RESEARCH ARTICLE

CME

Immunomodulatory Drugs Alleviate L-Dopa-Induced Dyskinesia in a Rat Model of Parkinson's Disease

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ABSTRACT: Background: Thalidomide and closely related analogues are used clinically for their immunomodulatory and antiangiogenic properties mediated by the inhibition of the proinflammatory cytokine tumor necrosis factor α. Neuroinflammation and angiogenesis contribute to classical neuronal mechanisms underpinning the pathophysiology of L-dopa-induced dyskinesia, a motor complication associated with L-dopa therapy in Parkinson's disease. The efficacy of thalidomide and the more potent derivative 3,6′-dithiothalidomide on dyskinesia was tested in the 6-hydroxydopamine Parkinson's disease model.

Methods: Three weeks after 6-hydroxydopamine infusion, rats received 10 days of treatment with L-dopa plus benserazide (6 mg/kg each) and thalidomide (70 mg/kg) or 3,6′-dithiothalidomide (56 mg/kg), and dyskinesia and contralateral turning were recorded daily. Rats were euthanized 1 hour after the last L-dopa injection, and levels of tumor necrosis factor-α, interleukin-10, OX-42, vimentin, and vascular endothelial growth factor immunoreactivity were measured in their striatum and substantia nigra reticulata to evaluate neuroinflammation and angiogenesis. Striatal levels of GLUR1 were measured as a L-dopa-induced postsynaptic change that is under tumor necrosis factor-α control.

Results: Thalidomide and 3,6′-dithiothalidomide significantly attenuated the severity of L-dopa-induced dyskinesia while not affecting contralateral turning. Moreover, both compounds inhibited the L-dopa-induced microgliosis and excessive tumor necrosis factor-α in the striatum and substantia nigra reticulata, while restoring physiological levels of the anti-inflammatory cytokine interleukin-10. L-Dopa-induced angiogenesis was inhibited in both basal ganglia nuclei, and L-dopa-induced GLUR1 overexpression in the dorsolateral striatum was restored to normal levels.

Conclusions: These data suggest that decreasing tumor necrosis factor-α levels may be useful to reduce the appearance of dyskinesia, and thalidomide, and more potent derivatives may provide an effective therapeutic approach to dyskinesia. © 2019 International Parkinson and Movement Disorder Society

Key Words: 3,6′-dithiothalidomide; dyskinesia; immunomodulation; L-dopa; thalidomide

Repurposing drugs that are well tolerated and approved for other indications is a highly pursued strategy to identify new treatments for Parkinson's disease (PD).[1,2] Thalidomide (TLD), despite its sordid history, is now recognized as an immunomodulatory drug (IMiD) and has emerged in the last decade as a useful treatment for several

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inflammatory diseases and cancer, consequent to its immunomodulatory and antiangiogenic properties.[3-5] In addition, recently synthesized more potent TLD derivatives are under investigation for neurological disorders.[6]

In PD management, a current primary concern is the development of abnormal involuntary movements that inexorably arise following long-term L-dopa therapy. This L-dopa-induced dyskinesia (LID) has proven difficult to pharmacologically treat, and its mitigation continues to be an urgent unmet therapeutic need for which interest is growing on repurposing drugs.[7] The pathophysiology of LID is complex and encompasses pre- and postsynaptic mechanisms involving basal ganglia nuclei, classically including enhanced corticostriatal glutamatergic transmission and altered synaptic plasticity, altered dopamine storage, postsynaptic changes in glutamate receptors composition and in gene expression in striatal neurons.[8-12] In addition, in the last decade increasing evidence has suggested that non-neuronal mechanisms may contribute to LID, such as glia-mediated neuroinflammation and angiogenesis.[8,13] Both these processes may undergo long-lasting adaptations that contribute to neuron function and brain plasticity.[14-16] Sustained microglia activation and neuroinflammation induce changes in gene expression related to synaptic plasticity,[17,18] suggesting that neuronal and non-neuronal mechanisms combine to drive the pathophysiology of LID. Neuroinflammation is a recognized key feature in PD pathogenesis, mainly mediated by microglial cells.[19-22] Multiple studies have reported that repeated L-dopa administration exacerbates the inflammatory environment in the striatum, with overproduction of the proinflammatory cytokine tumor necrosis factor (TNF)-α.[13,23-25] Although the mechanism underlying L-dopa-induced inflammatory response remains to be investigated, the increased dopamine metabolism and associated oxidative load may likely contribute.[13] Angiogenesis is also a recognized component of dyskinesia neuropathology, and the proangiogenic activity of L-dopa in the striatum as well as in the basal ganglia output nucleus substantia nigra reticulata (SNr) has been characterized in animal models of LID and in the parkinsonian brain.[26-30] Importantly, inflammation and angiogenesis are two events tightly interrelated, which regulate each other in physiological and pathological conditions.[31] Accordingly, previous studies have reported an antidyskinetic effect of drugs targeting the immune system, epitomized by corticosterone, the peroxisome proliferator-activated-gamma agonist rosiglitazone, the immunosuppressant rapamycine,[12,23,32] as well as the antidyskinetic property of the antiangiogenic compounds vandetanib and candesartan.[29,33]

Among inflammatory mediators, TNF-α is a potent activator of the immune system that facilitates the recruitment of resting microglia within the pathological microenvironment.[34,35] Importantly, TNF-α impacts recognized components of dyskinesia pathophysiology, such as angiogenesis and synaptic plasticity.[16,36-38] Moreover, the modulation of long-term synaptic plasticity by TNF-α involves the regulation of AMPA glutamate receptor subunit 1 (GLUR1) expression, which has been involved in dyskinesia.[10,39-42] TLD and derivatives act primarily through the inhibition of TNF-α production via post-translational mechanisms, with consequent dampening of the inflammatory cascade.[43-46] Furthermore, recent studies have established that the antiangiogenic activity of TLD relies on TNF-α inhibition.[47,48] The growing interest in TLD as a repurposed drug and the elucidation of its mechanistic targets has prompted the synthesis of more selective and potent derivatives, which may have an improved clinical profile in neurological disorders.[6] 3,6′-Dithiothalidomide (DTT) is a TLD derivative with more selective and potent TNF-α-lowering activity than the parent compound, while maintaining comparable blood–brain barrier (BBB) permeability.[46] Previous studies have reported that DTT was effective in mitigating inflammation and memory deficits in models of Alzheimer's disease.[49,50] Based on the current evidence, we hypothesized that TLD and DTT may be candidate drugs for ameliorating LID in PD, with the aim of investigating the potential of IMiD treatments for clinical translatability. We therefore evaluated the efficacy of these drugs in the development of LID in the 6-hydroxydopamine (6-OHDA) rat model of PD and found that both significantly attenuated LID severity. This antidyskinetic activity was associated with a dramatic reduction in the L-dopa-induced inflammatory response in the striatum and SNr. To further investigate the effect of selective TNF-α inhibition on mechanisms of dyskinesia, we evaluated L-dopa-induced angiogenesis and striatal expression of GLUR1 after treatment with DTT. The antidyskinetic action of DTT was associated with an antiangiogenic action in basal ganglia and with a restoration of L-dopa-induced striatal overexpression of GLUR1.

