

Soluble α -synuclein–antibody complexes activate the NLRP3 inflammasome in hiPSC-derived microglia

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Parkinson's disease is characterized by accumulation of α -synuclein (α Syn). Release of oligomeric/fibrillar α Syn from damaged neurons may potentiate neuronal death in part via microglial activation. Heretofore, it remained unknown if oligomeric/fibrillar α Syn could activate the nucleotide-binding oligomerization domain (NOD)-like receptor (NLR) family pyrin domain-containing 3 (NLRP3) inflammasome in human microglia and whether anti- α Syn antibodies could prevent this effect. Here, we show that α Syn activates the NLRP3 inflammasome in human induced pluripotent stem cell (hiPSC)-derived microglia (hiMG) via dual stimulation involving Toll-like receptor 2 (TLR2) engagement and mitochondrial damage. In vitro, hiMG can be activated by mutant (A53T) α Syn secreted from hiPSC-derived A9-dopaminergic neurons. Surprisingly, α Syn–antibody complexes enhanced rather than suppressed inflammasome-mediated interleukin-1 β (IL-1 β) secretion, indicating these complexes are neuro-inflammatory in a human context. A further increase in inflammation was observed with addition of oligomerized amyloid- β peptide (A β) and its cognate antibody. In vivo, engraftment of hiMG with α Syn in humanized mouse brain resulted in caspase-1 activation and neurotoxicity, which was exacerbated by α Syn antibody. These findings may have important implications for antibody therapies aimed at depleting misfolded/aggregated proteins from the human brain, as they may paradoxically trigger inflammation in human microglia.

neuroinflammation | Parkinson's disease | Lewy body dementia | Alzheimer's disease | antibody therapies

Parkinson's disease (PD) is characterized by accumulation of α -synuclein (α Syn; encoded by the *SNCA* gene) (1). Release of oligomeric/fibrillar α Syn from damaged neurons may potentiate neuronal cell death in part via microglial activation (2, 3). Moreover, misfolded proteins in general are thought to interact with brain microglia, triggering microglial activation that contributes to neurodegenerative disorders, although microglial phagocytosis may also initially clear aberrant proteins to afford some degree of protection (2, 4). Additionally, in Alzheimer's disease (AD), amyloid- β peptide (A β) is thought to trigger similar processes in microglia (5–7); however, the mechanism for this trigger is still poorly understood.

Microglial cells contribute to neuroinflammation, specifically that mediated by the inflammasome. In particular, the nucleotide-binding oligomerization domain (NOD)-like receptor (NLR) family pyrin domain-containing 3 (NLRP3) inflammasome has been associated with several neurodegenerative disorders, although other types of inflammation may also be important in this regard (8). The NLRP3

inflammasome is a multiprotein complex that responds to cell stress and pathogenic stimuli to promote activation of caspase-1, which in turn mediates maturation and release of proinflammatory cytokines, including interleukin-1 β (IL-1 β) and IL-18 (9–11). NLRP3 inflammasome activation is a two-step process, involving an initial priming step and a secondary trigger. Priming involves a proinflammatory stimulus, such as endotoxin, a ligand for Toll-like

Significance

Release of oligomeric/fibrillar α -synuclein (α Syn) from damaged neurons contributes to neuronal cell death in Parkinson's disease and other neurodegenerative disorders in part via microglial activation. Here, we show that α Syn activates the NLRP3 inflammasome in human induced pluripotent stem cell (hiPSC)-derived microglia (hiMG) via dual stimulation involving TLR2 engagement and mitochondrial damage. Oligomerized amyloid- β peptide (A β), as found in Alzheimer's disease brains, exacerbates this neuroinflammation. Importantly, we found that misfolded proteins such as α Syn and A β , when bound to antibody and presented to hiMG, result in enhanced proinflammatory response of the NLRP3 inflammasome. These findings may have important implications for antibody therapies aimed at depleting misfolded/aggregated proteins from the human brain, as they may paradoxically trigger neuroinflammation in human microglia.

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Competing interest statement: S.A.L. and G.H. are coauthors on a published consensus statement review of cell-death criteria along with several dozen other authors who are authorities in this field [N. M. C. Connolly et al., *Cell Death Differ.* 25, 542–572 (2018)]. That manuscript was published in order to help nonexperts in the field understand and use criteria for various types of cell death. They also published a similar type of review paper together 10 y ago [G. E. Hardingham, S. A. Lipton, *Antioxid. Redox Signal.* 14, 1421–1424 (2011)]. However, S.A.L. and G.H. have never formally collaborated or worked together on any laboratory-based scientific project, including the current work.

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receptor 4 (TLR4), that increases the abundance of NLRP3 and promotes de novo synthesis of pro-IL-1 β via nuclear factor κ B (11). The secondary trigger promotes inflammasome complex assembly and caspase-1 activation that in turn mediates the cleavage of pro-IL-1 β and subsequent release of mature IL-1 β . There are various secondary triggers, including adenosine triphosphate (ATP), microparticles, and bacterial toxins, all of which somehow lead to mitochondrial damage and release of oxidized mitochondrial DNA (11). Neuroinflammation has been reported in both human PD and AD brains (12–15), and NLRP3 inflammasome activation in particular has been observed in mouse models of PD and AD (7, 16). Importantly, in these PD models, dopaminergic (DA) neurons in the substantia nigra are resistant to damage in NLRP3-deficient mice compared with wild-type (WT) mice (16). Interestingly, a recent report identified an NLRP3 polymorphism that confers decreased risk in PD (17). Several groups have reported that fibrillar α Syn can activate the NLRP3 inflammasome in mice and in human monocytes (18–22), but it remains unknown if human brain microglia can be activated in this manner. Critically, antibodies targeting misfolded proteins are being tested in human clinical trials for several neurodegenerative diseases, including AD and PD; however, it is still unclear how antibodies to α Syn might affect this inflammatory response. In this study, we characterized the response of human induced pluripotent stem cell (hiPSC)-derived microglia (hiMG) to oligomeric/fibrillar α Syn in vitro and in vivo, using engraftment of hiMG in humanized mice. We used these immunocompromised mice because they prevent human cell rejection and express three human genes that support human cell engraftment (23). We show that α Syn and, even more so, α Syn-antibody complexes activate the NLRP3 inflammasome. Moreover, this process is further sensitized by the presence of A β and its cognate antibodies. These observations are of heightened interest because recent studies have shown that both misfolded A β and α Syn are present in several neurodegenerative disorders such as AD and Lewy body dementia (LBD), a form of dementia that can occur in the setting of PD (24–26).

