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1 **To: The Journal of Immunology**

2

3 **Microglial NLRP3 inflammasome activation upon TLR2 and TLR5 ligation by distinct α -**
4 **synuclein assemblies**

5

6 *Running title: NLRP3 inflammasome activation by α -syn in microglia*

7

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38 **Abstract**

39 Parkinson's disease (PD) is the second most common age-related neurodegenerative disorder
40 characterized by the formation of cellular inclusions inside neurons that are rich in an abnormal
41 form of the protein α -synuclein (α -syn). Microglia are the CNS resident immune cells that react
42 to misfolded proteins through pattern recognition receptor ligation and activation of signaling
43 transduction pathways. Here, we studied activation of primary microglia isolated from wild-type
44 mouse by distinct α -syn forms and their clearance. Internalization of α -syn monomers and
45 oligomers efficiently activated the NLRP3 inflammasome via Toll-like receptor-2 and -5 ligation,
46 thereby acting on different signaling checkpoints. We found that primary microglia effectively
47 engulf α -syn, but hesitate in its degradation. NLRP3 inhibition by the selective inhibitor CRID3
48 and NLRP3 deficiency improved the overall clearance of α -syn oligomers. Together, these
49 data show that distinct α -syn forms exert different microglial NLRP3 inflammasome activation
50 properties, thereby compromising its degradation which can be prevented by NLRP3 inhibition.

51

52

53 **Keywords**

54 microglia, α -synuclein, NLRP3 inflammasome, Toll-like receptors, clearance

55

56

57

58 **Key points**

- 59
- α -syn monomers and oligomers efficiently activated the NLRP3 inflammasome via
- 60 TLR2
- activation of the NLRP3 inflammasome compromised the α -syn clearance capacity
- 61
- NLRP3 inhibition improved the overall clearance of α -syn oligomers
- 62

63 Introduction

64

65 Parkinson's disease (PD) is the most common movement disorder and, after Alzheimer's
66 disease, the second most common neurodegenerative disease. PD is mainly characterized by
67 the presence of intraneuronal cytoplasmic inclusions called Lewy bodies (LB) rich in
68 aggregated α -synuclein (α -syn) (1, 2). α -syn is a 14 kDa protein with no defined structure (3).
69 The monomeric form of the protein progressively forms oligomeric structures and insoluble
70 fibrillary complexes that, together with crowded organellar components (1), accumulate in LBs
71 under pathological conditions. The fibrillar forms of α -syn interfere with neuronal functioning
72 by affecting the normal distribution of important membrane proteins (4), disrupting
73 neurotransmitter release (5), impairing mitochondrial function (6–8), blocking intracellular
74 vesicle transport (9, 10), affecting endo-lysosomal compartment integrity (11), and
75 compromising protein-degradation mechanisms (12), thereby inducing toxicity, eventually
76 leading to neuronal injury and degeneration. Even though α -syn aggregates are first found in
77 neurons evidence suggests that neuronal cell death and spreading of pathology will expose
78 these proteins to the environment, thereby activating surrounding immune cells.

79 Being the brain's primary innate immune cells, microglia contribute to cerebral homeostasis by
80 sensing changes in their immediate environment, clearing cellular debris and providing
81 neurotrophins (13). Upon ligation of pattern recognition receptors (PRRs), microglia become
82 activated and execute an inflammatory response which, in case it persists, causes chronic
83 neuroinflammation and neuronal damage (13, 14). Evidence for such a chronic
84 neuroinflammatory response can be found in brains of PD patients and other
85 synucleinopathies, where microglia activation occurs in all brain regions where aggregated α -
86 syn accumulates (15–17). In keeping with this, α -syn has been shown to induce excessive
87 microglial activation and inflammatory activity.

88 Interleukin-1 β (IL-1 β) is the most extensively studied pro-inflammatory cytokine, which has
89 been linked to various disorders of the CNS (18, 19). In PD, α -syn has been shown to induce
90 the microglia-derived IL-1 β secretion following NLRP3 inflammasome activation (20–22).
91 Likewise, NLRP3 inflammasome inhibition prevented α -syn pathology and dopaminergic
92 neurodegeneration in PD-related mouse models (23). *In vitro*, IL-1 β is synthesized by microglia
93 as a precursor (pro-IL-1 β) in response to pathogen-associated molecular patterns (PAMPs)
94 such as lipopolysaccharide (LPS) that is acting via Toll-like receptor (TLR) ligation (24, 25).
95 This step, which is referred as "priming" is required to induce the expression of the
96 inflammasome components: the pattern recognition receptor NOD-like receptor pyrin domain
97 containing 3 (NLRP3), apoptosis-associated speck-like protein (ASC), and the pro-caspase-1.
98 A second stimulus which is referred to as "activation" results in the formation and activation of
99 cytosolic multiprotein complexes called inflammasomes (19, 26, 27) that process the precursor

100 pro-IL-1 β to its mature IL-1 β form that is rapidly secreted from the cell into its microenvironment
101 (28).

102 To unravel microglial activation by different α -syn forms, we used structurally and functionally
103 well characterized human α -syn monomers, oligomers and two different fibrillar polymorphs
104 that have been shown to trigger distinct synucleinopathies (29, 30) and tested their potential
105 to activate microglia through PRR ligation. We further analyzed how microglia survival is
106 affected upon exposure to monomeric, oligomeric and fibrillar α -syn and how microglia deal
107 with the clearance of α -syn assemblies. Here, we present evidence that α -syn monomers and
108 oligomers are able to prime and activate the NLRP3 inflammasome through TLR2 and TLR5
109 ligation, leading to NLRP3 inflammasome-dependent IL-1 β secretion thereby inhibiting α -syn
110 clearance. In addition, the microglial survival was not affected by the different α -syn forms,
111 thus, largely excluding pyroptotic cell death within the tested time period.

112 **Material and Methods**

113

114 **Animals**

115 Wild-type (WT, Charles River Laboratories, Inc., Wilmington, MA, USA) and NLRP3-deficient
116 (Millenium Pharmaceuticals, Cambridge, MA, USA) animals were all of the C57BL/g genetic
117 background. Mice were housed under standard conditions at 22 °C and a 12 hrs light-dark
118 cycle with free access to food and water. Animal care and handling was performed according
119 to the guidelines of animal welfare as laid down by the German Research Council (DFG) and
120 approved by the local ethical committees.

121

122 **Cell culture**

123 Primary microglia cells were isolated by the method of Giulian and Baker (31). Briefly, brains
124 from neonatal mice were stripped of the meninges and dissociated using mechanical shearing
125 and trypsin (Life Technologies, Carlsbad, CA, USA). Cells of two brains were plated on poly-
126 L-lysine (PLL, Sigma-Aldrich by Merck KGaA, Darmstadt, Germany) coated T75 culture flasks
127 (Greiner bio-one, Kremsmünster, Austria) and cultivated in DMEM (Gibco by Thermo Fisher
128 Scientific, Waltham, MA, USA) supplemented with 10% heat-inactivated fetal calf serum (FCS;
129 Gibco) and 1% penicillin/streptomycin (P/S; Gibco). On the next day cells were washed three
130 times with DPBS (Gibco) to remove cellular debris and cultured with DMEM supplemented
131 with 10% FCS, 1% P/S and 1% L929 conditioned medium as a source of growth factors. After
132 7-10 days loosely attached mature microglia were shaken off the astrocytic layer with a
133 repetition of the harvesting procedure all two to three days for up to three times. For
134 experiments, primary microglia were seeded into well plates and allowed to adhere overnight
135 in DMEM supplemented with 10% FCS and 1% P/S. On the next day medium was changed to
136 serum-free DMEM complemented with 1% N-2 supplement (Gibco) and microglia were
137 allowed to rest for another 24 hrs before experiments were performed.

138

139 **α -synuclein assemblies generation**

140 Human wild-type α -syn was expressed in E. coli BL21 DE3 CodonPlus cells (Stratagene, San
141 Diego, CA, USA) and purified as described previously (32). To assemble human wild-type α -
142 syn into the fibrillar polymorph "Fibrils", the protein (100 μ M) was incubated in 50 mM Tris-
143 HCl, pH 7.5, 150 mM KCl at 37°C under continuous shaking in an Eppendorf Thermomixer set
144 at 600 rpm for 5 days (29). The assembly reaction was followed by withdrawing aliquots (20
145 μ l) from the assembly reaction at different time intervals, mixing them with Thioflavin T (10 μ M
146 final) and recording the fluorescence increase on a Cary Eclipse Fluorescence
147 Spectrophotometer (Varian Medical Systems Inc., Palo Alto, CA, USA) using an excitation
148 wavelength = 440 nm, an emission wavelength = 480 nm and excitation and emission slits set

149 at 5 and 10 nm, respectively. To label α -syn fibrils with extrinsic fluorophores, the fibrils were
150 centrifuged twice at 15,000 *g* for 10 min and re-suspended twice in PBS at 1,446 g/L and two
151 molar equivalents of ATTO-488 NHS-ester (Atto-Tec GmbH, Siegen, Germany, #AD 488-35)
152 fluorophore in DMSO were added. The mix was incubated for 1h at room temperature. The
153 labeling reactions were arrested by addition of 1mM Tris pH 7.5. The unreacted fluorophore
154 was removed by a final cycle of two centrifugations at 15,000 *g* for 10 min and resuspensions
155 of the pellets in PBS. The fibrillar nature of α -syn was assessed by Transmission Electron
156 Microscopy (TEM) after adsorption of the fibrils onto carbon-coated 200 mesh grids and
157 negative staining with 1% uranyl acetate using a Jeol 1400 transmission electron microscope.
158 The images were recorded with a Gatan Orius CCD camera (Gatan, Pleasanton, CA, USA).
159 The resulting α -syn fibrils were fragmented by sonication for 20 min in 2-ml Eppendorf tubes
160 in a Vial Tweeter powered by an ultrasonic processor UIS250v (250 W, 2.4 kHz; Hielscher
161 Ultrasonic, Teltow, Germany) to generate fibrillar particles with an average size 42-52 nm as
162 assessed by TEM analysis.

163

164 **Measurement of cytokine secretion**

165 Cytokine release was determined using the mouse IL-1 beta/IL-1F2 DuoSet ELISA (DY401,
166 R&D Systems, Minneapolis, MN, USA), mouse TNF-alpha DuoSet ELISA (DY410, R&D
167 Systems), mouse IL-6 DuoSet ELISA (DY406, R&D Systems), mouse IL-10 DuoSet ELISA
168 (DY417, R&D Systems), and mouse CXCL2/MIP-2 DuoSet ELISA (DY452, R&D Systems).
169 Primary microglia (7.5×10^4 cells/well) were seed into 96-well plates and allowed to adhere
170 overnight. Microglia were used unprimed or primed for 3 hrs prior to experiments with 10 ng/ml
171 (± 100 EU/ml) lipopolysaccharide (LPS; InvivoGen, San Diego, CA, USA) before cells were
172 washed with DPBS and treated with α -syn monomers and different assemblies and BSA as a
173 protein control. For TLR neutralizing experiments, microglia were treated 5 μ g/ml mouse anti-
174 TLR2 (InvitroGen), mouse anti-TLR4 (Invitrogen), or rat anti-TLR5 (InvivoGen) in conjunction
175 with 2 μ M α -syn assemblies. Supernatants were assayed after 24 hrs treatment according to
176 the manufacturer's protocol. Optical density was determined at 450 nm photometrically with a
177 microplate reader (Infinite M200, Tecan, Männedorf, Switzerland). Concentrations of the
178 secretion of the different cytokines were calculated by interpolation using a respective cytokine
179 specific standard curve.

180 The expression profile of different cytokines and chemokines in response to different α -syn
181 species was analysed using a Proteome Profiler™ Array (ARY020, R&D Systems). Primary
182 microglia (2×10^6 cells/well) were seed into 6-well plates and allowed to adhere overnight.
183 Microglia were used unprimed or primed for 3 hrs prior to experiments with 10 ng/ml (± 100
184 EU/ml) LPS before cells were washed with DPBS and treated with α -syn monomers and
185 different assemblies for 24 hrs. The Proteome Profiler™ Array was performed according to the

186 manufacturer's protocol using 1 ml of cell supernatant per condition. After overnight incubation
187 with the sample/antibody mixture, membranes were washed three times with the wash buffer
188 and incubated with the fluorescent near-infrared secondary antibody IRDye[®] 680LT
189 Streptavidin (1:10,000 in 3% BSA, LI-COR Biosciences, Lincoln, NE, USA) for 30 min at RT.
190 Capture spots were then visualized with the Odyssey CLx Imaging System (LI-COR
191 Biosciences) and quantified using Image Studio (LI-COR Biosciences).