Methods

Drugs

L-Dopa methyl ester and benserazide (6 mg/kg each; Sigma Aldrich, Milan) were dissolved in saline and administered subcutaneously. TLD (70 mg/kg body weight; Sigma Aldrich, Milan, Italy) and DTT (56 mg/kg body weight), synthesized and chemically characterized to >99% purity, were suspended in 0.5% carboxymethylcellulose and administered intraperitoneally. DTT was synthesized by a method slightly modified from the procedure originally described by Zhu et al (2003)[51] (for synthesis details, see Supplementary Material). The drug dose for DTT was chosen based on prior in vivo studies in rodents in which drug benefits were observed using this dose.[49,50,52-54] Because of the more potent anti-inflammatory actions of DTT over TLD, a higher TLD dose was used to compare similar activities. A pilot dose-

finding study revealed that doses higher than 100 mg/kg acutely induced some sedation in the rats, whereas the dose of 70 mg/kg was devoid of such effect, as also confirmed by the contralateral turning behavior recorded in rats treated with TLD in association with L-dopa. Therefore, the dose of 70 mg/kg was used for all further studies. Rats receiving these drug doses in the chronic regimen did not show signs of suffering or altered general behavior, except for some abdominal discomfort in the first 5 minutes after intraperitoneal TLD administration. DTT is not a US Food and Drug Administration (FDA)–approved compound and has not been tested in the clinic. TLD is currently a first-line treatment for multiple myeloma, with escalating doses from 200 to 400 mg/day,[55,56] and similar doses were used in a clinical trial in Alzheimer's disease patients.[57] Therefore, the TLD dose used in the present study, although on the high side, owns a clinical translatability significance.

Animals and Pharmacological Treatments

To achieve a full nigrostriatal lesion, male Sprague-Dawley rats (Harlan, Italy) weighting 275-300 g were deeply anesthetized with fentanyl (3 mg/kg intraperitoneally) and stereotaxically injected with 6-OHDA into the left medial forebrain bundle, as previously described.[58] The cylinder test and tyrosine-hydroxylase (TH) immunohistochemistry (IHC) in the substantia nigra pars compacta (SNc) were performed to assess dopamine depletion. Only animals showing an asymmetry score (number of affected limb wall touches/number of unaffected limb touches) < 0.25 and a nigrostriatal degeneration above 95% were included in the experiments (representative SNpc lesion in Fig. 1A).

All experimental procedures met the guidelines and protocols approved by the European Community (2010/63 UE L 276 20/10/2010), by the Ethical Commission for Animal Care and Use at the University of Cagliari, and the Italian Ministry of Health (pr. #1293/2015-PR).

Three weeks after 6-OHDA lesioning rats were treated for 10 days as follows: vehicle (Veh) rats received saline plus 0.5% carboxymethylcellulose (n = 11); Veh + dopa rats received vehicle and 6 mg/kg each of L-dopa + benserazide (n = 16); TLD + dopa rats received TLD (70 mg/kg) and L-dopa + benserazide (6 mg/kg each) (n = 11); DTT + dopa rats received DTT (56 mg/kg)and L-dopa + benserazide (6 mg/kg) (n = 7); TLD + Veh and DTT + Veh rats received TLD (70 mg/kg) or DTT (56 mg/kg), respectively, and vehicle (n = 4). TLD and DTT were administered daily 30 minutes before L-dopa. The 10-day administration regimen of L-dopa is a widely used protocol for assessing LIDs in rats and was chosen based on previous studies showing that after a daily drug treatment for 7–8 days, the severity of abnormal involuntary movements (AIMs) reaches a plateau, as shown in Supplementary Figure 1.[12,32] In a second experiment TLD was tested in the expression of AIMs. One group of rats received vehicle+ L-dopa + benserazide (Veh + dopa, 6 mg/kg each, n = 5) daily for 15 days; one other group received the same treatment for 10 days, followed by daily TLD (70 mg/kg) + L-dopa + benserazide (n = 5) for an additional 5 days.

Behavioral Studies

Limb and axial AIMs were recorded as a rodent model of LID daily during the 10-day treatment, together with contralateral turning behavior. Rats were individually monitored 1 every 20 minutes within 2 hours after L-dopa administration, as described.[25] The time (in seconds) spent in limb and axial AIMs and the number of contralateral turns were recorded (see Supplementary Material for details).

Immunohistochemistry

On the last day of pharmacological treatments, rats were anesthetized and transcardially perfused with 4% paraformaldehyde 1 hour after L-dopa methyl ester administration, when rats show the peak of AIM response (see Fig. 1C,D). Brains were postfixed, and 40-μm-thick coronal sections were vibratome-cut.[25] Striatal or midbrain sections were immunoreacted with primary antibodies against TH, GluR1, OX-42, TNF-α, interleukin (IL)-10, glial fibrillary acidic protein (GFAP), vimentin, and vascular endothelial growth factor (VEGF) for single or double immunolabeling and thereafter incubated with the appropriate fluorochrome-conjugated secondary antibodies as previously described[59] (see Supplementary Material for details on antibodies and reaction procedure).

Confocal Microscopy Analysis

Qualitative and quantitative analysis for GLUR1 expression, all markers of neuroinflammation, and angiogenesis immunoreactivity (IR) was performed using a Leica 4D confocal laser scanning microscope equipped with an argon-krypton laser. Confocal images were generated as previously described.[59,60] The volume occupied by GLUR1, cytokines/OX-42 colocalization, and vimentin or VEGF was determined by Imaris 7.3 (see Supplementary Material for details). The unlesioned side of Veh-treated brains was used as the inner control across studies.

