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PERSPECTIVE

Role of chronic neuroinflammation in neuroplasticity and cognitive function: A hypothesis

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Abstract

Objective: Evaluating the efficacy of 3,6'-dithioPomalidomide in 5xFAD Alzheimer's disease (AD) mice to test the hypothesis that neuroinflammation is directly involved in the development of synaptic/neuronal loss and cognitive decline.

Background: Amyloid-β (Aβ) or tau-focused clinical trials have proved unsuccessful in mitigating AD-associated cognitive impairment. Identification of new drug targets is needed. Neuroinflammation is a therapeutic target in neurodegenerative disorders, and TNF-α a pivotal neuroinflammatory driver.

New hypothesis: AD-associated chronic neuroinflammation directly drives progressive synaptic/neuronal loss and cognitive decline. Pharmacologically mitigating microglial/astrocyte activation without altering Aβ generation will define the role of neuroinflammation in AD progression.

Major challenges: Difficulty of TNF-α-lowering compounds reaching brain, and identification of a therapeutic-time window to preserve the beneficial role of neuroinflammatory processes.

Linkage to other major theories: Microglia/astroglia are heavily implicated in maintenance of synaptic plasticity/function in healthy brain and are disrupted by Aβ. Mitigation of chronic gliosis can restore synaptic homeostasis/cognitive function.

KEYWORDS
3,6'-dithioPomalidomide, 5xFAD mice, Alzheimer pathology, amyloid hypothesis, microglial/astrocyte activation, neuroinflammation, TNF-α

1 | OBJECTIVE

The controversial recent US Food and Drug Administration (FDA) approval of the Alzheimer's disease (AD) therapeutic Aducanumab, a monoclonal antibody that effectively clears aggregated soluble and insoluble forms of amyloid-β (Aβ) but affords little impact on cognitive decline,[1] has ignited contentious debates as to whether the amyloid hypothesis provides the best target to slow AD progression.[2,3] Considered central in AD initiation, once disease is established, would the clearance and lowering of Aβ be expected to impact cognition or do other mechanisms primarily drive cognitive decline?

To address the urgent need of the dementia field for alternative ideas on potential therapeutic strategies, one can consider neuroinflammation. Neuroinflammation is an invariable characteristic of AD and provides multiple potential targets for AD drug development. However, whether the development of neuroinflammation drives disease progression or is merely an epiphenomenon remains unknown. The objective of our study is to demonstrate the direct involvement of neuroinflammatory processes in the development of synaptic dysfunction and loss and consequent cognitive impairment. In order to accomplish this, we evaluate the efficacy of pomalidomide (Pom) and a novel analog, 3,6'-dithioPom (3,6'-DP) (Figure 1A, Figure S1-3 in the Supporting Information), in the 5xFAD animal model of AD. 5xFAD transgenic mice develop high levels of neuroinflammation consequent to abnormal Aβ peptide generation, together with cognitive deficits. The mice were evaluated over a 4-month period to assess whether the resolution of neuroinflammation induced by treatment with Pom and 3,6'-DP could mitigate this cognitive decline in the presence of progressive amyloid generation and deposition.

2 | BACKGROUND

2.1 | Historical evolution

AD is the most common neurodegenerative disorder worldwide. In the US alone, an estimated 5.8 million people aged 65 and older were affected in 2020, which is projected to increase to 13.8 million by 2050.[4] Because of the significant health, social, and economic burden associated with the disease, there have been numerous attempts to find a pharmacologic treatment able to effectively slow or prevent the onset and development of pathology. Despite these efforts, the clinical trial failure rate remains extremely high. The few currently approved and widely used AD drugs address symptomatic issues, and are able to only temporarily mitigate the cognitive impairment characteristic of the disorder; they are not effective in stopping or reversing disease progression.[5] In contrast, recently approved Aducanumab effectively clears Aβ deposits but has little impact on cognitive decline.[1]

Although AD is neuropathologically characterized chiefly by the extracellular accumulation of Aβ plaques and intracellular neurofibrillary tangle formations of phosphorylated tau protein, it would be simplistic to define AD by these two biomarkers alone. AD is associated with a series of other complex neuropathological mechanisms, etiologic processes, and risk factors that make research for potential therapeutic approaches exceptionally challenging, but importantly, provide alternative therapeutic targets to Aβ and tau. Neuroinflammation is increasingly considered a potential target for neurodegenerative disorders, including AD and dementia.[6] Significant evidence of inflammatory processes as a hallmark of AD is provided by the presence of inflammatory markers in cerebrospinal fluid, plasma, and post-mortem brain tissue of AD patients.[6,7]

Neuroinflammation has been shown across different phases of the pathology. From preclinical to late clinical stages[6,7] inflammatory markers, such as activated microglial cells, increased levels of pro-inflammatory cytokines, caspases, and chemokines, have been detected both in in vitro and in vivo preclinical studies, as well as in AD patients. However, the question as to whether or not they contribute to disease progression remains open in the light of anti-inflammatory drug clinical trial failures.[8]

The initial and acute activation of glial cells is a key element of the innate immune response to maintain homeostasis and to re-optimize brain function following a variety of pathological challenges. Indeed, it is considered an essential component of the healing process: promoting the removal of cellular debris, neurotoxic molecules, and dying cells, as well as ultimately supporting neuronal survival. However, an over-excessive and chronic neuroinflammation can assume a pivotal role in promoting the progression of neurodegenerative pathology. Preclinical and clinical studies show that microglial cells in AD brains are present in a phenotypically activated form and are highly associated with Aβ plaques, with their number and dimensions directly increasing in proportion to plaque size. This supports their potential contribution to Aβ fibril phagocytosis.[6,9] In parallel, activated microglia generate and release elevated levels of a series of pro-inflammatory mediators. These include cytokines, chemokines, complement factors, nitric oxide, reactive oxygen species, and other free radicals.[6,9,10] Such moieties are reported to be involved in the modulation of AD markers across cellular and animal studies.[11]

A key pro-inflammatory molecule involved in the onset and potential progression of AD, as well as other neurodegenerative disorders,

is TNF-α.[10,12,13] Under physiological conditions, low TNF-α levels are implicated in a series of processes mediating vascular function, fetal development, proliferation, and differentiation of macrophages, and immune mechanisms.[14,15] In brain, TNF-α is physiologically involved in regulation of blood-brain barrier function, synaptic plasticity, and scaling, glutamatergic transmission, and adult neurogenesis.[14,15] Treatment of human astrocytic cells with TNF-α results in an upregulation of APP gene transcription and translation via the 5'-untranslated region (UTR), with a consequent increase in APP and Aβ levels.[16] Additionally, TNF-α augments the conversion of APP into pathological forms of Aβ peptides,[17,18] both by increasing β-secretase levels as well as by acting as a potent stimulator of γ-secretase activity.[19,20] In turn, the presence of Aβ plaques induces microglial and astroglial activation,[21,22] which can further amplify production and release of TNF-α and other pro-inflammatory cytokines; this potentially creates a self-propagating cycle. Finally, a single nucleotide polymorphism in the TNF gene has been linked to late-onset AD.[23]

Consequent to its pivotal role in the progression of diffuse apoptotic neuronal cell death, TNF-α is considered a potential therapeutic target for neurodegenerative diseases.[10,12,13,18] Anti-TNF-α antibodies have demonstrated efficacy in the treatment of several systemic inflammatory conditions that include rheumatoid arthritis, psoriasis, and Crohn's disease. [10,13] However, their limited ability to enter brain[13] makes TNF-α antibody-based therapeutics of limited practical value in neurodegenerative conditions, except when administered by the perispinal route.[24] Given this restriction, an alternative means for targeting TNF-α is to use small molecules to inhibit or modulate TNF-α biosynthesis.