192

193 **Precipitation of supernatant samples**

194 For detection of the cleaved caspase-1 subunit p20 in the supernatant of treated primary
195 microglia cultures we performed a protein precipitation to concentrate the sample as described
196 by Jakobs and colleagues (33). Briefly, primary microglia (1.5×10^6 in 2 ml medium) were
197 cultured for 24 hrs in 6-well plates under control conditions or in medium containing chemical
198 compounds. After harvesting the supernatants 500 μ l methanol and 125 μ l chloroform were
199 added to 500 μ l supernatant and vortexed vigorously. After 5 min centrifugation at 13,000 g
200 the upper aqueous phase was removed and replaced by 500 μ l methanol. Samples were
201 vortexed again and centrifuged for 5 min at 13,000 g. Subsequently, supernatants were
202 removed and pellets were dried for 5 min in a vacuum dryer. The pellet was then resuspended
203 in 20 μ l 2x loading buffer and denatured for 3 min at 95 °C. Samples were then subjected to
204 Western blot analysis.

205

206 **Western blot**

207 For lysate collection, primary microglia (2×10^6) were cultured in 6-well plates under control
208 conditions or in medium containing different α -syn assemblies for 24 hrs before supernatants
209 (for protein precipitation) were collected and cells were washed and scraped off the well with
210 ice cold PBS containing 1x protease and phosphatase inhibitor cocktails (Thermo Fisher
211 Scientific). After pelleting the cells for 5 min at 10,000 g PBS was completely removed and
212 cells were lysed in ice cold RIPA buffer (50 mM Tris-HCl, 1% Triton X-100, 0.5% Na
213 deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 150 mM NaCl, pH 8.0) containing 1x
214 protease inhibitor cocktails for 15 min on ice. Lysates were centrifuged 5 min at 4 °C and
215 10,000 g and supernatants were frozen and kept in -20 °C until use.

216 Cell lysates and samples from protein precipitation were separated by a NuPAGE[®] 4-12% Bis-
217 Tris Gel (Invitrogen by Thermo Fisher Scientific) and transferred to a nitrocellulose blotting
218 membrane (0.2 μ m; GE Healthcare Life Sciences, Freiburg, Germany). Membranes were
219 washed with Tris-buffered saline supplemented with Tween-20 (TBST, 10 mM Tris-HCl, 150
220 mM NaCl, 0.05% Tween-20, pH 8.0). Membrane surface was blocked with 3% BSA in TBST
221 for 30 min at RT. Membranes were then incubated with the rat anti-caspase-1 antibody
222 (1:1,000; clone 4B4, Genentech, CA, USA), rabbit anti-Asc (AL177, 1:1,000, AdipoGen, San

223 Diego, USA), mouse anti-NLRP3 (1:500, AdipoGen), rabbit anti-MyD88 (1:500, Cell Signaling
224 Technologies), rabbit anti-NF- κ B p65 (1:500, Cell Signaling Technologies), or mouse anti- α -
225 tubulin (1:2,000; Thermo Fisher Scientific) overnight at 4 °C. After three washing steps á 5 min
226 with TBST the fluorescent near-infrared secondary antibodies IRDye® 800CW Goat anti-Rat
227 IgG (H + L) (1:10,000 in 3% BSA, LI-COR Biosciences, Lincoln, NE, USA) and IRDye® 680LT
228 Donkey anti-Mouse IgG (H + L) (1:10,000 in 3% BSA, LI-COR Biosciences) were applied for
229 30 min at RT. Proteins were then visualized with the Odyssey CLx Imaging System (LI-COR
230 Biosciences) and quantified using Image Studio (LI-COR Biosciences).

231

232 **Immunocytochemistry**

233 Cultures were fixed in 4% paraformaldehyde (PFA, Sigma-Aldrich) dissolved in PBS (Biochrom
234 GmbH, Berlin, Germany) for 15 min and permeabilized by washing them tree times for 5 min
235 with PBS containing 0.1% Triton X-100 (PTX). Blocking solution containing PTX and 5%
236 normal goat serum (Vector Laboratories, Burlingame, CA, USA) was applied for 30 min. The
237 primary antibodies rabbit anti-ASC (1:250; #D2W8U, Cell Signaling Technologies, Danvers,
238 MA, USA) and rat anti-CD11b (1:250; Serotec by Bio-Rad) were applied for 1 h followed by
239 three washing steps. The secondary antibodies goat anti-rabbit-AlexaFluor594 (1:250;
240 Invitrogen) and goat anti-rat-AlexaFluor594 (1:250; Invitrogen) were applied for 30 min. The
241 LysoTracker™ Red DND-99 (Thermo Fisher Scientific) was applied according to the
242 manufacturer's protocol. 4',6-Diamidino-2'-phenylindol-dihydrochloride (DAPI, Sigma-Aldrich)
243 was used for nuclear counterstaining at 0.1 mg/mL for 20 min in PBS. Images were taken using
244 a 60x oil-objective.

245

246 **Phagocytosis assay**

247 To assess microglial phagocytosis, primary microglia (3.5×10^5 cells/well) were seeded to 24-
248 well plates and allowed to adhere. Microglia were treated with 1 μ M Atto488-labeled α -syn
249 assemblies and incubated for 5-15 min. Phagocytosis was stopped by one washing steps with
250 PBS to remove free α -syn and cells were harvested using 0.5% trypsin (Gibco). Blocking
251 solution containing PBS and FCS (1:1 ratio) was applied for 10 min on ice. Cells were then
252 labelled with the APC anti-mouse/human CD11b antibody (1:100; #101212, BioLegend, San
253 Diego, CA, USA) for 30 min in FACS solution (PBS supplemented with 2% FCS) on ice.
254 Following labelling, cells were collected, resuspended in 300 μ l ice cold FACS solution, and
255 measured by flow cytometry using the FACS CANTO II and the FACSDIVA™ software (Becton
256 Dickinson, Heidelberg, Germany). Phagocytosis was then analysed and quantified using
257 FlowJo, LLC (v3.05470, Ashland, OR, USA).

258

259

260 **Microscopy and data analysis**

261 All experiments were examined with a Nikon Eclipse Ti fluorescence microscope (Nikon,
262 Tokyo, Japan). Acquired images were processed using NIS-elements 4 (Nikon) and Fiji
263 ImageJ (Wayne Rusband, National Institute of Health, USA). Data were evaluated using Graph
264 Pad Prism and presented as mean \pm SEM of at least three independent experiments with two
265 to three replicates. An outlier test was performed and data were analysed for Gaussian
266 distribution. When data passed the normality test statistical comparisons of controls versus
267 treatments were performed with one-way ANOVA or two-way ANOVA followed by a Tukey's
268 test. Otherwise, data were analysed with the Kruskal-Wallis test and a Dunn's post hoc test for
269 non-parametric data. Levels of significance are indicated as * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$;
270 **** $p < 0.0001$.

271 **Results**

272

273 We and others recently demonstrated that monomeric α -syn populates oligomeric species
274 (34–36) and assembles into structurally distinct fibrillar polymorphs that trigger when injected
275 to rodents the hallmarks of two distinct synucleinopathies, PD or MSA (29, 30). These
276 assemblies expose different polypeptide chains at their surfaces and as a consequence exhibit
277 differential tropism to neurons (37). How different α -syn assemblies, that form in the brain of
278 patients target and activate glial cells is still unclear. To investigate whether structurally
279 different α -syn polymorphs induce the NLRP3 inflammasome in microglia, we exposed primary
280 mouse microglia to well-characterized recombinant human α -syn assemblies (29, 30). To
281 investigate the potential impact on microglial activation of different forms of α -syn (Fig. 1 A-K),
282 we performed all experiments on microglia with and without pre-exposure to the inflammogen
283 LPS (10 ng/ml (\pm 100 EU/ml), 3 hrs) (Fig. S1 A). All α -syn species were initially validated as
284 endotoxin-free (Fig. S1 B) and dose response curve analysis were performed (Fig. S1 C). The
285 following stimulation experiments were performed using 2 μ M α -syn which has been the lowest
286 concentration inducing NLRP3 inflammasome activation in unprimed conditions (Fig. S1 C),
287 and has been confirmed as being non-cytotoxic using the XTT viability assay in conjunction
288 with an LDH assay (Fig. S1 D-E). BSA was tested in parallel to α -syn as a protein control.

289

290 **α -synuclein activates the NLRP3 inflammasome via TLR2 and TLR5 ligation and induces** 291 **the release of IL-1 β**

292 IL-1 β release is mediated by activation of the NLRP3 inflammasome upon assembly of NLRP3,
293 ASC, and pro-caspase-1. We assessed conditioned medium of primary microglia exposed to
294 monomeric, oligomeric or fibrillar polymorphs further referred as “fibrils” and “ribbons” for IL-
295 1 β concentrations at 6 and 24 hrs. Even though there was no detectable IL-1 β release at 6 hrs
296 (Fig. 1 L), exposure to α -syn monomers and oligomers was sufficient to induce pro-IL-1 β (Fig.
297 S2 A) and mature IL-1 β (Fig. 1 M) in unprimed microglia at 24 hrs. NLRP3 inflammasome
298 activation was confirmed by immunoblot detection showing increased levels of cleaved
299 caspase-1 compared to their controls (Fig. 1 N-P). Interestingly, α -syn monomers and
300 oligomers upregulated the expression of NLRP3 (Fig. 1 Q), while the expression of the adapter
301 protein ASC showed only a slight increase upon treatment with ribbons (Fig. 1 R). Using
302 immunoblot analysis we found the expression of the universal adapter protein MyD88
303 upregulated upon treatment with monomeric and oligomeric α -syn (Fig. 2 A), which further
304 activates the transcription factor NF- κ B. Even though there was no detectable difference in the
305 NF- κ B expression levels (Fig. 2 B), immunostainings revealed the translocation of NF- κ B p65
306 into the nucleus in microglia exposed to α -syn monomers (Fig. 2 C, arrows, Fig. 2 D).

307 To elucidate by which PRR ligation α -syn monomers and oligomers activate the NLRP3
308 inflammasome, three TLR neutralizing antibodies including anti-mouse TLR2, anti-mouse
309 TLR4, and anti-rat TLR5 were tested (Fig. 2). Unprimed microglia were exposed to different
310 forms of α -syn in the presence or absence of the respective TLR neutralizing antibodies (Fig.
311 2 E-H). Isotype specific antibodies were used as negative control. For a better clarity we
312 calculated the percent of neutralization to the α -syn treated control levels (\emptyset). We found that
313 anti-TLR2 and anti-TLR5 attenuated the IL-1 β release by about 50% to α -syn treated control
314 levels and isotype specific controls, in microglia exposed to α -syn monomers (Fig. 2 E) and
315 oligomers (Fig. 2 F). α -syn fibrils (Fig. 2 G) and ribbons (Fig. 2 H) did not increase IL-1 β levels.
316 We confirmed the inhibitory effects of TLR neutralization on NLRP3 inflammasome activation
317 by immunoblot analysis. Both, TLR2 and TLR5 neutralization inhibited the α -syn monomer-
318 and oligomer-induced cleavage of caspase-1 (Fig. 2 I-J), while only TLR2 neutralization
319 blocked the induction of NLRP3 (Fig. 2 I, K). Caspase-1 p45 and ASC expression remained
320 unchanged (Fig. S2 B-C). Importantly, the ability of α -syn to prime and activate the NLRP3
321 inflammasome was completely abolished in primary microglia derived from TLR2 knockout
322 animals (Fig. S2 D-E) as confirmed by immunoblot analysis and ELISA readings. Importantly,
323 caspase-1 cleavage inhibition by the NLRP3 inflammasome inhibitor CRID3 largely
324 suppressed NLRP3 inflammasome activation and IL-1 β secretion by α -syn treated unprimed
325 microglia (Fig. S3).

