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1 **Title**

2 Mutant huntingtin protein alters the response of microglial cells to inflammatory stimuli

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10 **Running Title**

11 Mutant huntingtin alters microglial responses

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20 **Abstract**

21 Huntington's disease (HD) is a progressive neurodegenerative disease that affects the striatum and
22 cerebral cortex. It is caused by a dominant CAG trinucleotide expansion in exon 1 of the *HTT* gene.
23 Mutant huntingtin protein (mHtt) is expressed in neurons and immune cells. HD patients demonstrate
24 altered blood cytokine profiles and altered responses of peripheral immune cells to inflammatory
25 stimuli. However, the effects of mHtt on microglial immune responses are not fully understood. Herein
26 we discuss the current understanding of how mHtt alters microglial inflammatory responses. Using
27 lentivirus, we expressed the N171 N-terminal fragment of wild-type or mhtt containing 18 and 82
28 glutamine repeats in cultured EOC-20 microglial cells. We then measured responses to
29 lipopolysaccharide or interleukin-6. Mutant huntingtin-expressing microglial cells produced less
30 interleukin-6 and nitric oxide in response to lipopolysaccharide stimulation than wild-type huntingtin-
31 expressing cells. However, mHtt-expressing microglia stimulated with interleukin-6 produced more nitric
32 oxide than wild-type cells. Mutant huntingtin-expressing cells had higher basal NF-κB and further
33 elevations of NF-κB after interleukin-6 but not lipopolysaccharide stimulation. Thus we demonstrate the
34 potential of mHtt to dampen responses to lipopolysaccharide but potentiate responses to interleukin-6.
35 This work adds to the emerging understanding that mHtt alters not only baseline status of cells but may
36 also result in altered immune responses dependent on the nature of the inflammatory stimuli. We also
37 present our perspective that in human HD the extent of inflammation may depend, in part, on altered
38 responses to varied inflammatory stimuli including environmental factors such as infection.

39 **Keywords**

40 Microglia, lipopolysaccharide, neuroinflammation, interleukin-6, Huntington's disease.

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42 **Inflammation in Huntington's disease**

43 Huntington's disease is caused by a genetically dominant CAG repeat expansion in the
44 huntingtin gene (*HTT*) that results in expression of polyglutamine-expanded mutant huntingtin protein
45 (mHtt) in neurons and microglial cells (Cisbani and Cicchetti, 2012). Neuroinflammation, marked by
46 microglial activation, is an early feature of Huntington's disease (HD) (Sapp et al., 2001, Tai et al., 2007).
47 Further, neuroinflammation may potentiate neurodegenerative processes and promote HD progression.
48 HD-positive individuals exhibit increased systemic inflammation, marked by elevated interleukin 6 and
49 other cytokines, that begins years before clinical onset (Björkqvist et al., 2008). However, the
50 relationship between peripheral and brain inflammation in HD is poorly understood. Further, the extent
51 to which inflammation in HD results from intrinsic effect of mHtt versus altered responses to
52 environmental stimuli is unclear.

53 **Monocytic cells in Huntington's disease**

54 Microglial and monocytes are innate immune cells that may contribute to HD pathogenesis.
55 Microglia are monocyte-like CNS-resident cells and express many of the same surface receptors and
56 markers as peripheral monocytes (Greter et al., 2015). Peripheral monocytes are cellular precursors of
57 tissue-resident macrophages, dendritic cells, monocyte-derived suppressor cells and infiltrating
58 microglial-like cells in the CNS (Greter et al., 2015). Both microglial cells and monocytes can be activated
59 by immune mediators as well as directly by microbial molecules.

60 Mutant huntingtin is expressed in both microglia and monocytes in HD patients (Weiss et al.,
61 2012). In the absence of external inflammatory stimulation, mHtt promotes cell-autonomous activation
62 of primary microglial cells (Crotti et al., 2014). Immune cells from pre-manifest HD patients and mouse
63 models demonstrate a pro-inflammatory phenotype as illustrated by increased levels of several
64 cytokines in blood, including interleukin-6 (IL-6) (Crotti et al., 2014, Björkqvist et al., 2008). Mutant

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65 huntingtin expression in human monocytes is associated with increased production of cytokines
66 interleukin-6 (IL-6), IL-1β, and TNF-α (Träger et al., 2014). IInterleukin-6 induces microglial proliferation
67 and is associated with increased microglia proliferation in response to mHtt-expressing neurons (Kraft et
68 al., 2012). Furthermore, monocytes isolated from HD patients and mice had reduced chemotactic
69 responses to ATP and complement protein C5a further demonstrating that mHtt exerts a modifying
70 effect on these cells (Kwan et al., 2012). Together, these data indicate that HD monocytes and microglia
71 have changes in basal activation state and that mHtt may alter how monocyte-derived cells respond to
72 innate immune stimuli and/or sensitivity to cytokine and chemokine stimulation.

