RNA-Seq of Huntington’s disease patient myeloid cells reveals innate transcriptional dysregulation associated with proinflammatory pathway activation

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Abstract

Innate immune activation beyond the central nervous system is emerging as a vital component of the pathogenesis of neurodegeneration. Huntington’s disease (HD) is a fatal neurodegenerative disorder caused by a CAG repeat expansion in the huntingtin gene. The systemic innate immune system is thought to act as a modifier of disease progression; however, the molecular mechanisms remain only partially understood. Here we use RNA-sequencing to perform whole transcriptome analysis of primary monocytes from thirty manifest HD patients and thirty-three control subjects, cultured with and without a proinflammatory stimulus. In contrast with previous studies that have required stimulation to elicit phenotypic abnormalities, we demonstrate significant transcriptional differences in HD monocytes in their basal, unstimulated state. This includes previously undetected increased resting expression of genes encoding numerous proinflammatory cytokines, such as IL6. Further pathway analysis revealed widespread resting enrichment of proinflammatory functional gene sets, while upstream regulator analysis coupled with Western blotting suggests that abnormal basal activation of the NFκB pathway plays a key role in mediating these transcriptional changes. That HD myeloid cells have a proinflammatory phenotype in the absence of stimulation is consistent with a priming effect of mutant huntingtin, whereby basal dysfunction leads to an exaggerated inflammatory response once a stimulus is encountered. These data advance our understanding of mutant huntingtin pathogenesis, establish resting myeloid cells as a key source of HD immune dysfunction, and further demonstrate the importance of systemic immunity in the potential treatment of HD and the wider study of neurodegeneration.
Introduction

Huntington’s disease (HD) is an incurable, autosomal dominant neurodegenerative disorder that is caused by a CAG repeat expansion in exon 1 of the huntingtin (HTT) gene (1). An expansion of 40 or more repeats is fully penetrant, resulting in production of the mutant (m)HTT protein that is the primary cause of cellular death and dysfunction (2). The central nervous system has traditionally been regarded as the primary site of HD pathology, with patients suffering from a range of symptoms including movement disorders, cognitive impairment and psychiatric disturbance. However, it is apparent that HD is in fact a disease of the whole body, with the mHTT protein being expressed in all cells and tissues that have been studied (3). Patients are affected by a range of peripheral pathologies including skeletal muscle wasting (4), and there is mounting interest in the immune system as a potential modifier of disease progression.

HD patients exhibit immune dysfunction both centrally, in the form of microglial activation (5), and peripherally, where elevated levels of proinflammatory mediators are detectable up to 16 years before the predicted onset of motor symptoms (6,7). These phenomena have recently been found to be correlated, suggesting a global immune response to the presence of mHTT whereby central pathology is mirrored peripherally (8). The innate immune system is thought to be the primary source of HD immune dysfunction, with myeloid cells comprising circulating monocytes and tissue macrophages the likely peripheral effector cells (9). HD myeloid cells ex vivo are hyper-reactive, producing significantly more IL-1β and TNFα after lipopolysaccharide (LPS) stimulation (10), and also exhibit functional deficits in their migratory and phagocytic capabilities (11,12). These changes contrast with T lymphocytes of the adaptive immune system, which do not display any intrinsic phenotypic defects as a result of mHTT expression (13). Such alterations in the innate immune system are mirrored in mouse models of HD (12), suggesting that they are caused by a common pathological effect of mHTT expression. Indeed, several studies have demonstrated that mHTT has a cell-autonomous effect on innate immune cell function, and that immune activation in HD is not simply a secondary response to neuronal pathology (10,14). For example, the hyper-reactive phenotype of human HD myeloid cells is reversible following HTT lowering with siRNA, while mHTT levels in peripheral immune cells correlate with disease burden scores in HD patients (15). Importantly, the peripheral innate immune system has been suggested to act as a modifer of HD progression. Dampening of the immune system using a peripherally restricted CB2 agonist improves the phenotype of an HD mouse model (16), as does the administration of a TNFα antagonist (17), a KMO inhibitor (18) and bone marrow transplantation (19). Although these data suggest that the study of the HD peripheral immune system has disease relevance far beyond its uses as a ‘window’ into the brain, the mechanisms underlying HD immune dysfunction remain incompletely understood.