Immunomodulatory imide drugs (IMiDs), a drug class that includes thalidomide and its derivatives, are able to reduce TNF-α production by targeting the 3'-UTR of TNF-α mRNA, and ultimately reduce its transcription and translation.[25,26] In addition to the TNF-α inhibitory effect and related anti-inflammatory actions, IMiDs are also characterized by anti-angiogenic and anti-proliferative properties[10,27] but also teratogenicity.[28] Tragically used and then withdrawn in the treatment of morning sickness in the 1950's, thalidomide was successfully repurposed for the treatment of skin disorders related to leprosy, and for multiple myeloma.[29]

2.2 | Rationale

As TNF-α is recognized as a key protein involved in the initiation and continuation of inflammatory processes in AD, we developed a series of thalidomide analogs able to mitigate the overexpression of this cytokine.[10] The third-generation thalidomide analog Pom is chemically characterized by presence of an amino group at the four-position carbon on the phthaloyl ring system[29] (Figure 1A). It is widely used in the treatment of relapsed/refractory multiple myeloma[29] and is reported to be more TNF-α potent and less neurotoxic, versus thalidomide,[30] but similarly teratogenic.[31] Pom is anti-inflammatory, and its neuroprotective properties have been characterized across in vitro and in vivo studies. Specifically, in rodent models of traumatic brain injury, Pom mitigated neuroinflammation as well as neuronal loss, and behavioral impairment.[32,33]

In the current study, we evaluated the potential of 3,6'-DP, a new dithionated Pom analog. It lacks cereblon downstream ubiquitination actions on key neo-substrates, which are essential for Pom's antineoplastic and teratogenic activity[34,35] but not for its anti-inflammatory actions, in 5xFAD transgenic mice. 3,6'-DP and Pom were assessed for their potential to mitigate cognitive impairments known to develop in 5xFAD mice; however, more specifically, we evaluated whether the Aβ-induced neuroinflammation known to develop in this particularly aggressive model of AD drives behavioral impairments or is merely an epiphenomenon.

RESEARCH IN CONTEXT

1. **Systematic review**: We reviewed the scientific literature using classical (eg, PubMed) sources relating to the role of neuroinflammation in Alzheimer's disease (AD) pathology, cognitive impairment, and the amyloid-β hypothesis. Whereas neuroinflammation is an unwavering feature of AD and induced by amyloid-β, whether its presence drives neurodegenerative processes or is an epiphenomenon remains unknown.
2. **Interpretation**: Using a novel immunomodulating imide drug as a pharmacological tool to quell neuroinflammation (demonstrated across cellular and animal inflammatory models), we show in 5xFAD mice that synaptic, neuronal, and behavioral impairments were mitigated in the absence of any change in multiple measures of amyloid-β generation/accumulation. We interpret this as amyloid-β-induced neuroinflammation providing an essential link in the cascade leading to neurodegeneration and cognitive loss, and potentially accounting for failure of amyloid-β centric treatments initiated after amyloid-β neuroinflammation has been triggered.
3. **Future directions**: Our study provides an avenue for future AD clinical studies by using the immunomodulating imide drug class to mitigate neuroinflammation, and a framework to evaluate the sequence of amyloid-β-initiated cascades in driving AD. Additionally, it accounts for past amyloid-β centric drug failures and the presence of high amyloid-β load in healthy individuals who may not have neuroinflammation.

3 | NEW OR UPDATED HYPOTHESIS

Memory deficits and cognitive decline in preclinical AD models as well as patients appear to be caused by synaptic dysfunction and loss in a neuronal degenerative process initiated by aberrant Aβ and other protein aggregation/clearance.[36] We hypothesize that neuroinflammation

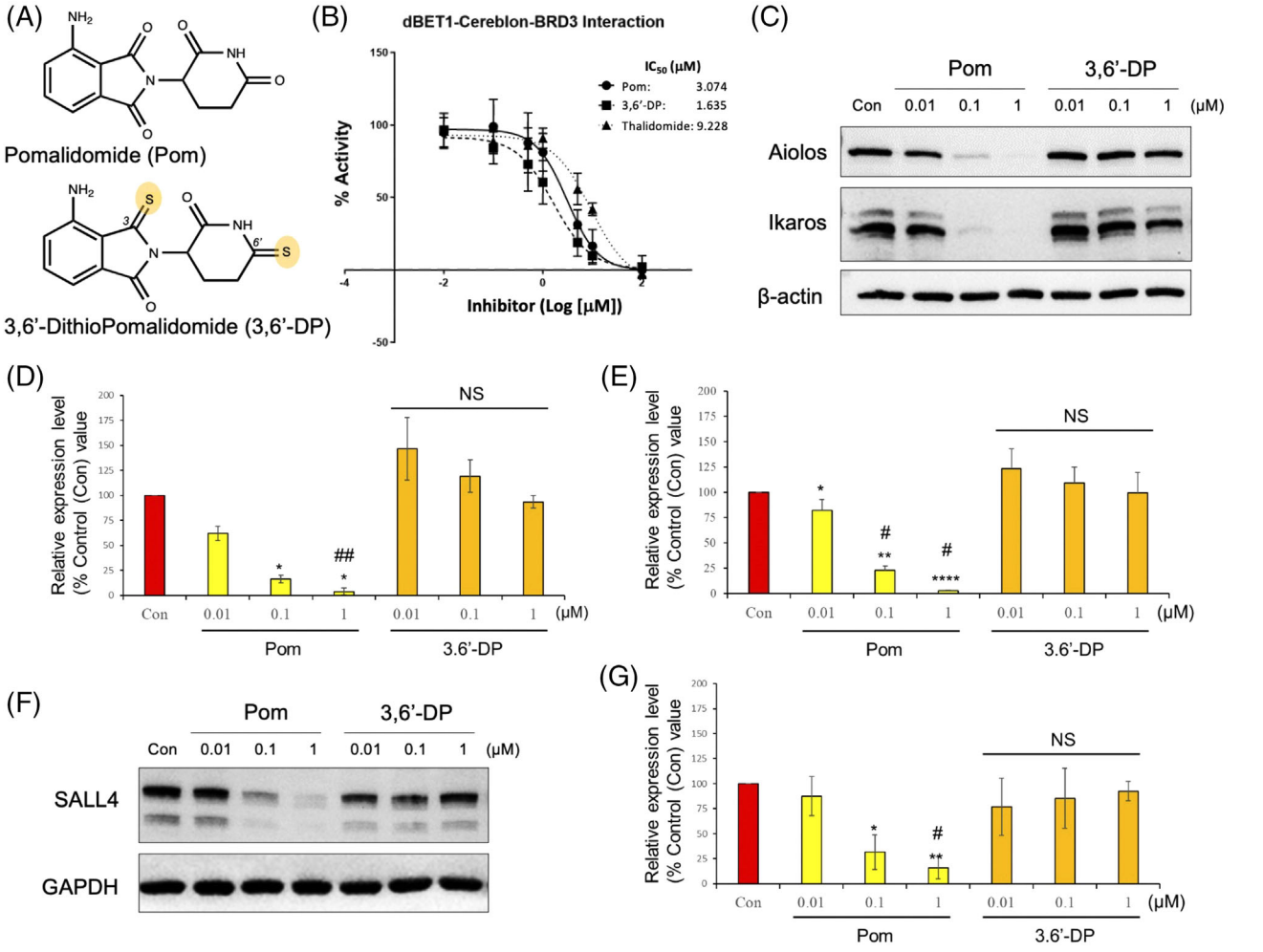

FIGURE 1 3,6'-DP and Pom bind cereblon, but 3,6'-DP does not lower downstream neo-substrates Ikaros, Aiolos, and SALL4. The binding of thalidomide analogs 3,6'-DP and Pom (A) to cereblon was examined by using a cereblon/BRD3 binding FRET assay (B). An initial concentration-dependent evaluation of binding between 3,6'-DP, Pom, and cereblon provided an IC_{50} value of 1.635 and 3.074 μM, respectively. Based on this, degradation of the downstream neo-substrates Ikaros and Aiolos was evaluated in MM.1S cells, and of SALL4 in Tera-1 cells. (C) Concentration-dependent challenge of MM.1S cells with Pom but not 3,6'-DP resulted in a reduction in Ikaros (D) as well as Aiolos (E). Likewise, a concentration-dependent challenge of Tera-1 cells with Pom but not 3,6'-DP (F and G) resulted in a decrease in SALL4. $^{*}P < .05$, $^{**}P < .01$, $^{****}P < .0001$, and NS (not significant) refer to the effects of treatments versus Control (Con). $\#P < .05$, $\#\#P < .01$ refer to the effects of Pom versus 3,6'-DP with the same concentration. Values are presented as mean ± S.E.M., of n observations (n = 2 or 3 per group)

is a key downstream determinant that drives and shapes this process and that excessively activated microglia represent a target underpinning a disproportionate inflammatory response. We hypothesize that 3,6'-DP and alike IMiDs are a means to potentially blunt this response and move it from a detrimental into a beneficial range.

