A novel pathogenic pathway of immune activation detectable before clinical onset in Huntington’s disease

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Huntington’s disease (HD) is an inherited neurodegenerative disorder characterized by both neurological and systemic abnormalities. We examined the peripheral immune system and found widespread evidence of innate immune activation detectable in plasma throughout the course of HD. Interleukin 6 levels were increased in HD gene carriers with a mean of 16 years before the predicted onset of clinical symptoms. To our knowledge, this is the earliest plasma abnormality identified in HD. Monocytes from HD subjects expressed mutant huntingtin and were pathologically hyperactive in response to stimulation, suggesting that the mutant protein triggers a cell-autonomous immune activation. A similar pattern was seen in macrophages and microglia from HD mouse models, and the cerebrospinal fluid and striatum of HD patients exhibited abnormal immune activation, suggesting that immune dysfunction plays a role in brain pathology. Collectively, our data suggest parallel central nervous system and peripheral pathogenic pathways of immune activation in HD.

Huntington’s disease (HD) is an incurable, autosomal dominantly inherited neurodegenerative condition caused by a CAG repeat expansion in the gene encoding huntingtin. The mutant protein causes neuronal dysfunction and death resulting in the cardinal disease features of movement disorder, cognitive decline, and psychiatric symptoms (1). Huntingtin is expressed ubiquitously (2), and HD includes several abnormalities outside the central nervous system (CNS), including up-regulation of immune proteins (3–6). The interactions between CNS pathology and changes detectable in peripheral tissues in HD are poorly understood but may be of importance in measuring or slowing disease progression. We previously demonstrated evidence of immune activation in peripheral plasma in manifest HD using proteomic profiling (6), but no significant differences between controls and premanifest mutation carriers have previously been shown.

The nature of the immune activation in HD remains incompletely explored. It is not known whether the innate or adaptive arm of
the immune system, or both, is activated in HD, and the alterations in each immunomodulatory cytokine at each disease stage are unknown. The cause of the immune activation peripherally is also unknown. IL-6, which triggers the acute phase response, is produced primarily by monocytes and lymphocytes, but this could be due to dysfunction of these cells caused by expression of mutant huntingtin (i.e., a cell-autonomous effect) or in response to inflammation-triggering events outside these cells, such as huntingtin-induced tissue damage or the mutant protein itself being interpreted as an antigen (i.e., non–cell-autonomous pathways). Of interest in this respect is the finding that the IkB kinase/NF-κB signaling pathway that triggers IL-6 release is up-regulated by mutant huntingtin, and this may contribute to neurotoxicity (7).

Critically, the relationship between peripheral inflammation and CNS pathology in HD is unknown. Certain inflammatory proteins, such as complement proteins and clusterin, are also up-regulated both peripherally and in the brain in HD (6, 8, 9). In vivo imaging, in vitro and postmortem studies have shown that microglia, the CNS counterpart of macrophages, are activated in premanifest (10) and manifest HD (11), that microglial activation correlates with disease severity (12), and that mutant huntingtin is expressed in microglia (13). Thus, inflammation is an established, though incompletely understood, feature of HD with likely pathogenic importance. Inflammatory changes in the CNS and peripheral tissues in HD may be due to independent effects of mutant huntingtin in both compartments, causing analogous derangements centrally and peripherally; or inflammatory activation may begin peripherally and spread to the CNS, or vice versa, through the passage of immunomodulatory molecules across the blood–brain barrier.