To date, studies on peripheral HD myeloid cells have almost exclusively demonstrated phenotypic abnormalities in response to LPS stimulation (10,11), and there is very little information available on whether these cells are also abnormal in their basal, unstimulated state. Indeed, there is currently no evidence that HD myeloid cells have increased resting expression of proinflammatory cytokines (10,14). It is therefore unclear whether mHTT only affects HD myeloid cells following stimulation, or if there is an underlying mechanism producing resting dysfunction that is responsible for the phenotypic changes seen once the cells are activated. Mutant HTT has been found to have a cell-autonomous priming effect on HD murine microglial cell lines (14), resulting in increased proinflammatory gene expression in the absence of stimulation. Although a similar effect is yet to be shown in primary human cells, it raises the possibility that mHTT’s potential baseline effects in HD myeloid cells are responsible for their hyper-reactive response to stimulation. This has important implications for targeting peripheral immunity as a therapeutic for HD, as intrinsic pathology is more likely to be treatable without the need to time intervention to coincide with inflammatory events.

Regardless of whether mHTT’s pathogenic effects are innate or only occur following stimulation, evidence will likely be detectable as changes in the cellular transcriptome; if mHTT has a tonic pathogenic effect then transcriptional changes should occur in unstimulated, resting cells. Transcriptional dysregulation is a central feature of HD pathogenesis, and has been consistently demonstrated in a range of HD tissues (20,21). Such broad effects on transcription could be caused by several underlying causative mechanisms, including sequestration of transcription factors by mHTT aggregates, impairment of transcription factor degradation by the ubiquitin-proteasome system, changes in histone modifications and the direct binding of mHTT to DNA (22). Moreover, the functions of upstream intracellular signalling pathways are likely affected. Mutant HTT activates the NFκB signalling pathway via a direct interaction with the IKK complex, a key regulator of the NFκB family of transcription factors (23). This is associated with hyper-reactivity in HD myeloid cells, which exhibit an exaggerated and prolonged NFκB signalling response to LPS (10). However, it is not clear whether HD myeloid cells also display increased NFκB activity in the resting state; this is likely key to determining whether HD myeloid cells are also abnormal in the absence of stimulation. Furthermore, signalling downstream of the LPS receptor TLR4 is complex, including as it does activation of signalling pathways such as those involving the mitogen-activated protein kinases (MAPKs) (24), the contribution of which to HD myeloid cell dysfunction is yet to be fully explored.

Previous gene expression studies have demonstrated numerous transcriptional changes in HD peripheral blood cells (21,25,26), but these have been largely carried out on heterogeneous cell populations with varying transcriptional profiles, and have rarely been validated between studies. Therefore, study of specific cell populations using modern sequencing technology is required to identify biologically relevant transcriptional changes associated with mHTT expression. Here, we use RNA sequencing (RNA-Seq) to investigate the human HD monocyte transcriptome under both resting and LPS-stimulated conditions. We demonstrate that the transcriptome of HD myeloid cells is abnormal even in the absence of stimulation, including previously unreported increases in the basal expression of proinflammatory cytokines. Pathway analysis to dissect the biological relevance of these expression changes identifies numerous proinflammatory functional gene sets that are altered in unstimulated HD myeloid cells, as well as specific upstream regulator molecules that may be driving transcriptional change; activation of potential regulator molecules is further investigated using Western blotting. These data provide new insight into the pathogenesis of mHTT expression in systemic innate immune cells, in addition to the potential for modulating the peripheral immune system to modify disease progression.