3.1 | Current and new experimental data to support our hypothesis

3,6'-DP and Pom bind cereblon, but 3,6'-DP does not lower downstream neo-substrates Ikaros, Aiolos, and SALL4. In the light of studies demonstrating that the major teratogenicity mechanism of thalidomide and clinical analogs results from their ability to bind cereblon and thereby trigger the ubiquitination and reduction of key downstream neo-substrates,[28] we investigated the interaction of 3,6'-DP with cereblon within the CRL4 complex. Evaluated by a cereblon/BRD3 binding FRET assay, 3,6'-DP and Pom potently bound cereblon with IC_{50} values of 1.635 and 3.074 μM, respectively, in comparison to thalidomide 9.228 μM (Figure 1B). Degradation of Ikaros and Aiolos was quantified in MM1S cells, and of SALL4 in Tera-1 cells (Figure 1C-G). Whereas Pom induced a concentration-dependent reduction in all three proteins, 3,6'-DP did not, which indicates that 3,6'-DP binding to cereblon did not trigger the ubiquitination of these key neo-substrates involved in the anticancer and teratogenic actions of the IMiD drug class, and providing a potentially safer drug to lower TNF-α levels.

3,6'-DP and Pom mitigate markers of Aβ-mediated inflammation, neuronal cell loss, and neurite network loss in primary cortical cultures. Aβ oligomers have been demonstrated to stimulate pro-inflammatory activation of primary microglia and to reduce neuronal survival.[37] With this background, we incubated primary cortical

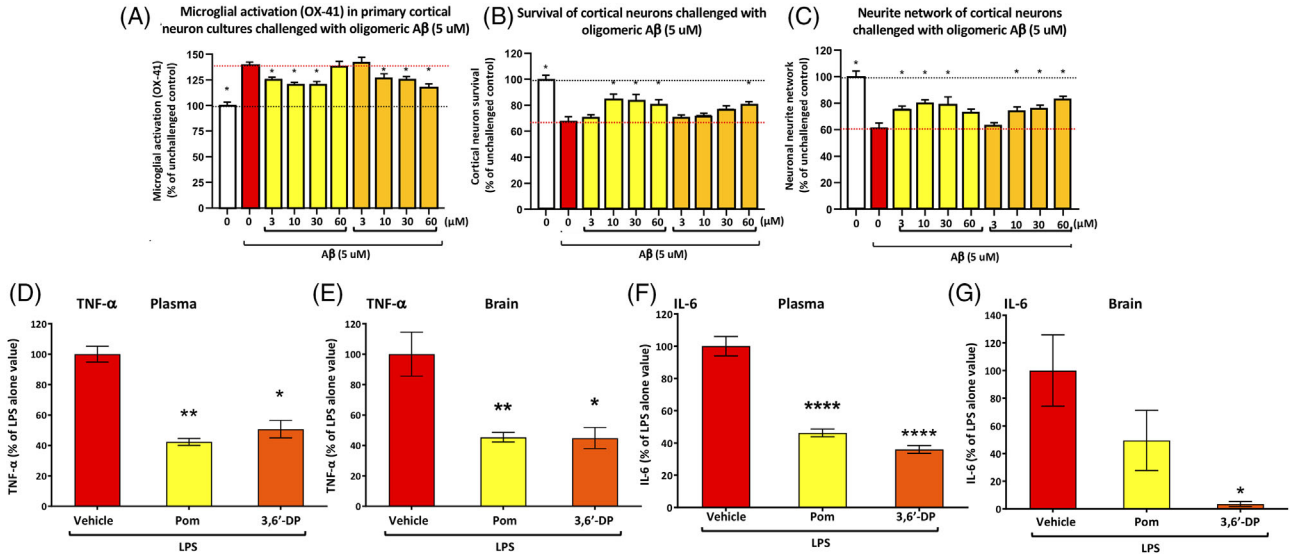

FIGURE 2 3,6'-DP and Pom mitigate inflammation in Aβ-challenged primary cortical cell cultures, and LPS-challenged rodents. (A) Mixed primary cortical cultures containing neurons and microglia were challenged with oligomeric Aβ (5μM) for 72 hours, and their inflammatory status was evaluated by OX-41 immunostaining, demonstrating microglial activation that was ameliorated by 3,6'-DP and Pom (3-60 μM). (B) Neuronal survival was quantified in the presence and absence of 3,6'-DP and Pom, as was (C) neurite number per cell. The addition of oligomeric Aβ (5 uM) to primary co-culture resulted in a reduction in parameters. Pretreatment with 3,6'-DP and Pom significantly mitigated these Aβ-induced effects. Mean ± S.E.M. (n = 5-6/group). *$P < .05$ versus the Aβ alone (red bar) group. Treatment of rodents in vivo challenged with LPS (1 mg/kg, i.p.) significantly elevated levels of proinflammatory cytokines, exemplified by TNF-α in (D) plasma and (E) brain (hippocampus), as well as IL-6 in (F) plasma and (G) brain (hippocampus). Pretreatment of animals with 3,6'-DP or Pom (29.5 or 26.4mg/kg, i.p., respectively) significantly mitigated this LPS-induced increase, thereby exhibiting their anti-inflammatory action. *$P < .05$, **$P < .01$, ****$P < .0001$ refer to the effects of treatments versus LPS + vehicle. Values are presented as mean ± S.E.M., of n observations (n = 4-5/group)

cultures with oligomeric Aβ (5μM x 72 hours) alone and with either 3,6'-DP or Pom in a concentration-dependent manner as an initial phenotypic screen to appraise potential anti-inflammatory and neuroprotective actions. The cellular composition of these primary cortical cultures at the time of treatment was ≈11% microglia, 48% astrocytes with the remains being neurons, in line with Zhang et al.[38] Aβ challenge induced a 33% loss in cell survival (Figure 2A). This was significantly mitigated by both 3,6'-DP and Pom that reduced this cell viability loss by 42.4% and 55%, respectively. In the surviving cortical neurons, neurite extensions were decreased by 38% by Aβ challenge (from 8750 ± 350 to 5385 ± 162 μm). This also was ameliorated by 3,6'-DP and Pom (Figure 2B), providing a maximal 52.7% and 47.3% mitigation of neurite network loss, respectively. Microglial activation, evaluated by OX-41 immunostaining, was significantly elevated by Aβ (Figure 2C) and this, likewise, was significantly abated by 3,6'-DP and Pom (maximally by 55% and 50%, respectively).

3,6'-DP and Pom lower LPS-induced TNF-α and IL-6 levels in plasma and brain. As an early assessment as to whether anti-inflammatory actions evident in cellular studies translate into animals, the ability of 3,6'-DP to diminish a LPS-induced elevation in TNF-α and IL-6 was evaluated in rodents. In accord with a former study,[39] systemic administration of a sub-maximal dose of LPS resulted in a significant rise in plasma TNF-α (Figure 2D), which was elevated from 6.9 ± 0.45 to 803.7 ± 41.7 pg/mL. 3,6'-DP and Pom mitigated this elevation by 49.3% and 57.6%, respectively ($P < .05$). Brain TNF-α levels were elevated by systemic LPS from 2.6 ± 0.7 to 32 ± 4.2 pg/mg tissue, which was mitigated by 55.2% and 54.6%, respectively by 3,6'-DP and Pom ($P < .05$, $P < .01$, Figure 2E). IL-6 plasma and brain levels, likewise, were elevated by LPS, and additionally lowered by 3,6'-DP and Pom (Figure 2F,G, $P < .05$ and $< .0001$). Levels of the anti-inflammatory cytokines IL-10 and IL-13 were less elevated than TNF-α in plasma and brain by LPS administration, and their levels were unaffected by 3,6'-DP and Pom (not shown). Taken together, these results suggest that 3,6'-DP and Pom possess TNF-α lowering anti-inflammatory actions that translate from cellular to in vivo models, with the ability of both agents to reduce markers of brain inflammation following systemic administration.

