HTT-lowering reverses Huntington's disease immune dysfunction caused by NFκB-pathway dysregulation

Running title: Reversing immune dysfunction in HD

Ulrike Träger[1], Ralph Andre[1], Nayana Lahiri[1], Anna Magnusson-Lind[1,2], Andreas Weiss[3], Stephan Grueninger[3], Chris McKinnon[1], Eva Sirinathsinghji[4], Shira Kahlon[5], Edith Pfister[6], Roger Moser[7], Holger Hummerich[1], Michael Antoniou[4], Gillian Bates[4], Ruth Luthi-Carter[7,8], Mark W Lowdell[9], Maria Björkqvist[2], Gary Ostroff[5], Neil Aronin[6] and Sarah J Tabrizi[1]

[1]UCL Institute of Neurology, Dept. of Neurodegenerative Disease, London, UK;
[2]Wallenberg Neuroscience Centre, Dept. of Experimental Medical Science, Brain Disease Biomarker Unit, Lund University, Lund, Sweden;
[3]Novartis Institutes for BioMedical Research, Novartis Campus, Basel, Switzerland;
[4]King's College London, Dept. of Medical and Molecular Genetics, Guy's Hospital, London, UK;
[5]University of Massachusetts Medical School, Program in Molecular Medicine, Worcester, MA, USA;
[6]University of Massachusetts Medical School, Dept. of Medicine, Division Endocrinology & Metabolism, Worcester, MA, USA;
[7]École Polytechnique Fédérale de Lausanne, Brain Mind Institute, Lausanne, Switzerland;
[8]University of Leicester, Biological Sciences and Psychology, College of Medicine, Dept. of Cell Physiology and Pharmacology, Leicester, UK;
[9]UCL, Department of Haematology, Royal Free Campus, London, UK;

Corresponding author: **Sarah J Tabrizi**

Department of Neurodegenerative Disease,

UCL Institute of Neurology,

Queen Square,

London,

WC1N 3BG, UK

Email = **s.tabrizi@ucl.ac.uk**

Tel: +44(0)203-448-4434

Fax: +44(0)207-676-2180

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Abstract

Huntington's disease is an inherited neurodegenerative disorder caused by a CAG repeat expansion in the huntingtin gene. The peripheral innate immune system contributes to Huntington's disease pathogenesis and has been targeted successfully to modulate disease progression, but mechanistic understanding relating this to mutant huntingtin expression in immune cells has been lacking. Here we demonstrate that human Huntington's disease myeloid cells produce excessive inflammatory cytokines due to cell-intrinsic effects of mutant huntingtin expression. A direct effect of mutant huntingtin on the NFκB pathway, whereby it interacts with IKKγ, leads to increased degradation of IκB and subsequent nuclear translocation of RelA. Transcriptional alterations in intracellular immune signalling pathways are also observed. Using a novel method of small interfering RNA delivery to lower huntingtin expression, we show reversal of disease-associated alterations in cellular function - the first time this has been demonstrated in primary human cells. Glucan-encapsulated small interfering RNA particles were used to lower huntingtin levels in human Huntington's disease monocytes/macrophages, resulting in a reversal of huntingtin-induced elevated cytokine production and transcriptional changes. These findings improve our understanding of the role of innate immunity in neurodegeneration, introduce glucan-encapsulated small interfering RNA particles as tool for studying cellular pathogenesis *ex vivo* in human cells and raise the prospect of immune cell-directed HTT-lowering as a therapeutic in Huntington's disease.

Keywords: Huntington's disease, immunology, myeloid cells, gene lowering

Introduction

Huntington's disease is an incurable, autosomal dominant neurodegenerative disorder caused by a CAG repeat expansion in exon 1 of the huntingtin (*HTT*) gene leading to an expanded stretch of 36 or more glutamine residues in the N-terminal region of the HTT protein (The Huntington's Disease Collaborative Research Group, 1993). The disease is characterised by progressive cognitive, psychiatric and motor impairments caused by neuronal dysfunction and cell death.

Whilst primary pathology in Huntington's disease is believed to arise from basal ganglia degeneration, HTT expression has been found in all tissues studied (Li et al., 1993). Indeed, numerous studies of Huntington's disease patients and mouse models have described abnormalities in peripheral tissues, including weight loss, muscle wasting, diabetes and changes in the neuro-endocrine system (van der Burg et al., 2009). Mutant HTT expression in non-neuronal cells in both the brain and the periphery may contribute to Huntington's disease neuropathology.

HTT is expressed in immune cells (Weiss *et al.*, 2012), and both central and peripheral immune system abnormalities have been shown in Huntington's disease patients (Soulet and Cicchetti, 2011). Microglia, the resident immune cells of the brain (Ransohoff and Perry, 2009), are sustained by self-renewal (Ajami *et al.*, 2007), however disrupting the blood brain barrier by irradiation has shown that blood monocytes are able to populate the brain (Simard and Rivest, 2004). Microglial activation, seen in post-mortem Huntington's disease brain tissue (Sapp *et al.*, 2001) and by PET imaging, occurs in Huntington's disease gene carriers before symptom onset (Tai *et al.*, 2007). We have previously demonstrated peripheral immune system dysfunction in Huntington's disease, including changes in innate immune proteins in patient plasma (Dalrymple *et al.*, 2007). Moreover, elevated plasma cytokine (Björkqvist *et al.*, 2008)

and chemokine (Wild *et al.*, 2011) levels in patients correlate with disease progression and can be detected years before disease onset. We have shown that primary human monocytes are hyper-reactive in response to lipopolysaccharide (LPS), producing increased levels of interleukin (IL)-6. This phenotype is replicated in murine mutant HTT expressing macrophages and microglia, demonstrating that peripheral cells could mirror pathology in the CNS in Huntington's disease (Björkqvist *et al.*, 2008).