73 Macrophages can be polarized in their response to be either “M1” or “M2” cells (Mills et al.,
74 2000). In HD, expression of CCR2 and production of the pro-inflammatory cytokine IL-12 are used to
75 identify M1 macrophages while CX3CR1 expression and immunosuppressive IL-10 production are used
76 to identify M2 macrophages (Di Pardo et al., 2013). Interestingly, HD patient monocytes are initially
77 more M1 polarized as evidenced by increased percentages of CCR2+ and IL-12+ macrophages before
78 disease onset, with a transition to M2 macrophages that express CX3CR1 and IL-10 later in the disease
79 course (Di Pardo et al., 2013). The M1-M2 polarization is partially mediated by nuclear factor kappa-
80 light-chain-enhancer of activated B-cells (NF-κB) (Tugal et al., 2013). Activation of the NF-κB p65 subunit
81 is critical for development of M1-associated functions in macrophages and NF-κB p65 is increased in HD
82 patients before clinical (Di Pardo et al., 2013). Thus, one putative mechanism of aberrant pro-
83 inflammatory responses observed during HD is the dysregulation of NF-κB activation in monocytes. The
84 switch of monocytes to acquire a more M2-like phenotype later in the disease course could indicate a
85 compensatory change to counteract the sustained NF-κB activation and M1-associated inflammatory
86 state early in HD.

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87 Compared to monocytes, less is known about how mHtt impacts the inflammatory status and
88 the responsiveness of microglial cells to immune stimulation. Mouse models of HD demonstrate that
89 mHtt can modify microglial responses. Lipopolysaccharide (LPS) treatment of YAC128 HD mice, which
90 express full-length mHtt, have increased microglial cell activation compared to wild-type litter-mates
91 (Franciosi et al., 2012). N171-82Q HD mice have increased brain indoleamine-2,3-dioxygenase, a
92 microglial enzyme activated by inflammation, and an altered response to the protozoan *Toxoplasma*
93 *gondii* (Donley et al., 2016). Therefore, based on previous studies, it is our perspective that mHtt
94 presence is sufficient to activate monocytes/microglia and alter both the inflammatory profile and
95 responses to extrinsic immune stimulation.

96 **NF-κB pathway activation in Huntington's disease**

97 Mutant huntingtin alters inflammatory responses in part through the NF-κB pathway as
98 demonstrated by decreased serum IL-6 in HD mice after NF-κB suppression (Garcia-Miralles et al., 2016).
99 It binds IKKγ, leading to increased IκB degradation and increased NF-κB signaling resulting in increased
100 pro-inflammatory cytokine levels, including increased IL-6 (Träger et al., 2014). Mutant huntingtin also
101 triggers mislocalization of NF-κB in synapses of neurons (Marcora and Kennedy, 2010). Decreasing NF-
102 κB nuclear translocation is protective in HD mice further demonstrating its importance (Garcia-Miralles
103 et al., 2016, Marcora and Kennedy, 2010). NF-κB signaling pathway can be triggered by pattern-
104 recognition receptors (PRR) on innate immune cells that recognize pathogen-associated molecular
105 patterns (PAMP) (Newton and Dixit, 2012, An et al., 2002). These PRR include the toll-like receptors
106 (TLRs). LPS is an immunogenic PAMP of gram-negative bacteria such as *E. coli* that is recognized by toll-
107 like receptor 4 (TLR4) resulting in NF-κB signaling and increased production of inflammatory mediators
108 including IL-6 and inducible nitric oxide synthase (iNOS) (Chanput et al., 2010, Thirunavukkarasu et al.,
109 2006, Libermann and Baltimore, 1990, An et al., 2002, Chow et al., 1999). Signaling from IL-6 (and other