Cytokine Analysis by Multiplex ELISA

For multiplex enzyme-linked immunosorbent assay (ELISA) the tissues were homogenized in a Tris-based lysis buffer with protease and phosphatase inhibitors (Halt protease & phosphatase inhibitor single-use cocktail; Thermo Scientific), centrifuged, and the supernatant placed into new tubes. The protein concentrations were measured by bicinchoninic acid (BCA) assay (Pierce BCA protein assay kit; Thermo Scientific). Equal

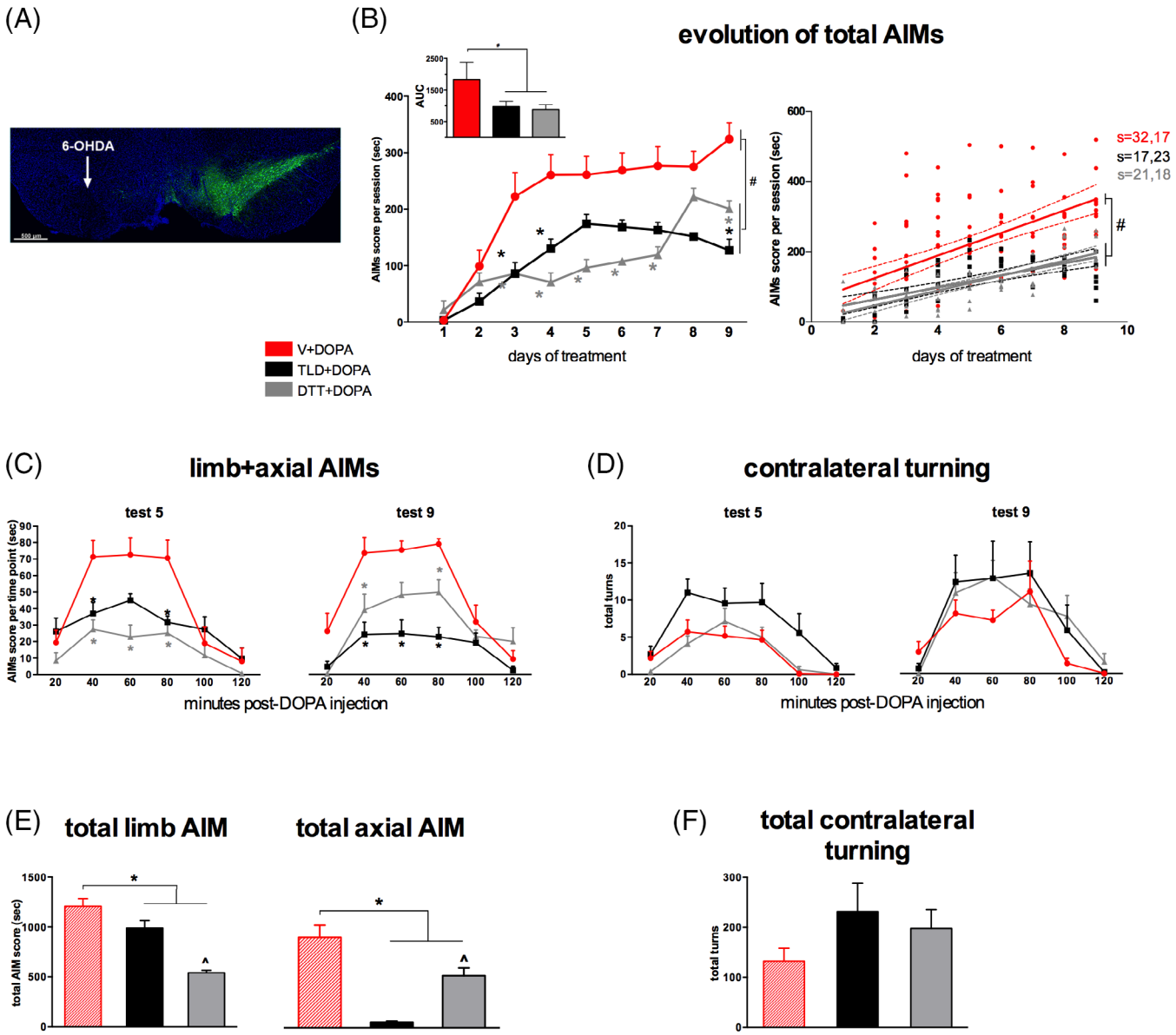


FIG. 1. TLD and DTT reduced the development of LID in hemiparkinsonian rats. Representative image of the 6-OHDA-infused SNc. (A) Development of AIMs during 10 days of treatment, score is shown as total seconds spent in AIMs in daily session, and linear regression of AIM development during 10 days of treatment (S, slope). (B) Development of AIMs (C) and contralateral turning (D) post-L-dopa injection during daily sessions (days 5 and 9); total limb and axial AIM score (E) and contralateral turns (F) in the whole treatment. Values represent the mean ± SEM. $^{\#}P < 0.0001$ for main treatment effect (B); $^{*}P < 0.05$ versus V + dopa (B, D, F, I); $^{\wedge}P < 0.01$ vs TLD + dopa (F). [Color figure can be viewed at wileyonlinelibrary.com]

protein loading concentrations (150 μg/well) were assessed in duplicate on the Mesoscale Discovery V-PLEX Proinflammatory Panel 2 for rat, using the protocol suggested by the manufacturer (see Supplementary Material for assay protocol details). Protein concentrations were expressed as pg/150 μg of tissue.

Statistical Analysis

All data were statistically analyzed by Statistica 8 (Stat Soft Inc., Tulsa, OK). Data from behavioral studies (AIMs and contralateral turning) were analyzed by two-way analysis of variance (ANOVA) with repeated-measures or one-way ANOVA, followed by Newman-Keuls post hoc tests. Linear regression was calculated to assess the development of AIMs. Immunohistochemistry and ELISA data were analyzed by one-way ANOVA followed by Tukey's honestly significant differences (HSD) post hoc test. Significance was set at $P < 0.05$.

Results

Motor Responses

We addressed the ability of TLD and DTT to counteract the development of L-dopa-induced AIMs. L-Dopa

induced a gradual development of limb and axial AIMs, whereas rats treated with TLD and DTT exhibited a significantly lower dyskinetic response to L-dopa (Fig. 1B). Two-way ANOVA revealed a main treatment effect ($F_{2,21}$ = 16.206, $P < 0.0001$), a main time effect ($F_{8,168}$ = 26.478, $P < 0.0001$), and a significant treatment/time interaction ($F_{16,168}$ = 3.743, $P < 0.0001$). The antidyskinetic effect of IMiDs was confirmed by linear regression analysis, which revealed a significant difference between the L-dopa and TLD/DTT slopes, indicating a different propensity to develop a sensitized behavioral response (F = 4.446, $P < 0.01$; Fig. 1B). TLD and DTT significantly attenuated the severity of limb and axial AIMs in response to L-dopa within the 120 minutes of recording, while not changing the duration of L-dopa effect ($P < 0.01$; Fig. 1C). Importantly, TLD and DTT did not affect the severity of contralateral turning, suggesting that IMiDs did not affect L-dopa therapeutic efficacy (Fig. 1D). Total limb ($F_{3,21}$ = 23,353, $P < 0.0001$) and axial ($F_{3,21}$ = 15,758, $P < 0.0001$) AIMs measured for the whole treatment were reduced by IMiDs, whereas the total turning response was unaffected (Fig. 1E,F). Interestingly, TLD was more efficient in reducing axial AIM ($P < 0.001$), whereas DTT was more effective in limb AIM ($P < 0.001$; Fig. 1E). Neither TLD nor DTT administered alone induced a motor response (data not shown). In contrast, when TLD was administered to rats with established AIMs, it failed to reduce AIMs severity (Supplementary Fig. 1).