Results

HD monocytes exhibit resting proinflammatory transcriptional changes

Although the phenotypic abnormalities associated with HD myeloid cells are relatively well characterized, considerably less
known about the transcriptional profile that underpins these changes. To address this, CD14+ monocytes were isolated from peripheral blood samples donated by thirty manifest HD patients and thirty-three control subjects, and cultured with and without stimulation with LPS and interferon gamma (IFN-γ). RNA-Seq of these samples was carried out to provide a quantitative analysis of the entire HD monocyte transcriptome. LPS-stimulation was confirmed by pooling the HD and control samples and performing differential expression analysis between the unstimulated and stimulated datasets; 12 599 genes were found to be differentially expressed (false discovery rate (FDR) < 0.05; Dataset S1).

Differential expression analyses were then performed to determine which genes are significantly altered between HD and control monocytes, whereby the unstimulated and stimulated monocytes were analysed separately. Analysis of the unstimulated monocyte data identified 130 genes that were differentially expressed (FDR < 0.05, 101 upregulated and 29 downregulated) in resting HD cells compared with control (Table 1; Dataset S2). Although the differentially expressed genes were associated with a wide range of effector functions, proinflammatory cytokines and chemokines were heavily featured, with HD monocytes having significantly increased expression of IL6, IL12B, IL19, IL23A, CCL9, CCL19, CCL20, CXCL6 and CSF2 gene transcripts. All of these genes had a log2 fold change of > 1, corresponding to a >2-fold increase in mRNA expression. This contrasted with the stimulated dataset, which showed comparatively little evidence of differential expression between HD and control cells, as only DNAJB13, STAC and RASEF were found to be differentially expressed (FDR < 0.05; Dataset S3). Each of these genes were also found to be differentially expressed in the unstimulated dataset (these genes are displayed as red dots in Fig. 1). Comparison of the log2 fold changes revealed a general trend whereby the relative expression differences between HD and control were lower in the stimulated compared with the unstimulated dataset; this was the case for 116 of the 130 differentially expressed genes (Fig. 1). This observation of more profound change in resting HD monocytes is in marked contrast to previous functional studies where a stimulus has been necessary to elicit phenotypic differences (10–12).

HD monocytes do not display significant changes in alternative splicing

Next, we investigated whether changes in alternative splicing underlie the expression differences seen in our dataset, as differential splicing has been shown to be a key pathogenic mechanism in tissues from a number of neurodegenerative diseases, including HD brain (27). However, analysis of individual exon, intron and transcript levels showed no significant differences in either the unstimulated or stimulated datasets (FDR < 0.05; Datasets S4–S9). This suggests that, in contrast to other tissues, differential splicing is not a major factor in mediating HD innate immune dysfunction.