Insights into CNS and peripheral immune system interactions in HD may provide new biomarkers and improve knowledge of key pathogenic mechanisms, possibly leading to novel therapeutic approaches. The present work seeks to elucidate further the nature of the peripheral inflammatory activation in HD through quantification of levels of key inflammatory and immunomodulatory molecules in human plasma, and serum from three different mouse models of HD. To investigate possible links between peripheral inflammation and neuronal dysfunction, we examined correlations between individual inflammatory molecules and clinical features of HD. We used targeted transcription profiling to examine expression of key immunomodulatory proteins in the HD striatum to determine whether the inflammatory activation seen peripherally is mirrored in the CNS. We examined expression of huntingtin in its WT and mutant forms in human monocytes to investigate the possibility that the immune activation is due to a disease–related cell–autonomous dysfunction of these cells. We confirmed this with functional studies of human monocytes, and macrophages and microglia from HD mouse models, demonstrating that there is disease–related dysfunction of CNS and peripheral inflammatory cells in HD.

RESULTS

We collected 194 plasma samples from HD mutation carriers ranging from premanifest to moderate HD and from control subjects (Table S1, available at http://www.jem.org/cgi/content/full/jem.20080178/DC1) and quantified levels of key inflammatory and immunomodulatory molecules using multiplex sandwich ELISAs and single radial immunodiffusion assays. We found an altered profile of cytokine levels in HD patients (Fig. 1). The most striking increases across subject groups from controls to progressing disease were in IL-6 and IL-8 (P < 0.0001 in each case). In addition, IL-4, IL-10, and TNF-α levels increased significantly with disease progression (Fig. 1 B). Moreover, IL-6 levels were significantly increased in premanifest subjects with an estimated mean of 16 yr until motor onset (Fig. 1 A) (14). Interestingly, the cytokines that were increased earliest in the disease course (IL-6 and IL-8) are involved in the innate immune response (15). IL-10 and IL-4, antiinflammatory cytokines involved in the adaptive immune response, increased significantly in moderate stage disease. There was no difference in levels of Igs (IgG, IgA, or IgM) at any disease
IL-8, and IL-10 best discriminated all HD expansion carriers (premanifest and manifest) from controls (AUC 0.82). IL-6, IL-8, and IL-10 together best discriminated between premanifest and manifest HD patients (AUC 0.85).

IL-6 triggers the acute phase response and is produced primarily by monocytes/macrophages and lymphocytes (15). To define the possible source of peripheral cytokines, we purified monocytes from whole blood from HD patients and control subjects by flow cytometric and magnetic sorting. Using RT–quantitative PCR (QPCR), we found monocytes from HD patients to express mutant huntingtin (Fig. 5, A and B). We then stimulated isolated monocytes with LPS and found that monocytes from premanifest HD mutation carriers behave abnormally, displaying excess IL-6 production compared with cells from control subjects (Fig. 6 A). Mutant huntingtin thus appears to produce functional overactivity of monocytes. We stimulated isolated macrophages from the yeast artificial chromosome (YAC)128 mouse model of HD with LPS. Echoing the results seen in human HD monocytes, macrophages from the YAC128 responded with enhanced...
secretion of IL-6 in response to stimulation compared with macrophages from WT mice (Fig. 6 B). To test whether the presence of mutant huntingtin per se is sufficient to produce dysfunction, we then examined the response to LPS stimulation in macrophages from the YAC18 mouse, which differs from the YAC128 only in the length of the polyglutamine stretch. Excessive IL-6 release was not seen in YAC18 cells (Fig. 6 C). To determine whether microglia, the CNS equivalent of monocytes/macrophages, are also dysfunctional in HD, we performed LPS stimulation of microglia isolated from the widely used R6/2 transgenic mouse model of HD (18). HD microglia, too, demonstrated hyperactivity in response to stimulation (Fig. 6 D).

We investigated expression of inflammatory transcripts in postmortem human striatal tissue using RT-PCR and found markedly increased expression of IL-6, IL-8, and TNF-α in HD (Fig. 7), mirroring the key changes seen in plasma much earlier in the disease.

To investigate the relationship between central and peripheral inflammatory processes, we measured IL-6 and IL-8 in matched plasma and cerebrospinal fluid (CSF) samples from HD patients and controls using ELISA. CSF and plasma levels of IL-6 and IL-8 correlated closely (Fig. 8; R = 0.74 and R = 0.66, respectively; P < 0.0001 for both).