326 Since NLRP3 inflammasome mediated IL-1 β production usually requires a priming step, we
327 investigated if a classical priming condition would influence the degree and pattern of α -syn-
328 induced NLRP3 activity. Therefore, we pre-stimulated primary microglia for 3 hrs with 10 ng/ml
329 LPS before treating the cells with α -syn. LPS priming did not change the IL-1 β release within
330 6 hrs of treatment (Fig. 3 A), but largely increased the secretion of IL-1 β at 24 hrs when
331 microglia were exposed to α -syn monomers, oligomers or fibrils (Fig. 3 B). We performed
332 immunoblot analysis to check for the activation of the NLRP3 inflammasome (Fig 3 C). Even
333 though caspase-1 p45, NLRP3, and ASC expression levels remained unchanged after
334 exposure to α -syn monomers and assemblies for 24 hrs (Fig. 3 C-G), we found significantly
335 increased secretion levels of the active caspase-1 subunit p20 in response to monomers and
336 oligomers (Fig. 3 E). Most importantly, NLRP3 knockout and caspase-1 cleavage inhibition by
337 CRID3 largely suppressed NLRP3 inflammasome activation and IL-1 β secretion by α -syn
338 treated LPS-primed microglia (Fig. S4). In addition to caspase-1 cleavage, inflammasome
339 activation is characterized by formation of ASC specks (38) that can be visualized by intra- and
340 extracellular ASC-immunopositive specks (Fig. 3 H). LPS-primed microglia displayed an
341 increase of ASC specks at 24 hrs after exposure to α -syn monomers (Fig. 3 I) with a partial
342 co-localization of α -syn monomers and ASC specks (Fig. 3 J). Again, we used TLR

343 neutralization antibodies to check for PRR ligation of α -syn monomers and distinct assemblies
344 under primed conditions. For a better clarity we calculated the percent of neutralization to the
345 α -syn treated control levels (\emptyset). Using anti-TLR neutralization antibodies on LPS-primed α -
346 syn treated microglia we confirmed that the IL-1 β release of microglia treated with α -syn
347 monomers (Fig. 3 K), oligomers (Fig. 3 L) and fibrils (Fig. 3 M) was attenuated upon
348 neutralization of TLR2, whereas ribbons neither increased IL-1 β nor did TLR neutralization
349 showed any effect (Fig. 3 N). TLR4 and TLR5 neutralization did not change the α -syn-induced
350 IL-1 β release of LPS-primed microglia (data not shown).

351 In addition to IL-1 β , we checked for the release of other pro- and anti-inflammatory cytokines
352 (Fig. S5). Initially, we exposed supernatants of primary microglia with or without prior LPS-
353 priming to a proteome profile array to get an overview about the inflammatory profile of the
354 cells (Fig. S5 A). We found that α -syn monomer, oligomers and fibrils augmented the release
355 of various pro-inflammatory cytokines (Fig. S5 A, left panel) which was further amplified by
356 prior LPS-priming (Fig. S5 A, right panel). Ribbons showed no effect on the cytokine secretion.
357 To confirm these findings and to quantify the cytokine release in more detail we selected
358 certain cytokines and performed ELISA readings on supernatants of primed and unprimed
359 microglia exposed to α -syn monomers and distinct assemblies.

360 Interestingly, we found that α -syn monomers, oligomer and fibrils induced the release of TNF α
361 (Fig. S5 B), IL-6 (Fig. S5 C), IL-10 (Fig. S5 D), NO (Fig. S5 E), and CXCL2 (Fig. S5 F) whereby
362 the release was further amplified by prior LPS priming. Again, ribbons had no effect on the
363 cytokine secretion. In order to confirm the involvement of TLRs in α -syn-induced immune
364 signalling, we performed TLR neutralizing experiments on unprimed microglia and used
365 CXCL2 secretion as another read-out (Fig. S5 G-J). For better clarity we calculated the percent
366 of neutralization to the α -syn treated control levels (\emptyset). Again, we found that neutralization of
367 TLR2 and TLR5 attenuated the release of CXCL2 from microglia exposed to α -syn monomers
368 (Fig. S5 G) and oligomers (Fig. S5 H). In line with that, TLR2 and TLR5 neutralization
369 decreased the CXCL2 release in response to fibrils (Fig. S5 I) and ribbons (Fig. S5 J). We did
370 not find any indication for an involvement of TLR4 in α -syn-induced CXCL2 release.

371 Together, our data show a TLR2- and TLR5-mediated inflammatory signalling in microglia
372 dependent on α -syn structural characteristics. Moreover, to our knowledge, this is the first
373 evidence for an involvement of TLR5 in monomeric and oligomeric α -syn-induced microglial
374 activation. In addition to IL-1 β , we found various other inflammatory agents upregulated by α -
375 syn including TNF α , IL-6, IL-10, NO, and CXCL2 further underlining differential pro-
376 inflammatory properties of distinct α -syn species (Fig. S5).

377

378 **NLRP3 modulates the uptake and degradation of α -syn in microglia**

379 To characterize and compare the microglial uptake of different α -syn forms, cells were exposed
380 for 5, 10 or 15 min to fluorescently labelled α -syn monomers, oligomers, fibrils or ribbons prior
381 to uptake assessment analysis (Fig. 4). Using flow cytometry analysis, we found that microglia
382 took up the different forms of α -syn as soon as they were exposed to them; however, α -syn
383 monomers and distinct assemblies were taken up to a different extent. While about 10% of
384 microglia had taken up α -syn monomers (Fig. 4 A) and oligomers (Fig. 4 B), around 90% of
385 the cells were immunopositive to fibrils (Fig. 4 C) and ribbons (Fig. 4 D) after 5 min of exposure.
386 At time 15 min, 64 and 75% of microglia had incorporated α -syn monomers and oligomers,
387 respectively, while 98% of cells contained fibrils or ribbons. The uptake of α -syn was blocked
388 by the actin polymerization inhibitor cytochalasin D (CytD) in all cases indicating active α -syn
389 phagocytosis by microglia. The engulfment of BSA was performed in parallel as a phagocytosis
390 and degradation control (Fig. S6 A-D). Immunocytochemical analysis of microglia exposed to
391 the different α -syn forms revealed limited internalization of monomers and oligomers as
392 compared to massive uptake of fibrils and ribbons (Fig. 4 E, Fig. S6 E).

393 To determine whether microglia process α -syn after phagocytosis, we allowed microglia to
394 take up monomers, oligomers, fibrils or ribbons for 15 min and then washed the cells twice
395 with warm PBS and further incubated them for 24 hrs in fresh α -syn-free culture medium. We
396 found that about 50% of all the exogenously applied α -syn monomers and different assemblies
397 remained inside microglia after 24 hrs (Fig. 4 F). Consistently, immune staining of microglia
398 exposed to α -syn oligomers, fibrils or ribbons exhibited aggregates after allowing degradation
399 for 24 hrs (Fig. S6 F) that were largely co-localized to lysosomes (Fig. 4 G-H).

400 Several studies have shown that cytokines, including NLRP3 inflammasome-derived IL-1 β
401 impair β -amyloid uptake (39). In keeping with this, suppression of inflammatory cytokine
402 production resets microglial phagocytosis capacity in the APP/PS1 AD mouse model. To test
403 whether NLRP3 inflammasome activity and the resulting IL-1 β release play a similar role in α -
404 syn phagocytosis, the latter was analysed in WT microglia, WT microglia treated with the
405 NLRP3 inflammasome inhibitor CRID3 and NLRP3-knockout microglia (Fig. 5). FACS analysis
406 revealed that α -syn monomers (Fig. 5 A) and oligomers (Fig. 5 B) were engulfed to a
407 significantly greater extent by WT microglia treated with the NLRP3 inflammasome inhibitor
408 CRID3 or NLRP3-knockout microglia compared to WT cells, whereas NLRP3 inhibition or
409 deficiency did not change the engulfment of fibrils (Fig. 5 C) and ribbons (Fig. 5 D).
410 Interestingly, those α -syn monomers and oligomers were previously shown to activate the
411 NLRP3 inflammasome even under basal conditions (Fig. 1). A similar NLRP3-dependent
412 engulfment behaviour was found in microglia exposed to BSA (Fig. S6 D). By expressing the
413 degradation as a function of what was phagocytosed, we found that the degradation of α -syn
414 monomers (Fig. 5 E) was unaffected, while the degradation of oligomers was largely improved

415 in cells deficient for a functional NLRP3 inflammasome (Fig. 5 F). Also, the degradation of α -
416 syn fibrils did not change in WT or NLRP3-deficient microglia (Fig. 5 G), but CRID3-treatment
417 improved the degradation of ribbons, while NLRP3 deficiency did not show any effects (Fig. 5
418 H). Together, these data suggest that there is an inverse relation between microglial NLRP3
419 inflammasome activation (Fig. 1) and its negative regulation on oligomeric α -syn uptake and
420 clearance (Fig. 5).

421 **Discussion**

422

423 Abnormal α -syn accumulations and microglial activation represent key pathological hallmarks
424 of synucleinopathies. Even though α -syn aggregates are first found in neurons, evidences
425 suggest that spreading of pathology will expose such protein aggregates to surrounding
426 microglia. Microglia are responsible for the clearance of misfolded and aggregated proteins
427 from the brain and represent the main drivers of inflammatory processes within the CNS. They
428 are equipped with PRRs which enable them to respond to α -syn aggregates and their
429 activation has been widely observed in PD (40). Once activated, microglia initiate a range of
430 inflammatory responses including the release of immune mediators such as cytokines and
431 complement factors which, once chronically present, collectively contribute to neuronal
432 dysfunction and degeneration. They also initiate response mechanisms for phagocytic
433 clearance of the respective protein aggregates. Since α -syn proteins can adopt distinct
434 conformations with noticeable differences in their aggregation phenotype, this may cause
435 quantitative and qualitative differences in microglial immune response. The aim of this work is
436 to delineate the different response patterns microglia show when exposed to various α -syn
437 assemblies.

438

439 **Bent out of shape: α -synuclein misfolding and its inflammatory consequences**

440 NLRP3 inflammasome activation by misfolded proteins, has emerged as important mechanism
441 for neurodegeneration (21) and misfolded α -syn has been reported to cause IL-1 β release
442 following microglial NLRP3 inflammasome activation (23). The pathological impact of this
443 finding is further supported by the fact that NLRP3 inhibition prevents α -syn-mediated
444 pathology in multiple rodent PD models (23). Native α -syn exists as a monomer however,
445 under pathological conditions, it forms aggregates with different inflammatory features. Most
446 studies have been focussing on the induction of inflammation by various α -syn oligomers and
447 inhomogeneous fibrillar assemblies. None however addressed the possible pro-inflammatory
448 effects of α -syn monomers and homogeneous and structurally distinct fibrillar polymorphs. Due
449 to variable experimental procedures and concentrations used throughout different reports, a
450 direct comparison between the effects of different α -syn forms on NLRP3 inflammasome
451 activation is therefore impossible. For example, it has been demonstrated elsewhere that 10
452 μ M fibrillar α -syn largely upregulated the assembly of the NLRP3 inflammasome complex in
453 primary microglia that were pre-stimulated with 200 ng/ml LPS (23). In contract, another study
454 reported the induction of the NLRP3 inflammasome upon treatment with 3.5 - 7 μ g/ml α -syn
455 upon pre-stimulation of primary microglia with 1 μ g/ml LPS (41). Comparing those studies,
456 both, the α -syn concentration and the LPS concentration vary greatly and may possibly
457 complicate any interpretation. Most importantly, both reports do not take possible actions of

458 the monomeric form of α -syn and its potential to activate the NLRP3 inflammasome complex
459 into account. We therefore set out to compare structurally well-characterized recombinant α -
460 syn monomers, oligomers and two fibrillar polymorphs, fibrils and ribbons, for their capacity to
461 induce microglial inflammatory activation (Fig. 1 A-I). Compared to both reports mentioned
462 above, we used microglia that were unprimed or primed with a minimal dose of LPS (10 ng/ml)
463 to avoid microglial over-activation and selected an α -syn concentration that was just sufficient
464 to trigger IL-1 β secretion (Fig. S1 C).

465 *In vitro*, microglia show no or just minimal activation of the NLRP3 inflammasome and its
466 priming is usually required as first signaling step of activation by inducing the transcription of
467 its components and substrates including NLRP3, the adaptor ASC, pro-caspase-1 and pro-IL-
468 1 β . This is usually followed by a secondary posttranscriptional activation step, that leads to the
469 assembly of the inflammasome complex followed by cleavage of its substrates and cytokine
470 release (21, 26). Here, we provide evidence that α -syn monomers and oligomers are able to
471 effectively activate the inflammasome without the requirement of a prior priming step (Fig. 1 L-
472 R). In contrast, α -syn fibrils showed NLRP3 inflammasome assembly and IL-1 β release only
473 after initial priming (Fig. 3 A-G), while ribbons did not affect the NLRP3 inflammasome in
474 microglia.