Results

Generation and Characterization of hiPSC-Derived Microglia-Like Cells. To study neuroinflammatory pathways in molecular detail in a human context, we modeled the normal yolk sac and erythromyeloid embryonic program of brain microglial development to produce a simple 21-d differentiation protocol that generates microglia-like cells from hiPSCs more rapidly than previously reported procedures (27–30). A schematic of the differentiation process is provided in Fig. 1A. Differentiation was initiated with formation of floating embryoid bodies (EBs). Starting from days 3 to 4 of differentiation, cystic EBs were formed and continued to expand in size over the next 4 d (SI Appendix, Fig. S1A). EBs harbored a heterogeneous population of cells, a subset of which displayed coexpression of the yolk sac markers CD41, c-Kit, CD144 (VE-cadherin), and CD235a (glycophorin) (31) (SI Appendix, Fig. S1B).

Throughout the differentiation procedure, the cells exhibited temporal patterns of gene expression corresponding to successive stages of microglial embryonic development (SI Appendix, Fig. S1C). For example, at early stages of differentiation (day 4), cells expressed mix paired-like homeobox (*MIXL1*) (32) and apelin receptor (*APLNR*) (33). Between days 4 and 11, cells displayed messenger RNA expression of kinase insert domain receptor [*KDR*; also known as *FLK1* (31)], platelet-derived growth factor subunit A (*PDGF α*), and ETS variant 2 [*ETV2*; also known as *ER71* (33)], and, subsequently, runt-related transcription factor 1 (*RUNX1*) and TAL bHLH transcription factor 1, erythroid differentiation factor [*TAL1*; also known as *SCL* (34)]. Notably, from days 17 to 21, the cells expressed high levels of colony-stimulating factor 1 receptor (*CSF1R*), interferon regulatory factor 8 (*IRF8*), crystallin β B1 (*CRYBB1*), MAF bZIP

transcription factor B (*MAFB*), and *CD14*, a group of microglia-specific factors that were recently shown to represent mature microglia (35). Interestingly, by day 21, the cells also exhibited decreased levels of *CSF1*, a phenomenon indicative of microglia maturation (35) (SI Appendix, Fig. S1C). After 21 d of differentiation, the adherent cell population resulted in hiMG exhibiting morphology and markers indistinguishable from human brain-derived microglia (36, 37). They manifested an irregular shape with ramified processes that varied in size and length, and all of the cells expressed the microglial markers transmembrane protein 119 (TMEM119), Iba1, and purinergic receptor P2Y12 (P2RY12) (Fig. 1B–D) (38–41). TMEM119 and P2RY12 are associated with a homeostatic microglia state, suggesting that our hiMG cells were not activated when cultured and required additional stimulation to acquire a disease-associated microglial phenotype and proinflammatory state (39, 40). Moreover, flow cytometry confirmed the high purity of the hiMG population in our cultures, with >80% of the cells expressing CD11b, CSF1R, and CD14 (Fig. 1E and SI Appendix, Fig. S1D).

To identify genes activated in hiMG cells, we performed RNA sequencing (RNA-seq). In comparison with parental hiPSCs, we identified 2,100 genes with significantly lower and 2,232 genes with significantly higher expression in hiMG (Dataset S1A). As expected, we found lower expression of genes associated with pluripotency in hiMG, including *POU5F1* (*OCT4*), *NANOG*, *SOX2*, and *DNMT3B*. In support of a bona fide microglial identity, genes with elevated expression in hiMG were enriched for functional annotations consistent with cytokine/chemokine function, immune response, Toll receptor signaling, integrin signaling, interleukin signaling, and constituents of lysosomes (SI Appendix, Fig. S2A and Dataset S1B). We analyzed additional RNA-seq data from published human brain microglial cells and other monocyteoid cells to offer further support for the identity of our hiMG (SI Appendix, Fig. S2 and Dataset S2).

Next, we examined the function of hiMG in contexts relevant to their activation in the brain. Microglia serve as phagocytic cells and are thus involved in removal of pathogens, isolation of aggregated/misfolded proteins, and pruning of synapses (42). Indeed, indicative of their phagocytic capability, our hiMG engulfed zymosan-pHrodo bioparticles, displaying fluorescent signal only in lysosomes (SI Appendix, Fig. S3A). Another functional characteristic of microglia is proinflammatory activation. Under basal conditions, our hiMG did not produce proinflammatory cytokines and demonstrated gene expression reminiscent of quiescent microglia. Lipopolysaccharide (LPS or endotoxin) stimulation resulted in a dose-dependent increase in secretion of proinflammatory cytokines, including IL-6 and tumor necrosis factor (TNF). Additionally, LPS engendered a dose-dependent increase in mRNA expression of IL-1 β (SI Appendix, Fig. S3B) and a concomitant decrease in CSF1R and fractalkine receptor CX₃CR1 (SI Appendix, Fig. S3C); CSF1R and CX₃CR1 expression are known to negatively correlate with proinflammatory activation (43, 44). Interestingly, LPS also induced dose-dependent decreases in TREM2 and CD33 (SI Appendix, Fig. S3C), whose down-regulation has been shown to be associated with brain inflammation in AD (45). Thus, while our in vitro hiMG may not completely mimic gene expression of human brain microglia in vivo (46, 47), they do provide a platform to study the mechanistic details of inflammatory activation since the expression level of these genes is similar (SI Appendix, Figs. S1 and S2).