Neuroinflammation

The generation of LID was associated with a marked proinflammatory response in microglia in the lesioned dorsolateral striatum (Str) and SNr, which was significantly attenuated by treatment with IMiDs.

OX-42 IR. OX-42 was analyzed as a classical marker of reactive microglia in the Str and SNr. The 6-OHDA lesion induced an increase of OX-42 IR that was significant in the Str only ($P < 0.0001$), and that was exacerbated by the chronic L-dopa treatment in both areas ($P < 0.0001$; Fig. 2A–C,H).[25] Both TLD and DTT significantly reduced the OX-42 IR ($P < 0.0001$) induced by L-dopa, restoring control levels in the Str and SNr (Fig. 2D–H). In the unlesioned contralateral Str, L-dopa induced a nonsignificant increase in OX-42 IR, whereas TLD and DTT significantly reduced OX-42 IR (Supplementary Fig. 2).

We also analyzed levels of the proinflammatory cytokine TNF-α and the anti-inflammatory cytokine IL-10, colocalized with OX-42, to investigate changes in microglial phenotype. L-dopa caused an imbalance in the microglial content of both cytokines that was restored by both IMiDs (Figs. 2 and 3).

TNF-α. Dopamine depletion induced a slight, but not significant rise of TNF-α IR colocalized with OX-42 in both Str and SNr, whereas chronic L-dopa caused a large increase in TNF-α ($P < 0.0001$), indicating the augmentation of proinflammatory microglia (Fig. 2A–C,H). A slight but significant increase in TNF-α was induced by L-dopa in the unlesioned Str (Supplementary Fig. 2). The coadministration of TLD or DTT with L-dopa fully restored TNF-α/OX-42 colocalization to control values, in agreement with their mechanism of action and indicating a reduction of proinflammatory microglia (Fig. 2D,E,H, and Supplementary Fig. 2).

IL-10. In contrast to TNF-α, the 6-OHDA lesion significantly reduced levels of the anti-inflammatory cytokine IL-10 in microglia compared with the unlesioned Str (Fig. 3B, C,I; $P < 0.0001$). Moreover, chronic L-dopa administration further reduced IL-10/OX-42 colocalization below 6-OHDA levels ($P < 0.0001$; Fig. 3D), whereas the coadministration of either TLD or DTT restored IL-10 to 6-OHDA values (Fig. 3E,F,I). Therefore, repeated L-dopa treatment dampened anti-inflammatory microglia, whereas both IMiDs significantly attenuated such effect. Results shown in Figure 3L compare the % volume of striatal OX-42-positive cells colocalized with TNF-α or IL-10, and the related proportion is shown as a ratio to highlight overall imbalance in the microglia phenotype. Under physiological conditions microglia containing IL-10 prevailed over microglia containing TNF-α, whereas dopamine depletion inverted this ratio from 0.33 to 3.57. L-dopa further exacerbated this imbalance by elevating the ratio to 121.95, inducing a dramatic increase in proinflammatory microglia over anti-inflammatory. Of note, both IMiDs restored the ratio to near control values (9.62 and 1.78 for TLD and DTT, respectively); however, DTT was more effective than TLD, a feature in agreement with the drug's greater potency in lowering TNF-α levels (Fig. 3L).

In a separate experiment we extended the investigation of inflammatory markers by multiplex ELISA, and found that several cytokines were expressed at higher levels in L-dopa-treated rats compared with the unlesioned side of vehicle-treated rats (IL-6, IL-1β,IL-5, CXCL1, IL-10), whereas TLD reduced IL-6 and IL-1β levels compared with unlesioned-treated rats (Supplementary Table 1).

Angiogenesis Markers

As elevations in TNF-α protein and angiogenesis are coupled, and as the development of dyskinesia is linked with abnormal angiogenesis occurring mainly within the SNr,[26-28,61] we investigated new vessel formation in this area and in the Str by analyzing vimentin and VEGF as markers of angiogenesis.[62] The astroglial marker GFAP showed that astrocytes enwrapped vessels expressing different levels of vimentin and VEGF, depending whether they were preexisting or newly formed vessels (arrows in Fig. 4A