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110 cytokines) then results in upregulation of iNOS via JAK/STAT (IL-6 specifically via STAT3) signaling,
111 leading to elevated nitric oxide (NO) production (Dawn et al., 2004, Yu et al., 2003). Interleukin-6 can
112 also stimulate NF-κB independent of LPS (Wang et al., 2003). Whether IL-6 directly upregulates NF-κB
113 through JAK/STAT family transcription factor or through an indirect mechanism such as regulation of IKK
114 is not clear (Lee et al., 2009, Wang et al., 2003, Yang et al., 2007). However, the evidence suggests that
115 STAT3 synergizes with NF-κB resulting in increased pro-inflammatory cytokines including TNF-α, IL-1,
116 and IL-6 that are increased in HD (Yang et al., 2007, Grivennikov and Karin, 2010, Björkqvist et al., 2008).

117 These findings support the possibility that if mHtt increases activation of NF-κB then synergy
118 with other pathways, such as STAT signaling, could promote inflammation in HD. Evidence suggests that
119 STAT-dependent cytokine signaling may synergize with NF-κB-activating PRR signaling to produce a
120 hyper-responsive state in HD monocytes and microglia treated with LPS (NF-κB-activating) and IFN-γ
121 (STAT-activating) (Björkqvist et al., 2008). However, it is unknown which signaling mechanism(s), PRR or
122 JAK/STAT, are altered by mHtt and whether mHtt differentially impacts these pathways. Therefore, here
123 we tested whether mHtt expressing microglial cells have altered responses to LPS to model PRR
124 stimulation, and also to IL-6 stimulation to model STAT-mediated immune stimulation.

125 **Mutant huntingtin expression alters microglial responses to immune stimuli**

126 The mouse microglial cell line EOC-20 (American Type Culture Company, CTRL-2469) was utilized
127 as they have previously been used to study responses to inflammatory stimulus (Hensley et al., 2003,
128 Mencel et al., 2013, Guadagno et al., 2013, Walker et al., 1995). Cells were cultured at 37°C and 5% CO_2
129 in high glucose DMEM media supplemented with 20% v/v LADMAC-conditioned media according to
130 ATCC guidelines. LADMAC cells are bone marrow-derived cells that produce high amounts of colony
131 stimulating factor-1 needed to support EOC-20 cell growth (Olivas et al., 1995). Conditioned media was
132 collected and frozen at -20°C until use. Wild-type (N181-18Q) or mHtt (N171-82Q) protein, or eGFP were

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133 expressed in EOC-20 cells using independent lentiviruses that use the phosphoglycerate kinase
134 promoter. Huntingtin constructs expressed the first 3 introns of human HTT, including the CAG tract in
135 exon 1. For each of the three viruses, a four-plasmid system was used; plasmids were transfected into
136 HEK293T cells, virus was harvested from the media, quantified, and stored as previously described (Fox
137 et al., 2015).

138 We evaluated viral transduction efficiency using eGFP-encoding virus. We identified 77.8% ± 2.5
139 (means ± SE, n=4) GFP-positive cells 24 hours post transduction (**Fig. 1A**). We further verified N171-
140 18/82Q expression using the MAB5492 (EMD Millipore) (**Figs. 1B-C).** Inclusions were not observed.
141 Because the antibody also identifies endogenous murine huntingtin we quantified total cellular
142 fluorescence on a per-cell basis in mock transduced and N171 huntingtin expressing cells. N171-18/82Q
143 expression resulted in a greater than 2-fold increase in expression over levels attributed to endogenous
144 huntingtin protein (**Fig. 1D**). There was no effect of N171-18/82Q expression on cell viability compared
145 to GFP as measured using a lactate dehydrogenase release assay (**Fig. 1E**). The microglial culture model
146 therefore provides a way to assess the effects of mHtt on intrinsic responses to inflammatory stimuli.