Furthermore, several recent studies have suggested that the peripheral immune system can act as a modifier of Huntington's disease neuropathology. Transplantation of wild-type bone marrow into Huntington's disease mice partially rescues their motor defects, increases synaptogenesis and reduces elevated plasma cytokine levels (Kwan *et al.*, 2012). Peripheral administration of a kynurenine 3-monooxygenase (KMO) inhibitor extends lifespan, prevents synaptic loss and decreases microglial activation in Huntington's disease mice. As the drug cannot cross the blood brain barrier, the neuroprotective effect is secondary to inhibition of KMO in peripheral immune cells (Zwilling *et al.*, 2011). Furthermore, treatment with a cannabinoid receptor 2 (CB2) agonist known to dampen immune responses, suppresses motor deficits and CNS inflammation whilst extending life span in a Huntington's disease mouse model. This positive effect can be blocked with an antagonist that is restricted to the periphery, demonstrating the importance of peripheral immune cells in modulating pathogenesis (Bouchard *et al.*, 2012). These studies provide strong evidence that the immune system plays a disease-modifying role in Huntington's disease neuropathogenesis, but the mechanism(s) by which mutant HTT expression in immune cells causes this dysfunction has not yet been established.

Intracellular signalling pathways leading to the activation of the transcription factor NFκB are important regulators of cytokine production and play a key role in inflammation. Events such

as the activation of Toll-like receptors (TLRs) lead to signal transduction via adapter proteins MyD88 and IRAK1, leading to the phosphorylation and activation of IKK. This kinase phosphorylates IκB, which is then ubiquitinated and degraded by the proteasome, whereby it dissociates from the NFκB transcription factor subunits (RelA, RelB, cRel, NFκB1, NFκB2) that it sequesters in an inactive state in the cytoplasm. The free NFκB molecules can then translocate into the nucleus and activate gene transcription (Hayden and Ghosh, 2012). The NFκB pathway has previously been implicated in Huntington's disease, with Khoshnan et al. having shown in inducible PC12 cells and striatal extracts from R6/2 Huntington's disease mice that overexpression of mutant HTT exon 1 can activate the NFκB pathway by directly interacting with IKKγ (Khoshnan *et al.*, 2004). Similarly, a recent study has shown enhanced NFκB signalling in astrocytes isolated from R6/2 mice (Hsiao *et al.*, 2013). It remains to be shown that this interaction also occurs in a human system with expression of full-length HTT at normal allelic expression levels.

The present work seeks to identify the mechanism of dysfunction in primary human Huntington's disease monocytes and macrophages *ex vivo*. We have characterised immune cell dysfunction by detailed cytokine profiling and study of upstream intracellular signalling pathways, identifying NFκB pathway dysregulation as the cause of immune dysfunction. We have used overexpression studies and a novel small interfering RNA (siRNA)-mediated knock-down technique to investigate the role cell-intrinsic HTT plays in human Huntington's disease monocyte and macrophage function, demonstrating the feasibility of reversing peripheral immune dysregulation by cell-targeted HTT-lowering.

Materials and methods

Collection and classification of human samples

All human experiments were performed in accordance with the Declaration of Helsinki and approved by University College London (UCL)/UCL Hospitals Joint Research Ethics Committee. All subjects provided informed written consent. Classification of patients is detailed in the Supplementary experimental procedures. Subjects' demographic are provided in Table S1.

Isolation of human monocytes and macrophages

Cells were isolated from whole blood, as previously described (Björkqvist *et al.*, 2008) and in Supplementary experimental procedures. Cells were cultured in RPMI culture medium supplemented with 10% FCS, 2 mM L-glutamine, 50 U/ml penicillin and 50 μg/ml streptomycin (Invitrogen). Monocytes were allowed to rest for 16 h before experimental use. Culture medium was supplemented with 20 ng/ml GM-CSF for 6 days to differentiate monocytes into macrophages.

Mutant HTT expression in U937 cells

U937 cells (Sundström and Nilsson, 1976) were transduced with lentiviral constructs containing human *HTT* exon 1 sequences with either 29, 71 or 129 CAG repeats, together with GFP, or a control vector containing GFP but no *HTT* exon 1. For details of vectors, viral production and transduction, see Supplementary experimental procedures. Transduced U937 cells were tested for HTT protein expression using a time resolved fluorescence resonance energy transfer (TR-FRET) immunoassay. *HTT* exon 1 expressing U937 cells were seeded into 24-well plates at 5x 10^5 cells per well and differentiated into mature monocytes using 10 nM phorbol 12-myristate 13-acetate (PMA) for 3 days (Alciato *et al.*, 2010) before cytokine profiling.

HTT silencing

Monocytes and macrophages were incubated with β1,3-D-glucan-encapsulated siRNA particles (GeRPs) for 4 hours, after which fresh medium was added to the cultures. GeRP uptake was visualised by seeding $1x10^5$ monocytes per 13 mm coverslip, incubating them with empty green fluorescent GeRPs for 12 h and mounting onto slides with 1 μg/ml DAPI. Images were acquired using a Zeiss 510 meta microscope (Objective 63x/1.4 Oil DIC, 1024x1024), overlaying the bright-field image of the cells with the 405 nm and 488 nm fluorescence channels for DAPI and green fluorescence, respectively. Macrophages, which were transfected on day three of the differentiation protocol, were transfected with green fluorescent GeRPs containing no siRNA at various ratios (1-1; 1-3; 1-10) before uptake rates were measured by flow cytometry. Cells were fixed with 3.7% paraformaldehyde for 10 min, washed with FACS buffer (PBS containing 1% FCS and 0.02% sodium azide) and resuspended in 200 μl FACS buffer for analysis by flow cytometry (FACSCalibur with CellQuest Pro BD Bioscience). Data analysis was performed using FlowJo 7.2.5 (Tree Star). To examine the effects of *HTT* knockdown on cytokine production, macrophages were treated with either scrambled or anti-*HTT* siRNA containing GeRPs at a 1:10 cell: particle ratio on day 3 of the differentiation protocol; stimulation of the cells took place three days later. To examine the effects of *HTT* knockdown on transcriptional dysregulation, monocytes were treated with either scrambled or anti-*HTT* siRNA containing GeRPs at a 1:10 cell: particle ratio, before qPCR analysis three days later.

Cytokine profiling

All cells were seeded at $5x10^5$ cells per well in 24-well plates and isolated/ differentiated/ transduced as described above. For stimulation, medium was changed to fresh cell culture medium containing 10 ng/ml IFNγ (R & D Systems) and 2 μg/ml LPS (Sigma-Aldrich, E.coli

055:B5, strain 1644-70. Cat. number L6529). After 24 h, supernatants were harvested and analysed using MSD multiplex assays, according to manufacturer's instructions (MesoScale Discovery). For monocytes the pro-inflammatory (7-plex) assay was used, however IFNγ measures were not analysed as we used IFNγ as stimulus. For all other cell types, the pro-inflammatory II (4-plex) assay was used and all data is shown. Monocyte data was adjusted to basal cytokine levels, whilst all other cell types were normalised to total protein concentration in each well. Cells were lysed in 50 mM Tris pH 8, 150 mM NaCl, 0.5% sodium deoxycholate, 0.5% Triton X-100 and assayed for total protein concentration using a Bradford-based protein assay (Bio-Rad).