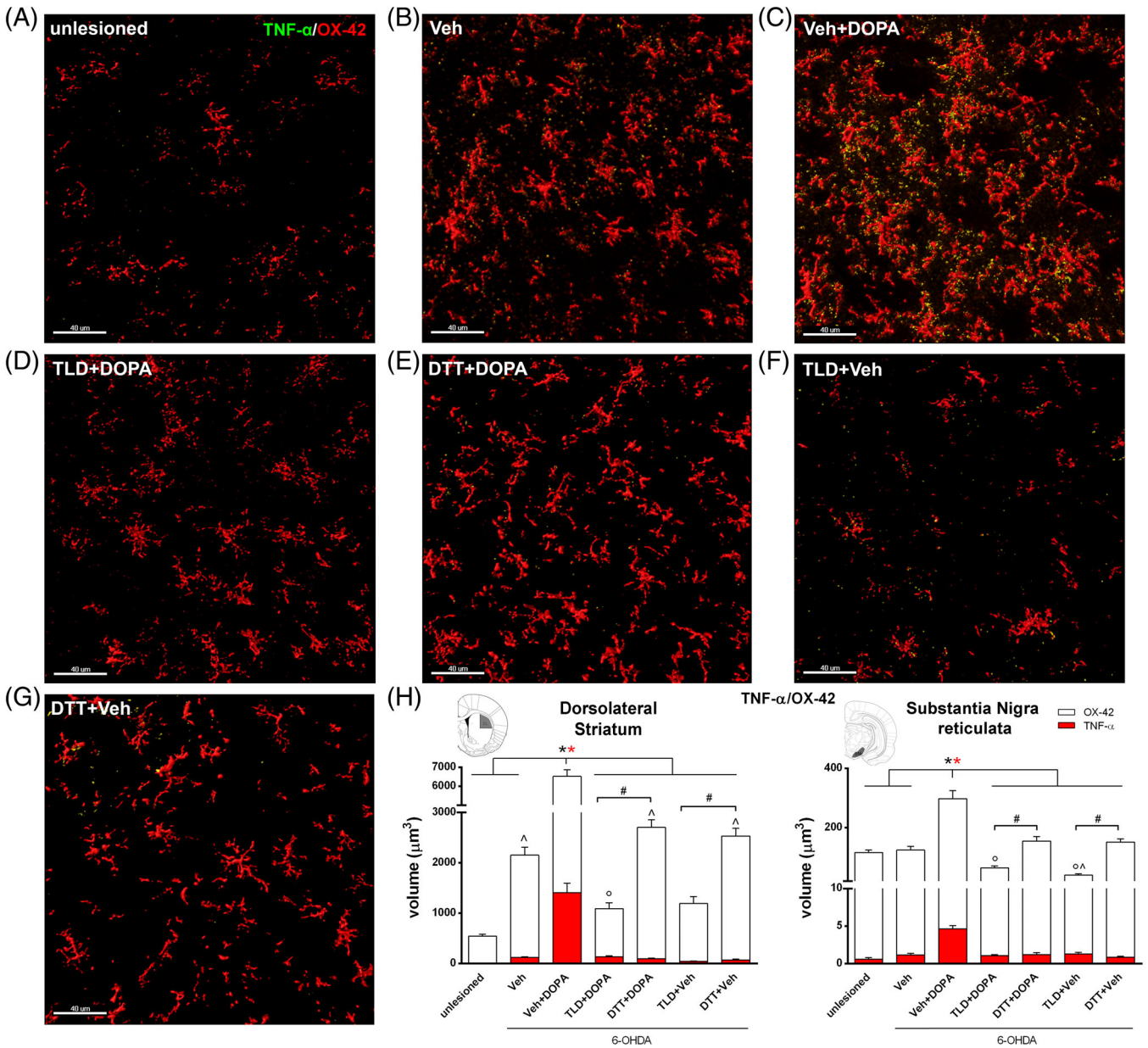
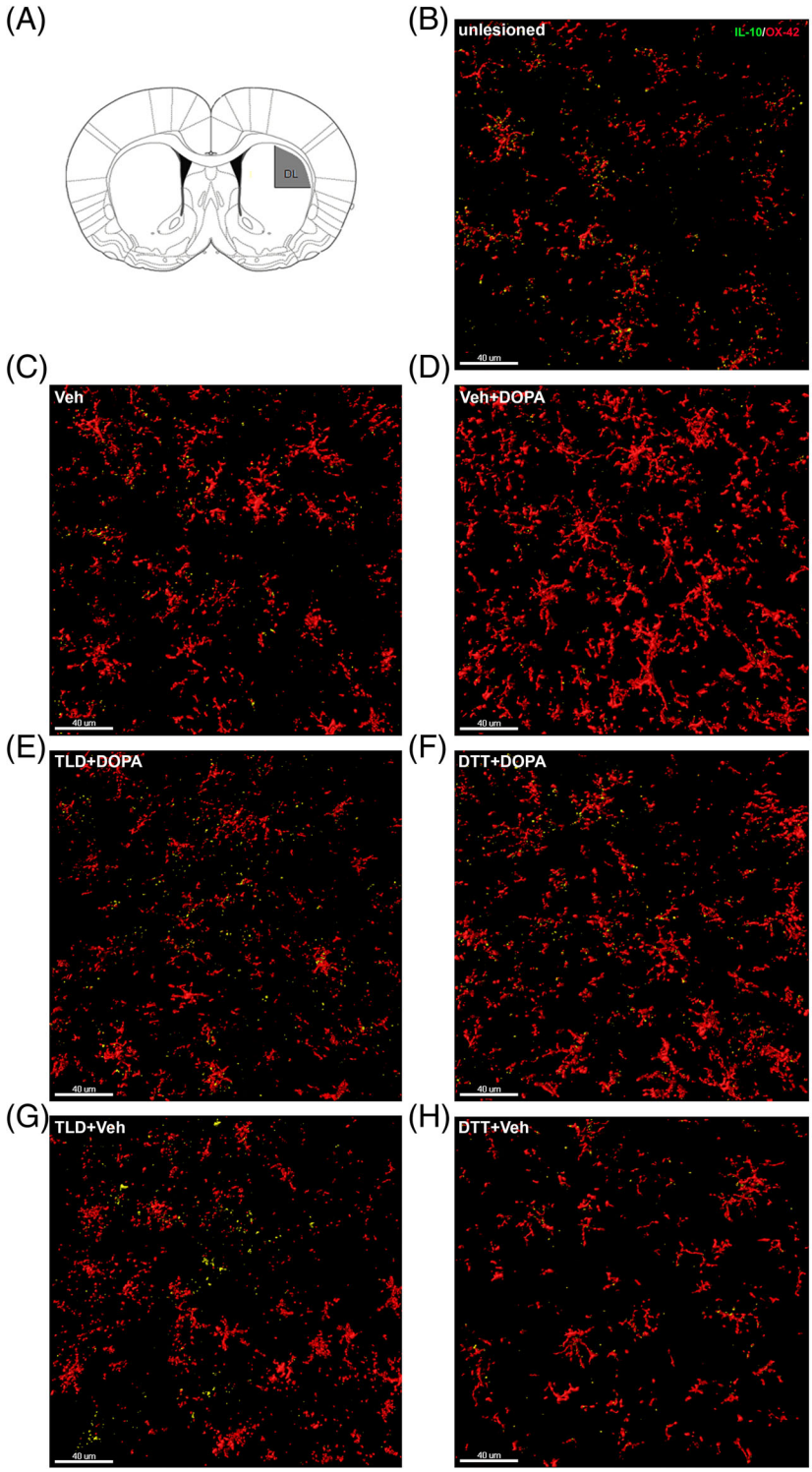


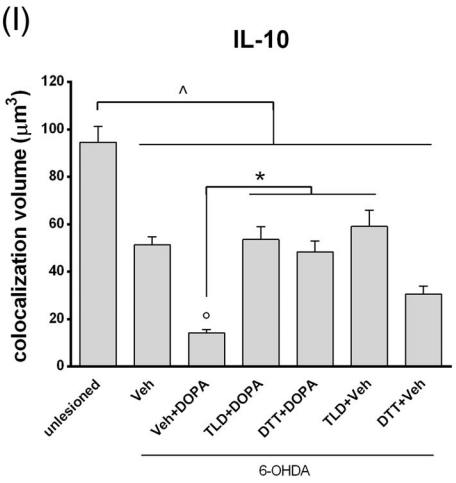
FIG. 2. TLD and DTT reduced L-dopa-induced microgliosis and microglial TNF-α content in the dopamine-depleted Str and SNr. Representative confocal images showing TNF-α (yellow) in OX-42 (red)-positive cells in the Str (A–G); total volume occupied by OX-42 and colocalized with TNF-α (red columns) in the Str and SNr (H). Values represent the mean ± SEM. $^{*}P < 0.0001$; $^{\#}P < 0.05$; $^{\wedge}P < 0.0001$ versus unlesioned; $^{\circ}P < 0.05$ versus Veh (1-way ANOVA followed by Tukey HSD post hoc test). [Color figure can be viewed at wileyonlinelibrary.com]

and Fig. 4C,D, respectively). We found a slight, not significant increase in both vimentin and VEGF expression after the 6-OHDA lesion, compared with the unlesioned hemisphere, which was in line with the observed changes in TNF-α protein in Figure 2 (Fig. 4A,B,F,G,H,N and Supplementary Fig. 3). Chronic L-dopa dramatically increased the formation of new vessels, as clearly shown by the increased vimentin and VEGF expression in Figure 4C,F,I,N and Supplementary Figure 3 ($P < 0.0001$). DTT prevented L-dopa-induced angiogenesis, in line with the restoration of physiological TNF-α protein levels and with the significant attenuation of AIMs (Fig. 4D,F,L, N; $P < 0.0001$).