**Table 1. The top 20 gene expression changes in resting HD monocytes (ranked by FDR)**

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Ensembl ID</th>
<th>RPKM (control)</th>
<th>RPKM (HD)</th>
<th>Log2 fold change</th>
<th>P-value</th>
<th>FDR</th>
<th>Protein function</th>
</tr>
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<tbody>
<tr>
<td>FAM124A</td>
<td>ENSG00000150510</td>
<td>0.242</td>
<td>0.812</td>
<td>2.684</td>
<td>4.61E-08</td>
<td>6.05E-04</td>
<td>Functionally uncharacterized</td>
</tr>
<tr>
<td>IL19</td>
<td>ENSG00000142224</td>
<td>0.586</td>
<td>1.473</td>
<td>2.358</td>
<td>1.15E-07</td>
<td>7.57E-04</td>
<td>Proinflammatory cytokine</td>
</tr>
<tr>
<td>IL23A</td>
<td>ENSG00000110944</td>
<td>1.914</td>
<td>3.597</td>
<td>1.576</td>
<td>7.84E-07</td>
<td>2.55E-03</td>
<td>Proinflammatory cytokine</td>
</tr>
<tr>
<td>FAM213B</td>
<td>ENSG000001357870</td>
<td>7.837</td>
<td>5.501</td>
<td>0.604</td>
<td>8.99E-07</td>
<td>2.55E-03</td>
<td>Prostaglandin metabolism</td>
</tr>
<tr>
<td>TGFA</td>
<td>ENSG00000163235</td>
<td>0.665</td>
<td>1.392</td>
<td>1.550</td>
<td>9.69E-07</td>
<td>2.55E-03</td>
<td>Growth factor; promotes cell proliferation</td>
</tr>
<tr>
<td>FZD7</td>
<td>ENSG00000155760</td>
<td>0.397</td>
<td>0.246</td>
<td>-0.721</td>
<td>1.39E-06</td>
<td>3.04E-03</td>
<td>Wnt protein receptor</td>
</tr>
<tr>
<td>C6orf23</td>
<td>ENSG00000181577</td>
<td>0.063</td>
<td>0.297</td>
<td>2.127</td>
<td>1.92E-06</td>
<td>3.60E-03</td>
<td>Functionally uncharacterized</td>
</tr>
<tr>
<td>PROCR</td>
<td>ENSG00000101000</td>
<td>3.695</td>
<td>10.872</td>
<td>1.552</td>
<td>3.61E-06</td>
<td>5.69E-03</td>
<td>Receptor for activated protein C</td>
</tr>
<tr>
<td>NT5E</td>
<td>ENSG00000135318</td>
<td>0.210</td>
<td>0.725</td>
<td>2.082</td>
<td>4.24E-06</td>
<td>5.69E-03</td>
<td>Hydrolases extracellular nucleotides</td>
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<tr>
<td>SMO</td>
<td>ENSG00000128602</td>
<td>0.259</td>
<td>0.124</td>
<td>-1.161</td>
<td>4.33E-06</td>
<td>5.69E-03</td>
<td>G protein-coupled receptor</td>
</tr>
<tr>
<td>CISH</td>
<td>ENSG00000114737</td>
<td>2.723</td>
<td>5.161</td>
<td>1.439</td>
<td>5.90E-06</td>
<td>7.04E-03</td>
<td>Negative regulation of JAK/STAT signalling</td>
</tr>
<tr>
<td>C6orf165</td>
<td>ENSG000000213204</td>
<td>0.038</td>
<td>0.086</td>
<td>1.205</td>
<td>6.57E-06</td>
<td>7.20E-03</td>
<td>Functionally uncharacterized</td>
</tr>
<tr>
<td>CCL19</td>
<td>ENSG00000172724</td>
<td>0.518</td>
<td>1.767</td>
<td>1.763</td>
<td>8.79E-06</td>
<td>8.89E-03</td>
<td>Chemoattraction of T and B lymphocytes</td>
</tr>
<tr>
<td>PTGS2</td>
<td>ENSG00000073756</td>
<td>2.319</td>
<td>38.604</td>
<td>1.813</td>
<td>9.52E-06</td>
<td>8.93E-03</td>
<td>Prostaglandin synthase enzyme</td>
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<tr>
<td>HPSE</td>
<td>ENSG00000173083</td>
<td>11.322</td>
<td>24.941</td>
<td>1.142</td>
<td>1.10E-05</td>
<td>9.68E-03</td>
<td>Extracellular matrix remodelling</td>
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<tr>
<td>VEGFA</td>
<td>ENSG00000121751</td>
<td>5.475</td>
<td>14.251</td>
<td>0.898</td>
<td>1.35E-05</td>
<td>1.11E-02</td>
<td>Promotes angiogenesis and endothelial cell growth</td>
</tr>
<tr>
<td>ANXA11</td>
<td>ENSG00000122359</td>
<td>136.875</td>
<td>105.621</td>
<td>-0.374</td>
<td>1.72E-05</td>
<td>1.33E-02</td>
<td>Phospholipid-binding protein</td>
</tr>
<tr>
<td>CDK2</td>
<td>ENSG00000123374</td>
<td>5.534</td>
<td>7.186</td>
<td>0.390</td>
<td>1.88E-05</td>
<td>1.39E-02</td>
<td>Cell cycle regulation</td>
</tr>
<tr>
<td>R3HCC1</td>
<td>ENSG00000104679</td>
<td>15.225</td>
<td>11.394</td>
<td>-0.421</td>
<td>2.18E-05</td>
<td>1.45E-02</td>
<td>Nucleic acid binding</td>
</tr>
<tr>
<td>PGAP3</td>
<td>ENSG00000161395</td>
<td>2.943</td>
<td>2.310</td>
<td>-0.364</td>
<td>2.28E-05</td>
<td>1.45E-02</td>
<td>GPI-specific phospholipase</td>
</tr>
</tbody>
</table>
Due to overlapping gene sets there is redundancy in the analysis, and that multiple points along a pathway may be suggested as upstream regulators, even if only one is driving the change. This effect is quantified by the activation z-score statistic, which increases or decreases depending on the inferred activation/inhibition state of a particular upstream regulator.