Neuroinflammatory markers are age-dependently elevated in 5xFAD mice, in line with elevations in Aβ levels and amyloid plaques. In the light of the 3,6'-DP and Pom mitigation of Aβ-induced inflammation and palliation of Aβ's action on survival and neurite loss in our mixed primary cortical culture studies, we evaluated the age-dependent development of neuroinflammation in brain regions pertinent to AD (hippocampus dentate gyrus and cerebral cortex) in 5xFAD mice (for methods see the Supplemental Materials in the Supporting Information). This is an animal model developed to time-dependently generate excessive quantities of human Aβ.[40]

Aβ amyloid plaques were evident in 5xFAD mice as young as 4-months-old, and were particularly apparent in cerebral cortex. They showed a trend to age-dependently increase in hippocampus dentate

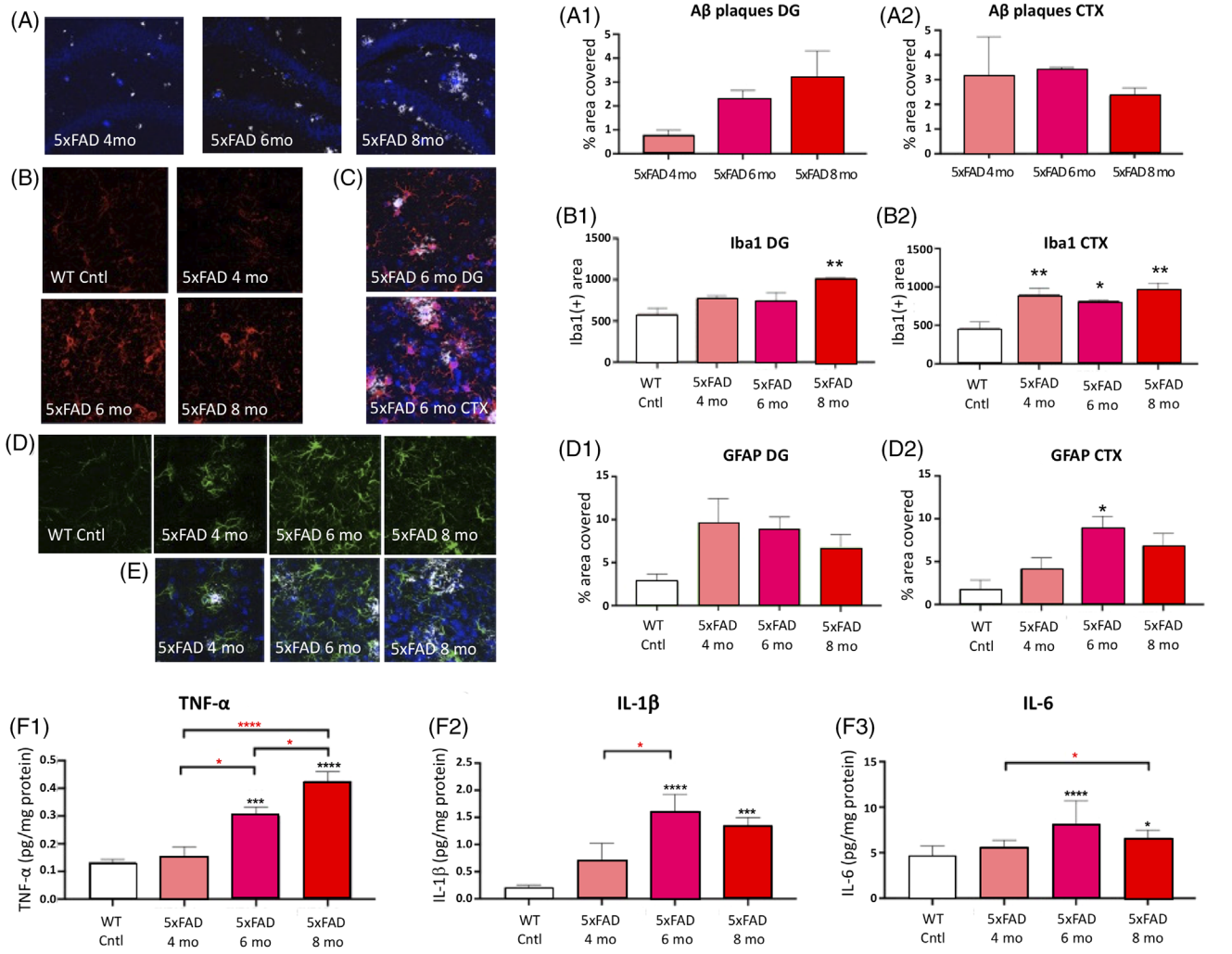

FIGURE 3 Aβ amyloid plaques, activated microglia, elevated expression of glial fibrillary acid protein (GFAP) associated with astrogliosis, and elevated proinflammatory cytokine levels are evident in 5xFAD Tg6799 C57BL6 female mice by 4-months (mo) of age, particularly in cerebral cortex and dentate gyrus, and demonstrate a trend to age-dependently increase. Aβ amyloid plaques: (A) Representative photomicrographs in dentate gyrus from 5xFAD mice of increasing age (4 to 8-months) (white: Aβ amyloid plaques; blue: DAPI [nucleus]). (A1) Quantification of the percent brain area covered by Aβ amyloid plaques across age in dentate gyrus (DG) and (A2) cerebral cortex (CTX). Activated microglia: (B) Representative photomicrographs of Iba1 expression in cerebral cortex from WT (wild type) and 5xFAD mice of increasing age (4 to 8 months). (C) Co-localization of Iba1 positive cells with Aβ amyloid plaques in the cerebral cortex of 6 mo 5xFAD mice (red: Iba1 [microglia]; blue: DAPI [nucleus]; white: Aβ amyloid plaques). (B1) Quantification of the percent brain area covered by Iba1 positive cells across age in dentate gyrus (DG) and (B2) cerebral cortex (CTX). GFAP-associated astrocytosis: (D) Representative photomicrographs of GFAP expression in cerebral cortex from WT and 5xFAD mice of increasing age (4 to 8 months). (E) Co-localization of GFAP positive cells with Aβ amyloid plaques in the cerebral cortex of 6 mo 5xFAD mice (green: GFAP [astroglia]; blue: DAPI [nucleus]; white: Aβ amyloid plaques). (D1) Quantification of the percent brain area covered by GFAP positive cells across age in dentate gyrus (DG) and (D2) cerebral cortex (CTX). Pro-inflammatory cytokines: (F1) TNF-α, (F2) IL-1β and (F3) IL-6 levels were quantified in hippocampal brains samples from WT and 5xFAD 4-, 6-, and 8-month-old mice, and were found to change age-dependently. Protein loading concentration = 200 μg. $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$, and $^{****}P < 0.0001$ for 5xFAD mice versus WT control (cntl) mice. $^{*}P < .05$, $^{****}P < .0001$ for comparison to 5xFAD 4 mo mice. Values are presented as mean ± S.E.M., of n observations (n = 4-5 per group)

gyrus (Figure 3A-A2). These Aβ amyloid plaques were accompanied by microglial cell activation, as evidenced by increased Iba1 expression (Figure 3B-B2,C), and gliosis, as substantiated by an elevated expression of GFAP (Figure 3D-D2,E). Age-dependent increases in brain TNF-α protein levels were apparent by 8-months, accompanied by rises in IL-1β and IL-6 (Figure 3F1-F3). No changes were evident in the levels of IL-2, IL-4, IL-5, or IL-10 either between WT and 5xFAD mice, or across age in 5xFAD mice (data not shown).