TR-FRET quantification of HTT

TR-FRET immunoassay quantification of total HTT and soluble mutant HTT was performed as previously described (Baldo *et al.*, 2012) and is detailed in the Supplementary experimental procedures.

PCR arrays

SABioscience Human NFκB Signaling Pathway RT²*Profiler*™ PCR Arrays were used in combination with the QIAGEN RNeasy Mini Kit for RNA isolation from $2x10^6$ cells. RNA integrity was evaluated using 2100 RNA Bioanalyser chips (Agilent). RNA was reverse transcribed using the RT^2 First Strand kit for cDNA transcription, before the RT^2 SYBR Green qPCR Mastermix and pre-primer coated PCR plates were used for qPCR (SABioscience). All kits were used according to manufacturer's instruction.

For standard SYBR Green protocols and bioinformatics used for Figure S6, see Supplementary experimental procedures.

Proximity ligation assays

Proximity ligation assays (PLA) were conducted on monocyte-derived macrophages seeded on 13 mm coverslips. Cells were fixed in 4% PFA for 10 min and permeabilised with 100% ice cold methanol at -20°C for 15 min. Coverslips were blocked with 10% BSA for 30 min at 37°C before staining with primary antibodies was performed for 1 h at 37°C (mouse anti-HTT 4C9, 1:300, kind gift from Novartis; rabbit anti-IKKγ, 1:100, Santa Cruz; rabbit anti-IKKα/β, 1:25, Santa Cruz). Instead of using fluorescently labelled secondary antibodies, a proximity ligation approach was applied following manufacturer's instructions (Sigma). Briefly, samples were incubated with secondary antibodies conjugated with DNA probes (minus anti-mouse and plus anti-rabbit DNA probes). Probes were hybridised and ligated, followed by amplification of the DNA template in a rolling circle amplification reaction. Detection solution was added to identify amplified DNA. Signals were detected using a Zeiss LSM 710 confocal microscope (Objective plan-apachromat 40x/1.4 Oil DIC M27, 1024x1024). Spots were quantified using Volocity (PerkinElmer) on at least seven fields of view per subject, taken of random sides of each coverslips.

Immunoblot analysis of IκB degradation

Monocytes were seeded at $1x10^6$ cells/well into 24-well plates and rested for 16 h. Cells were stimulated with 2 μg/ml LPS over a 2 h or 24 h time-course before extraction of lysates for western blotting (see Supplementary experimental procedures for detail).

NFκB RelA translocation

$CD14^+$ monocytes were seeded at $2x10^6$ into 6-well plates and left to rest for 16 h. The cells were stimulated with 2 μg/ml LPS before being scraped off the plates. Pelleted cells were fixed for 15 min and permeabilised for 10 min using the eBioscience Fix/Perm solutions, before NFκB p65/RelA XP antibody (1:200; Cell Signaling) diluted in permeabilisation buffer was

added. After 30 min incubation shaking at 4°C, cells were washed twice with FACS buffer and spun 5 min at 300 xg. Secondary anti-rabbit IgG PE (eBioscience) was added at 1:100 in FACS buffer and incubated for 30 min before washing the cells twice with FACS buffer. Cells were resuspended in 80 μl FACS buffer and stained with 1 μg/ml DAPI just before analysis. Samples were run on the ImageStreamX (Amnis) and analysed using the IDEAS software. Briefly, gating on single cells, the similarity feature (Similarity_Erode(Object (M04,BF,Tight)2)_Dapi _RelA) was used to establish the rate of RelA translocation by measuring the overlap of DAPI and RelA staining. Translocation rate was normalised to baseline levels for each subject.

Statistical analysis

For cytokine profiling data, inter-group differences were identified by one-way ANOVA with post-hoc Tukey HSD testing to allow for multiple comparisons. Data were corrected for age and gender before analysis. Linear regression with log10 transformed data was used to establish whether cytokine production by primary human monocytes and macrophages correlate with CAG repeat length. Cytokine profiling data from U937 cells and knock-down cells were analysed by two-way ANOVA with Bonferroni post-tests. Gene expression changes measured by qPCR were analysed using unpaired two-tailed student t tests. Paired two-tailed student t tests were used to analyse the effects of anti-*HTT* siRNA compare to scrambled siRNA in cells from the same individual. All error bars represent standard error of the mean.

Results

Huntington's disease patient monocytes and macrophages are hyper-reactive after LPS stimulation

Previously we have shown that Huntington's disease patient monocytes produce increased levels of IL-6 upon stimulation with LPS (Björkqvist *et al.*, 2008). To extend these findings to other cytokines, we collected blood samples from a large cohort (n=53) of *HTT* gene carriers ranging from pre-manifest to moderate disease-stages and control subjects (n=27) (Table S1). $CD14^+$ monocytes were isolated, primed with IFNγ and stimulated with LPS *ex vivo*. Monocytes from Huntington's disease gene carriers at each disease-stage were found to produce more IL-6 and TNFα than control cells (Figure 1A). Furthermore, IL-1β production by pre-manifest monocytes was significantly increased. IL-8, IL-10 and IL-12 levels did not differ between Huntington's disease and control cells (Figure 1A).

When monocytes migrate into tissues, they differentiate into macrophages capable of eliciting effective immune responses to localised inflammatory signals (Gordon and Taylor, 2005). To test whether Huntington's disease macrophages are abnormal too, blood monocytes were differentiated into macrophages using GM-CSF and stimulated with IFNγ and LPS to assess their cytokine profile. In keeping with our previous findings, macrophages from all Huntington's disease stages produced significantly higher levels of TNFα than control cells (Figure 1B). Furthermore, IL-8 levels were also significantly increased in Huntington's disease macrophages (Figure 1B). This marks a shift in the pro-inflammatory cytokines elevated, when comparing to the pattern seen in monocytes (Figure 1A), where IL-8 was not changed. This is likely due to the distinct functions of the two cell types.