Significance in IPA is typically attributed to upstream regulators that have an overlap p-value of < 0.01 and an activation z-score of > 2 or < -2; 155 upstream regulators fulfilled these criteria in our unstimulated dataset (Table 2; Dataset S13). Of these, 125 were predicted to be significantly activated, while thirty were predicted to be significantly inhibited. Due to the redundancy associated with IPA it is unlikely that this many are actually affected, so we decided to narrow our focus to the subset of upstream regulators with the highest activation z-scores, and therefore the strongest evidence for their mediating transcriptional dysregulation. Crucially, a large number of molecules associated with intracellular signalling pathways downstream of the TLR4 receptor were represented in this group (Fig. 3). Consistent with previous data showing NFκB dysregulation in HD myeloid cells, both RELA and the NFκB complex were featured in the top ten most significant results ranked by activation z-score. Other notable potential regulators included NFκB1, the ERK and p38 MAPKs, in addition to the transcription factor STAT3. These data suggests that the transcriptional changes observed in the RNA-Seq dataset are related to the abnormal activation of specific upstream signalling molecules responsible for driving gene expression in resting HD myeloid cells.

**NFκB but not ERK or p38 MAPK signalling is abnormally activated in resting HD myeloid cells**

Previously we demonstrated that HD myeloid cells exhibit an exaggerated NFκB signalling response to LPS, both in the magnitude of the initial activation and the prolonged time taken for activation to return to baseline (10). However, it is unclear whether NFκB dysfunction also extends to the cells in their basal, resting state. To follow up the upstream regulator analysis, we next investigated whether the NFκB pathway is abnormally activated in unstimulated HD myeloid cells. Monocyte-derived macrophages were obtained from manifest HD and control subjects, before Western blotting was carried out to quantify the expression of IκBα. IκBα is a cytoplasmic inhibitor of the NFκB family of transcription factors; lower IκBα levels are used to demonstrate increased NFκB activity, as reduced inhibition will lead to increased translocation of NFκB to the nucleus. Consistent with the upstream regulator analysis, we found that resting HD myeloid cells express significantly less IκBα than control cells (Fig. 4). This demonstrates that the abnormal NFκB activity previously described in stimulated HD myeloid cells also exists basally.

However, the contribution of alternative signalling pathways to HD myeloid cell dysfunction is yet to be fully characterized. The ERK and p38 MAPKs were selected for investigation as they both play a major role in signalling downstream of the LPS receptor TLR4 (24), and were identified by IPA as potentially significant transcriptional regulators in resting HD monocytes. ERK and p38 are both activated by phosphorylation; quantification of phosphorylated relative to total protein therefore allows their activation states to be investigated. Monocyte-derived macrophages were obtained from manifest HD and control subjects, before cells from each subject were pulsed with LPS and INF-γ for 15, 30, 60 and 120 min, or left unstimulated. Western blotting...
to quantify the expression of the total and active, phosphorylated forms of both ERK and p38 showed no significant differences in activation either basally or at any of the time points following LPS stimulation. Furthermore, no significant differences were seen in the expression of total ERK or p38 compared with the reference protein β-actin. This suggests that, in contrast to NFκB, HD myeloid cells do not exhibit abnormally activated ERK or p38 signalling, and that these pathways are unlikely to drive the transcriptional changes seen in the RNA-Seq dataset.