Four-month 3,6'-DP and Pom treatment was well tolerated in 5xFAD mice and mitigated behavioral impairments. In the light of clear evidence of neuroinflammation and gliosis expression and their co-localization with Aβ plaques in 5xFAD mice, together with prior studies and reported accompanying behavioral impairments,[40,41] we assessed whether Aβ generation and deposition directly induce behavioral impairments or do so via neuroinflammation and gliosis. We therefore evaluated the action of 3,6'-DP and Pom on behavioral

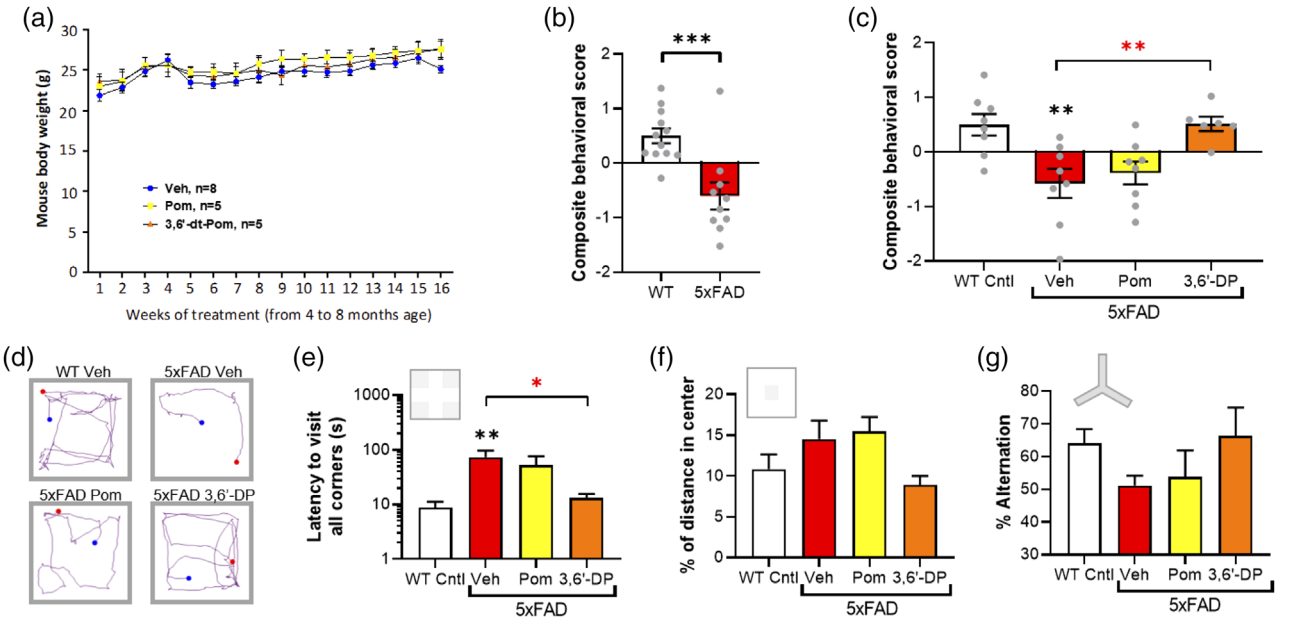

FIGURE 4 3,6'-DP and Pom were well tolerated and the former mitigated behavioral impairments in 5xFAD mice following 4 months treatment. (A) Weekly body weights were no different between 5xFAD mice administered vehicle (1% CMC in 0.9% saline) and those provided a systemic daily dose of 3,6'-DT (29.5 mg/kg) or Pom (26.4 mg/kg). (B) Composite behavioral score in a naïve cohort of 8 to 10-month-old female 5xFAD mice and wild-type littermates. Composite behavioral score was derived by combining behavioral measures depicted in panels E-G below. ***$P < .001$, Student's t-test. (C) Composite behavioral score in a separate cohort of mice following 4 months of daily drug administration. (D) Sample tracks of mice in first 30 seconds of exploration in the open field test. (E) Latency for mice to visit each of the four corners of apparatus. (F) Percent of distance traveled that was in the center zone of the open field. (G) Spontaneous alternation behavior in the Y-maze. **$P < .01$ 5xFAD veh versus WT veh groups, *$P < .05$ 5xFAD veh versus 5xFAD 3,6'-DP groups, **$P < .01$ 5xFAD veh versus 5xFAD 3,6'-DP groups by one-way ANOVA followed by Dunnett's post-hoc tests

measures that are altered by the presence of 5xFAD transgenes resulting in Aβ generation and deposition. We reasoned that a behavioral improvement associated with a selective mitigation of neuroinflammation and gliosis would suggest the direct involvement of these processes in AD-associated cognitive loss, whereas a lack of behavioral improvement in the presence of abated neuroinflammation and gliosis would indicate that these were secondary phenomena. We hence evaluated the ability of 3,6'-DP and Pom to mitigate neuroinflammation and gliosis, and selected the dose found effective in lowering plasma and brain TNF-α levels in our prior LPS study (equivalent to 25 mg/kg thalidomide). This dose proved well-tolerated when administered once daily to 4-month-old mice for 4-months' duration, as appraised by weight gain (Figure 4A). Additional well-being markers (evaluated from subjective measures that included body mass, grooming, and appearance, righting skills, ambulation, and blinking reflex[42]) were evaluated daily, and were maintained across treatment and control groups.

Two behavioral paradigms were evaluated in 5xFAD mice, based on initial testing in a separate, age-matched cohort of 5xFAD mice (Figure S4). Alternation rate in the Y-maze was used to assess spatial working memory. We also found two measures in the open field test highly sensitive in discriminating wild-type from 5xFAD mice. We furthermore measured percent of distance traveled in the center region, which is indicative of reduced anxiety-like behavior in 5xFAD transgenic mice. Additionally, we observed that wild-type mice rapidly patrolled the perimeter when first placed in the open field, whereas 5xFAD mice were delayed to respond. We quantified this by measuring latency to visit each of four 12 × 12 cm corner zones (Figure S4E), an outcome that is likely affected by impairments in working memory, stress reactivity, and motor function. Log_{10} transformation was performed on this measure to obtain a normal distribution. Importantly, abnormalities in spontaneous alternation, anxiety, and motor function have previously been shown to develop in the 6 to 9-month age range of female 5xFAD mice on a C57BL/6J background, similar to the animals used here.[40,43] All three of these metrics were combined to form a composite behavioral score that had greater sensitivity than any individual test measure in detecting 5xFAD behavioral dysfunction. This composite behavioral score differed between wild-type and 5xFAD mice in the validation cohort (Figure 4B; Student's *t* test, $P = .0006$) and between vehicle-treated wild-type and 5xFAD mice in the drug experiment (Figure 4C; Dunnett's post-hoc, $P = .003$) (Figure 4B). Strikingly, 4-months of treatment with 3,6'-DP prevented cognitive behavioral impairments from developing in 5xFAD mice ($P < .01$ vs 5xFAD vehicle).

Four-month 3,6'-DP and Pom treatment mitigates brain inflammation in 5xFAD mice, and abates synaptic loss and neurodegeneration. Microglial activation was evaluated in relation to morphological changes as well as cell proliferation. 5xFAD vehicle-treated animals demonstrated an increase in Iba1 immunoreactivity (IR) in both hippocampus ($P < .001$) and cortex ($P < .0001$), as compared to the