We used multiplex ELISA to determine whether peripheral immune activation is present in serum in HD mouse models and found increased levels of several cytokines in the R6/2 transgenic mouse and the full-length knock-in model of HD (Hdh150Q150) (19). In 12-wk R6/2 mice, IL-6, IL-10, IL-1β, and IL-12p70 were significantly increased (Fig. 9 A). In 22-mo knock-in Hdh150Q150 mice, IL-6, IL-10, and IL-12p70 were significantly elevated (Fig. 9 B). In the YAC128 mouse model of HD at 12 mo of age, we saw similar elevations in serum IL-6 and mKC, a mouse functional homologue of IL-8 (20) (Fig. 9 C). 12-mo YAC128 animals are phenotypically equivalent to early HD (21), and these animals therefore model the human patients whose plasma was studied.

**DISCUSSION**

Collectively, our data show that immune activation in HD is widespread and detectable in peripheral plasma across disease stages. Key cytokines of the innate immune system are up-regulated both centrally and peripherally, and robust changes

Figure 5. Human monocytes express WT and mutant huntingtin. (A) RT-QPCR studies of human monocytes obtained by flow cytometry demonstrated huntingtin expression in 100% of monocyte samples tested from controls (n = 2) and HD patients (n = 3). Expression ratios are relative to B2M. Graph shows mean expression ratios with standard error bars. (B) PCR amplification of CAG repeat tracts from huntingtin mRNA reveals that HD monocytes express both WT and mutant huntingtin, supporting the possibility of cell-autonomous dysfunction resulting in immune activation. WT and mutant CAG repeat lengths are shown. +, p4G6E4.0 plasmid expressing HTT exon 1 with 18 CAG repeats.

Figure 6. HD monocytes, macrophages, and microglia are overactive when stimulated. (A) No IL-6 was detectable in the supernatant of monocytes from control (n = 9) or premanifest HD subjects (n = 8) in the unstimulated state or after priming with IFN-γ. Monocytes stimulated by the addition of both IFN-γ and 2 μg/ml LPS expressed IL-6, but expression levels were significantly higher from HD monocytes. (B) Alveolar macrophages from the YAC128 HD mouse model have similarly altered function when stimulated. YAC128 macrophages stimulated by the addition of both IFN-γ and 100 ng/ml LPS expressed significantly more IL-6, n = 3 WT and 4 YAC128. (C) Macrophages from YAC18 mice, which differ from YAC128 cells only in the number of CAG repeats, behaved no differently from WT cells (P = 0.231; n = 4 per genotype) in response to stimulation at the same LPS concentration, suggesting that the hyperactivity in the YAC128 is due to mutant huntingtin. (D) Microglia isolated from neonatal R6/2 mice are also hyperactive when stimulated by 10 ng/ml LPS (n = 4 per group). Graphs show mean concentrations with standard error bars. ND, not detected. Unpaired t tests: *, P < 0.05; **, P < 0.01.
even take place in premanifest HD mutation carriers many years before the onset of motor abnormalities. To our knowledge, the elevated IL-6 level seen in premanifest subjects with a mean of 16 yr until predicted clinical onset represents the earliest plasma abnormality identified to date in HD. The peripheral changes correlate well with clinical variables and are accompanied by alterations in striatal gene expression.

Although cytokines such as IL-4 and IL-10 are increased later in the disease, normal Ig levels throughout the disease course suggest that there is no generalized activation of the adaptive immune response. Whereas IL-6 and IL-8 production are triggered by NF-κB activation (7, 22), IL-4 and IL-10 act to down-regulate NF-κB (15). The late involvement of these cytokines may reflect an adaptive response to chronic immune activation, possibly involving altered interactions between monocytes/macrophages and Th2 cells.