475 Cell priming occurs through ligation of PRRs which usually respond to a relatively restricted
476 ligand spectrum (42). Various receptors have been implicated in binding to and mediating α -
477 syn signalling in microglia including TLR2, MHCII, Fc receptor and fractalkine receptor (43–
478 46). Using TLR neutralization antibodies we found that monomers and oligomers exert their
479 effects on the NLRP3 inflammasome via TLR2 and TLR5 (Fig. 2). Interestingly, neutralization
480 of TLR2 largely blocked the α -syn-mediated NLRP3 protein expression in unprimed microglia,
481 indicating an important role of TLR2 for NLRP3 inflammasome priming (Fig. 2 G). In addition,
482 TLR5 neutralization prevented caspase-1 cleavage in response to α -syn monomers and
483 oligomers (Fig. 2 F). Thus, our data indicate that TLR2 ligation by α -syn monomer and
484 oligomers implement the inflammasome priming step, whereas TLR5 ligation results in the
485 activation of the inflammasome under basal conditions (Fig. 6). A recent screening of TLR
486 expression dynamics in mouse models of neurodegenerative disease including PD and DLB
487 revealed the upregulation of TLR2 (47), resulting in the activation of microglial inflammation
488 (45, 48, 49). Also, increased TLR2 levels have been found in the SNpc of PD patients (50)
489 further suggesting a pathogenetic role of TLR2 in synucleinopathies. In contrast, TLR5 has not
490 yet been described as a PRR involved in microglial activation by α -syn, this PRR is rather
491 known to be activated by flagellin, a monomer present on the outer cell wall of gram-negative
492 flagella bacteria. While it remains unknown whether flagellin and α -syn monomers and
493 oligomers share a similar TLR recognition site our data provide the first insights into TLR5-

494 mediated inflammatory activation mechanisms by which α -syn monomers and oligomers prime
495 and activate the NLRP3 inflammasome.

496

497 **The inverse relation between the NLRP3 inflammasome and α -synuclein clearance**

498 Effective microglial clearance of misfolded and aggregated proteins may play an important role
499 in neurodegenerative disease. α -syn has been shown to be released from neurons and are
500 detectable in biological fluids including plasma and CSF (51, 52), as well as in conditioned
501 media of α -syn expressing neurons (53). Microglia are the main cell type responsible for the
502 uptake and degradation of misfolded or aggregated proteins within the CNS thereby avoiding
503 the spreading of pathology between neighbouring neurons. We therefore assessed the efficacy
504 of microglial uptake and degradation of the aforementioned α -syn species. Microglia quickly
505 internalized the protein in an assembly- and time-dependent manner (Fig. 4), but seemed to
506 fail to fully degrade their cargo (Fig. 5) resulting in the accumulation of internalized α -syn
507 aggregates (Fig. S6 E-F). Several studies provide evidence for impaired clearance
508 mechanisms in PD leading to the idea that α -syn can block its own clearance (54, 55).
509 Alternatively, the microglial activation state may alter its intracellular capacity to effectively
510 degrade the taken-up protein aggregates (56). In response to persistent neurodegeneration,
511 microglia adopt a chronically activated phenotype resulting in the sustained release of
512 cytokines, including IL-1 β , that have been found to impair its own clearance functions (57). We
513 therefore analysed the impact of NLRP3 inflammasome inhibition by CRID3 and NLRP3
514 depletion on the phagocytic and degrading capacity of microglia. A large increase in oligomeric
515 α -syn phagocytosis was found in CRID3-treated WT or NLRP3-knockout microglia (Fig. 5 B).
516 Interestingly we showed that oligomeric α -syn has the potential to activate the inflammasome
517 (Fig. 1), suggesting that NLRP3 inflammasome activation deteriorates microglial phagocytosis
518 (58). In addition, degradation experiments revealed that NLRP3 deficiency substantially induce
519 the depletion of oligomeric α -syn (Fig. 5 B, F). Monomeric α -syn uptake was barely increased
520 in NLRP3 deficient cells (Fig. 5 A), whereas its degradation did not show any NLRP3
521 dependency (Fig. 5 E). In contrast, uptake and degradation of fibrils and ribbons did not show
522 NLRP3 dependency (Fig. 5).

523 Taken together, we provide first evidence that α -syn monomers rather than oligomers or
524 fibrillar polymorphs largely induce microglial NLRP3 inflammasome activation. Both, α -syn
525 monomers and, to a lower extent, oligomers had the potential to prime and activate the NLRP3
526 inflammasome via TLR2 and TLR5 ligation without the requirement of an initial inflammogen
527 (LPS) treatment. Activation of the NLRP3 inflammasome compromised α -syn clearance
528 capacity (Fig. 6). Likewise, NLRP3 inflammasome inhibition or NLRP3 depletion improved α -
529 syn phagocytosis and degradation of α -syn monomers and oligomers. These findings highlight

530 the potential of targeting the NLRP3 inflammasome or related intracellular signalling
531 mechanisms to control excessive cytokine release mediated by α -syn monomers even before
532 the development of fibrillar complexes.

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547

548 **Author Contributions**

549 H.S. and M.T.H. designed all experiments; H.S. performed experiments and analysed data
550 with assistance of A.G., S.S. and M.T.H.; L.B. and R.M. generated and characterized all α -
551 synuclein assemblies and provided advice; E.L. provided chemical compounds; H.S. and
552 M.T.H. wrote the manuscript with input from all co-authors.

553

554 **Disclosures**

555 Michael T. Heneka serves as an advisory board member at IFM Therapeutics, Alector and
556 Tiaki. He received honoraria for oral presentations from Novartis, Roche and Biogen. The other
557 authors declare that there is no conflict of interest with regard to the experimental part of this
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711

712

713 Figure Legends

714

715 Figure 1

716 **Different forms of α -syn prime and activate the NLRP3 inflammasome under basal conditions.**

717 **(A)** Schematic drawing of the α -syn species used throughout this study. **(B-I)** Characterization of the α -
718 syn species used throughout this study. **(B)** The monomeric nature of α -syn was assessed by analytical
719 ultracentrifugation. **(C)** The assembly of monomeric α -syn (100 μ M) into the fibrillar polymorphs Fibrils
720 (red) and Ribbons (green) was monitored in triplicate using Thioflavin T binding as this assay shows the
721 lag phase is short and the fluorescence at steady state high for the polymorph fibrils as compared to
722 that of the polymorph ribbons. **(D-H)** Electron micrographs of oligomeric and fibrillar α -syn species after
723 negative staining with Uranyl acetate (1%). **(D)** On fibrillar assembly pathway oligomeric α -syn purified
724 through a Superose 6 column. **(E-F)** The fibrillar polymorph fibrils before and after fragmentation into
725 particles with an average size 40 nm, respectively. **(G-H)** The fibrillar polymorph ribbons before and after
726 fragmentation into particles with an average size 40 nm, respectively. **(I-K)** Proteolytic patterns upon
727 exposure to Proteinase K of the α -syn oligomers **(I)**, and the fibrillar polymorphs Fibrils **(J)** and Ribbons
728 **(K)** after SDS-PAGE and Commassie blue staining. The arrowheads on the right point to bands that
729 distinguish the polymorph Fibrils from the polymorph Ribbons. **(L-M)** IL-1 β levels in conditioned medium
730 of primary microglia treated for 6 hrs **(L)** or 24 hrs **(M)** with 2 μ M α -syn monomers, oligomers, fibrils, and
731 ribbons. BSA was used as protein control. (for A: n=3; for B: n=7-10 independent experiments with
732 triplicate treatments for all conditions). **(N)** Immunoblot of microglia cell lysates and supernatants
733 exposed to 2 μ M α -syn for 24 hrs, stained for caspase-1, NLRP3, ASC and α -tubulin. (for lysates: n=7-
734 9; for supernatants: n=5 independent experiments). Quantification of caspase-1 p45 **(O)**, secreted
735 cleaves caspase-1 p20 **(P)**, NLRP3 **(Q)**, and ASC **(R)** of cell lysates and supernatants of microglia
736 exposed for 24 hrs to 2 μ M α -syn. All graphs are presented as mean \pm SEM and were analysed by one-
737 way ANOVA followed by Tukey's multiple comparison post hoc test (O-R) or Kruskal-Wallis test for
738 nonparametric data (L-M). Levels of significance are: ****p < 0.0001, ***p < 0.001, **p < 0.01, *p < 0.05.
739 Scale bars: 20 μ m. See also Fig. S1 and Fig. S3.

740

741 Figure 2

742 **Toll-like receptor 2 (TLR2) and TLR5 neutralization suppress α -syn-mediated IL-1 β release**
743 **following NLRP3 inflammasome activation in primary microglia.**

744 **(A)** Immunoblot analysis and quantification of the expression of MyD88 after exposure to 2 μ M α -syn
745 for 24 hrs. (n=3 independent experiments). **(B)** Immunoblot analysis and quantification of the expression
746 of NF κ B in microglia cell lysates exposed to 2 μ M α -syn for 24 hrs. **(C)** Representative staining with
747 NF κ B (magenta) of microglia exposed to α -syn show the translocation of NF κ B from the cytoplasm
748 when treated with LPS or α -syn monomers for 24 hrs. Arrows point toward the translocation of NF κ B
749 into the nucleus (DAPI, blue). **(D)** Quantification of the translocation of NF κ B from the cytosol to the
750 nucleus in microglia exposed for 24 hrs to 2 μ M α -syn. IL-1 β levels in conditioned medium of unprimed
751 primary microglia treated for 24 hrs with TLR2-, TLR4-, and TLR5-neutralizing antibodies or the
752 respective isotype controls in parallel to stimulation with α -syn monomers **(E)**, oligomers **(F)**, fibrils **(G)**
753 and ribbons **(H)**. The baseline release of IL-1 β is indicated by the red dashed line for all graphs. (n = 3-
754 4 per group with duplicate or triplicate treatments for all conditions). **(I)** Immunoblot for caspase-1 p45,
755 cleaved caspase-1 p20, NLRP3, ASC and α -tubulin. Quantification of secreted cleaved caspase-1 p20
756 **(J)** and NLRP3 **(K)** in microglia treated with the different α -syn forms alone (\emptyset) or in combination with
757 TLR2-, TLR4-, TLR5-neutralizing antibodies or the respective IgG controls. (n=4 independent
758 experiments). All graphs are presented as mean \pm SEM and were analysed by one-way ANOVA
759 followed by Tukey's multiple comparison post hoc test. Levels of significance are: ****p < 0.0001, ***p <
760 0.001, **p < 0.01, *p < 0.05. See also Fig. S2.

761

762 Figure 3

763 **Different forms of α -syn activate the NLRP3 inflammasome in LPS-primed microglia.**

764 IL-1 β levels in conditioned medium of LPS-primed primary microglia treated for 6 hrs **(A)** or 24 hrs **(B)**
765 with 2 μ M α -syn monomers, oligomers, fibrils, and ribbons. BSA was used as protein control. (for A:
766 n=3; for B: n=7-10 independent experiments with triplicate treatments for all conditions). **(C)** Immunoblot
767 of primed microglia cell lysates and supernatants exposed to 2 μ M α -syn for 24 hrs, stained for caspase-
768 1, NLRP3, ASC and α -tubulin. (for lysates: n=7-9; for supernatants: n=5 independent experiments).
769 Quantification of caspase-1 p45 **(D)**, secreted cleaves caspase-1 p20 **(E)**, NLRP3 **(F)**, and ASC **(G)** of
770 cell lysates and supernatants of LPS-primed microglia exposed for 24 hrs to 2 μ M α -syn. **(H)**

771 Representative staining with ASC (red) of LPS-primed microglia exposed to α -syn-Atto488 (green) show
772 the formation of ASC specks after 24 hrs. **(I)** Quantification of the number of ASC-speck containing cells.
773 (n=3 independent experiments). **(J)** Co-localization analysis of ASC specks and α -syn after 24 hrs
774 exposure. (n=3 independent experiments). IL-1 β levels in conditioned medium of LPS-primed primary
775 microglia treated for 24 hrs with TLR2-neutralizing antibody or the respective isotype control in parallel
776 to stimulation with α -syn monomers **(K)**, oligomers **(L)**, fibrils **(M)** or ribbons **(N)**. The baseline release
777 of IL-1 β is indicated by the red dashed line for all graphs. (n = 3-6 per group with duplicate or triplicate
778 treatments for all conditions). All graphs are presented as mean \pm SEM and were analysed by one-way
779 ANOVA followed by Tukey's multiple comparison post hoc test (D-I, K-N) or Kruskal-Wallis test for
780 nonparametric data (A, B, J). Levels of significance are: ****p < 0.0001, ***p < 0.001, **p < 0.01, *p <
781 0.05. Scale bars: 20 μ m. See also Fig. S4.