Oligomeric/Aggregated α Syn Activates the NLRP3 Inflammasome. To investigate the effect of soluble α Syn assemblies on NLRP3 inflammasome activation in our hiMG, we generated monomeric α Syn and oligomeric/aggregated α Syn preparations, which are known to contain fibrils (48) (Fig. 2A). The oligomeric preparation exhibited a higher molecular mass than monomers and contained larger particles based on dynamic light scattering (DLS)

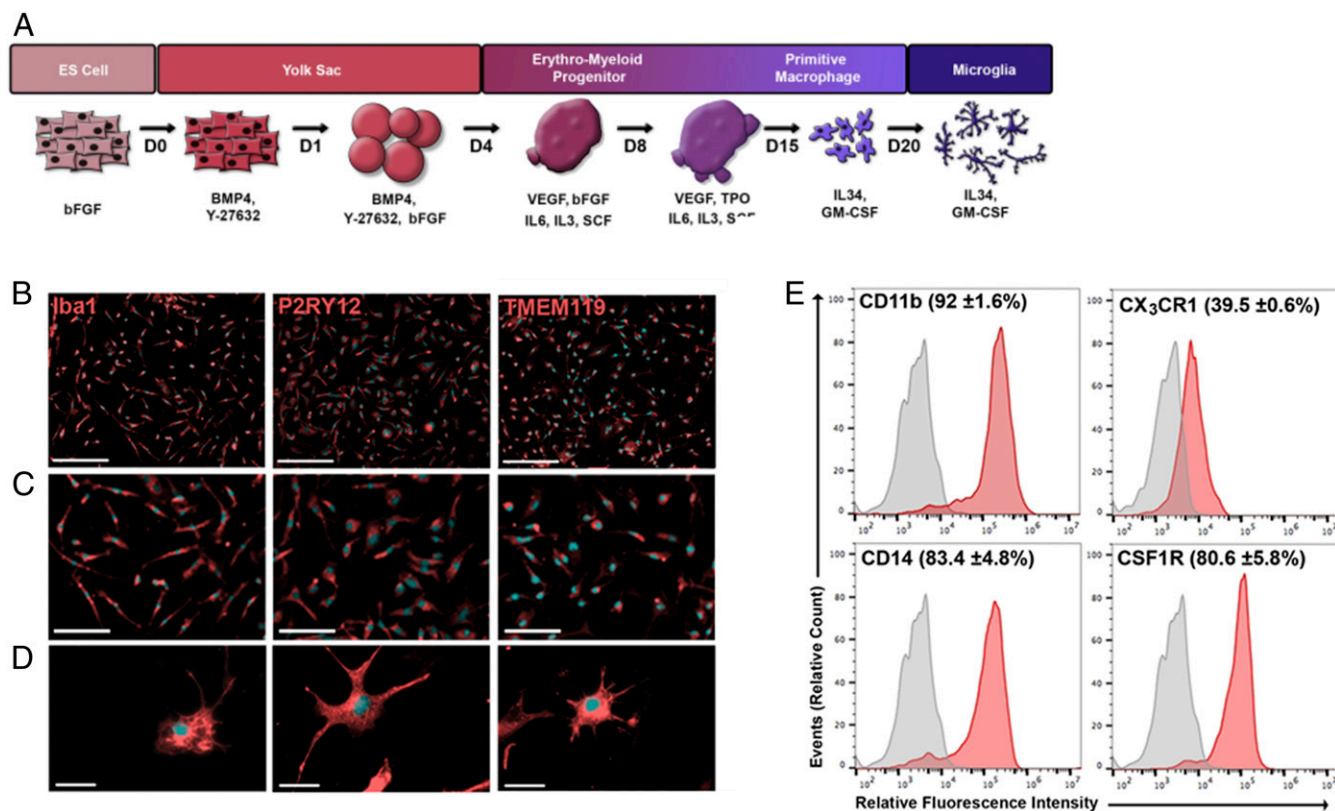


Fig. 1. Differentiation of human iPSCs into microglia-like cells. (A) Schematic representation of the differentiation protocol showing media composition and culture conditions. (B) Immunocytochemical characterization of hiMG (red) and Hoechst nuclear staining (blue). (Scale bars, 200 μ m.) (C) Higher magnification showing marker expression is evident in virtually every cell. (Scale bars, 100 μ m.) (D) Even higher magnification images showing individual cell morphology and marker expression. (Scale bars, 25 μ m.) (E) Flow cytometry analysis of cell-surface microglial markers. Representative histograms of CD11b-APC, CX₃CR1-PE, CD14-APC, and CSF1R-APC; primary antibody (red) and unstained control (gray). Labels above each histogram indicate the mean percentage of positive cells and SEM ($n = 3$ biological replicates).

(Fig. 2A and *SI Appendix*, Fig. S3D and E). We then examined NLRP3 inflammasome activation in hiMG using well-established primers and activators, such as LPS and Alum/Nigericin/ATP (11), all of which increased IL-1 β secretion (Fig. 2B). Importantly, incubation of hiMG with oligomeric α Syn stimulated IL-1 β secretion and caspase-1 activation in a dose-dependent manner, and was far more active than monomeric α Syn (Fig. 2C and D and *SI Appendix*, Fig. S3F). This effect was NLRP3-dependent since inhibiting NLRP3 by small interfering RNA knockdown blocked IL-1 β release (Fig. 2E and *SI Appendix*, Fig. S3G and H). Additionally, MCC950, a pharmacological NLRP3 antagonist (49), attenuated IL-1 β release (*SI Appendix*, Fig. S3J). This finding is consistent with the hypothesis that α Syn oligomers provide both of the necessary triggers for NLRP3 inflammasome priming and activation, and thus does not require a priming signal, consistent with a previous observation in mice (21). After exposure to oligomeric α Syn, we also found increased secretion of TNF and IL-6, further indicating proinflammatory activation (*SI Appendix*, Fig. S3J). Of note, the minor degree of activation observed with α Syn monomers may well have resulted from incipient oligomer formation, as observed within 2 h of incubation at 37 $^{\circ}$ C (*SI Appendix*, Fig. S3E).

α Syn has been reported to bind to TLR2 and TLR4 (50, 51). To determine which TLR is involved in α Syn-mediated inflammasome activation, we used neutralizing antibodies. We found that neutralizing TLR2 nearly completely inhibited IL-1 β release (Fig. 2F). TLR2-neutralizing antibody also reduced IL-6 and TNF release following activation induced by α Syn oligomers (*SI Appendix*, Fig. S3J). In contrast, neutralizing TLR4 had no effect on IL-1 β release (Fig. 2F). This lack of effect was not the result

of inefficient neutralization of TLR4 because the TLR4-neutralizing antibody completely blocked LPS-induced IL-6 and TNF release (*SI Appendix*, Fig. S3K). These results suggest that α Syn binding to TLR2 leads to hiMG release of IL-1 β , and may be responsible for priming (the initial step in NLRP3 inflammasome activation).