The correlation we show between plasma and CSF levels of IL-6 and IL-8 links the central and peripheral immune activation in HD. We show that IL-6 and IL-8 are increased in plasma and the striatum. These cytokines are not thought to cross the healthy blood–brain barrier in the acute setting (23, 24), and studies of brain penetration of specific molecules in HD have not identified alterations of the blood–brain barrier (e.g., reference 25). Mutant huntingtin therefore probably induces parallel dysfunction in both compartments (Fig. 10). The immune dysfunction we demonstrate in monocytes from premanifest HD gene carriers may reflect similar central changes in HD microglia and therefore act as a window onto central disease pathogenesis at this very early stage in the disease process. Given the dramatic changes seen in striatal cytokine expression and the chronic nature of these changes, passage of cytokines from the CNS into blood is a possibility we cannot exclude, although cytokines are rapidly broken down (26) and would likely be subject to considerable dilution in plasma. However, the presence of primary abnormalities of both peripheral and central cytokine-producing
ever, it is possible that HD also causes differences in the level of background immune stimulation, which could contribute to the later up-regulation of the adaptive immune response cytokines IL-4 and IL-10.

There is a need for markers of progression ("state biomarkers") in HD and other neurodegenerative diseases (29). Our results suggest that inflammatory changes detected in peripheral plasma may be biologically relevant and mirror the neurodegenerative process occurring in the CNS (Fig. 10). Indeed, combined peripheral markers of inflammation were recently suggested to be biomarkers for diagnosis and progression in Alzheimer’s disease (29). Remarkably, peripheral inflammatory changes may also reveal early pathogenic events in HD, occurring more than 15 yr before the onset of neurological manifestations. The inflammatory changes seen in patients are echoed in mouse models of HD. Importantly, they may therefore provide translational biomarkers for the use of HD mouse models in the development of therapeutic interventions. Finally, the mechanism of early innate immune activation in HD warrants further study as a potential source of targets for disease-modifying therapies.

MATERIALS AND METHODS

Ethical approval. All human experiments were performed in accordance with the declaration of Helsinki and approved by University of British Columbia (UBC) Clinical Research Ethics Board (Canadian patients) or University College London (UCL)/UCL Hospitals Joint Research Ethics Committee (UK patients), as appropriate. All subjects gave informed written consent. All animal experiments were performed in accordance with relevant legislation and approved by local and national regulatory authorities (Institutional Review Board of the University of Washington, King’s College London Animal Care and Use Committee, or UBC Committee on Animal Care and the Canadian Council on Animal Care, as appropriate).

Collection and processing of human plasma samples. Blood samples were obtained from control subjects and genetically diagnosed HD patients.
and processed as described previously (6). Subjects with inflammatory or in- 
fective conditions were excluded. A subset of subjects was assessed on the 
UHDRS (16) by a neurologist experienced in assessment of HD patients. 
Subjects’ demographic and clinical data are given in Table S1.

Collection of matched CSF and blood samples. CSF donors were re- 
cruited through the UBC HD Medical Clinic. 20 HD patients and 10 con- 
trol subjects, age-matched and lacking the HD mutation, were recruited 
(Table S1). Mutation-positive subjects were staged early or moderate ac- 
cording to independence score. CSF and matched blood samples were ob- 
tained within 1 h and plasma was extracted as described previously (6).

Collection of mouse serum samples. For the present experiments, 
Hdh<sup>Q150/Q150</sup> knock-in and R6/2 exon 1 models that develop comparable 
and widespread molecular phenotypes (19) were used. R6/2 (18) and 
Hdh<sup>Q150/Q150</sup> mice (original nomenclature, CHL2) (30) were bred and serum 
samples were collected as described previously (19). All animals had unlim- 
ited access to water and breeding chow (Special Diet Services) under a 12-h 
light–12-h dark cycle. YAC128 mice were maintained on the FVB/N strain 
background (21). Numbers and ages of animals are shown in Table S4 (avail-
able at http://www.jem.org/cgi/content/full/jem.20080178/DC1).