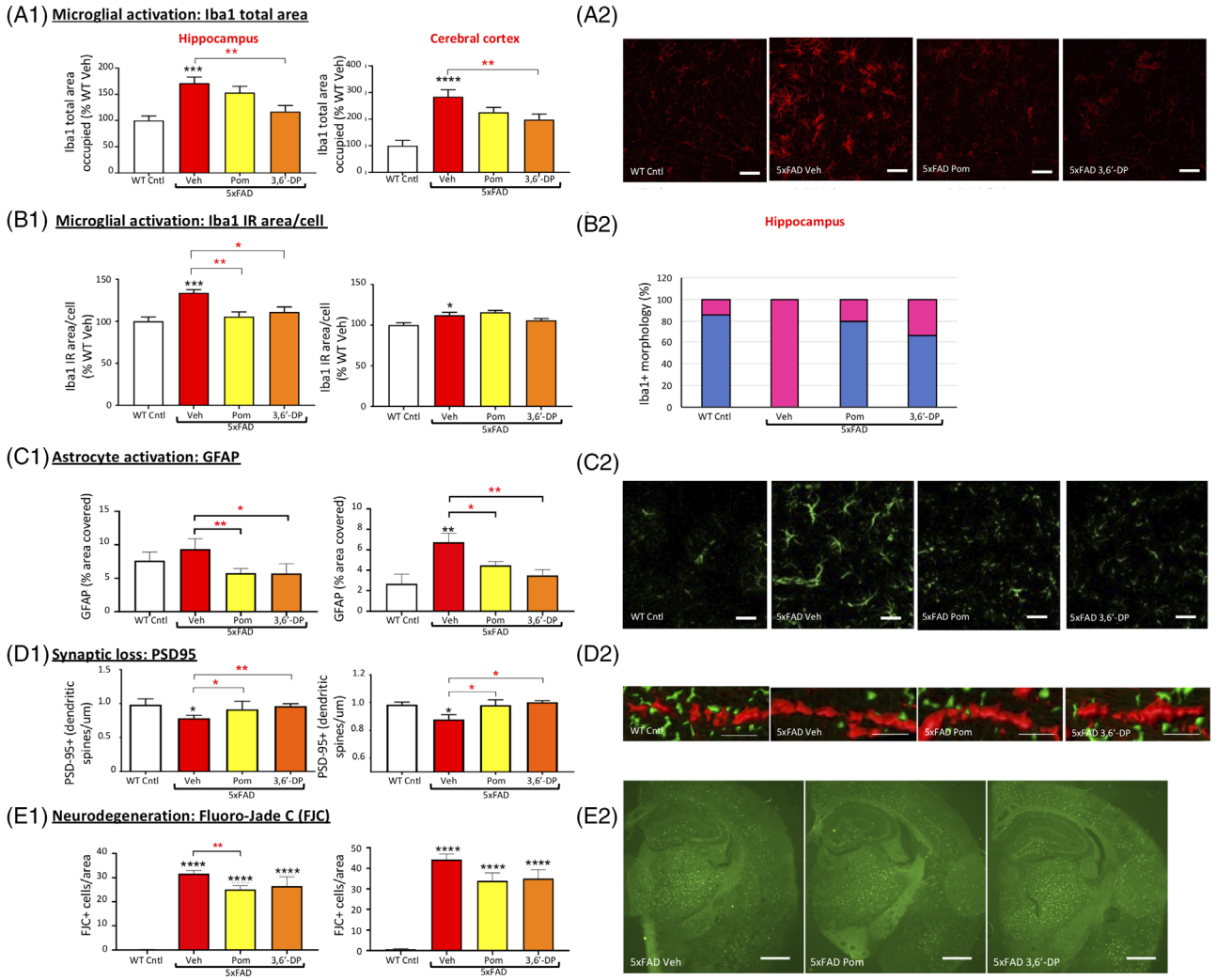

FIGURE 5 3,6'-DP and Pom mitigate brain inflammation in 5xFAD mice, and abate synaptic loss and neurodegeneration. (A) Microglial activation was evaluated by Iba1 immunohistochemistry in both hippocampus and cerebral cortex across WT vehicle (Veh) and 5xFAD animals treated with Veh, 3,6'-DT (29.5mg/kg) or Pom (26.4mg/kg) i.p. daily for 4 months. (A1) Quantification of Iba1 total area occupied as a percent of the WT Veh group, (A2) representative photomicrographs (scale bar = 30 μm). (B) Microglial activation was additionally evaluated in relation to (B1) Iba1 immunoreactivity (IR) per cell, as well as by their morphology into activated (pink) versus quiescent (blue) phenotypes (B2). (C) Gliosis was evaluated by GFAP immunohistochemistry in hippocampus and cerebral cortex across groups. (C1) Quantification of GFAP as a percent of brain area occupied, (C2) representative photomicrographs (scale bar = 30 μm). (D) Dendritic/synaptic loss was evaluated immunohistochemically with postsynaptic density protein 95 (PSD-95) in hippocampus and cerebral cortex across groups. (D1) Quantification of the number of PSD-95+ dendritic spines/μm, (D2) representative images showing PSD-95+ spines (green) in MAP2+ dendrites (scale bar = 3 μm). (E) Neurodegeneration was evaluated by Fluoro-Jade C (FJC) staining in hippocampus and cerebral cortex across animal groups. (E1) The total number of FJC-(+) cells/area of equal neuroanatomical selected areas were calculated for three brain sections per sample. (E2) Representative images of FJC staining (20×, tile scan over entire area of the brain section, scale bar = 1 mm). *$P < .05$, **$P < .01$, ***$P < .001$, ****$P < .0001$ versus WT Veh group; *$P < .05$, **$P < .01$ versus 5xFAD Veh group by one-way ANOVA followed by Dunnett's post-hoc test. (n = 4-5/group)

WT vehicle control group (Figure 5A1,A2). Treatment with 3,6'-DP attenuated this microglial activation in both brain regions (expressed as total area occupied, $P < .01$). Pom demonstrated a trend to mitigate 5xFAD microglial activation that failed to reach statistical significance ($P = .0665$ for cortex; $P = .5574$ for hippocampus). In a further characterization of microglial cell inflammatory status in response to AD, the Iba1 IR area/cell was determined to define phenotype/activation stage. In response to neuronal injury, microglia swell, and reduce the size of their processes to, thereby, transform into a "reactive" form (Figure 5B1) associated with inflammatory and phagocytic features.[44] Based on their morphology and IR, Iba1-positive cells were classified into different morphological states: ramified and intermediate types as the resting/surveillance state and amoeboid and round types as the activated/reactive state (Figure 5B2). AD progression induced an elevation in the Iba1 IR area/cell, consistent with an active/reactive phenotype. Treatment of 5xFAD mice with 3,6'-DP or Pom mitigated this elevation and, to a large part, returned microglial cells to a quiescent/surveillant morphology (Figure 5B1,B2).

Gliosis was evaluated by quantifying the total area covered by GFAP-(+) cells, which encompassed both changes in number and the morphology of astrocytes, and is manifested as an increase in astrocyte activation in vehicle-treated 5xFAD animals ($P < .01$ cerebral cortex). Treatment with either 3,6'-DP or Pom reduced this astrocyte activation ($P < .01$ for 3,6'-DP; $P < .05$ for Pom) (Figure 5C1,C2).

To appraise whether the behavioral improvements observed in 5xFAD mice treated with 3,6'-DP were associated with changes in synaptic integrity, we evaluated expression of the post-synaptic protein PSD-95 in cortex and hippocampus (Figure 5D1). Whereas vehicle-treated 5xFAD mice demonstrated a 20% decline in the number of PSD-95+ dendritic spines in both hippocampus and cortex, compared to WT vehicle animals ($P < .05$), treatment with 3,6'-DP fully reversed this loss in hippocampus and cerebral cortex ($P < .01$ and $P < .05$, respectively), as did Pom ($P < .05$ for both brain regions) (Figure 5D1,D2).

Finally, FJC, a fluorescein-based dye that predominantly labels degenerating neuronal cells,[45] was used to evaluate cellular loss in the brain of 5xFAD mice. As illustrated in Figure 5E1,E2, whereas minimal degenerating neuron numbers were apparent in the WT, there were substantial degenerating neurons in the 8-month-old 5xFAD group. 3,6'-DP and Pom mitigated this by ≈20%, which reached statistical significance for Pom in hippocampus ($P < .05$).

3,6'-DP and Pom showed mitigation of neuroinflammation, neuronal, and synaptic loss, and improved behavioral outcome in 5xFAD mice occur in the absence of actions on amyloid plaque and Aβ burden. Given the very high levels of Aβ generation in the brain of 5xFAD mice, and in accord with prior studies by Oakley et al.,[40] robust amyloid plaque deposition was noted in hippocampus and cerebral cortex of 5xFAD mice at 8-months age (Figure 6A). Evident in Figure 6A,B, treatment with 3,6'-DP or Pom had no effect on amyloid plaque load (evaluated by the percent brain area covered). In line with large amyloid plaque accumulation, high levels of Aβ42 and 40 were evident in brain, and increased age-dependently from 4 to 8-months (by 6.7- and 3.7-fold, respectively ($P < .0001$, $P < .01$) (Figure 6C1,C2), thereby age-dependently elevating the Aβ42/40 concentration ratio ($P < .01$) (Figure 6C3). Notably, drug treatment had no impact on concentrations of either soluble or insoluble Aβ42 or 40, or the ratio of these peptides (Figure 6D-D3,E1-E3).

Accordingly, we found that the composite behavioral score, which was greatly different between 5xFAD and wild-type mice, did not correlate with any amyloid-related measurements (Figure 6F). We included in this analysis all six individual amyloid measurements as well as the first principal component of a PCA analysis, which accounted for 86.3% of variances within the amyloid data. Interestingly, there was a non-significant trend ($r = -0.67$, $P = .10$) for cortical Aβ40 tissue content to correlate with the behavioral composite score when assessed only within the AD vehicle group (Figure 6G). The correlation was in the expected direction of effect; that is, more amyloid protein was associated with stronger behavioral impairment. This correlation was greatly diminished when assessed among all 5xFAD mice together ($r = -0.18$, $P = .45$). Even when drug treatment was included as a linear model term, no amyloid measurement correlated with the composite behavioral score in 5xFAD mice (Table S2), suggesting that 3,6'-DP treatment prevents the development of cognitive behavioral symptoms through a mechanism independent of the amyloid secretory pathway.