782 **Figure 4**

783 **Microglia take up α -syn within minutes but hesitate in its degradation.**

784 Flow cytometric analysis and quantification of the percentage of α -syn-positive cells after exposure to 2
785 μ M monomers **(A)**, oligomers **(B)**, fibrils **(C)**, and ribbons **(D)**. (n=5 independent experiments). Active α -
786 syn uptake was verified by inhibiting the engulfment with the phagocytosis inhibitor cytochalasin D
787 (CytD). **(E)** Representative immunostainings for CD11b-positive microglia (red) containing distinct forms
788 of α -syn-Atto488 (green). DAPI (blue) was used as a counterstain. **(F)** Quantification of the percentage
789 of α -syn degradation after 24h in microglia treated with 2 μ M α -syn monomers (grey), oligomers (blue),
790 fibrils (red), or ribbons (green). (n=4 independent experiments with duplicated treatments for all
791 conditions). Representative immunostaining **(G)** for LysoTracker (red) and α -syn-Atto488 (green) and
792 quantification **(H)** of the Lysosome : α -syn co-localization calculated by the Pearson's correlation
793 coefficient. Graphs in (A-D) are presented as mean \pm SEM and were analysed by one-way ANOVA
794 followed by Tukey's multiple comparison post hoc test (B-D) or Kruskal-Wallis test for nonparametric
795 data (A). Graphs in F are presented as mean \pm SEM and were analysed by an unpaired t-test. Levels
796 of significance are: ****p < 0.0001, ***p < 0.001, **p < 0.01, *p < 0.05. Scale bars: 20 μ m. See also Fig.
797 S6.

798 **Figure 5**

799 **Microglia take up α -syn monomers and oligomers in a NLRP3 dependent manner.**

800 Quantification and comparison of the relative uptake and degradation of α -syn monomers **(A)**, oligomers
801 **(B)**, fibrils **(C)**, and ribbons **(D)** by WT microglia (filled bars), WT microglia treated with 200 nM of the
802 NLRP3 inhibitor CRID3 (striped bars), and NLRP3-deficient microglia (empty bars). Expression of the
803 degradation as a function of α -syn monomers **(E)**, oligomers **(F)**, fibrils **(G)** and ribbons **(H)** that have
804 been phagocytosed by WT microglia (filled bars), WT microglia treated with 200 nM of the NLRP3
805 inhibitor CRID3 (striped bars), and NLRP3-deficient microglia (empty bars). (n=6-7 independent
806 experiments). All graphs are presented as mean \pm SEM and were analysed by one-way ANOVA
807 followed by Tukey's multiple comparison post hoc test. Levels of significance are: ****p < 0.0001, ***p <
808 0.001, **p < 0.01, *p < 0.05. See also Fig. S6.

809 **Figure 6**

810 **The inverse relationship between microglial NLRP3 inflammasome activation and α -syn clearance.**

811 The schematic was created with BioRender (<https://biorender.com>). TLR2 ligation by α -syn monomer
812 and oligomers induce the expression of NLRP3, ASC and caspase-1 p45 following translocation of
813 NF κ B to the nucleus. Ligation of TLR5 by α -syn monomer and oligomers acts as a "activation" stimulus
814 resulting in the assembly of the NLRP3 inflammasome and the release of IL-1 β . Activation of the NLRP3
815 inflammasome blocks the phagocytosis and degradation of α -syn oligomers, and to a lower extent α -
816 syn monomers, resulting in protein accumulation.