To determine how α Syn leads to the second signal for NLRP3 inflammasome activation in hiMG, we examined whether oligomeric α Syn induced mitochondrial damage by assessing production of mitochondrial reactive oxygen species (mtROS), maintenance of mitochondrial membrane potential ($\Delta\Psi$ m), and release of mtDNA into the cytosol, the three mitochondrial signals linked to inflammasome activation (11, 52). We found that oligomeric/aggregated α Syn induced a significant increase in mtROS generation (Fig. 2G and H), a decrease in $\Delta\Psi$ m (Fig. 2I and J), and an increase in cytosolic mtDNA (Fig. 2K). Previously, α Syn overexpression has been reported to induce mitochondrial damage in neurons (53), and α Syn mutant-initiated cardiolipin exposure has been reported to trigger mitochondrial damage and mitophagy (54). Hence, the mitochondrial damage observed in hiMG in the present study may contribute to NLRP3 inflammasome activation.

Neuronal α Syn and α Syn-Antibody Complexes Induce Inflammasome-Related Cytokine Production by hiMG. To study the interplay of hiMG and neurons mechanistically, we utilized a “disease-in-a-dish” model of human PD; for this purpose, we cocultured hiMG with hiPSC-derived A9-type DA neurons bearing the A53T α Syn mutation or with gene-corrected isogenic control neurons (WT), each plated on a bed of astrocytes (55, 56). In these cocultures,