147 Effects of wild-type huntingtin and mHtt on the microglial cells was assessed by measuring
148 responses to *E. coli* LPS (Sigma) and interleukin-6 (Biolegend). We measured nitrite, iNOS and NF-κB
149 levels in response to these; additionally we measured IL-6 production after LPS stimulation. Nitrite is an
150 oxidative product of NO that is further oxidized in medium to nitrate. Nitrates were reduced to nitrite
151 using *Aspergillus* nitrate reductase and the Griess test was used to quantify nitrite (Guevara et al., 1998,
152 Gilliam et al., 1993). Total nitrite/nitrate (hereafter called nitrite) is a surrogate for iNOS activity.
153 Standards were made using sodium nitrite in cell culture media. IL-6 was quantified using an ELISA
154 according to manufacturer's guidelines (Biolegend). Relative iNOS was quantified using an ELISA
155 (MyBioSource) (Mendonca et al., 2017). Activation of NF-κB was quantified using an ELISA for total and

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156 phosphorylated p65 subunit levels (Thermo Fisher) (Roth-Walter et al., 2014). The ratio of
157 phosphorylated to total NFκB p65 is presented with increasing ratio indicative of activation. As expected
158 both GFP and N171-18Q expressing cells demonstrated significantly increased IL-6 and nitrite levels in
159 response to LPS. However, N171-82Q expressing cells lacked a response to LPS (**Figs. 2A-B**). Consistent
160 with decreased nitrite levels following LPS stimulation, iNOS levels in N171-82Q cells did not increase
161 following LPS treatment (**Fig. 2C**). Since LPS activates NF-κB which then upregulates expression of IL-6
162 and iNOS we quantified NF-κB activation in our cells with and without LPS stimulation. Baseline NF-κB
163 activation measured by p65 subunit phosphorylation was increased in N171-82Q expressing cells
164 compared to GFP and N171-18Q expressing cells (**Fig. 2D**). Low dose LPS increased NF-κB in GFP and
165 N171-18Q expressing cells only while high does LPS increased NF-κB in all groups compared to no LPS
166 controls (**Fig. 2D**).

167 We then tested the effect of STAT pathway-inducing IL-6 on iNOS and NF-κB activation. IL-6 did
168 not have a significant effect in GFP and N171-18Q groups; however, N171-82Q cells demonstrated
169 significantly increased nitrite production and iNOS levels (**Figs. 2E-F**). N171-82Q expressing cells also
170 demonstrated increased NF-κB activation after IL-6 stimulation (**Fig. 2G**) and had a greater level of NF-
171 κB activation compared to low dose LPS (**Fig. 2D**). These results indicate that mHtt sensitizes these cells
172 to IL-6 stimulation, an effect opposite to that of LPS (see model **Fig. 2H**).

173 **Mutant Huntingtin impacts microglial immune signaling**

174 Mutant huntingtin promotes increased inflammation and microglial cell activation in human HD
175 (Crotti et al., 2014, Björkqvist et al., 2008, Tai et al., 2007). However, the ways that mHtt drives this
176 process, especially in microglial cells is unclear. Mutant huntingtin could affect microglial cell
177 inflammatory processes in the absence of immune stimulation, and/or alter their responses to immune
178 stimulants. Here we assessed the effect of mHtt on cultured microglial cell functional responses to LPS

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179 and IL-6-mediated stimulation. Not only did mHtt alter microglial immune responses, but the direction
180 of the response depended on the nature of stimulation (**Fig. 2**).

181 LPS signals TLR4-mediated NF-κB activation results in induction of iNOS and IL-6 (Libermann and
182 Baltimore, 1990, Arias-Salvatierra et al., 2011). The low dose of LPS we used is established to activate
183 NF-κB (Sung et al., 2014). The lack of increased NF-κB activation with low dose LPS stimulation in N171-
184 82Q expressing cells together with decreased nitrite and IL-6 suggests that mHtt induces tolerance
185 similar to LPS-induced models of tolerance in macrophages. LPS-induced macrophage tolerance is
186 characterized, among other mechanisms, by increases in the suppressive p50 NF-κB subunit that
187 reduces inflammation by blocking NF-κB-p65 (Kastenbauer and Ziegler-Heitbrock, 1999, Rajaiah et al.,
188 2013). Therefore, while NF-κB-p65 is increased, cells are "tolerized" as displayed by decreased
189 functionality which can be overcome by high doses of LPS stimulation.