These data show that myeloid cells isolated from Huntington's disease patients are hyper-reactive, producing elevated levels of several key pro-inflammatory cytokines following stimulation.

Correlating production of individual cytokines to CAG repeat length showed a significant association (p=0.048) of CAG repeat length with TNFα produced by Huntington's disease monocytes (Figure S1). There was no correlation between CAG repeat length and levels of any other cytokine in either Huntington's disease monocytes or macrophages.

Lowering HTT levels reverses Huntington's disease myeloid cell hyper-reactivity

Lowering HTT expression using siRNA is a promising therapeutic approach for Huntington's disease (Sah and Aronin, 2011). Therefore, we investigated whether lowering total HTT levels can reverse the hyper-reactive phenotype in primary Huntington's disease monocytes and macrophages. Using a novel approach that takes advantage of these cells' ability to phagocytose (Aouadi *et al.*, 2009), β1,3-D-glucan-encapsulated siRNA particles (GeRPs) were packaged with, previously validated (DiFiglia *et al.*, 2007), anti-*HTT* siRNA for delivery into human *ex vivo* monocytes or monocyte-derived macrophages. Monocytes cultured with the GeRPs readily ingested them via phagocytosis (Figure 2A), without effect on cell viability (Figure S2). Testing different macrophage-to-GeRP ratios, up to 90% of macrophages phagocytosed the green fluorescent GeRPs when they were added at a ten-fold particle to cell ratio, demonstrating high transfection efficiency at this concentration (Figure 2B).

The efficacy of the anti-*HTT* siRNA GeRPs was tested three days after siRNA delivery in macrophages, using both qPCR for *HTT* RNA levels and TR-FRET immunoassay for HTT protein levels. Macrophages treated with anti-*HTT* siRNA GeRPs had 60-70% less *HTT* mRNA and 50% less HTT protein, compared to macrophages treated with scrambled siRNA-

containing GeRPs (Figure 2C). As expected, the decrease in HTT levels was the same in both control and disease macrophages (Figure 2C).

Next, we examined the effect of lowering total HTT levels on cytokine production. After treating primary human monocyte-derived macrophages with anti-*HTT* or scrambled siRNA GeRPs for three days, IFNγ-primed macrophages were stimulated with LPS and cytokine production was measured. Validating our previous findings, IL-8 and TNFα levels were significantly higher in Huntington's disease than in control cells, when both had been treated with scrambled siRNA (Figure 3). However, lowering HTT levels in Huntington's disease macrophages using anti-*HTT* GeRPs rescued this increase by significantly decreasing the production of IL-6, IL-8 and TNFα (Figure 3). IL-1β production showed a similar trend that did not reach significance. Interestingly, lowering HTT levels also significantly reduced IL-6, IL-8 and TNFα levels in control cells, suggesting a role of wild-type HTT in cytokine production.

Thus, lowering HTT levels by 50% using this novel method of siRNA delivery can reverse the hyper-reactivity of Huntington's disease patient macrophages. The use of GeRPs to achieve cell-targeted gene knockdown has to date shown significant promise in mice, but this is the first report showing efficient siRNA delivery, pathogenic gene knock-down and rescue of a deleterious phenotype using this method in primary human immune cells.

The use of HTT-lowering in Huntington's disease patient myeloid cells demonstrates that their production of cytokines in response to stimuli is regulated by HTT. This suggests that immune cell dysfunction is caused by the cell-intrinsic expression of mutant HTT, rather than being secondary to extracellular disease-associated factors. To confirm this, we transduced histiocytic lymphoma U937 cells, a commonly-used model of monocytes (Alciato *et al.*, 2010), with lentiviral vectors expressing human *HTT* exon 1. The constructs contained either wild-

type *HTT* exon 1 with 29 CAG repeats, or mutant *HTT* exon 1 with 71 or 129 CAG repeats. Sorting the transduced cells using the co-expressed GFP resulted in 99% pure cultures (Figure S3) and HTT expression in the transduced cells was confirmed using TR-FRET (Figure 4A). The *HTT* exon 1-expressing U937 cell lines were differentiated with PMA for 3 days to induce a mature monocyte phenotype before stimulating the cells with IFNγ and LPS, and analysing their cytokine profile. Stimulated U937 cells expressing either 71 or 129Q mutant *HTT* exon 1 produced significantly increased levels of IL-6 and TNFα compared to those expressing the 29Q wild-type *HTT* exon 1 construct (Figure 4B). Cells expressing 129Q produced significantly higher IL-1β levels compared to control, whereas IL-8 levels did not differ.

Taken together, modulating HTT levels via overexpression of mutant *HTT* exon 1 in a myeloid cell line and by knock-down of HTT in primary human peripheral immune cells demonstrates that cell-intrinsic expression of mutant *HTT* causes the hyper-reactive immune phenotype observed in monocytes and macrophages from Huntington's disease patients.

Mutant HTT interacts with the NFκB pathway in human Huntington's disease myeloid cells

That Huntington's disease patient monocytes and macrophages resemble normal cells when unstimulated, but are hyper-reactive in response to LPS, suggests that mutant HTT affects the signalling cascade induced by LPS. Expression of the main LPS receptor, TLR4, was unaltered (Figure S4), suggesting downstream effects. The NFκB pathway, a key signalling cascade downstream of TLR4, has previously been shown to interact with mutant HTT exon 1 in mice (Khoshnan *et al.*, 2004).

To test whether this interaction occurs in human primary immune cells, peripheral blood mononuclear cells (PBMCs) from early-stage Huntington's disease patients and control

subjects were isolated for co-immunoprecipitation experiments. Full-length HTT was detectable in both the control and Huntington's disease samples with two anti-HTT antibodies (2B7 and MAB2166), whereas co-precipitation of IKKγ was observed only in the Huntington's disease sample (Figure S5A). Given the high background signal in these experiments due to poor antibody performance in the immunoprecipitation, we performed more sensitive proximity ligation assays to detect native IKKγ-HTT interactions in the cells. As shown in Figure 5A, specific IKKγ-HTT protein interactions, represented by red spots can be detected in both control and disease macrophages. Quantification of the number of spots per cell demonstrated more interaction between IKKγ and HTT in Huntington's disease patient cells compared to controls (Figure 5B). Further evidence for a CAG repeat dependent interaction between HTT and the IKK complex was given by an increased number of interactions between HTT and the IKKα/β subunits in Huntington's disease samples (Figure 5B). The fact that classical immunoprecipitation did not pick up an interaction of the proteins in control individual's cells is likely to be due to the method being less sensitive. These data demonstrate for the first time a direct interaction between the IKK complex and full-length HTT expressed at normal allelic expression levels in primary human cells.