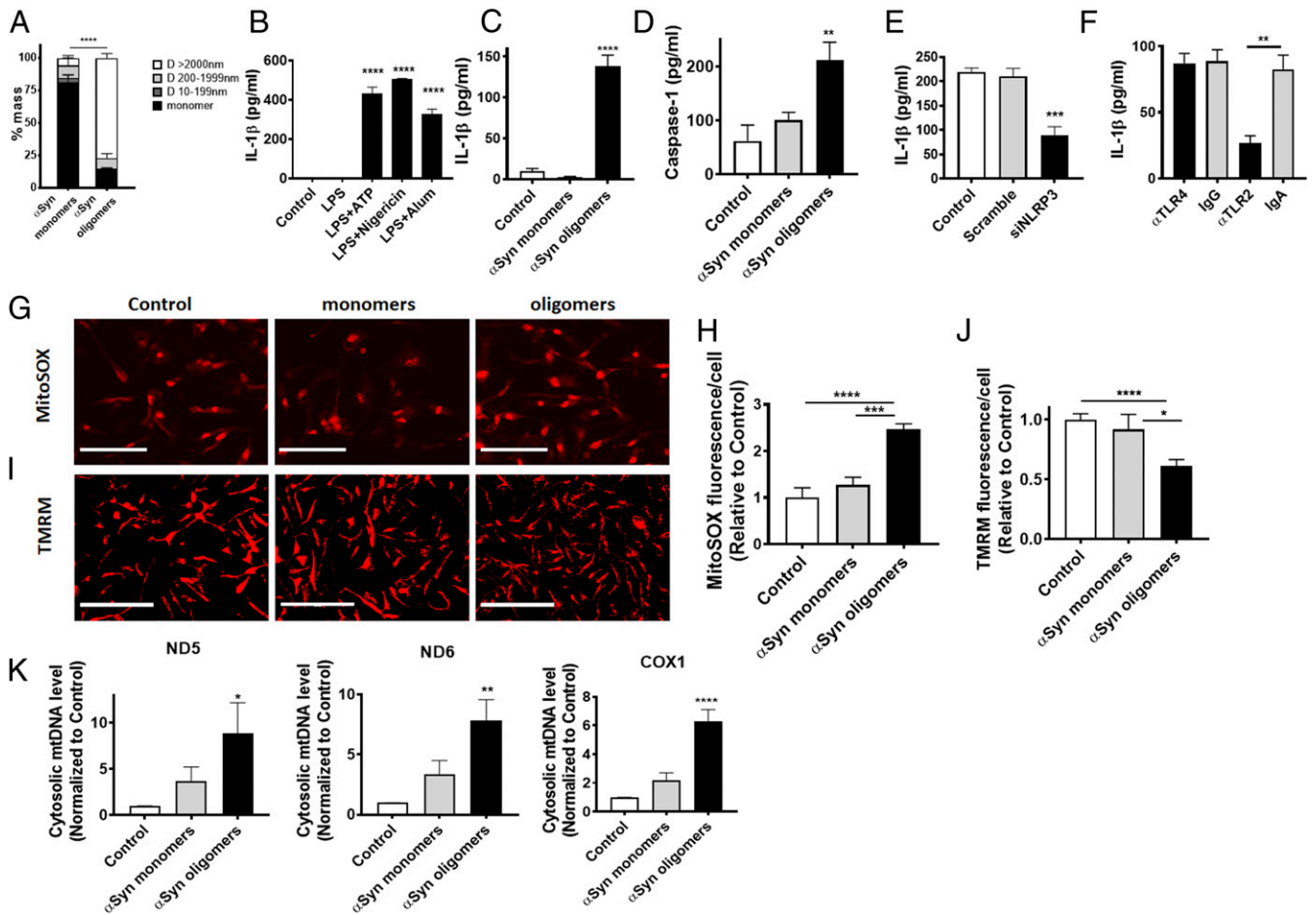


Fig. 2. Oligomeric α Syn provides two signals for NLRP3 inflammasome activation. (A) Dynamic light scattering analysis of the particle size of the two types of α Syn preparations used in this study— α Syn monomers and oligomer/aggregates. Fractions of monomer vs. aggregated α Syn categorized by hydrodynamic diameter (D) on DLS are shown in different shading. Two-way ANOVA followed by Sidak's multiple-comparisons test revealed a significant difference between the two preparations for monomeric α Syn as well as aggregates $>2,000$ nm. No significant difference was found for aggregates of 10 to 200 and 200 to 2,000 nm (light gray and dark gray bars). (B) Quantification of IL-1 β secretion following inflammasome activation with LPS + ATP/Alum/Nigericin ($n = 5$ per group). (C) IL-1 β secretion following exposure to α Syn monomers and oligomers (750 nM, $n = 10$ per group). (D) Cleaved caspase-1 (p20) release following exposure to α Syn monomers and oligomer (750 nM, $n = 5$ to 9). (E) IL-1 β secretion following NLRP3 knockdown and exposure to oligomeric α Syn (750 nM, $n = 5$ per group). (F) Quantification of IL-1 β following treatment with neutralizing antibodies against TLR2 and TLR4 or controls (IgA and IgG, respectively) ($n = 6$ per group). (G) Representative images of MitoSOX fluorescence reflecting mitochondria-generated ROS following vehicle (control) and monomer and oligomer α Syn exposure (750 nM). (Scale bars, 100 μm .) (H) Quantification of MitoSOX fluorescence after exposure to monomeric or oligomeric α Syn (750 nM, $n = 9$ per group). (I) Representative images of tetramethylrhodamine methylester (TMRM) fluorescence, assessing mitochondrial membrane potential, following control and monomer and oligomer α Syn exposure (750 nM). (Scale bars, 200 μm .) (J) TMRM quantification after exposure to monomeric or oligomeric α Syn (750 nM, $n = 5$ to 10). (K) mtDNA release of ND5, ND6, and COX1 following exposure to α Syn monomers and oligomers (750 nM, $n = 3$ to 5). Graphs indicate mean \pm SEM. Statistical analysis was performed using two-way ANOVA with Bonferroni post hoc test (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$).

hiMG developed a highly ramified morphology, reminiscent of microglia in vivo (Fig. 3A). To investigate the effect of endogenous α Syn on the NLRP3 inflammasome in this system, we collected conditioned medium (CM) from control or A53T DA neuronal cultures, which manifest increased production of mutant, aggregated α Syn (Fig. 3B) (57). We found that medium from A53T neurons (A53T CM) induced significantly more IL-1 β release from hiMG than CM from isogenic controls (Fig. 3C). To show that the inflammatory effect of A53T neuronal CM was indeed triggered by α Syn, we performed an in vitro immunodepletion experiment on the CM by using anti- α Syn antibody (Ab) coupled to protein-A/G beads. Since this technique should decrease α Syn, it would be expected to decrease inflammasome activation by the CM. We confirmed that α Syn was depleted from the CM by enzyme-linked immunosorbent assay (Fig. 3D). As predicted, this resulted in decreased IL-1 β release from hiMG (Fig. 3E). Next, in

order to more closely mimic treatment in humans, we added a variety of anti- α Syn Abs to the media without depleting them using beads. Unexpectedly, adding these antibodies, including humanized anti- α Syn single-chain variable fragment (scFv) Ab, known to bind monomeric and oligomeric α Syn (58), further increased IL-1 β release rather than preventing it. In contrast, isotype-matched control immunoglobulin G (IgG) manifested no such effect. This deleterious effect of α Syn Abs was observed in the presence of either oligomerized recombinant α Syn or A53T CM (Fig. 3 F and G). Importantly, anti- α Syn Ab alone did not induce IL-1 β release (SI Appendix, Fig. S44), suggesting that this effect was specific to the Ab- α Syn complex and not to a direct effect of antibody. Moreover, scFv lacks the Fc domain that binds to Fc receptors on microglia (59), providing further support for a specific effect of the Ab- α Syn complex. Similar increases in inflammatory cytokines were observed with misfolded protein complexed to antibodies