Figure 1

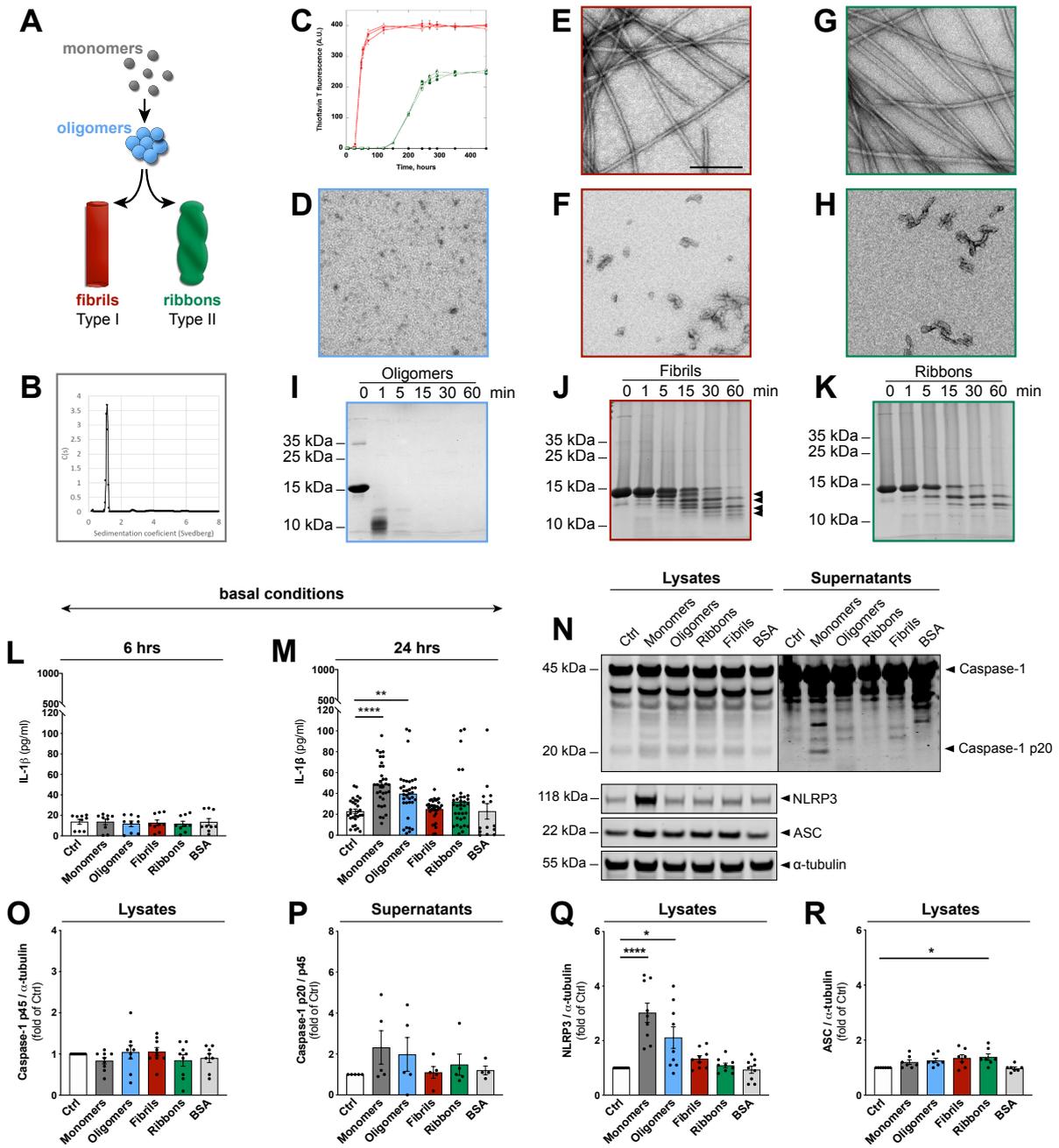


Figure 2

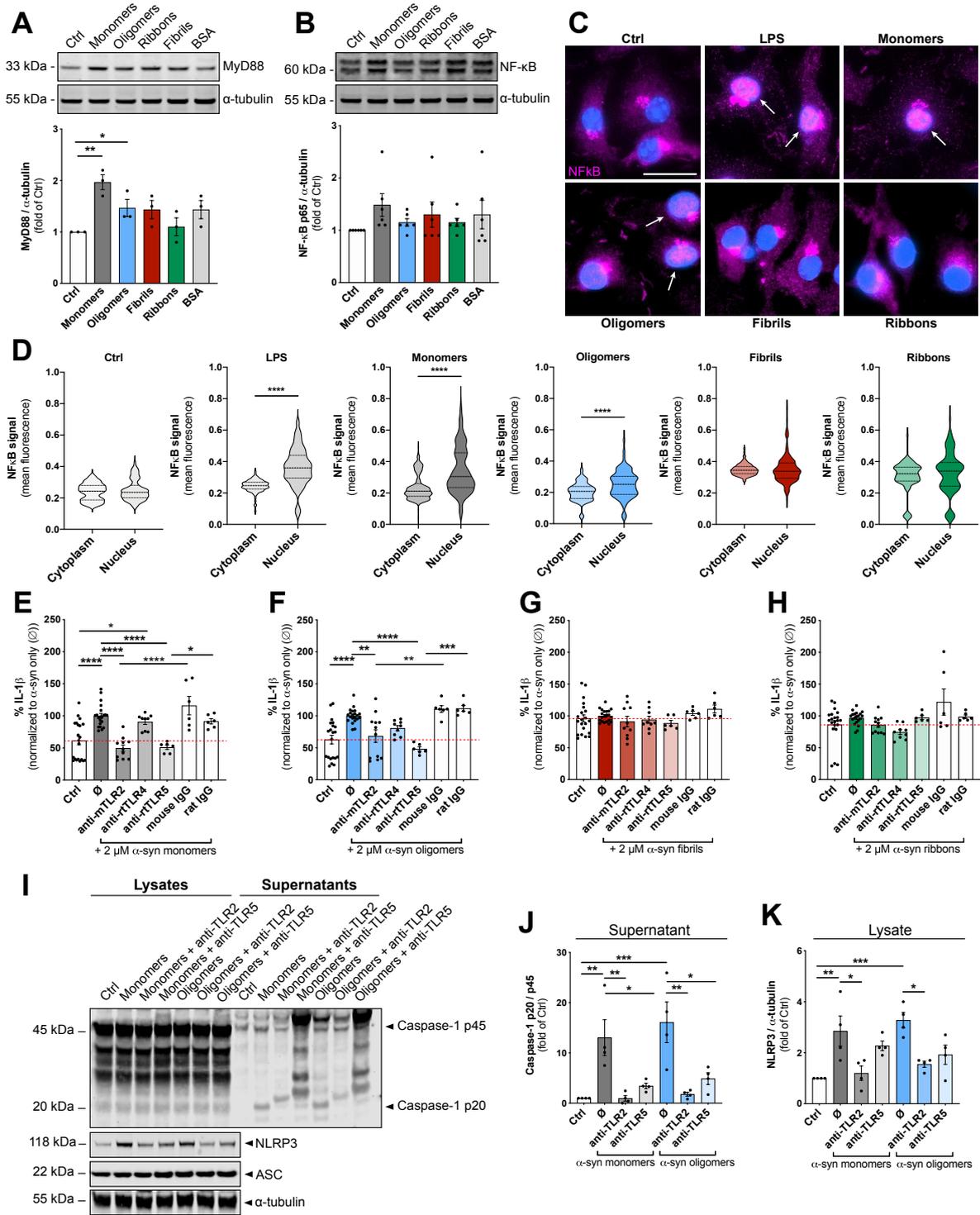


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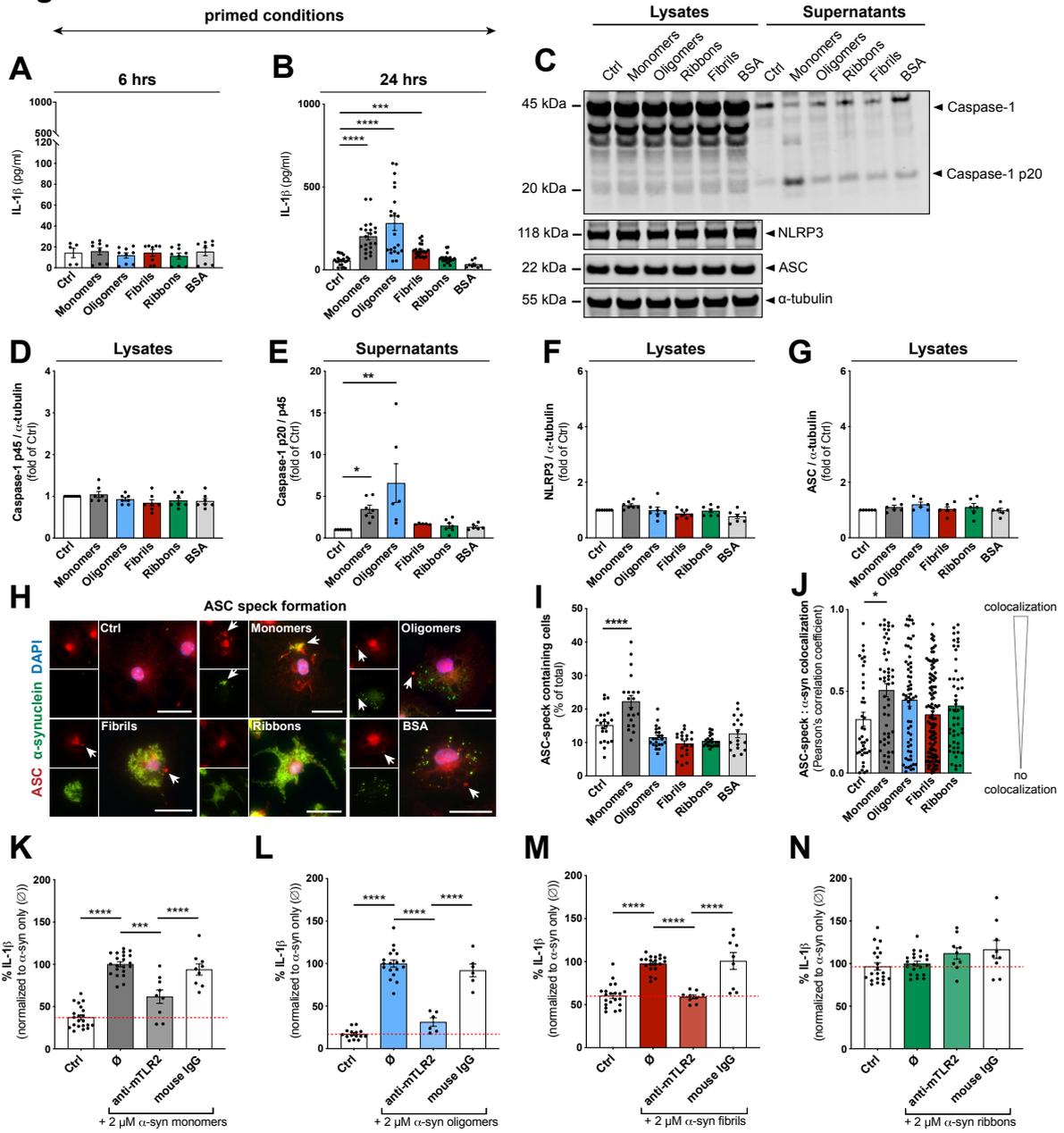


Figure 4

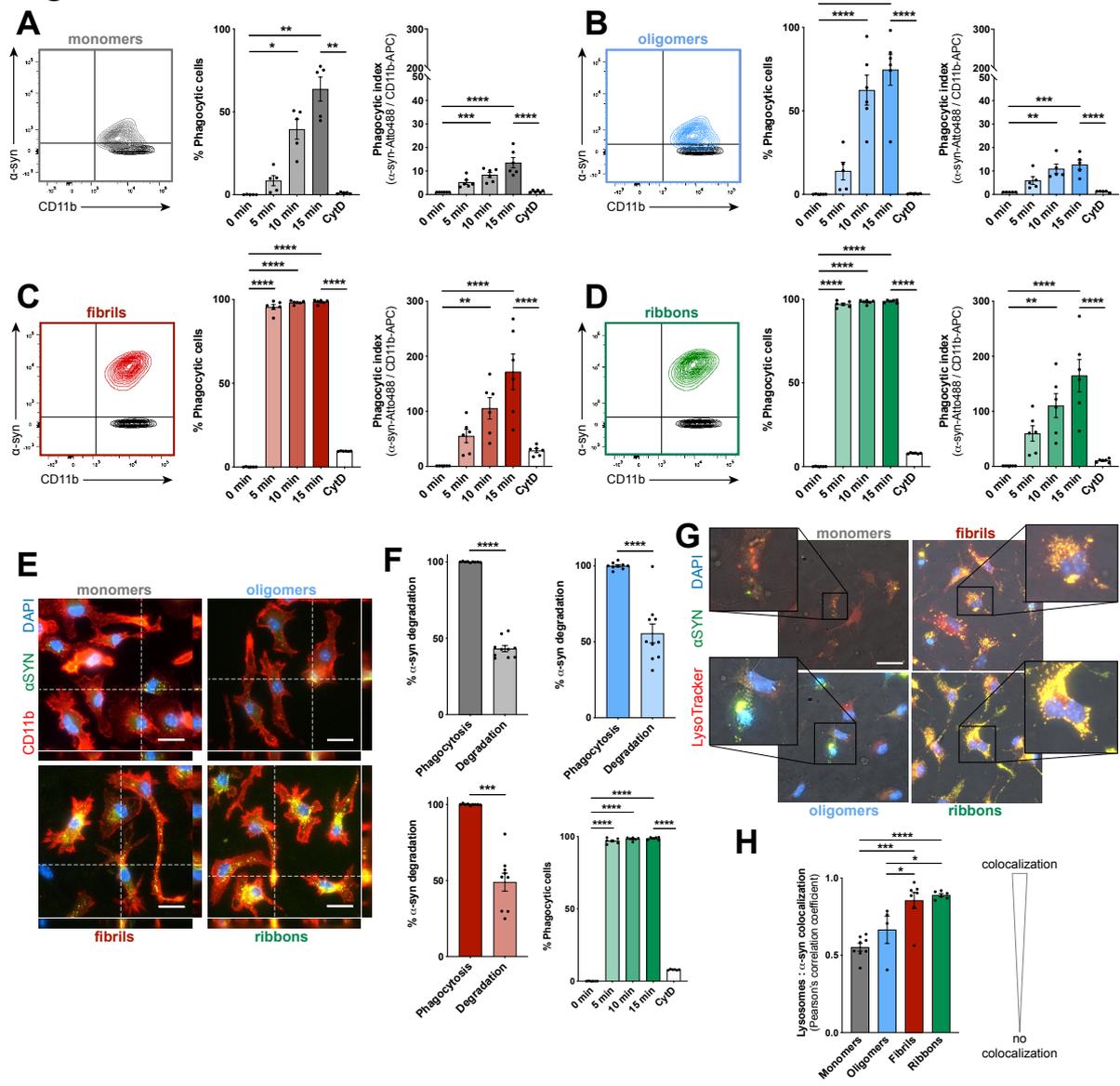


Figure 5

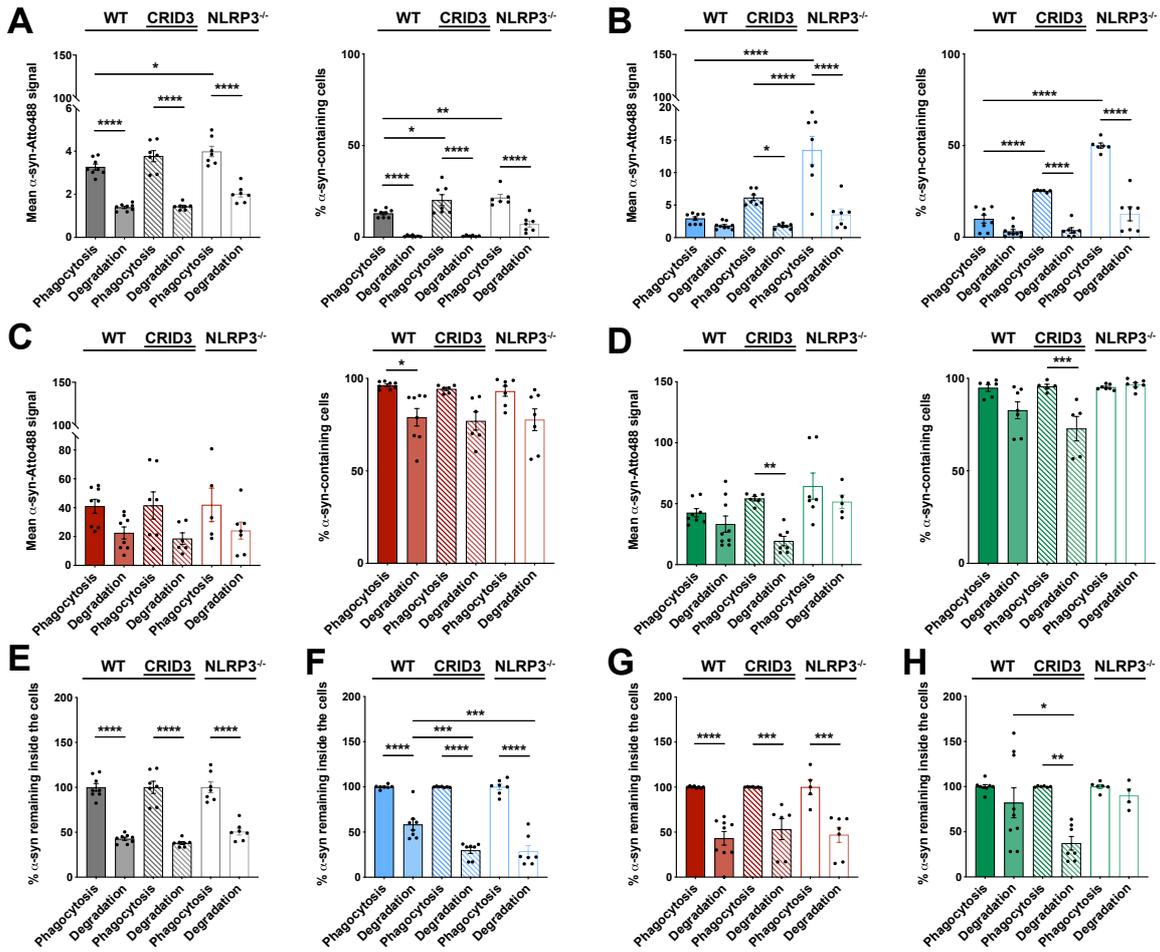
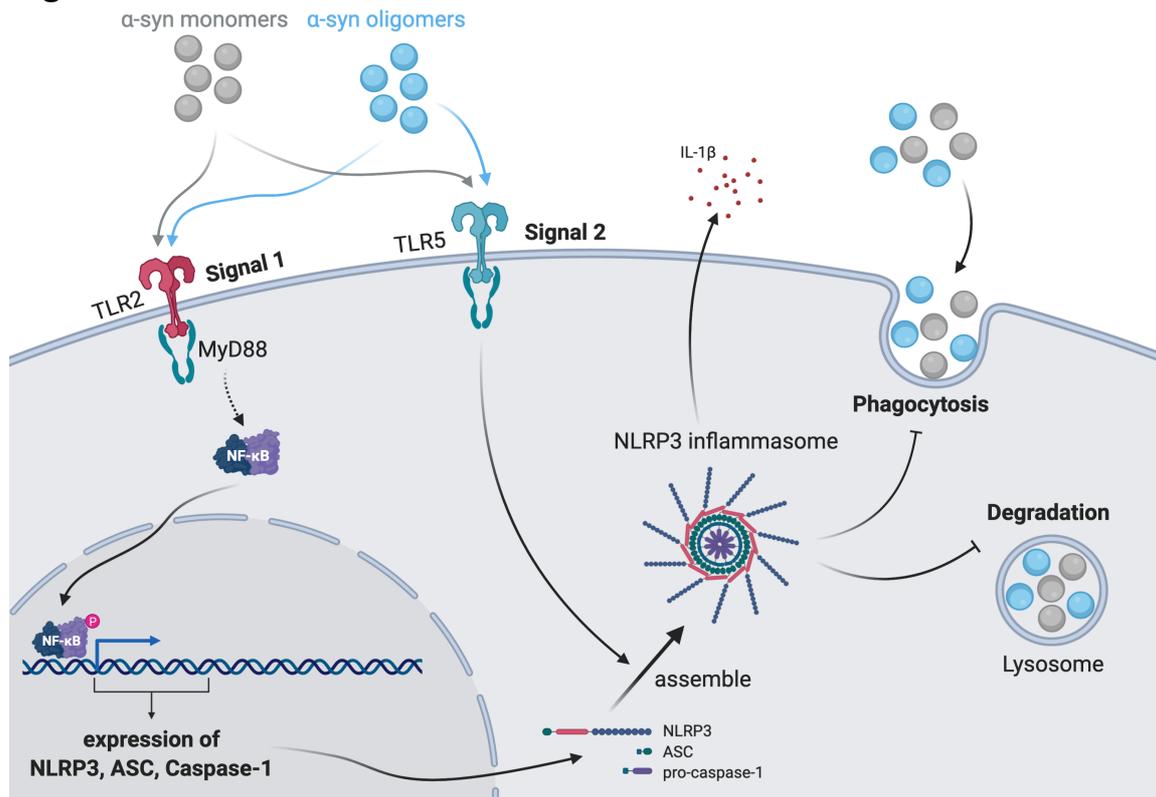