Striatal GLUR1 Protein Levels

Changes in GLUR1 protein levels were investigated in the Str (see insert in Fig. 5A) as a postsynaptic neuronal event that is under TNF-α regulation. The confocal analysis showed that GLUR1 IR remained unchanged after the 6-OHDA lesion (Fig. 5B). Moreover, L-dopa induced





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Group	TNF-α (%)	IL-10 (%)	TNF-α/IL-10
unlesioned	2,46	7,42	**0,33**
6-OHDA +vehicle	6,72	1,88	**3,58**
6-OHDA+DOPA	25,61	0,21	**121,95**
6-OHDA+TLD+DOPA	15,49	1,61	**9,62**
6-OHDA+DTT+DOPA	3,65	2,05	**1,78**
6-OHDA+TLD	8,91	1,98	**4,5**
6-OHDA+DTT	3,05	1,93	**1,58**

FIG. 3. TLD and DTT reverted L-dopa-induced decrease of IL-10 content in the dopamine-depleted Str (A). Representative confocal images showing IL-10 (yellow) in OX-42 (red)-positive cells (B–H); volume of OX-42 colocalized with IL-10 (I). Total OX-42-occupied volume is shown in Figure 2. TNF-α/IL-10 volume ratio expressed as % versus OX-42 volume (L). Values represent the mean ± SEM. *$P < 0.0001$; °$P < .0001$ versus Veh. DL, dorsolateral striatum. [Color figure can be viewed at wileyonlinelibrary.com]

a significant increase in striatal GLUR1 protein, whereas the administration of DTT in association with L-dopa restored control levels ($P < 0.001$; Fig. 5A,B). DTT administered alone did not change striatal GLUR1 levels.

Discussion

Previous studies support an active role of L-dopa-induced striatal inflammation in the development of

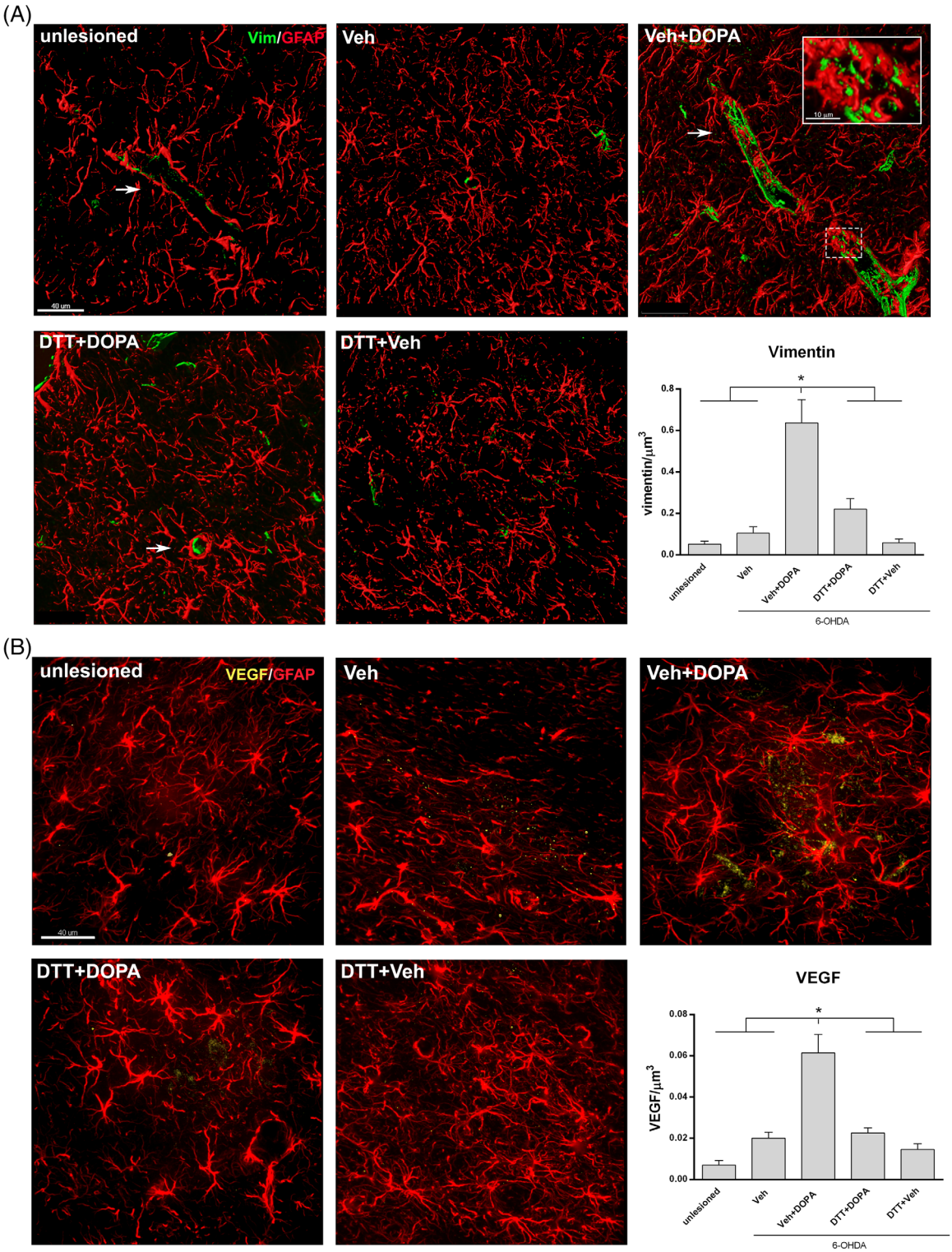

FIG. 4. DTT prevented L-dopa-induced angiogenesis in the SNr. Representative confocal images showing GFAP (red) and vimentin (green, A–E) or VEGF (yellow, G–M) in the SNr, and total volume occupied by vimentin (F) and VEGF (N). Values represent the mean ± SEM. *$P < 0.0001$. [Color figure can be viewed at wileyonlinelibrary.com]