Serum and plasma analyses. Cytokine levels were quantified using Meso 
Scale Discovery (MSD) assays as per the manufacturer’s protocol and ana- 
yzed on a SECTOR 2400 instrument (MSD). The operator was unaware of 
the disease state of each sample during processing, and statistical analysis was 
performed independently. Serum levels of IgG, IgM, and IgA were determined by single radial immunodiffusion assays (The Binding Site Ltd) 
according to the manufacturer’s protocol.

Human monocyte huntingtin expression study. Whole blood was col- 
clected from HD patients, and controls were matched for age and sex (Table S5, 
available at http://www.jem.org/cgi/content/full/jem.20080178/DC1). Leukocytes were isolated by density gradient centrifugation over Lympho- 
prep solution (Axis-Shield). Monocytes were obtained by flow cytometry. 
In brief, mononuclear cell suspensions were labeled with anti-CD45 FITC 
and anti-CD14 PE (Becton Dickinson), and viable monocytes were sorted 
flow cytometrically by immunophenotype (CD45<sup>+</sup>/CD14<sup>+</sup>) and forward 
age light scatter signals (FACSAria high speed cell sorter; Becton Dickin-
son) to at least 95% purity (Fig S1 A). Monocytes were counted, and 5 × 10<sup>5</sup> cells 
were added into 24-well culture plates in RPMI culture medium supple- 
mented with 5% FBS, 2 mM t-glutamate, and 1% penicillin/streptomycin 
(Invitrogen). Cells were incubated for 16 h before stimulation. The medium 
was then changed to fresh cell culture medium with or without 10 ng/ml 
IFN-γ (R&D Systems). For LPS stimulation, 2 μg/ml LPS was added to the 
medium (Sigma-Aldrich). After 24 h, supernatants were harvested from two 
separate wells for each subject/condition. The cells remaining were lysed 
in 50 mM Tris, pH 8.5, 150 mM NaCl, 0.5% sodium deoxycholate, and 0.5% 
Triton X-100 and assayed for total protein concentration using a protein assay 
kit according to the manufacturer’s instructions (Bio-Rad Laboratories). 
IL-6 concentrations in supernatants were determined using the MSD assay 
and adjusted for total protein concentration.

Functional study of tissue macrophages. Alveolar macrophages were isolated from 12-mo-old WT, YAC18, and YAC128 mice, all maintained 
on a pure FVB/N strain background. The YAC128 (line 53) mouse line ex- 
presses high levels of full-length human huntingtin with ~128 polygluta- 
amine repeats and is a well-established model of HD. These mice develop an 
age-dependent phenotype similar to that seen in HD patients, including cog- nitive deficits, motor dysfunction, and selective neurodegeneration; 12-mo 
mice are equivalent to early human HD (21). YAC18 mice (line 212) express 
transgenic human WT huntingtin and do not exhibit any disease phe- 
notypic relative to their WT littermates. They differ from YAC128 mice 
only in the length of the polyglutamine tract.

Animals were killed using 10 mg avertine via i.p. injection. Blood was 
drawn from the inferior vena cava, and serum samples were obtained by 
two-stage centrifugation. Alveolar macrophages were extracted by intratra- 
cheal infusion of ice-cold PBS (Invitrogen) followed by centrifugation and 
resuspension of extracted cells. Cells were counted and seeded at 1.5 × 10<sup>5</sup> 
cells/ml onto 96-well gelatin-coated plates and incubated in culture media 
containing 5% FBS and 1% penicillin/streptomycin (Invitrogen). After 24 h, 
the medium was changed to fresh 1% medium or 1% medium containing 
10 μg/ml IFN-γ (R&D Systems) with or without 100 μg/ml control standard endotoxin (Avasa- 
aces of Cape Cod). After 24 h, IL-6 concentrations were measured in su- 
pernatants from two independent wells from each animal for each condition, 
using a commercial mouse IL-6 ELISA kit according to the manufacturer’s 
instructions (Bioscience). Numbers of animals used in each experiment are 
given in Table S4.
brief, 4-d-old mice were decapitated. The brains were removed and sub-
merged in ice-cold Hank’s saline. The meninges and blood vessels were re-
moved before the tissue was trypsinized, carefully dissociated with a 5-mL
pipette, resuspended, and filtered twice (100-μm diameter Falcon filter; Bec-
ton Dickinson) before seeding the cells in 5 mL of medium per flask (one
brain/flask). Cells were cultured in poly-ornithine–coated 25-cm² flasks in
DME and supplemented with 10% FBS (D10F).