Activation of the IKK complex leads to the phosphorylation and degradation of IκB, the endogenous inhibitor of NFκB (Hayden and Ghosh, 2012). To evaluate whether the increased interaction of mutant HTT with IKKγ leads to increased IKK complex activation and subsequent changes in IκB degradation, we stimulated Huntington's disease and control monocytes with LPS over a time-course of two hours to analyse IκB levels by western blot. Control monocytes demonstrated a drop in IκB levels over the first 15 minutes, followed by a recovery of IκB levels over the next two hours, representing a normal pattern of NFκB activation upon stimulation (Figure 5C) (Gross and Piwnica-Worms, 2005). Following

stimulation of Huntington's disease monocytes, we observed a different pattern: IκB levels dropped within 5 minutes and did not recover to baseline levels within the two hour time-course (Figure 5C). This demonstrates that IκB is degraded more rapidly and over a prolonged period of time in primary human Huntington's disease monocytes due to IKK activation. Similarly, levels of phosphorylated IκB were increased over the two hour period in monocytes isolated from Huntington's disease patients compared to controls (Figure S5B). To investigate by which time IκB levels return to baseline in Huntington's disease patients, we performed a prolonged time-course over 24 h and found that IκB levels returned to baseline levels or above (due to high level re-synthesis of the protein) by 4 h post-stimulation (Figure S5C). These findings demonstrate a transient effect of mHTT expression on IκB levels following stimulation.

Under steady-state conditions, IκB binds NFκB and blocks its translocation to the nucleus. Degradation of IκB allows the NFκB transcription factors to enter the nucleus and influence transcription (Beinke and Ley, 2004). In order to test whether increased IκB degradation in Huntington's disease monocytes leads to more rapid nuclear translocation of NFκB, we analysed translocation of RelA, one of five DNA-binding NFκB subunits, in Huntington's disease and control monocytes using imaging flow cytometry. ImageStream technology, combining the high image content information of microscopy with the high throughput analysis of flow cytometry, is used to overcome the limitations of conventional assays to produce highly reproducible and statistically robust data (Maguire *et al.*, 2011). Cells were stained with DAPI to mark the nucleus and with anti-RelA antibodies (Figure 5D). Analysis of the levels of RelA and DAPI co-localisation showed significantly higher levels of RelA translocation in Huntington's disease than in control monocytes at 45 and 90 minute post LPS stimulation (Figure 5E).

Thus, we demonstrate in primary Huntington's disease patient cells that mutant HTT binds IKKγ and causes increased NFκB activity by increased IκB degradation and subsequent NFκB translocation. We hypothesise that this causes altered transcription of NFκB target genes, leading to increased cytokine production by immune cells.

Transcriptional changes affect signalling pathways in Huntington's disease myeloid cells

Transcriptional dysregulation is a central pathogenic mechanism in Huntington's disease (Hodges *et al.*, 2006). Therefore, we tested whether basal differences in transcription play a role in mutant HTT induced immune hyper-reactivity by analysing differences in the expression of genes related to the NFκB pathway. The mRNA expression of 84 genes was tested in untreated human monocytes using the SABioscience NFκB signalling pathway PCR array. We identified seven genes that were significantly up-regulated (*TLR2, LTBR, CD40, TMED4, AKT1, IL10, FR2*) and one gene that was significantly down-regulated (*CHUK*) in Huntington's disease compared to control monocytes (Table 1). Four of the up-regulated genes showed a ≥1.5-fold change: *CD40* (1.5); *AKT1 (*1.5); *IL10* (1.85) and *F2R* (2.23). Furthermore, the adaptor molecules *IRAK1*, *TICAM2*, *MYD88*, and *TRADD*, were also up-regulated (Table 1 and Figure S6). Interestingly, *CHUK*, which encodes for IKKα, was found to be down-regulated, whilst all other parts of the IKK complex, IκB and the NFκB transcription factors were unchanged.

The array also screened intracellular signalling pathways closely linked to the NFκB pathway, such as MAPK and PI3K/AKT pathways. Increased AKT protein levels have been found in Huntington's disease patient lymphoblasts (Colin *et al.*, 2005) and *AKT1* is one of the genes up-regulated in Huntington's disease monocytes (FC=1.5, p=0.031). Moreover, the genes

composing the transcription factor AP-1, *JUN* and *FOS*, are also up-regulated in Huntington's disease monocytes (Table 1 and Figure S6). Therefore, both of these pathways may also contribute to the increased immune response observed after stimulation of Huntington's disease monocytes.

To validate our findings, six candidate genes chosen on the basis of array fold changes and their importance within the NFκB signalling cascade (*CD40*, *AKT1*, *IRAK1*, *JUN*, *IL6* and *IL10*) were quantified by qPCR using different primer sets and cells from a different patient cohort. The relative changes in gene expression when comparing Huntington's disease and control human monocytes matched our previous findings for all six genes (Figure S7). Expression levels for *CD40*, *IRAK1* and *IL10* were significantly increased in Huntington's disease compared to control monocytes, whilst expression changes in *AKT1*, *JUN* and *IL6* demonstrated an upwards trend, not reaching statistical significance due large inter-individual differences.

Lowering HTT levels reverses transcriptional changes in Huntington's disease myeloid cells

Finally, we investigated whether lowering HTT using anti-*HTT* siRNA GeRPs could reverse the transcriptional changes observed in Huntington's disease monocytes. *IRAK1* (the main adapter molecule between TLR4 and NFκB), *CD40* (immunomodulatory molecule giving co-stimulatory signals to both innate and adaptive immune cells) and *JUN* (part of the AP-1 transcription factor) expression were all increased in Huntington's disease monocytes (Table 1). Following three days of treatment with GeRPs containing either scrambled or anti-*HTT* siRNA, we analysed the expression of these candidate genes by qPCR. HTT mRNA levels were assessed to validate the knock-down. Treatment with anti-*HTT* siRNA resulted in a 50% reduction in *HTT* levels in both Huntington's disease and control monocytes (Figure 6A/B).