**Discussion**

Previous studies of peripheral HD myeloid cells have primarily demonstrated phenotypic dysfunction in the stimulated state (10–12). In contrast, very little is known about the extent to which these cells also display abnormalities in their basal, resting state. Here, use of RNA-Seq demonstrates that the basal transcriptome of resting HD monocytes is significantly altered, suggesting that mHTT expression has a state-independent effect occurring via a mechanism that is not restricted to the dysregulation of intracellular signalling pathways following stimulation. This has previously been shown in a wide range of tissues including brain, skeletal muscle and whole blood (22), reinforcing the observation that mHTT consistently affects the basal transcriptome in both patients and animal models of HD. Crucially, HD monocytes expressed basally elevated levels of numerous proinflammatory cytokine gene transcripts, for the first time showing increased resting cytokine expression at either the mRNA or protein level. HD patients have elevated levels of cytokines and chemokines in their peripheral blood, with increased IL-6 being detectable up to 16 years before the predicted onset of motor symptoms (6,7); the upregulated IL6 expression seen in our dataset strongly suggests that peripheral myeloid cells are a key source. The existence of proinflammatory transcriptional changes in the absence of stimulation was confirmed by GSEA, whereby significant enrichment of numerous proinflammatory cytokine gene transcripts, for the first time showing increased resting cytokine expression at either the mRNA or protein level. HD patients have elevated levels of cytokines and chemokines in their peripheral blood, with increased IL-6 being detectable up to 16 years before the predicted onset of motor symptoms (6,7); the upregulated IL6 expression seen in our dataset strongly suggests that peripheral myeloid cells are a key source. The existence of proinflammatory transcriptional changes in the absence of stimulation was confirmed by GSEA, whereby significant enrichment of numerous functional gene sets relating to innate immunity, inflammation and cytokine production was found in resting HD monocytes. This is important as it allows us to assign wider biological relevance to the expression changes seen in our dataset, while avoiding any investigator bias that could occur from looking at specific genes in isolation. Consistent with a previous RNA-Seq study on post-mortem brain, we also found that the majority of...
the transcriptional changes in HD monocytes involved upregulation (30).

Mutant HTT expression has been shown to promote cell-autonomous dysfunction in murine microglial cultures and human monocyte models (10,14). Furthermore, in a murine HD microglial line it has been suggested to prime resting cells, whereby the enrichment of proinflammatory molecules in the resting state leads to an exaggerated response once a stimulus is encountered. This is thought to occur via increased expression of proinflammatory transcription factors including PU.1 (14). The transcriptional dysregulation we have demonstrated in resting HD monocytes suggests that mHTT expression has a similar priming effect on primary human myeloid cells. This is a possible explanation for the increased cytokine release seen after LPS stimulation (10), and may have significant relevance to the pathobiology of HD in humans. Although HD patients have elevated levels of circulating proinflammatory cytokines (6), there is no evidence that they have an increased incidence of infectious or inflammatory events. In addition, the phenotypic improvement produced by modulation of the immune system in animal models of HD is not dependent on ameliorating artificial inflammatory stimuli (16–18). This suggests that the proinflammatory peripheral changes observed in HD in vivo are not dependent on, or primarily caused by, excessive cytokine