Littermate heterozygote R6/2 and WT mice were used. Each brain was pro-
cessed and cultured individually to prevent cross-contamination between
animals. Genotype and CAG repeat length were determined by PCR from
tail samples taken at the time of CNS culture preparation (Laragen). Once
astrocytes reached confluence (5–7 d), D10F medium was supplemented
with 2 ng/mL GM-CSF. Microglial cells were collected, pooled according to
genotype, and seeded in 96-well Primaria plates (2.5 × 10⁵ cells in 250 μL
D10F per well; Benton Dickenson). Cultures >95% pure as assessed by
CD11b immunostaining. For each experiment, WT and R6/2 microglial
cells were processed in parallel.

24 h after plating, the cells were serum starved (MSEM, 0.2 ng/ml GM-
CSF) for an additional 24 h. They were then stimulated with 10 U IFN-γ ±
10 ng/ml LPS or carrier control. After 24 h of stimulation, supernatant
was collected and stored at −80°C for further analysis.

IL-6 concentration was measured by Lumines bead array system (QIA-
GEN). 60 μL of supernatant of three representative experiments (n = 4
for each condition) was thawed and processed using the BioPlex platform
(Bio-Rad Laboratories).

Striatal gene expression study. Total RNA was isolated and purified
from striatal samples obtained from The New Zealand Neurological Foun-
dation Human Brain Bank and the New York Brain Bank at Columbia
University (6 controls and 17 patients with pathological grades as shown in
Table S3, which is available at http://www.jem.org/cgi/content/full/jem.
20080178/DC1). RNA was reverse transcribed to cDNA using random
primers that monocytes, macrophages, and microglia produce more IL-6 than
WT animals when stimulated with LPS.

Online supplemental material. Fig. S1 shows representative flow cy-
tometry plots demonstrating purity of cells obtained by flow cytometry
and magnetic sorting. Table S1 shows the characteristics of subjects in each
human biofluid study. Table S2 shows plasma cytokine levels by disease
stage measured by multiplex ELISA assay. Table S3 shows characteristics
of subjects in the postmortem striatal expression study. Table S4 shows details
of animals used for mouse experiments. Table S5 shows details of the sub-
jects whose blood was used for the monocyte huntingtin expression study.
Table S6 shows the primers used for the human monocyte expression
study. The online supplemental material is available at http://www.jem.
org/cgi/content/full/jem.20080178/DC1.

We thank the patients and controls who donated samples; the staff of the
multidisciplinary HD clinics in London and Vancouver; Professor Richard Fault
of Auckland University and the New York Brain Bank at Columbia University
for supplying human postmortem samples; Ms. Janet North for flow cytometry
assistance; Dr. Martin R. Stämpfli (McMaster University, Hamilton, ON) for
assistance with serum assays; and Professor Chris Frost for statistical advice.

This study was financially supported by CHDI (previously the High G
Foundation), New York, and also in part by the Medical Research Council (UK),
the Wellcome Trust (66270), the Canadian Institutes for Health Research and the
Huntington Society of Canada. It was undertaken in part at UCH/UCLH, which
received a proportion of funding from the UK Department of Health’s NIHR
Biomedical Research Centres funding scheme. M.R. Hayden is a Killian University
Professor and holds a Canada Research Chair in Human Genetics.

The authors have no conflicting financial interests.

Submitted: 25 January 2008
Accepted: 6 June 2008

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