However, only the Huntington's disease monocytes demonstrated a significant 20-30% reduction in *IRAK1, CD40* and *JUN* expression when treated with anti-*HTT* siRNA (Figure 6A). Lowering *HTT* in control cells did not affect levels of *IRAK1, CD40* and *JUN* transcript expression (Figure 6B), suggesting that the transcriptional dysregulation of these genes in Huntington's disease myeloid cells is caused specifically by a gain of mutant HTT function, rather than loss of wild-type protein function.

Discussion

Plasma pro-inflammatory cytokine levels are elevated in Huntington's disease patients, even in the pre-manifest stages of the disease (Björkqvist *et al.*, 2008). Here we demonstrate that Huntington's disease PBMCs are the likely source of the increased pro-inflammatory cytokines, as both monocytes and macrophages isolated from Huntington's disease patients and stimulated with LPS produce significantly more IL-6, IL-8 and TNFα compared to controls. Supporting our previous finding that plasma cytokine levels are already elevated in pre-manifest subjects with a mean of 16 years to clinical onset (Björkqvist *et al.*, 2008), myeloid cells isolated from pre-manifest Huntington's disease patients were hyper-reactive to the same degree as cells isolated from late-stage disease patients. Cytokine production seems CAG length independent and suggests an early deficit that is already present many years before disease onset, which may be a marker of when to intervene with potential modulatory therapies. Modulating HTT expression by overexpression of mutant *HTT* exon 1 in a monocyte-like cell line and lowering HTT levels in primary human monocytes/macrophages demonstrated that this hyper-reactive phenotype is due to a cell-intrinsic effect of mutant HTT expression and not non-cell autonomous secondary factors.

Importantly, we have been able to show that lowering total HTT levels partially rescues this hyper-reactive phenotype, with a reversal of both elevated cytokine production and transcriptional changes observed in human Huntington's disease myeloid cells *ex vivo*. This is the first report showing that lowering HTT in cells freshly isolated from Huntington's disease patients can reverse cellular dysfunction caused by mutant HTT expression – an important first demonstration of the reversibility of cellular dysfunction after HTT-lowering in human tissue. HTT-lowering was achieved using a novel phagocytosis dependent approach, in which siRNAs are packaged into glucan particles isolated from yeast (Aouadi *et al.*, 2009). This study is the

first to use this technique in primary human macrophages and demonstrates that a 90% transfection rate can be achieved, much higher than the 10-20% transfection rate achieved by traditional methods such as lentiviral transduction.

Our findings, that lowering total HTT levels by only 50% in primary human Huntington's disease macrophages and monocytes can reverse the increased cytokine production and transcriptional changes, respectively, validate the potential of HTT-lowering therapy as well as the possibility of using peripheral cells to test siRNA efficiency, safety and efficacy. Interestingly, cytokine release was also decreased in control macrophages treated with anti-*HTT* siRNA, indicating either that HTT regulates cytokine production in a CAG dependent manner or that wild-type HTT influences cytokine production in parallel to mutant HTT. Wild-type HTT has been shown to play a role in both actin remodelling (Munsie *et al.*, 2011, Kwan *et al.*, 2012) and microtubule-mediated transport (Gauthier *et al.*, 2004). As both processes are needed for the trafficking of cytokines to the cell surface membrane for release (Lacy and Stow, 2011), a reduction of wild-type HTT levels might exert a loss of function by hindering normal actin and microtubule remodelling causing changes in cytokine release. A future study using allele-specific silencing of mutant but not wild-type HTT will help determine the exact contributions loss of wild-type HTT and gain of mutant HTT function have on the myeloid cell dysfunction in Huntington's disease.

The NFκB pathway has been previously implicated in Huntington's disease in murine studies (Khoshnan *et al.*, 2004, Thompson *et al.*, 2009, Steffan, 2010, Hsiao *et al.*, 2013). Investigating this pathway, we found that HTT binds the IKK complex in a CAG repeat length dependent manner. Testing HTT binding to both IKKγ and IKKα/β subunits, we detected a stronger interaction between HTT and IKKγ, suggesting this subunit as the direct interaction partner. IKKγ is the regulatory subunit of the IKK trimer, consisting of one regulatory (γ) and two

kinase subunits (α and β), and is a critical component without which cells are unresponsive to all upstream stimuli (Israël, 2000). During signal transduction, polyubiquitin chains form the scaffold on which TAK1/TAB2/3 and IKKα/β/γ complexes are formed to induce TAK1 dependent activation of IKKβ (Miyamoto, 2011). In agreement with previously described findings using non-primary human cell model systems (Khoshnan and Patterson, 2011), we have shown in primary human cells that HTT can function as an alternative scaffold for the NFκB pathway. The CAG repeat dependent binding of HTT to IKKγ is associated with increased IKK complex formation and downstream signal transduction following LPS stimulation in Huntington's disease myeloid cells (Figure 7). Previously, this interaction has only been observed in cultured tumour cells (Khoshnan *et al.*, 2004) or mouse models expressing exon 1 mutant HTT (Khoshnan *et al.*, 2004, Hsiao *et al.*, 2013). Here we demonstrate that the interaction also takes place in primary human *ex vivo* cells expressing the full-length protein at normal allelic expression levels. That mutant HTT exon 1 fragments have previously been shown to bind IKKγ is consistent however with our finding that an N-terminal human exon 1 mutant HTT fragment can induce elevated cytokine production in a histiocytic cell line and our recent report demonstrating increasing N-terminal fragmentation of mutant HTT in human myeloid cells as the disease progresses (Weiss *et al.*, 2012).

Interestingly, a recent study showed that activating the immune modulator CB2, which is thought to dampen NFκB signalling (Rajesh *et al.*, 2007), reduces increased serum IL-6 levels while extending life span and reducing motor deficits in Huntington's disease mouse models (Bouchard *et al.*, 2012). Our finding that mutant HTT alters the NFκB pathway in human Huntington's disease monocytes is likely to be relevant to other cell types and tissues, including those of the CNS. We previously showed that hyper-reactivity of Huntington's disease peripheral myeloid cells is mirrored in microglia (Björkqvist *et al.*, 2008). The NFκB pathway

is present and active in both neurons and glial cells (O'Neill and Kaltschmidt, 1997). Pharmacological inhibition of NFκB impairs memory and learning (Mattson and Meffert, 2006) and NFκB pathway activation is critical for neuronal survival and neurite outgrowth (Teng and Tang, 2010). Increased levels of NFκB activity have been shown in both Alzheimer's disease (AD) (Kaltschmidt *et al.*, 1997) and Parkinson's disease (Hunot *et al.*, 1997). Blocking NFκB function in mutant HTT exon 1 expressing PC12 cells leads to reduced mutant HTT toxicity, implying that the NFκB pathway contributes to neurotoxicity in Huntington's disease (Khoshnan *et al.*, 2004). Indeed, a recent study showing that mutant HTT enhances NFκB-mediated inflammation in astrocytes to cause toxicity in the brain of Huntington's disease mice underlines the potential importance of NFκB in non-neuronal cells during neurodegeneration (Hsiao *et al.*, 2013).