<table>
<thead>
<tr>
<th>Upstream regulator</th>
<th>Activation z-score</th>
<th>P-value of overlap</th>
<th>Function</th>
</tr>
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<tbody>
<tr>
<td>TNF</td>
<td>5.764</td>
<td>2.18E-13</td>
<td>Proinflammatory cytokine</td>
</tr>
<tr>
<td>IL1B</td>
<td>5.754</td>
<td>8.08E-14</td>
<td>Proinflammatory cytokine</td>
</tr>
<tr>
<td>MyD88</td>
<td>4.761</td>
<td>2.92E-12</td>
<td>Cytoplasmic adaptor protein for the TLR family</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>4.635</td>
<td>3.09E-07</td>
<td>Cytokine involved in mediating the innate immune response</td>
</tr>
<tr>
<td>IL1A</td>
<td>4.536</td>
<td>1.69E-11</td>
<td>Proinflammatory cytokine</td>
</tr>
<tr>
<td>TLR3</td>
<td>4.323</td>
<td>1.99E-09</td>
<td>Cell surface receptor involved in the activation of the innate immune response</td>
</tr>
<tr>
<td>RELA</td>
<td>4.122</td>
<td>5.86E-08</td>
<td>Transcription factor; one of five NFκB family members</td>
</tr>
<tr>
<td>NFXB (complex)</td>
<td>4.077</td>
<td>2.63E-08</td>
<td>A family of five transcription factors and their regulators that mediate the inflammatory response</td>
</tr>
<tr>
<td>TLR9</td>
<td>3.773</td>
<td>4.33E-07</td>
<td>Cell surface receptor involved in the activation of the innate immune response</td>
</tr>
<tr>
<td>TLR2</td>
<td>3.770</td>
<td>3.94E-09</td>
<td>Cell surface receptor involved in the activation of the innate immune response</td>
</tr>
<tr>
<td>STAT3</td>
<td>3.701</td>
<td>2.34E-07</td>
<td>Transcription factor</td>
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<tr>
<td>IL6</td>
<td>3.654</td>
<td>7.39E-06</td>
<td>Proinflammatory cytokine</td>
</tr>
<tr>
<td>TICAM1/TRIF</td>
<td>3.651</td>
<td>2.59E-08</td>
<td>TLR cytoplasmic adaptor protein; mediates an alternative pathway to MyD88</td>
</tr>
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<td>ERK1/2</td>
<td>3.636</td>
<td>5.33E-06</td>
<td>Protein kinase intracellular signalling molecules</td>
</tr>
<tr>
<td>TLR4</td>
<td>3.610</td>
<td>7.68E-12</td>
<td>Cell surface receptor involved in the activation of the innate immune response</td>
</tr>
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</table>

IPA was used to identify transcriptional regulators with significant target gene overlap with changes in the resting HD monocyte transcriptome. A P-value cut-off of < 0.01 was used to determine which genes were included in the analysis. The upstream regulators were ranked by activation z-score and the 15 most significant were included in the table.

Figure 3. Specific intracellular signalling pathways are predicted to be abnormally activated in resting HD monocytes. IPA analysis of upstream regulators of gene transcription identified numerous signalling pathway components downstream of the TLR4 receptor which are predicted to be abnormally activated in resting HD monocytes. Upstream regulators with significantly inferred activation are shown in orange (darker colour = higher activation z-score). The activation z-score for each component is also shown (>2 or < -2 is considered to be significant).
release from repeatedly stimulated cells. Instead, a chronic increase in the basal release of proinflammatory mediators allows these observations to be reconciled independently of stimulation. Furthermore, a number of the cytokines known to be upregulated in HD peripheral blood are pre-synthesized in the resting state. TNFα is synthesized as a membrane bound precursor that is cleaved in response to stimulation (28), while IL-8 undergoes vesicular storage prior to release (29). Priming of HD myeloid cells by mHTT could therefore cause increased cytokine storage ready for release following stimulation, or indeed increased basal release in the absence of stimulation. Indeed, their increase in basal cytokine expression suggests that primed myeloid cells are likely to have the latter effect. It is likely that this phenomenon is also present in pre-manifest HD patients, as immune changes including increased cytokine release are detectable long before the onset of motor symptoms (6), and the short life span of peripheral blood cells precludes the possibility of a chronic effect built up over many years. This is supported by the clear existence of intrinsic mHTT-related dysfunction in HD myeloid cells, as it has been shown that peripheral immune changes occur independently of neurodegeneration, and therefore are unlikely to be solely linked to advancing central disease. The fact that numerous studies have demonstrated a disease-modifying role for the peripheral immune system raises the possibility that HD myeloid cells may even play a role in modifying disease onset (16–19), but this will require further investigation in mouse models of the disease.