LID. Here, we show for the first time that the immunomodulatory drug TLD and its analogue DTT significantly reduced the severity of LIDs in the 6-OHDA model of PD. Moreover, both IMiDs dramatically diminished the neuroinflammatory response induced by L-dopa in the Str and the SNr. As DTT possesses a more potent TNF-α inhibitory profile than TLD,[51] we broadened our investigation to characterize altered GLUR1 protein levels in the underlying antidyskinetic mechanism of DTT treatment and found that DTT

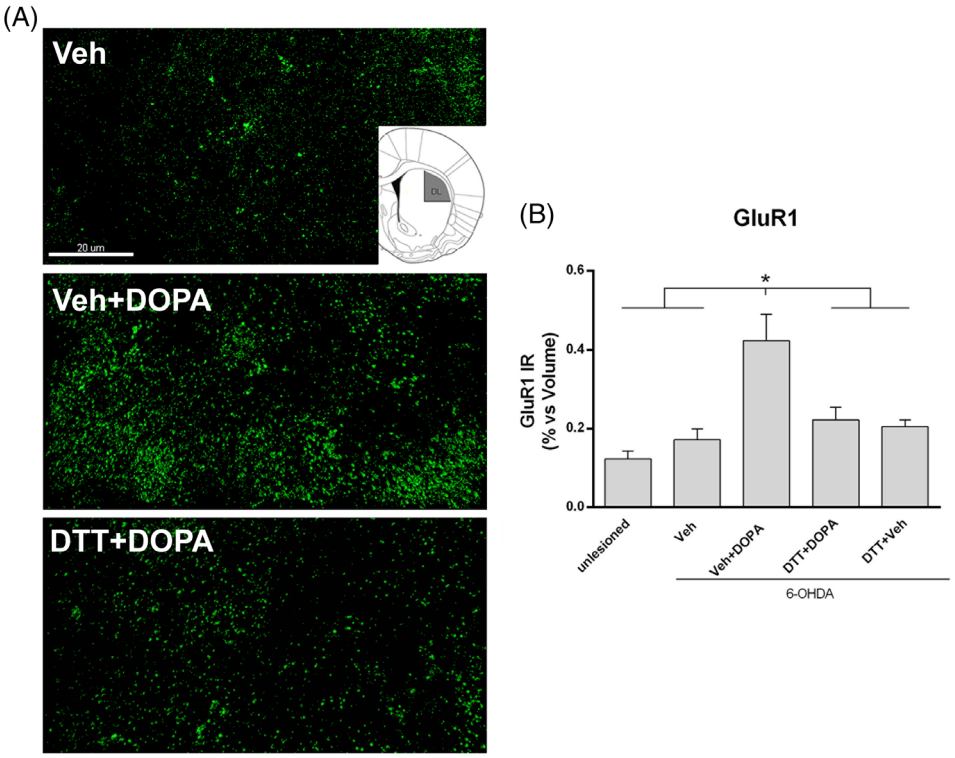

FIG. 5. Representative confocal images of GLUR1 protein expression in the dopamine-depleted Str (A) and GLUR1 levels expressed as % of the analyzed volume (B). [Color figure can be viewed at wileyonlinelibrary.com]

reduced L-dopa-induced increases in GLUR1 protein.[10] We also found that DTT strongly attenuated L-dopa-induced angiogenesis. These findings are of clinical relevance for PD, because TLD and several close analogues are FDA-approved drugs, currently in clinical trials for several central nervous system disorders (ClinicalTrials.gov Identifier: NCT00140452, NCT00231140, NCT01094340, NCT02415153 and NCT01553149).

Both TLD and DTT reduced the AIM score during the whole treatment. This effect resulted from the induction of less intense AIMs when L-dopa was administered in association with IMiDs, whereas the duration of the pharmacological effect was not affected. This effect was further highlighted by the linear regression analysis showing that both IMiDs reduced the development of AIMs response upon repeated L-dopa injections and suggesting a dampening of striatal mechanisms underlying sensitization.[63] Of note, treatment with IMiDs did not affect the severity of contralateral turning behavior in L-dopa-treated animals, suggesting that these drugs did not interfere with L-dopa therapeutic efficacy. TLD induced a slightly more intense turning behavior than did L-dopa, likely a consequence of the dramatic suppression of axial AIMs, which mostly impede motor responses. Interestingly, DTT effects were similar to those of TLD, albeit that DTT was used at a lower dose. This is in line with the 10- to 30-fold higher potency of DTT against TNF-α synthesis compared with TLD.[64] We also found that DTT inhibited the striatal overexpression of GLUR1 induced by repeated L-dopa treatments. The GLUR1 subunit confers Ca++-permeability to the AMPA channel and is highly expressed in the striatum. Several reports suggest that the AMPA receptor plays a role in dyskinesia.[40-42] An increase of PKA-dependent phosphorylation of GLUR1 and alterations in alternative splicing of AMPA receptor subunits were associated with LIDs in rats,[65,66] whereas the inhibition of the Ca++-permeable AMPA receptor was associated with antidyskinetic effects.[10,41] Of note, TNF-α has been shown to increase the cell surface expression of GLUR1 protein.[67] Therefore, results show that increased TNF-α levels induced postsynaptic neuronal changes in the striatum, suggesting a functional link between neuroinflammation and altered neuronal activity.

As previously reported, L-dopa induced an intense microgliosis and increased TNF-α levels in microglia in the dopamine-depleted striatum, whereas in the intact striatum the drug only induced a mild inflammatory response. This suggests that the preexisting inflammatory milieu, related to the neurodegenerative process dramatically amplified an otherwise mild inflammatory response to L-dopa. Moreover, a similar inflammatory reaction extended to the SNr, the output basal ganglia structure that also mediates motor effects and LIDs.[68-70] The microglial overproduction of TNF-α was