Given the manifold roles NFκB signalling plays in the different cell types, inhibiting the pathway to lower hyper-reactive immune function in Huntington's disease may also affect other cell types. For example, compounds that target NFκB activity need to be closely evaluated as to whether they cross the blood brain barrier and with regard to negative effects on synaptic activity and plasticity. However, drugs that target this pathway will not necessarily have negative effects. Laqinimod for example, a immunomodulatory compound inhibiting NFκB activity (Brück et al., 2012) was well tolerated and showed decreased progression rates in multiple sclerosis patients in clinical trials (Comi et al., 2012). Furthermore, targeting the NFκB pathway further downstream, for example at the level of cytokine secretion is also a possible therapeutic target. In a Huntington's disease mouse model, peripherally administrated anti-IL-6 antibody treatment has shown improvement of disease progression (Bouchard *et al.*, 2012), while perispinal administration of a TNFα inhibitor improves disease in Alzheimer's disease patients (Tobinick *et al.*, 2006), clearly demonstrating the positive effect of immunomodulatory therapy for neurodegeneration.

We have identified gene expression changes in key molecules involved in immune signalling in Huntington's disease patients' monocytes. Several adapter proteins downstream of TLR4, such as *IRAK1, TICAM2* and *MyD88* were found to be slightly elevated in native Huntington's disease patients' monocytes. A cumulative baseline increase in expression of several of these adapter proteins may lead to increased signal transduction from TLR4 to NFκB, further increasing NFκB pathway dysregulation. Another gene found to be up-regulated in Huntington's disease monocytes was *CD40* which, together with its ligand CD154, mainly expressed on T cells, regulates the immune response on several levels. Monocytes are activated leading to up-regulated cytokine production and antigen presentation, and priming of the adaptive immune system (Grewal and Flavell, 1998). This points to further functional abnormalities in the immune system of Huntington's disease patients, suggesting a possible deficit in the communication between antigen presenting cells and the adaptive immune system. Furthermore, CD40 mediates cell adhesion needed for leukocyte trafficking (Alderson *et al.*, 1993). Given recent studies showing defective migration in Huntington's disease due to defective actin remodelling (Kwan *et al.*, 2012), the increase in CD40 expression could be a compensatory response of immune cells to counteract their decreased migrative ability. *FOS* and *JUN*, subunits of the AP-1 transcription factor, were also upregulated in primary human myeloid Huntington's disease cells. Interestingly, FOS and JUN levels have been found to be increased in AD patient brains (Anderson *et al.*, 1994), and the MAP kinase needed for JUN activation, JNK, is elevated and involved in neurotoxicity in Huntington's disease mouse (Fan *et al.*, 2012) and rat models (Perrin *et al.*, 2009). Thus, we cannot exclude that dysregulation in these signalling pathways may also contribute to the Huntington's disease immune phenotype.

In addition to the pathways identified in this study, we cannot exclude other previously described mechanisms, which may contribute to the transcriptional dysregulation we found in

primary human Huntington's disease myeloid cells. For example, mutant HTT is known to bind and thereby deplete transcription factors such as CBP and p53 from their normal location causing changes in the genes they control (Steffan *et al.*, 2000, Nucifora *et al.*, 2001). Furthermore, HTT may alter DNA conformation upon direct binding, affecting transcription factors binding to their promoter regions (Benn *et al.*, 2008).

This study demonstrates that the cellular dysregulation observed in hyper-reactive immune cells in Huntington's disease can be reversed by HTT-lowering and represents the first demonstration of phenotypic reversibility on HTT-lowering in primary human cells in Huntington's disease. It also identifies the underlying intracellular mechanisms of immune dysfunction in human cells in Huntington's disease. This is important as the immune system has been shown to be a powerful modifier of Huntington's disease pathogenesis in various mouse models (Zwilling *et al.*, 2011, Bouchard *et al.*, 2012, Kwan *et al.*, 2012). There is currently a search for genetic and environmental modifiers of Huntington's disease as the CAG repeat expansion only explains 50-70% of variance in age of onset, and its role in modulating disease progression is variable (Andrew *et al.*, 1993, Brinkman *et al.*, 1997). The remainder of the variance is likely due to environmental and other genetic factors (Wexler *et al.*, 2004). The immune system may be a powerful modifier of Huntington's disease age of onset and progression, with an interaction of both genetic and environmental factors. This has already been shown to be the case in large GWAS in AD where several key genes involved in the innate immune system were shown to increase susceptibility to developing AD (Harold *et al.*, 2009, Lambert *et al.*, 2009, Guerreiro *et al.*, 2013).

Our novel method of siRNA delivery has potential therapeutic relevance to Huntington's disease and other diseases where immune dysregulation is a feature. Glucan particles are a versatile phagocytic cell targeted delivery system and have been administered by oral,

subcutaneous, intraperitoneal and intravenous routes in mice and rats. In our future studies, we plan to administer GeRPs loaded with anti-*HTT* siRNA to reverse the inflammatory phenotype via intrathecal administration to directly target phagocytic microglial cells and infiltrating monocyte/macrophages, and via intravenous administration to target circulating monocytes and PMNs, a precursor pool for inflammatory cells trafficking into inflamed brain sites.

Finally, our work also suggests a potential new therapeutic target for Huntington's disease through modulating NFκB activation and downstream targets. The muscle wasting, weight loss and depression that occurs in Huntington's disease (van der Burg *et al.*, 2009) may be related to increased peripheral cytokine levels. Therefore, modulating the immune system may have beneficial effects in both the CNS and the periphery. Indeed, a peripherally administered anti-inflammatory, anti-IL-6 antibody treatment in R6/2 mice has already been show to improve both weight loss and disease progression (Bouchard *et al.*, 2012). This work therefore has implications for both understanding the role of the innate immune system as a modifier of neurodegeneration and modulation of the immune system as a possible therapeutic in Huntington's disease.