3.2 | Additional and future experiments for validation data

The anti-inflammatory potential of 3,6'-DP has been validated in other animal models of neuroinflammation. In order to assess its ability to lower systemic and central levels of pro-inflammatory cytokines, 3,6'-DP was administered intraperitoneally (i.p.) together with a dose of LPS (1mg/kg, i.p.), a classic model of inflammation in rats. 3,6'-DP mitigated LPS-induced elevations in pro-inflammatory TNF-α and IL-6 protein levels in both plasma and brain (Figure 2D-G). Moreover, in a controlled cortical impact model of moderate traumatic brain injury, 3,6'-DP inhibited microglial and astroglial activation in the injured area both acutely (24 hours[46]) and longer-term (7 days[47]), and fully mitigated elevations of mRNA and protein level expressions of TNF-α, IL-6, and IL-1β.[46] Consequent to its anti-inflammatory action, 3,6'-DP demonstrated neuroprotective actions by reducing neuronal death and the volume loss in the brain region affected by the controlled cortical impact, as well as mitigating the behavioral impairment.[46,47] This, in part, cross-validates the ability of 3,6'-DP to lessen neuronal cell death evident in our AD model (Figure 5E). This TBI model is of particular relevance to our AD study as an increased risk of dementia in individuals with a TBI diagnosis has been reported in numerous but not all epidemiological evaluations.[48–50] Finally, behavioral impairments in 5xFAD mice were validated in a separate cohort of mice in Figure S4.

Our future studies are focused on the fundamental requirements that 3,6'-DP should meet in order to be considered as a potential human drug candidate. Our recent studies demonstrate that 3,6'-DP lacks genotoxicity, as assessed by the in vitro chromosomal aberration assay and bacterial reverse mutation (AMES) assay, and that it appears safe in cardiac cells as appraised by hERG patch clamp assay (data not shown). Nevertheless, extensive toxicity and toxicokinetic studies in both male and female small and large animal species, in line with FDA guidelines, are essential to evaluate the translational potential of 3,6'-DP to humans, and drug to support these studies is currently being generated.

4 | MAJOR CHALLENGES FOR THE HYPOTHESIS

A primary challenge in targeting neuroinflammation as a potential therapeutic strategy relates to an inability of many systemically administered anti-inflammatory drugs, and particularly those targeting TNF-α, to effectively enter and achieve therapeutic concentrations in brain without inducing systemic adverse events. Notably, 3,6'-DP has a favorable CNS multiparameter optimization score of 5.5 that is predictive for an agent possessing desirable drug-like properties for neurological action, and it appears to readily enter the brain (brain/plasma concentration ratio 0.8).[46] A recent Phase 2 AD clinical

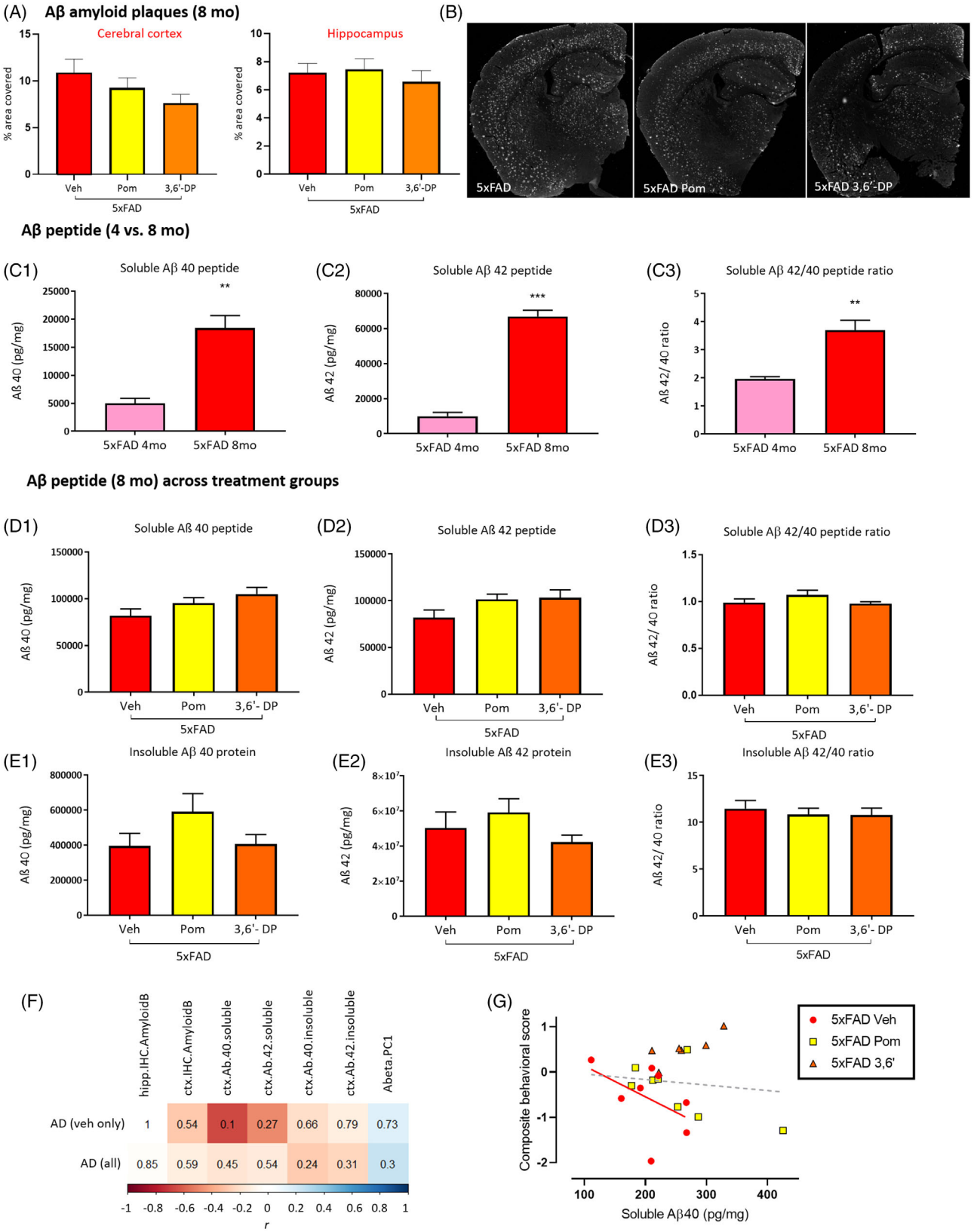

FIGURE 6 3,6'-DP and Pom mediated mitigation of neuroinflammation, neuronal, and synaptic loss, and improved behavioral outcome in 5xFAD mice occur in the absence of actions on amyloid plaque and Aβ burden. Aβ plaque load was evaluated by immunohistochemistry using a polyclonal anti-β-amyloid antibody (Cell Signaling, #24545) in both hippocampus and cerebral cortex across WT vehicle (Veh) (no activity and not shown) and 5xFAD animals treated with Veh, 3,6'-DT (29.5 mg/kg) or Pom (26.4 mg/kg) i.p. daily for 4 months. (A) Quantification of Aβ plaque total area occupied, (B) representative photomicrographs. There was no statistical difference across groups ($P > .05$). Brain (cerebral cortex) Aβ peptide concentrations were quantified by ELISA for both Aβ42 and Aβ40 forms across age (4 to 8 mo) as well as by treatment. (C) By age: Aβ42 (C1) and

trial of the first-generation IMiD, thalidomide, failed likely due to the development of adverse events prior to achieving the targeted dose to attain a meaningful reduction in TNF-α and proinflammatory cytokine levels.[13] Pom and analogs are more potent TNF-α lowering IMiDs[35] and appear less neurotoxic than thalidomide.[30]

Another possible challenge relates to the dual healing/detrimental nature of inflammatory mechanisms. The neuroinflammatory process can be considered as a double-edged sword, with both a protective and a toxic role. Targeting the inflammatory response as a therapeutic approach requires the identification of a balance between when to intervene and to what extent, in order to preserve the beneficial role of inflammatory mechanisms.