associated with decreased levels of the anti-inflammatory cytokine IL-10 in the same cell type. When the cytokine profile was evaluated by multiplex ELISA, we found an increase of inflammatory cytokines IL-6 and IL-1β, and of CXCL1 and IL-10, whereas several other cytokines were unaltered by L-dopa, including TNF-α. Moreover, TLD reversed IL-6 and IL-1β levels. Therefore, ELISA results suggest that the L-dopa and TLD effect extended to additional inflammatory cytokines. Interestingly, the ELISA assay also produced some mismatching results when compared with IHC data. This was not surprising because ELISA could not discriminate different cell types, and it was performed in the whole substantia nigra, including the pars compacta, where the neuroinflammatory environment was profoundly affected by the 6-OHDA lesion, in contrast to the focused analysis in single cell types performed by IHC. Moreover, in the ELISA assay the TNF-α levels were in the very low range of detection, whereas IL-6 and IL1-β levels were easily measured. Altogether the results suggest that repeated L-dopa treatment induced a chronic imbalance in the microglial phenotype, with a prevalence of pro-inflammatory over anti-inflammatory microglial phenotypes. This phenomenon thus provides a solid scientific rationale for using IMiDs in the treatment of LIDs. Accordingly, TLD and DTT were able to reduce the microgliosis and restore the cytokine imbalance induced by L-dopa to near control levels, indicating that these drugs drove the activity of microglial cells to that of an anti-inflammatory phenotype. TLD and DTT rapidly inhibit TNF-α protein production at the posttranscriptional level via key elements within the 3′ untranslated region,[43] as well as transcriptionally by downregulating NF-kB and myeloid differentiation factor 88.[71] TNF-α is a key inflammatory cytokine that is rapidly upregulated and released by microglial cells on receiving inflammatory stimuli. If this response is not restored to normal levels in a timely manner, TNF-α can trigger a cascade of events that generate a chronic pro-inflammatory microenvironment and affect the activity of neurons driving neuronal dysfunction.[6] Moreover, TNF-α is a master regulator of the inflammatory response inducing the expression of other cytokines, chemokines, and growth factors.[72] IL-10, in turn, is constitutively present in the brain microenvironment, holding a pivotal role in maintaining homeostasis in microglial cells; moreover, it is an important contributor to remission of inflammation, downregulating the expression and secretion of TNF-α and other pro-inflammatory cytokines and growth factors.[72,73] Whereas L-dopa disrupted the complex cross talk between cytokines promoting the proliferation of inflammatory microglia, IMiDs restored the balance to dampen neuroinflammation, braking the self-fueling circle instigated by L-dopa. Again, the lower dose of DTT used here reduced TNF-α levels to an extent similar to TLD, in accordance with DTT's higher potency as a TNF-α inhibitor. In this regard, DTT displays a greater potency (10- to 30-fold increased activity[64] at lowering TNF-α protein compared with TLD, reaffirming that TNF-α inhibition is the pharmacological target of these IMiDs for LID attenuation.[51,74] The doses used in our present study compare favorably with prior preclinical research,[49,50,74] and are in line with translational doses used in humans in which up to 1200 mg singly and 1000 mg daily of TLD have been administered.[75] More routine in humans is a daily TLD dose on the order of 200 to 400 mg, and this dose proved to be poorly tolerated and inefficacious in a recent clinical trial in Alzheimer's disease.[57] Common TLD-related adverse events reported in oncologic patients are hematologic such as mild neutropenia, peripheral sensory neuropathy, constipation, and infection. These adverse effects may represent a concern for a possible drug repurposing of TLD, and safety/tolerability of the drug should be scrutinized in the parkinsonian population yet reiterating the need for well-tolerated and more potent analogues.

Several studies have provided convincing evidence for a neuroinflammatory component in LID neuropathology.[13] Initial explorative studies showed an intensified inflammatory environment in the striatum of L-dopa-treated dyskinetic rats,[23,24] which was associated with a dramatic increase in the microglial production of TNF-α.[25] In turn, dyskinesia was exacerbated by an inflammatory stimulus.[25] Of note, striatal neuroinflammation was linked with the dyskinetic outcome of L-dopa, because the continuous nondyskinetic treatment was devoid of inflammatory effects.[25] Interestingly, previous studies have shown that LIDs were more intense in aged than in young rats,[76] which may relate to the physiological presence of senescent reactive-like microglia in the elderly brain.[77] Moreover, a recent study showed that rats overexpressing brain-derived neurotrophic factor, a TNF-α-induced growth factor, were more prone to develop LIDs.[78] As shown by seminal studies by Picconi et al, LIDs are associated with impaired synaptic plasticity at corticostriatal synapses that become unable to undergo depotentiation.[79] Of note, TNF-α has a critical neuromodulatory function, and increased TNF-α levels have been related to synaptic deficits and altered synaptic plasticity associated with neurodegeneration.[16,38,80-82] In line with a TNF-α neuromodulatory role, in the present study excessive production of this soluble cytokine in dyskinetic animals may have contributed to impaired synaptic plasticity at corticostriatal synapses.

In addition to immune functions, TNF-α is a potent proangiogenic cytokine acting via the NF-kB transcription factor to stimulate VEGF production and new blood vessel formation.[31,36,37] Using vimentin and VEGF as markers of angiogenesis, we report that L-dopa was highly proangiogenic, and inhibition of TNF-α production by IMiDs completely prevented such an effect. The contribution of angiogenesis in the development of

dyskinesia has been elegantly demonstrated in several studies. Elevated angiogenesis was reported in the basal ganglia of dyskinetic PD patients compared with non-dyskinetic patients undergoing L-dopa treatment.[28,33,84] Moreover, preclinical studies have shown that L-dopa dose-dependently induced angiogenesis, whereas LIDs were attenuated by VEGF inhibition with vandetanib and candesartan.[29,33,83,84] Here, we have demonstrated a causal relationship between angiogenesis and L-dopa-induced inflammation and suggest that contrasting the latter by inhibiting TNF-α with IMiDs may be a useful tool to diminish both angiogenesis and dyskinesia. In line with our results, the inhibition of nitric oxide, another proangiogenic product that is potently lowered by IMiDs,[85] reduced the severity of LID in the 6-OHDA rat model.[24] Vimentin was analyzed as a marker of mesenchymal cells in newly forming endothelial tissue.[62] Interestingly, vimentin has been described as part of the epithelial-mesenchymal transition process associated with chronic inflammation, a sophisticated pathway enrolled in growing of tissue under inflammatory stresses.[86] Moreover, factors released from degenerating neurons such as lactate dehydrogenase A induce brain angiogenesis through an increase of VEGF in a vimentin-dependent manner.[87] confirming the role of vimentin in the pathological central nervous system angiogenesis.

In conclusion, the present study shows that TNF-α is a critical player in the pathophysiology of LIDs, and pharmacological strategies aimed at decreasing TNF-α levels may be useful to reduce the occurrence or severity of clinical LID. The inhibition of TNF-α production by TLD and its analogue DTT attenuated the severity of dyskinesia by breaking the chronic inflammatory cycle induced by L-dopa and restoring the cytokines to near-physiological levels. Mechanistically, the alleviation of dyskinesia by IMiDs may occur at multiple levels, involving inhibition of angiogenesis as well as removal of L-dopa-induced postsynaptic changes in striatal neurons. TLD and the more recently synthetized analogues are FDA-approved drugs for several chronic inflammatory treatments. In addition, both TLD and derivatives display good BBB permeability and have shown beneficial effects in models of neurological diseases. Taken together, these data suggest that these compounds may be clinically repurposed as an effective therapeutic treatment approach to clinical LIDs in PD. ●

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Supporting Data

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