190 HD monocytes and microglia have increased IL-6 production when stimulated with both LPS and
191 IFN-γ, a STAT activator (Björkqvist et al., 2008). In the studied microglial cell line, mHtt had disparate
192 effects on these responses (**Fig. 2H**). We observed that mHtt rendered the microglial cells
193 hyporesponsive to low-dose LPS, but hypersensitive to IL-6, a cytokine that activates STAT pathways
194 (**Figs. 2E-H**). STAT3 signaling downstream of IL-6 can synergize with NF-κB to promote inflammation
195 (Grivennikov and Karin, 2010, Yang et al., 2007). Therefore, one possibility is that mHtt, despite reducing
196 PRR signaling downstream of LPS stimulation, could promote synergy of NF-κB (from a PRR agonist) and
197 STAT transcription factors (from cytokine signaling) and enhance immune activation. Our data suggests
198 the possibility of a novel mechanism underlying HD-associated neuroinflammation driven by interaction
199 of NF-κB and STAT signaling pathways and/or the differential effect of mHtt on these pathways.

200 The findings also suggest a possible mechanism where IL-6 downstream of LPS stimulation is
201 necessary in microglial cells to drive iNOS activity and NO production. When mHtt-expressing cells are

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202 stimulated with IL-6, NF-κB activation and nitrite are greatly increased. Given these data, we think that
203 the IL-6 requirement for iNOS activity in microglia is not a complete explanation of our results. Therefore
204 our perspective, taking into consideration previously published studies, is that mHtt has disparate
205 effects on PRR signaling and STAT signaling pathways that differentially impacts sensitivity of cells to
206 extrinsic stimuli which impacts microglial activation. Cell culture studies may not replicate all aspects of
207 endogenous microglia, therefore additional studies are needed to determine if similar effects of mHtt on
208 microglia occur in other microglial culture models and *in vivo*.

209 **Conclusion**

210 HD is only caused by a mutation within the *HTT* gene. However, there is significant variability in
211 age of onset, after adjusting for the size of the CAG repeat size, that is partly explained by
212 environmental factors (USVCRP and Wexler, 2004). There is considerable evidence that mHtt results in
213 increased baseline inflammation (Björkqvist et al., 2008, Träger et al., 2014, Crotti et al., 2014).
214 However, recent evidence also points towards altered responses to a common infection in a mouse
215 model of HD (Donley et al., 2016). Here we demonstrate disparate effects of LPS and IL-6 on microglial
216 responses in mHtt expressing microglial cells. These finding suggest that mHtt may also alter responses
217 to other immune molecules, microbial antigens, or neurotropic pathogens. If these mechanisms apply to
218 human HD then these could contribute significantly to modifying age of onset or progression by altering
219 systemic and brain inflammatory pathways. More work is clearly needed to fully understand the
220 mechanisms underlying altered immune responses in HD cells. However, the findings contribute to
221 understanding mechanisms of mHtt-induced neuroinflammation and neurodegeneration as well as the
222 variability in human HD.