Figure 6



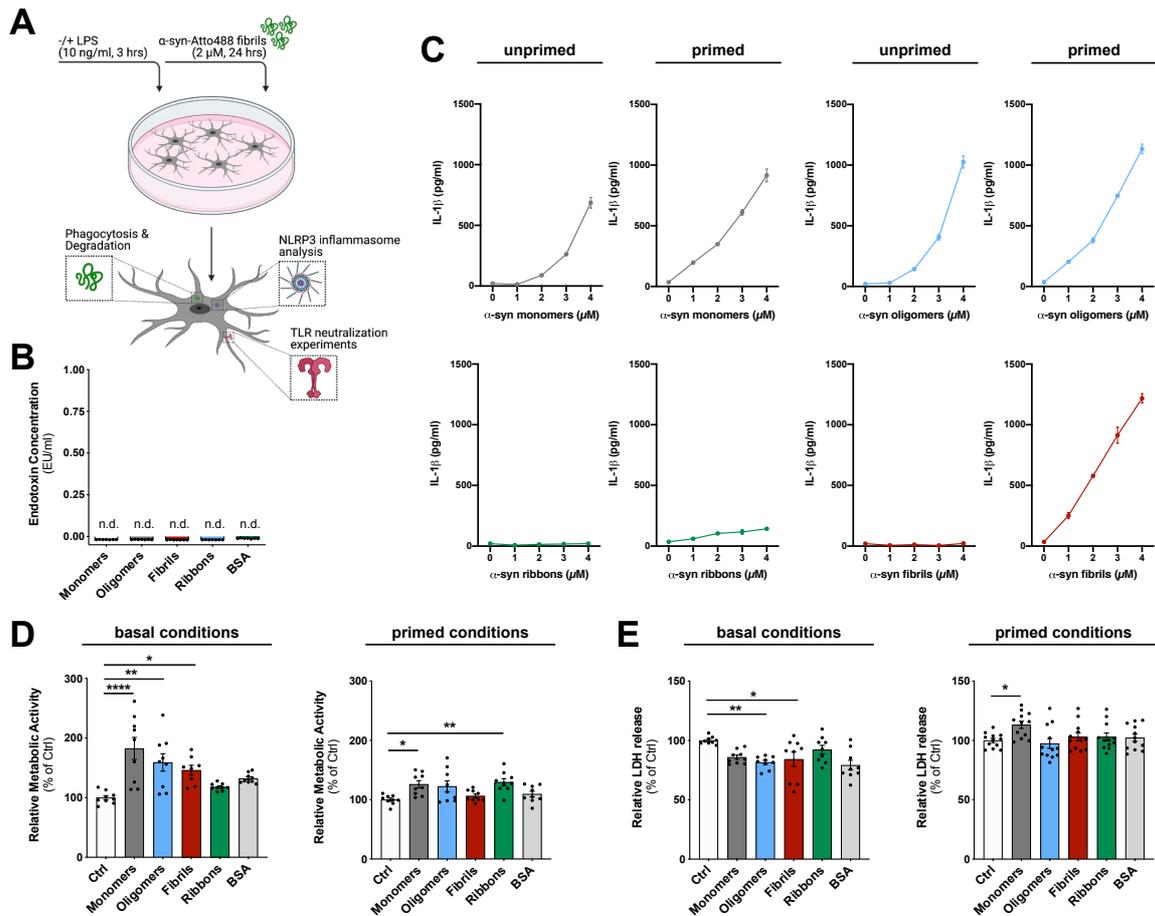


Figure S1

(A) Schematic drawing of the experimental setup used in this study. (B) Quantification of the different α -syn forms for endotoxin verify the preparations as endotoxin-free. (n=3 independent experiments with triplicated measurements). (C) Quantification of IL-1 β levels in conditioned medium of primary microglia treated for 24 hrs with different concentrations of distinct α -syn forms. (n=4 independent experiments with duplicated measurements). Quantification of the relative metabolic activity (D) and the relative LDH release (E) by microglia treated with 2 μ M α -syn under basal conditions or after pre-exposure to LPS. See also Fig. 1. All graphs are presented as mean \pm SEM and were analysed by one-way ANOVA followed by Tukey's multiple comparison post hoc test. Levels of significance are: ****p < 0.0001, ***p < 0.001, **p < 0.01, *p < 0.05.

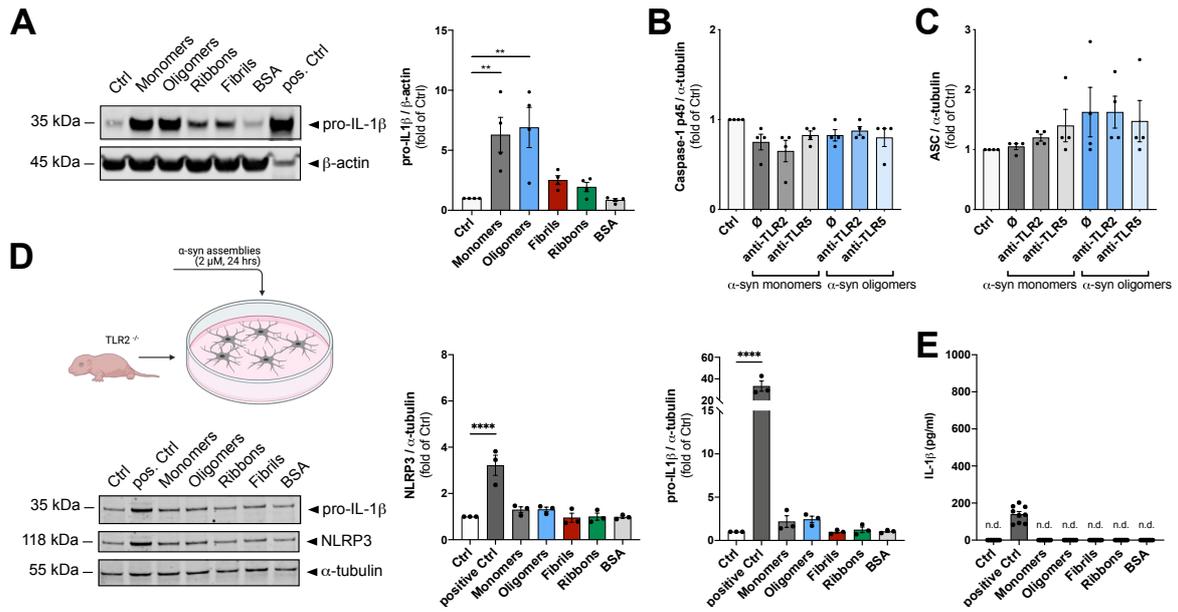


Figure S2

(A) Immunoblot analysis and quantifications of pro-IL1β levels in microglia treated with different α-syn assemblies. Quantification of caspase-1 p45 (B) and ASC (C) expression upon treatment with TLR2 and TLR5 neutralizing antibodies. (n=4 independent experiments). (D) Immunoblot analysis and quantifications of pro-IL1β and NLRP3 levels in microglia derived from TLR2^{-/-} mice treated with different α-syn assemblies. (n=3 independent experiments). (E) Quantification of IL-1β levels in conditioned medium of primary microglia derived from TLR2^{-/-} mice treated for 24 hrs with 2 μM distinct α-syn forms and 200 nM CRID3. (n=3 independent experiments with triplicated measurements). See also Fig. 2. All graphs are presented as mean ± SEM and were analysed by one-way ANOVA followed by Tukey's multiple comparison post hoc test (A, D-E) or Kruskal-Wallis test for nonparametric data (B-C). Levels of significance are: ****p < 0.0001, ***p < 0.001, **p < 0.01, *p < 0.05.

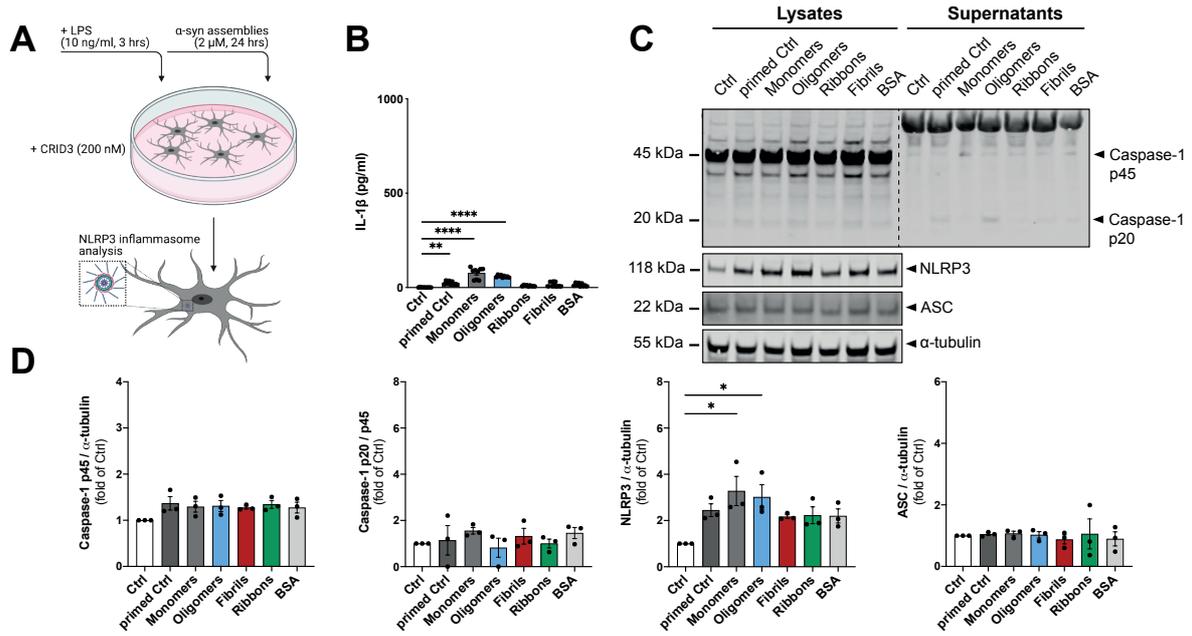


Figure S3

(A) Schematic drawing of the experimental setup used for the following experiments (B-D). (B) Quantification of IL-1 β levels in conditioned medium of LPS-primed primary microglia treated for 24 hrs with 2 μ M distinct α -syn forms and 200 nM CRID3. (n=3 independent experiments with triplicated measurements). (C-D) Immunoblot and quantification of primed microglia cell lysates and supernatants exposed to 2 μ M α -syn and 200 nM CRID3 for 24 hrs, stained for caspase-1, NLRP3, ASC and α -tubulin. All graphs are presented as mean \pm SEM and were analysed by one-way ANOVA followed by Tukey's multiple comparison post hoc test. Levels of significance are: ****p < 0.0001, **p < 0.01, *p < 0.05.