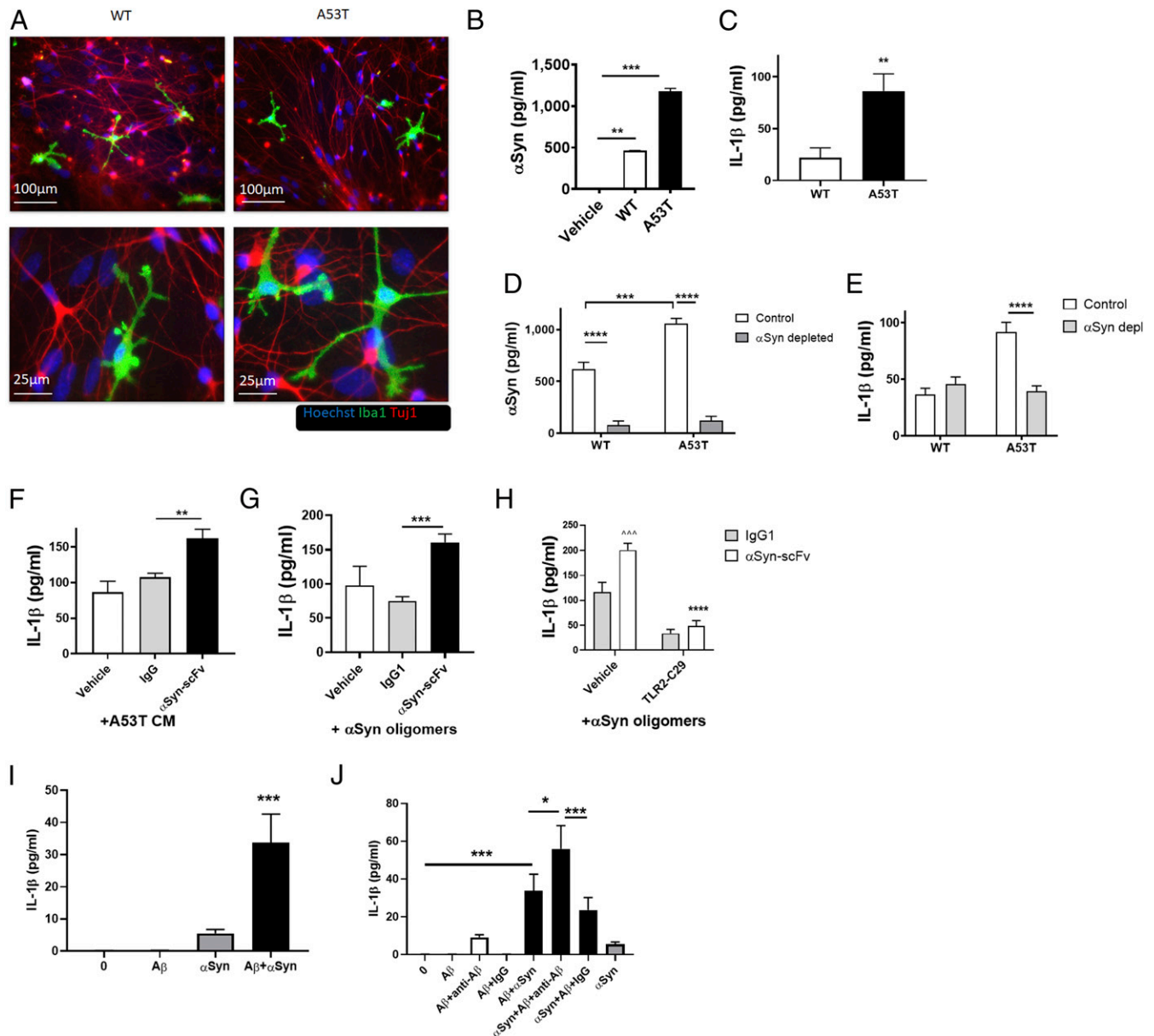


Fig. 3. A53T α Syn mutant CM from hiPSC-derived DA neurons and Ab- α Syn complex activate the inflammasome in hiMG. (A) Representative images of astrocyte/DA neuron/microglia coculture of A53T and isogenic control (WT) neurons. Iba1 (green), Tuj1 (red), and Hoechst (blue). (A, Top) Low magnification. (Scale bars, 100 μ m.) (A, Bottom) Higher magnification. (Scale bars, 25 μ m.) (B) α Syn levels in CM from WT and A53T CM ($n = 4$). (C) IL-1 β release from hiMG exposed to CM from WT and A53T DA neurons ($n = 6$ to 8). (D) Secreted α Syn levels in α Syn-immunodepleted CM from isogenic corrected (WT) and A53T DA neurons ($n = 4$ per group). (E) IL-1 β release from hiMG exposed to α Syn-immunodepleted CM from isogenic corrected (WT) and A53T DA neurons ($n = 7$ to 11). (F) IL-1 β release from hiMG exposed to CM from isogenic corrected (WT) and A53T DA neurons pretreated with humanized α Syn scFv or human IgG1 control. Note the increased effect of Ab on CM from A53T over isogenic corrected (WT), consistent with their increased production of α Syn oligomers ($n = 6$ to 8). (G) Quantification of IL-1 β from hiMG exposed to oligomeric α Syn (750 nM) pretreated with humanized α Syn scFv or human IgG1 control ($n = 5$ to 10). (H) IL-1 β secretion from hiMG exposed to oligomeric A β (10 μ M) and low concentrations of oligomeric α Syn (100 nM) with anti-A β Ab, compared with IgG control. ^Comparison with vehicle-IgG. *Comparison with vehicle-scFv ($n = 7$ to 9). (I) IL-1 β release from hiMG following TLR2 inhibition. ($n = 7$). (J) IL-1 β release from hiMG exposed to oligomeric A β (10 μ M), low concentrations of oligomeric α Syn (100 nM), or a combination, showing a synergistic effect ($n = 7$ per group). Graphs indicate mean \pm SEM. Statistical analysis was performed using a two-way ANOVA with Bonferroni post hoc test (* $P < 0.05$, ** $P < 0.01$, ^^^ P or **** $P < 0.001$, **** $P < 0.0001$).

generated in mouse or rabbit (SI Appendix, Fig. S4 B and C). Importantly, this inflammatory effect was not seen in mouse primary microglia with any of the antibodies (e.g., mouse, rabbit, sheep, or goat) (SI Appendix, Fig. S4D). Furthermore, TLR2 inhibition with TLR2-IN-C29, an inhibitor of TLR2/1 and TLR2/6 signaling (60), ameliorated NLRP3 inflammasome activation in hiMG in this setting (Fig. 3H), implying that TLR signaling was involved mechanistically.

A β Oligomers and A β -Antibody Complexes Induce Inflammasome-Related Cytokine Production by hiMG. Next, to generalize this effect to other misfolded proteins and other diseases, we exposed hiMG to oligomeric A β . We found that A β oligomers alone did not provide the two signals necessary for activation of the inflammasome. Notably, however, the combination of misfolded A β with very low concentrations of α Syn, which alone did not induce inflammasome activation, resulted in robust, synergistic inflammasome activation

(Fig. 3*I*). A β has been shown to contribute to activation of the NLRP3 inflammasome in a process dependent on TLR4 (61). The synergistic effect of A β and α Syn could result from engagement of both TLR2 and TLR4 to induce a stronger immune response. For example, A β may provide additional secondary signal for NLRP3 inflammasome activation (62). Moreover, addition of anti-A β scFv-Fc Ab increased IL-1 β in a similar manner to anti- α Syn Ab, suggesting a generalized effect of antibody treatment on inflammasome activation in hiMG (Fig. 3*J*). Taken together, these findings are consistent with the notion that the Ab- α Syn complexes are in fact immunoinflammatory by acting on hiMG, and this effect may be unique to human microglia as it was not observed with mouse microglia. Moreover, A β and the A β -Ab complex may intensify this effect on hiMG, which is relevant given recent findings that A β and α Syn aggregates are both often found in the human brain in neurodegenerative diseases such as AD and LBD (24–26).

Antibody- α Syn Complex Induces Inflammation and Neuronal Cell Death in hiMG Engrafted into Mouse Brain. To investigate whether hiMG can also induce inflammasome-like activity *in vivo*, we engrafted hiMG into the brains of humanized mice; these mice support human cells by expressing three human genes: IL-3, CSF2, and KITLG (23). In this series of experiments, we stereotactically injected hiMG into the ventricles of adult mice at 4 wk of age. We injected either hiMG alone, hiMG together with α Syn oligomers, or hiMG with α Syn oligomers and antibody. We then evaluated the mice 2 wk later for inflammasome activation and neuronal cell death. Engraftment of hiMG with α Syn triggered caspase-1 activity, as indicated by the presence of cleaved caspase-1 in the engrafted hiMG. Notably, α Syn oligomers injected with humanized anti- α Syn scFv Ab induced a significant increase in the caspase-1 response. In contrast, unstimulated hiMG manifested minimal caspase-1 activity after engraftment in the brain (Fig. 4*A* and *B*). Moreover, engrafted hiMG demonstrated the presence of apoptosis-associated speck-like protein