223 **Acknowledgements**

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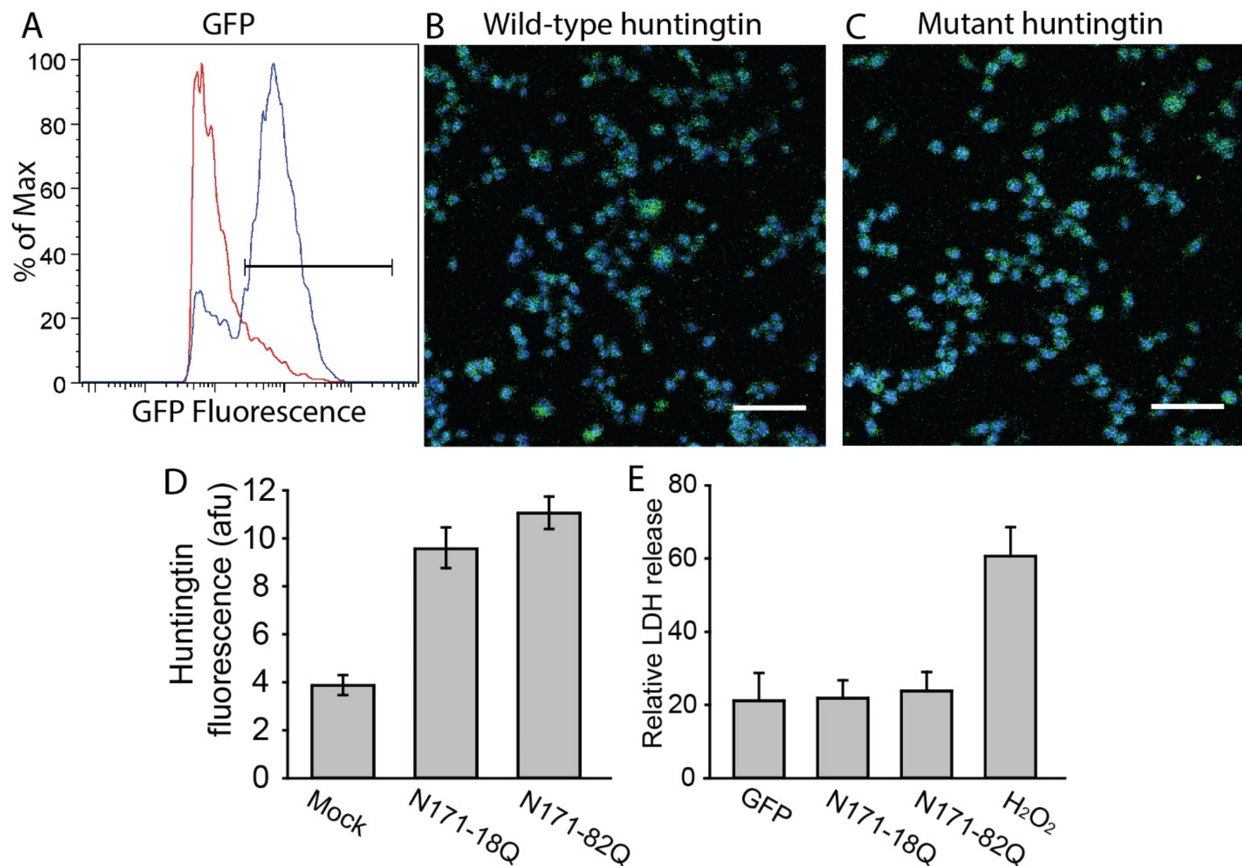
228 of laboratory equipment.

229 **Conflict of Interest**

230 The authors have no conflict of interest to report. The funding agents had no role in study design, data
231 collection and analysis, decision to publish, or preparation of the manuscript.



232

233 **Figure 1. Lentiviral-mediated huntingtin expression in cultured microglial cells.** EOC-20 cells were
234 transduced for 24 hours by adding 10 ng/ml p24 viral equivalents to the media with 8 μg/ml

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235 hexadimethrine bromide (Sigma). **A**. Transduction efficiency as determined by the proportion of GFP-
236 expressing cells, quantified using a Guava easyCyte 12HT flow cytometer. After transduction in 6-well
237 plates, cells were scraped in PBS, counted, and plated for analysis at 5.0×10^5 cells/well. Dead cells were
238 labeled with Fixable Near-IR Live/Dead stain (Life Technologies) and gated from the final analysis.
239 Representative fluorescence histogram shows the gating strategy for identifying GFP expressing cells.
240 Red = mock transduced cells; blue = eGFP transduced cells. **B-C**. Confocal photomicrographs showing
241 N171-18Q (B) and N171-82Q (C) huntingtin expression in EOC-20 cells. Blue = dapi, green = huntingtin;
242 scale bars = 50 μm. **D**. Fluorescence was quantified on a per-cell basis using DAPI to identify cells from
243 immunofluorescence images. N=152 cells from three mock transduced wells, n=170 cells from three
244 N171-18Q transduced wells, and n=199 cells from three N171-82Q transduced wells. Bars represent
245 means ± 95% CI. **E**. Expression of N171-82Q does not alter cell viability as determined by LDH release.
246 Lactate dehydrogenase (LDH) activity was measured in the cell fraction and medium. Relative LDH
247 release was determined as the activity in the supernatant divided by the total activity in supernatant
248 and cell fraction. Two percent hydrogen peroxide was used as a positive control. N=7 for GFP, N171-
249 18Q, N171-82Q groups and N=4 for H_2O_2 control. Bars represent means ± standard errors.