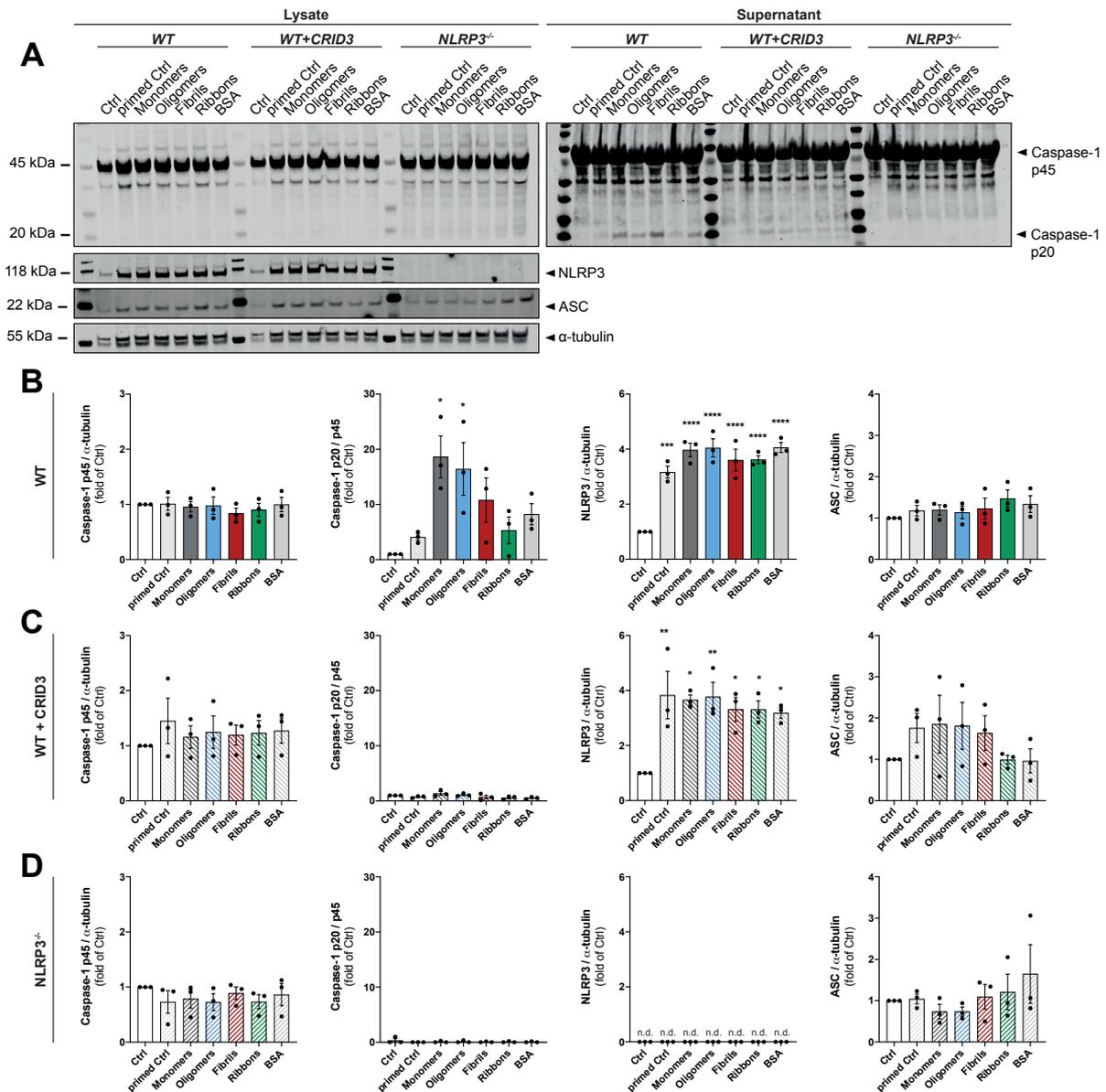


Figure S4

(A) Immunoblot of cell lysates (left) and supernatants (right) of primary microglia derived from WT or NLRP3^{-/-} mice exposed to 2 μM α-syn and 200 nM CRID3 for 24 hrs. Blots were stained for caspase-1, NLRP3, ASC and α-tubulin. Quantification of Caspase-1 p45, cleaved Caspase-1 p20, NLRP3, and ASC in WT (B), WT+CRID3 (C), and NLRP3^{-/-} (D). n=3 independent experiments. All graphs are presented as mean ± SEM and were analysed by one-way ANOVA followed by Tukey's multiple comparison post hoc test. Levels of significance are: ****p < 0.0001, ***p < 0.001, **p < 0.01, *p < 0.05.

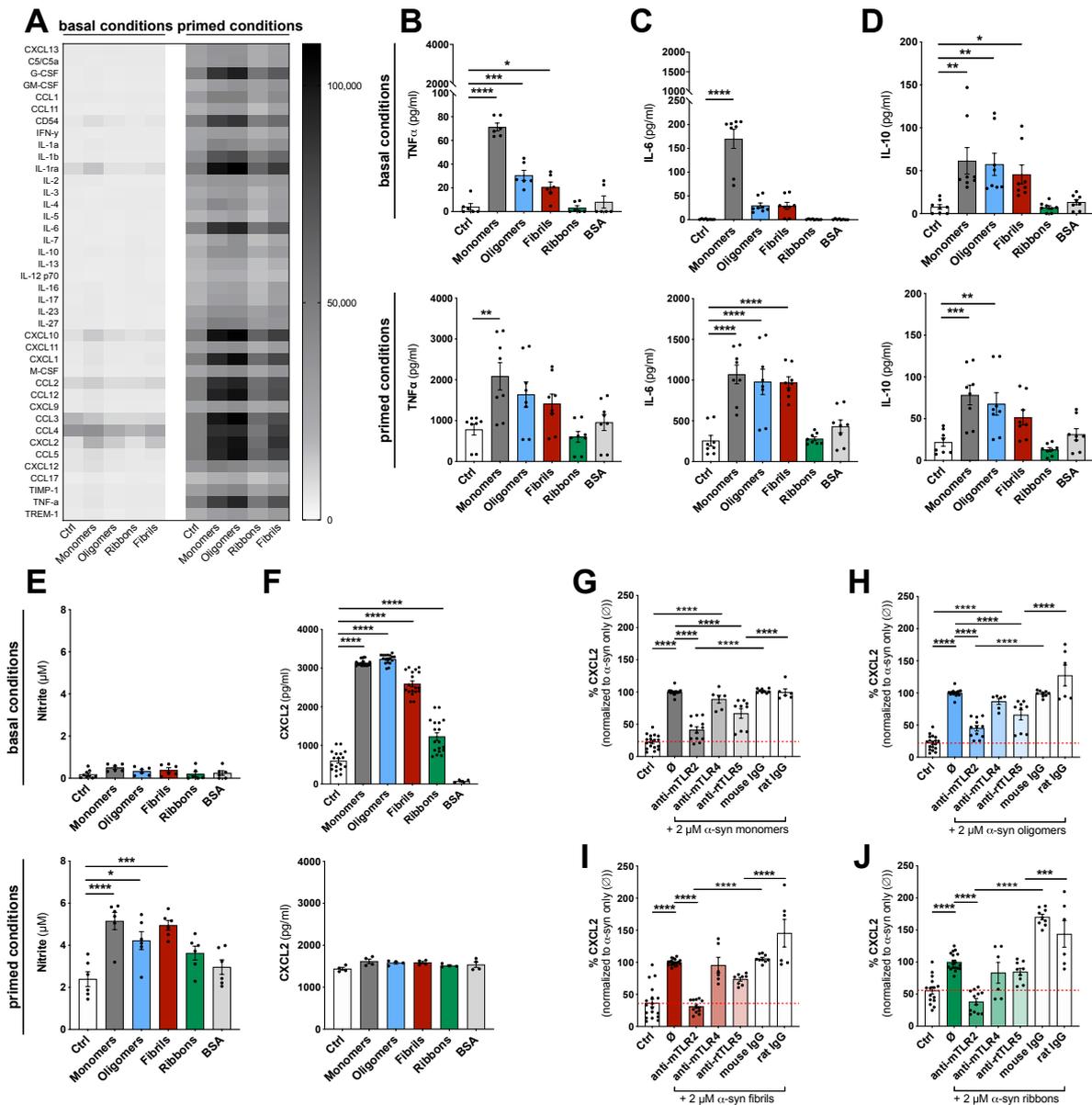


Figure S5

(A) Proteome profiling array on supernatants of primary microglia treated with 2 μ M distinct α -syn forms without or with pre-exposure to LPS for 3 hrs. Quantification of TNF α (B), IL-6 (C), IL-10 (D), Nitrite (E), and CXCL2 (F) in supernatants of primary microglia treated with 2 μ M distinct α -syn forms without (upper panel) or with (lower panel) pre-exposure to LPS for 3 hrs. (n=3-4 independent experiments with single, duplicate or triplicate measurements). CXCL2 levels in conditioned medium of unprimed primary microglia treated for 24 hrs with TLR2-, TLR4, and TLR5-neutralizing antibodies or the respective isotype controls in parallel to stimulation with α -syn monomers (G), oligomers (H), fibrils (I) and ribbons (J). (n=4-6 independent experiments with duplicate or triplicate measurements). All graphs are presented as mean \pm SEM and were analysed by one-way ANOVA followed by Tukey's multiple comparison post hoc test. Levels of significance are: ****p < 0.0001, ***p < 0.001, **p < 0.01, *p < 0.05.

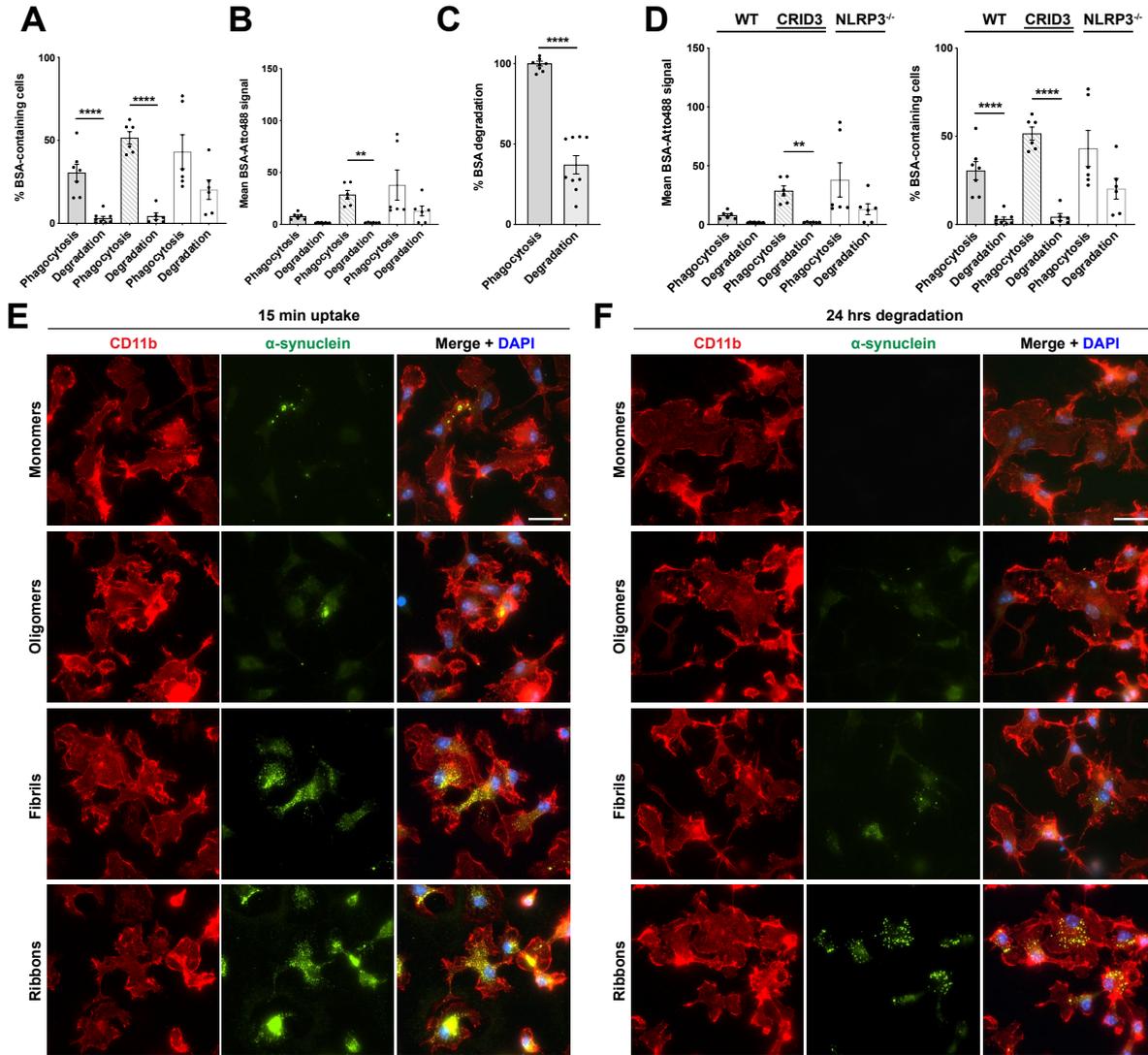


Figure S6

Quantification of the percent of phagocytic cells (**A**), the phagocytic index (**B**), and the percentage of degradation (**C**) of primary microglia exposed to BSA as protein control. (n=5-6 independent experiments). For comparison see also Fig. 4 and 5. (**D**) Quantification and comparison of phagocytosis and degradation of BSA by WT microglia (filled bars), WT microglia treated with the NLRP3 inflammasome inhibitor CRID3 (striped bars), or NLRP3-deficient microglia (empty bars). Representative immunostainings for CD11b-positive microglia (red) and distinct forms of α -syn-Atto488 (green) after 15 minutes of phagocytosis (**E**) or 24 hours of degradation (**F**). All graphs are presented as mean \pm SEM and were analysed by one-way ANOVA followed by Tukey's multiple comparison post hoc test. Levels of significance are: ****p < 0.0001, ***p < 0.001, **p < 0.01, *p < 0.05. Scale bar: 20 μ m.