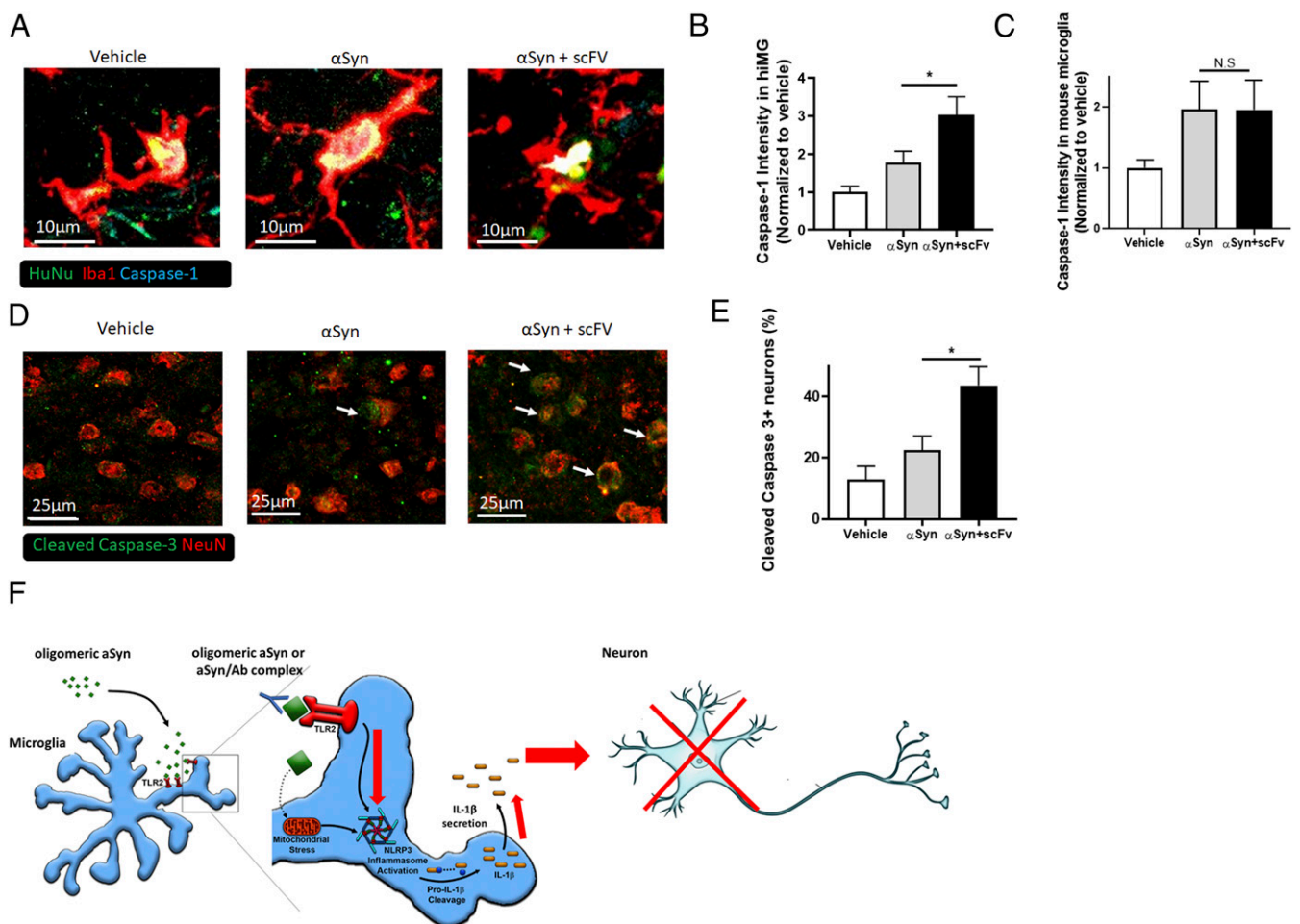


Fig. 4. Ab- α Syn complex activates the inflammasome and induces neuronal death in hiMG-engrafted mice. (A) Representative images showing cleaved/activated caspase-1 staining in transplanted hiMG: hiMG only (Left), hiMG with α Syn (Middle), or hiMG with α Syn and Ab scFv (Right). Human nuclear antigen (HuNu; green), Iba1 (red), and activated caspase-1 (cyan). (Scale bars, 10 μ m.) (B) Quantification of caspase-1 intensity in engrafted hiMG (Iba1⁺/HuNu⁺ cells) ($n = 6$ per group). (C) Quantification of caspase-1 intensity in endogenous mouse microglia (Iba1⁺/HuNu⁻ cells) ($n = 6$ per group). (D) Representative images of cleaved/activated caspase-3 staining in endogenous mouse neurons after transplantation of hiMG only (Left), hiMG with α Syn (Middle), or hiMG with α Syn plus scFv (Right). Cleaved caspase-3 (green, indicated by arrows) and NeuN (red). (Scale bars, 25 μ m.) (E) Quantification of cleaved caspase-3 in neurons (NeuN⁺ cells) ($n = 6$ per group). (F) Schema showing the effect of α Syn on NLRP3 inflammasome activation. Oligomeric/fibrillar α Syn is released from DA neurons, and more so from A53T DA neurons. Oligomeric/fibrillar α Syn activates the NLRP3 inflammasome by providing two triggers: Stimulation of TLR2 provides the priming trigger, and induction of mitochondrial damage and/or ROS production provides the secondary trigger. This leads to the assembly of the NLRP3 inflammasome, which results in secretion of IL-1 β and caspase-1, and contributes to caspase-3-associated neuronal cell death. Ab- α Syn complexes enhance inflammasome-mediated IL-1 β secretion in a TLR2-dependent manner. Red arrows indicate increased response to the Ab- α Syn complexes. Graphs indicate mean \pm SEM. Statistical analysis was performed using a two-way ANOVA with Bonferroni post hoc test (* $P < 0.05$; N.S., not significant, $P > 0.9$).

containing a caspase activation and recruitment domain (CARD) specks (63), indicating inflammasome activation by the presence of complexes of apoptosis-associated speck-like protein containing a CARD (*SI Appendix, Fig. S5A*). Remarkably, mouse microglia contiguous to hiMG did not manifest an increase in caspase-1 activation or ASC specks after injection of Ab- α Syn complexes compared with α Syn alone (Fig. 4C), indicating that enhanced inflammation in response to Ab- α Syn is unique to human microglia. These findings are consistent with the notion that α Syn-stimulated hiMG induce inflammasome activation and Ab- α Syn complexes exacerbate this response in vivo, similar to the effect that we had found in vitro.

