involving iPSC transplantation.

MATERIALS AND METHODS

A complete description of the methods is provided in the Supplementary Methods.

Study Approval

All animal protocols were reviewed and approved by the Animal Research Committee, Graduate School of Medicine, Kyoto University, Center for iPS Cell Research and Application, Kyoto University, and Graduate School of Medical Sciences of Kanazawa University.

Statistics

Results obtained from more than three experiments are expressed as the means ± SEM. Statistically significant differences were determined using a two-tailed, unpaired Student's test, or one-way analysis of variance (ANOVA) followed by a Tukey-Kramer comparison test. A P value less than 0.05 was considered statistically significant and indicated with a single asterisk (*); a P value less than 0.01 was indicated with a double asterisk (**).

Image analysis

Cells were plated on 96-well PureCoat amine–coated plates (Corning) and treated as desired for each specific experiment. After immunolabeling was complete, automated image acquisition (at ×20 magnification, 2 × 2 charge-coupled device binning, 25 fields per well), and analysis were performed using an Arrayscan VTI (Thermo Fisher Scientific) with a Cellomics Compartmental Analysis module (for BrdU incorporation) or Opera Phenix (Perkin Elmer) with Harmony software (for cyclin D1/p21/Nrf2 quantification). Image acquisition was also performed using a fluorescence microscope (BZ-9000; Keyence) or confocal microscope (SP-8; Leica).

Animal models

C57black/6J male mice at 8 to 9 weeks old were intraperitoneally injected four times with MPTP (Sigma-Aldrich) at a dose of 20 mg/kg with 10-ml/kg volume at 2-hour intervals (*38*). LPS (O55:B5) (Sigma-Aldrich, L2880) was intraperitoneally injected at 1 mg/kg once daily for 4 days. Human iPSC-derived DA progenitors were transplanted into the striatum of severe combined immunodeficient (SCID) male mice at 4 weeks of age. Drug treatment was performed 1 hour before MPTP, or LPS, or iPSC transplantation.

SUPPLEMENTARY MATERIALS

Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/6/46/eabc1428/DC1

View/request a protocol for this paper from *Bio-protocol*.

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