5 | LINKAGE TO OTHER MAJOR THEORIES

Neuroinflammation is increasingly being considered as a potential therapeutic target in AD. In response to CNS insults, microglia can modify their cellular phenotype, migrate, proliferate, and profoundly change their gene expression profile. There is a resulting generation and release of signaling factors.[10,51,52] These responses can, at first, be neuroprotective but often lead to later harmful, chronic neuroinflammation when a protracted insult remains unresolved and results in microglia that excessively secrete pro-inflammatory proteins[51,52] and induce inflammatory phenotypes in astrocytes.[53] Although the effect of neuroinflammation at various disease stages is still disputed, recent findings suggest that inflammatory processes are not just a simple consequence of the pathology-associated damage but have a more central role on the initiation and development of disease-related changes. This role of neuroinflammation as a disease trigger could be a consequence of disproportionate pro-inflammatory factor generation as well as a compromise of their routine homeostatic function.

The neuroinflammatory response in AD is not an isolated mechanism independent from the other pathophysiological events occurring during the development of the disease. Specifically, the cellular and molecular mediators of inflammatory processes can indirectly affect cognitive function, being involved in the control of synaptic plasticity. Glial cells, in particular, play a pivotal role in the maintenance of synaptic development and function. Astrocytes are known to be structurally and functionally associated with synaptic elements. They are involved in postnatal synaptogenesis, from the initial phases of synapse formation through their maturation and strengthening.[54] Astrocytes stimulate structural synaptic shaping by releasing a series of chemical signals, including thrombospondins TSP1 and 2 and SPARCL1/hevin.[55] In addition, astrocytes contribute to functional synapse formation by positively regulating AMPA receptor levels in postsynaptic terminals through the secretion of glypican 4 and 6.[56] Microglial cells, likewise, play an important part in regulating synaptic plasticity, inducing axon outgrowth and dendritic spine formation,[57] as well as controlling neural activity by creating direct contacts with axon terminals through their processes.[58] Microglial cells also are fundamental to the process of pruning; removing weak or non-functioning synapses is essential to continuously preserve and optimize brain function in the developing and mature brain by actively shaping neural circuitry.[57] A primary mechanism through which microglial cells mediate their many actions is via cytokine release.[10,14,15,52]

In light of these considerations, it would not be surprising that an altered glial status, such as a condition of chronic activation as seen in neurodegenerative diseases, may lead to disruption of modulation of synaptic activity and, ultimately, to synaptic dysfunction. Numerous cytokines and complement system components, particularly C1q and C3, are markedly upregulated in AD rodent models, mediating early synapse loss.[59] The depletion of selected pro-inflammatory cytokines and of C1q and C3 can reduce synapse loss in AD animal models.[60,61] Several human observational studies have evaluated the association of TNF-α inhibitor use as a common treatment of rheumatoid arthritis with the later development of dementia and have found mixed results, in part, hampered by small sample size and short follow-up time.[62] However, a recent retrospective case-control study of the electronic health records from 56 million adult patients demonstrated systemic inflammation was associated with a higher risk for AD, and the use of TNF-α blocking agents significantly reduced AD risk.[63] Furthermore, in a recent retrospective evaluation with follow-ups of up to 20 years, TNF-α inhibitor use was associated with lower long-term risk of dementia/AD among US veterans.[62] Microglia also appear involved in direct Aβ-mediated mechanisms of synapse elimination. In rodent models of AD, the presence of soluble Aβ oligomers is associated with neuronal glutamate uptake interference; this contributes to an impairment in long-term potentiation[64] and facilitation of long-term depression, ultimately leading to a weakening of synaptic networks. One of several proposed mechanisms involves a "tagging" action of Aβ oligomers on dendritic spines that leads to a recruitment of microglial cells to eliminate these Aβ-tagged synapses.[65] In line with this literature, 5xFAD mice in our study demonstrated a substantial decrease in

Aβ40 (C2) levels increased age-dependently, as evaluated at 4 through 8 months. A greater elevation in Aβ42 levels resulted in an increase in the Aβ 42/40 concentration ratio at 8 versus 4 months. $^{**}P < .01$, $^{****}P < .0001$ versus 4 mo group (n = 4-5/group). Protein loading concentration = 25 μg. (D, E) By treatment (initiated at 4 mo age and dosed to and evaluated at 8 mo): No statistically significant differences were evident either in soluble Aβ42 (D1) and Aβ40 (D2) or in insoluble Aβ42 (E1) and Aβ40 (E2) levels across treatment groups (Veh, 3,6'-DT (29.5mg/kg) or Pom (26.4mg/kg) i.p. daily for 4 months). The ratios of the Aβ 42/40 forms were calculated in the soluble (D3) and insoluble (E3) fractions, and likewise, were not statistically different across treatment groups ($P > .05$, n = 4-5/group). Protein loading concentration for detection of soluble Aβ = 25 μg. Protein loading concentration for detection of insoluble Aβ = 1μg. (F) Correlation matrix for all seven amyloid-related measurements (columns) assessed within 5xFAD Veh only mice (top row) or all 5xFAD mice (bottom row). Pearson correlation coefficient is indicated by panel color, with legend at bottom. Number within each panel indicates *P*-value for each comparison. (G) Scatterplot of composite behavioral score versus cortical soluble Aβ40. Red line is best-fit line for 5xFAD veh group only; dashed gray line is fit for all 5XFAD mice

their number of PSD-95 dendritic spines in hippocampus and cortical layer V (Figure 5D1), as compared to WT mice of the same age. 3,6'-DP countered dendritic spine loss in AD mice in both analyzed regions (Figure 5D1/D2), and did so more effectively than Pom.

Together these findings argue that microglia are key factors determining changes in neuronal viability and synaptic function that underpin impaired cognition in AD, and are causative in this detrimental cascade. Our cellular studies are in line with this notion, and demonstrate 3,6'-DP- or Pom-mediated reduction of microglial activation in cortical cultures, challenged with a neurotoxic concentration of Aβ, reduces the loss of both dendritic neural networks and neuronal loss (Figure 2). In translational studies in 5xFAD mice, 3,6'-DP reduction of neuroinflammation, likewise, resulted in mitigation of synaptic loss, neuronal degeneration, and notably, cognitive decline occurring in the "absence" of changes in Aβ (40 or 42) production or amyloid plaque formation (Figure 6) (ie, in the constantly present and unchanged challenge of Aβ). These data, importantly, place neuroinflammation downstream of Aβ and both upstream and causative, rather than an epiphenomenon, in the processes of neuronal dysfunction, loss, and behavioral impairment. In support of this, global manipulation of microglial phenotype during aging has been reported to improve cognition,[66] and microglia elimination in AD mice reduced neuronal loss, augmented memory function, and partially prevented AD pathology progression, similarly with little effect on Aβ levels and plaque load.[67,68] Microglial elimination provides a powerful tool to elucidate their role in health, aging, and disease, but may never be translatable to humans. Blunting persistent, excessive microglial, and astrocyte activation mediated by Aβ and secondary factors with a new generation of IMiDs, such as 3,6'-DP, likewise provides a tool to understand where neuroinflammation fits within the cascade of synaptic and neuronal loss that leads to AD cognitive and behavioral impairments. Our studies place neuroinflammation as a direct effector and provides a therapeutic strategy that complements and extends the amyloid hypothesis.

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CONFLICT OF INTERESTS

3,6'-Dithiopomalidomide (3,6'-DP) is protected under US Patent 8,927,725 to support its development for the treatment of neurodegenerative disorders. DT, WL and NHG are named inventors on this patent and have assigned all their rights to the National Institutes of Health (US Government), and hence have no ownership of 3,6'-DP or other agents within US Patent 8,927,725. DSK is the founding scientist and CEO of Aevis Bio, Inc., and its US division AevisBio, Inc., which have a Cooperative Research and Development Agreement with the National Institute on Aging, National Institutes of Health, in relation to the evaluation of thalidomide analogs in preclinical models of neurodegenerative disorders. IH, YKK and SK are employees of Aevis Bio, Inc. All other authors declare that they have no competing interests.

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