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Figure 1: Pro-inflammatory cytokine production by monocytes and macrophages is elevated in Huntington's disease patients. Innate immune regulators such as IL-6, IL-8 and TNFα were elevated in Huntington's disease patient **(A)** blood monocytes and **(B)** macrophages collected from two independent patient cohorts, stimulated *in vitro* with 10 ng/ml IFNγ and 2 μg/ml LPS for 24 h. Data show mean concentrations +/- SEM, n= individual biological repeats, ANOVA with post-hoc Tukey HSD test. *p>0.05; **p>0.01, ***p>0.001.

Figure 2: Glucan encapsulated siRNA particles (GeRPs) can effectively knock-down total *HTT* in primary human immune cells. (A) GeRPs deliver siRNA efficiently when phagocytosed by myeloid cells, as shown in primary human monocytes after 12 h incubation in culture (GeRPs = green; DAPI = blue). **(B)** 90% of macrophages take up GeRPs when incubated at 1:10 cell: particle ratio for 12 h as quantified by flow cytometry. Data shown as mean (n=2 for controls and n=3 for Huntington's disease) +/- SEM. **(C)** Total *HTT* RNA measured by qPCR and protein levels measured by TR-FRET were reduced by 70 and 50%, respectively, in macrophages treated for 3 days with GeRPs containing anti-*HTT* siRNA. Data shown as mean HTT levels (each combining two independent experiments, n stands for individual biological repeats) +/- SEM. Data is normalised to the scrambled siRNA treated condition for each genotype.

Figure 3: Knock-down of total HTT reverses the hyper-reactive cytokine production by Huntington's disease macrophages. Huntington's disease and control macrophages were treated with either anti-*HTT* or scrambled siRNA for 3 days, before the cells were stimulated

with 10 ng/ml IFNγ and 2 μg/ml LPS for 24 h. Measuring cytokine production with multiplex ELISA assays showed that lowering HTT levels reduces IL-6, IL-8 and TNFα levels following stimulation. Data shown as mean concentrations (n=9 for controls and n=8 for Huntington's disease, combined from 3 independent experiments, n stands for individual biological repeats) +/- SEM, two-way ANOVA with Bonferroni post-tests. *p>0.05; **p>0.01, ***p>0.001.

Figure 4: Expression of mutant HTT induces elevated cytokine production. U937 cells were lentivirally-transduced with (m)HTT exon 1 containing either 29, 71 or 129 glutamine (Q) repeats or an empty vector. **(A)** Expression of (m)HTT protein post-transduction was confirmed, with increased levels of soluble HTT in all three cell lines expressing exogenous *HTT*. **(B)** Innate immune regulators were elevated in PMA-differentiated mutant HTT expressing U937 cells stimulated for 24 h with 10 ng/ml IFNγ and 2 μg/ml LPS. Data shown as mean concentrations (n=3 technical repeats for all conditions) +/- SEM, two-way ANOVA with Bonferroni post-tests, *p>0.05; **p>0.01, ***p>0.001. Experiment was repeated three times independently showing similar results.

Figure 5: HTT interacts directly with the NFκB pathway, which is dysregulated in Huntington's disease. (A) HTT interacts directly with IKK, as shown by PLA. Monocyte-derived macrophages were differentiated on glass cover slips and stained for HTT and IKKγ or IKKα/β, before antibodies binding in close proximity were visualised using PLA-probes as red spots, shown here. Cells stained with a single primary antibody did not result in red spots. **(B)** Quantification of the number of spots per cell using the Volocity software shows increased binding between IKKγ and HTT in Huntington's disease compared to control cells (p=0.06).

Binding of HTT to the α and β subunit of IKK showed a similar, but smaller trend (p=0.1). Two-tailed unpaired t test used for statistical analysis. **(C)** While in control cells LPS-induced degradation of IκB occurred within 15 min of stimulation with LPS and recovered within 2 h, Huntington's disease monocytes demonstrate a more rapid loss of IκB and no recovery of the protein. Shown is an example blot of samples from one control and one Huntington's disease subject. **(D)** Translocation of the NFκB transcription factor RelA to the nucleus after LPS stimulation was measured using imaging flow cytometry; example images are shown here. In untranslocated cells the green RelA staining surrounds the nuclear DAPI staining, while in cells demonstrating translocation of RelA the colours merge. **(E)** Increased RelA translocation into the nucleus following LPS stimulation was observed in Huntington's disease monocytes (n=7) compared to controls (n=8). n= individual biological repeats. Data shown as mean concentrations +/- SEM, two-way ANOVA with Bonferroni post-tests, *p>0.05; **p>0.01. All experiments were repeated at least twice with the same results.

Figure 6: Lowering total HTT levels reverses transcriptional changes found in Huntington's disease monocytes. Huntington's disease and control monocytes were incubated with either scrambled or anti-*HTT* siRNA containing GeRPs for 3 days before RNA isolation. Using qPCR, efficient *HTT* knock-down was demonstrated as well, as lowering of key NFκB pathway molecules *IRAK1*, *CD40* and *JUN* in **(A)** Huntington's disease patient cells but not **(B)** controls. Data shown as relative gene expression (n= 10 individual biological repeats for controls and Huntington's disease) +/- SEM, paired t test *p>0.05; **p>0.01.

Figure 7: Mechanism of immune dysfunction in Huntington's disease. (A) In normal wild-type HTT expressing myeloid cells, LPS binds the TLR4 receptor activating the NFκB pathway triggering production of pro-inflammatory cytokines such as IL-6 and TNFα. **(B)** Mutant HTT interferes with the NFκB pathway by two distinct mechanisms. The mutant protein binds IKKγ to directly cause increased IκB degradation and NFκB transcription factor translocation, allowing increased transcription of target genes such as IL-6 and TNFα. Moreover, mutant HTT causes transcriptional changes leading to increased expression of key molecules within the signalling cascade likely to increase signalling transduction rate.

Table 1: The top twenty gene changes within the NFκB pathway observed in Huntington's disease monocytes. Data presented as fold change calculated from delta-delta-CT values, unpaired two-tailed t test used as statistical method.