Critically, we also found that neurons in the hiMG- plus α Syn-injected brains underwent increased cell death, as evidenced by the presence of cleaved/active caspase-3 in the neurons, and this effect was further exacerbated with the addition of antibody (α Syn + scFv) (Fig. 4D and E). Unstimulated (control) hiMG did not induce significant neuronal cell death under similar conditions. As a control for an effect of inflammatory hiMG vs. misfolded α Syn by itself on the mouse brain, when hiMG were preexposed to α Syn aggregates and then extensively washed prior to engraftment, these hiMG also induced neuronal cell death (*SI Appendix, Fig. S5B–E*). This finding is consistent with the notion that under these conditions the increase in neurotoxicity was a result of hiMG inflammatory activation rather than merely a direct effect of α Syn on the neurons or other cells in the mouse brain. Collectively, these results provide evidence that α Syn-stimulated hiMG can induce NLRP3 inflammasome activation and neuronal cell death in vivo, and these adverse effects are markedly increased by the presence of Ab- α Syn complexes.

Discussion

The data presented here show that α Syn aggregates, particularly when complexed to cognate antibodies, activate the inflammasome in hiMG. As such, these results have important potential implications for immunotherapies aimed at neurodegenerative disorders because misfolded protein-antibody complexes may trigger a profound microglial inflammatory response in the human context but not in the mouse, where most preclinical experiments have been performed. Along these lines, in prior work on α Syn-overexpressing mouse models of PD, antibodies directed against α Syn were shown to attenuate synaptic and axonal damage, reduce loss of tyrosine hydroxylase fibers, and improve neurobehavioral outcomes, while also decreasing cell-to-cell propagation of α Syn and microglial neuroinflammatory markers such as Iba1 (64). Such findings have led to human clinical trials with antibodies against aberrant misfolded proteins, but we are concerned that our results of increased microglial activation triggered by α Syn protein-antibody (and further enhanced by A β -antibody complexes) in a human context with hiMG (Fig. 4F) may not be faithfully reproduced in these mouse models. For example, we suggest that the recent failure of a human phase II clinical trial of α Syn antibodies (65) may, at least in part, have been due to increased inflammation, and human clinical trials with A β antibodies may be suffering a similar fate (but see ref. 66). Further along these lines, recent work engrafting human microglia into humanized mice has demonstrated that human microglia manifest distinct transcriptome differences from their mouse counterparts (67). In addition, human microglia have been shown to be more heterogeneous than mouse microglia, displaying a more varied cell repertoire, transcriptome, and proteome (46, 68).

While differences between mouse and human microglia have been reported, another potential explanation of the difference observed here is the difference in the source of the cells, namely primary cells or hiPSC-derived cells. Despite the similarity between hiMG and in vivo microglia, there are some differences. Future studies to test this may use mouse iPSC-derived microglia and human ex vivo microglia. There have been several differentiation

protocols for generating mouse iPSC-derived microglia (69, 70), although many of them do not follow the yolk sac pathway used here. Ex vivo human microglia have been isolated in previous reports (46, 71), although they also manifest differences from the in vivo cells, particularly when considering the age of the subjects used as donors.

Our finding of hiMG-induced neurotoxicity is supported by recent reports of inflammasome activation in mouse models of PD expressing the A53T α Syn mutation (21) as well as in genetic AD models (62). The inflammatory side effect mediated by hiMG could potentially be averted if protein-antibody complexes could somehow be removed, or if other immune modulators could be used to prevent the inflammation induced by the complexes. Targeting TLR2 signaling to block inflammasome activation might be beneficial in preventing the adverse effects of antibody treatment, as we show in vitro, because inhibition of TLR2 was recently shown to alleviate some of the harmful effects of α Syn in vivo in the mouse brain (72). Protein-antibody complexes could potentially exacerbate the immune response in several ways, for example by changing the kinetics of fibril formation or by exposing different epitopes of the protein (73). This could result in stronger activation of TLRs, raising the potential of offsetting the adverse effect of antibody treatment by directly inhibiting these TLRs.

In addition to activating the microglial inflammasome, oligomeric/fibrillar α Syn may also initiate an immune cascade via microglial presentation of these peptides to other immune cells such as T cells, which further contribute to the immune pathology of PD (48, 74). Interestingly, human PD patients reportedly have a higher frequency of an IL-1 β gene polymorphism that increases IL-1 β expression (75); this may result in higher susceptibility to inflammation, and thus contribute to PD pathology via microglial secretion. Collectively, the present study shows that the α Syn- and particularly α Syn/A β -driven pathogenesis may manifest a component of neuroinflammation mediated by human microglia that, in contrast to mouse, is enhanced by anti- α Syn or anti-A β antibodies.

Materials and Methods

hiPSC Cultures and Reagents. The use of human cells was approved by the institutional review boards of the Scintillon Institute and The Scripps Research Institute. hiPSCs were generated from normal human fibroblasts (Hs27, ATCC CRL-1634, and Coriell GM02036) using an integration-free reprogramming method (76). hiPSCs were differentiated into hiMG, as described in *SI Appendix, Extended Materials and Methods*, and subsequently used for characterization and experiments. α Syn oligomers were prepared by shaking in a thermomixer at 1,400 rpm for 6 d at 37 °C, as described in *SI Appendix, Extended Materials and Methods*.

Detailed information on culture conditions and cell maintenance, reagents, experimental design of in vivo animal studies, analysis of RNA-seq, and immunohistochemistry is given in *SI Appendix, Extended Materials and Methods*.

Data Availability. The RNA-seq data reported in this article have been deposited in the Gene Expression Omnibus (accession no. [GSE169065](https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE169065)) (77). Source code used to generate results that are reported in the paper is available in GitHub as "FINALcode.r," using the following link: <https://github.com/doritrud/hiMG-analysis> (78).

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