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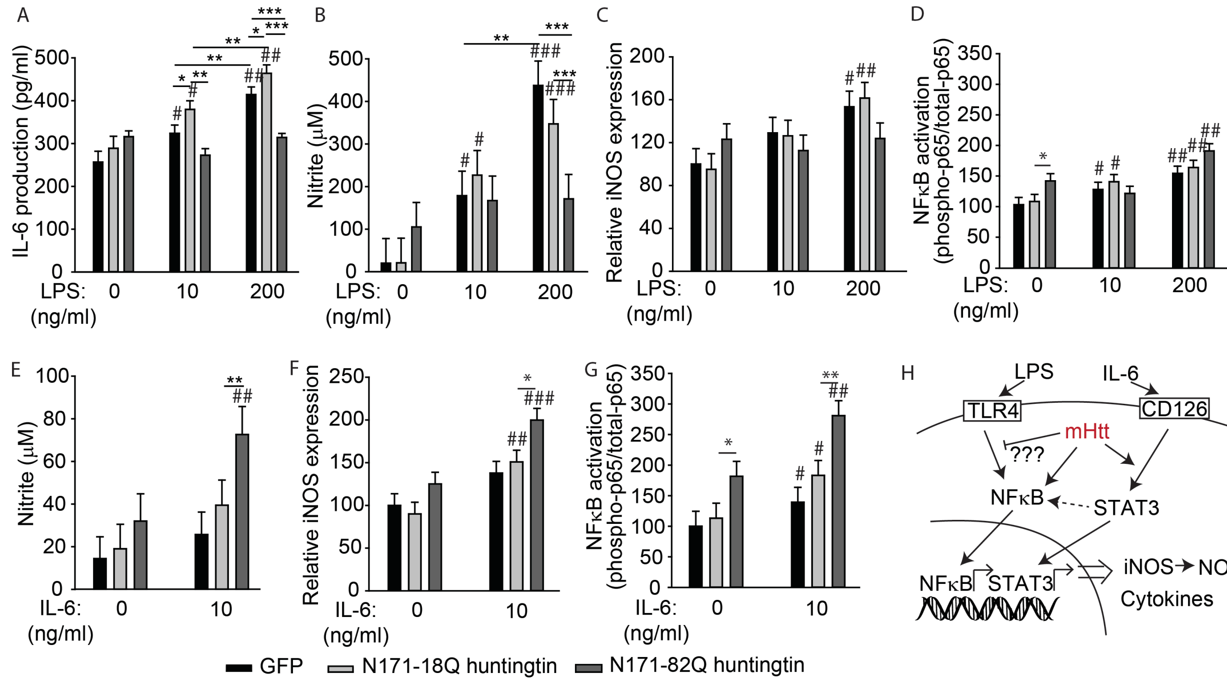

250

251 **Figure 2. Mutant huntingtin alters the response of cultured microglial cells to immune stimulation.**

252 Culture media was changed 24 hours after transduction then EOC-20 cells were stimulated with LPS or

253 IL-6 for 12 hours (**A-C, E-F**) or 2 hours (**D, G**) before analysis. Cells were lysed then IL-6 and nitrite

254 measured in the supernatant fractions. Experimental values for each well were determined from

255 technical duplicates. n=independent samples. Bars: left to right; black=GFP, light gray=N171-18Q, and,

256 dark gray=N171-82Q. **A-B**. GFP and N171-18Q expressing cells dose-dependently respond to LPS

257 stimulation by increasing IL-6 and nitrite production. In contrast, N171-82Q-expressing cells fail to

258 upregulate IL-6 in response to LPS (**A**, n=6) and have less nitrite production (**B**, n=8). **C**. GFP and wild-

259 type huntingtin-expressing, but not mHtt-expressing cells upregulate iNOS in response to LPS (n=5). **D.**

260 Cells expressing mHtt have higher NF-κB activation at baseline and altered responses to LPS compared

261 to wild-type expressing cells (n=6). **E**. Cells expressing mHtt have increased nitrite production after IL-6

262 stimulation (n=6). **F.** Mutant huntingtin-expressing cells have increased iNOS in response to IL-6

263 treatment compared to wild-type huntingtin-expressing cells. **G**. Cells expressing mHtt have higher NF-

264 κB activation at baseline and with IL-6 stimulation compared to wild-type expressing cells (n=6).

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265 Bars=means ± SE. P-values: #<0.05, ##<0.01, and ###<0.001 comparison to no LPS or IL6 treatment
266 within the same group. *=p<0.05, **=p<0.01, ***=p<0.001. Analyses were performed using the GLM
267 procedure in SAS. Both iNOS and NF-κB relative levels were normalized to GFP-transduced cells. **H**.
268 Model figure. Mutant huntingtin alters inflammatory signaling in microglia resulting in decreased
269 responses to LPS and increased response to IL-6 stimulation.

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