Surprisingly, the transcriptome of stimulated HD monocytes was comparatively unaffected in terms of differential gene expression. Comparison of the unstimulated and stimulated log2 fold changes revealed that, while the directions of effect were largely consistent, the relative size of the majority of the fold

Figure 4. NFκB but not ERK or p38 MAPK signalling is abnormally activated in resting HD myeloid cells. Monocyte-derived macrophages were isolated from HD and control peripheral blood samples and pulsed with LPS and IFNγ for 15, 30, 60 and 120 min, or left unstimulated. Western blotting was then carried out on the unstimulated samples to quantify the basal levels of IκBα, while additional Western blotting was carried out on all samples to quantify the levels of total and active, phosphorylated ERK and p38 MAPK. (A) Resting HD myeloid cells express significantly reduced IκBα protein compared with control (control n = 7, HD n = 9). However, no significant differences were seen in (B) the ratio of phosphorylated to total enzyme, or (C) the ratio of total enzyme to β-actin for either ERK or p38 at any of the time points that were studied (n = 10). Error bars represent ± SEM, *P < 0.05.
changes was reduced under stimulated conditions to the point that they were no longer significant. This may be due to the extremely strong activating response provided by LPS, resulting in a plateau in transcriptional activity as the cellular machinery reaches the limits of its mRNA synthesis capability. This is likely to result in the masking of biological differences between HD and control monocytes, as the proinflammatory phenotype displayed by resting HD monocytes is ablated by the stronger inflammatory stimulus provided by LPS. Regardless, the lack of differentially expressed genes in the stimulated samples suggests that the proinflammatory transcriptional profile displayed by resting HD monocytes is likely to be even more crucial in mediating their hyper-reactive response to LPS. Interestingly, a number of gene sets were found to be enriched among the downregulated genes in LPS-stimulated HD monocytes. The enrichment for downregulation in numerous gene sets relating to protein localization and targeting is consistent with known cytoskeletal defects in HD (30), while further enriched gene sets were associated with organelles which are known to be affected by mHTT, including the mitochondria and lysosome (31,32). Although this analysis demonstrates that the transcriptome of stimulated HD monocytes is affected by mHTT, the relative lack of significant individual gene expression changes suggests this gene set enrichment may not have the same biological significance as that seen in resting HD myeloid cells.

Although this dataset suggests that mHTT expression has a priming effect on the transcriptome of HD monocytes, understanding the underlying mechanism requires investigation of the intracellular signalling pathways that are driving the transcriptional changes. Previous work has demonstrated that the proinflammatory transcriptional profile displayed by resting HD monocytes is likely to be even more crucial in mediating their hyper-reactive response to LPS. Interestingly, a number of gene sets were found to be enriched among the downregulated genes in LPS-stimulated HD monocytes. The enrichment for downregulation in numerous gene sets relating to protein localization and targeting is consistent with known cytoskeletal defects in HD (30), while further enriched gene sets were associated with organelles which are known to be affected by mHTT, including the mitochondria and lysosome (31,32). Although this analysis demonstrates that the transcriptome of stimulated HD monocytes is affected by mHTT, the relative lack of significant individual gene expression changes suggests this gene set enrichment may not have the same biological significance as that seen in resting HD myeloid cells. However, a previous study found no evidence of increased JAK/STAT activation in a mouse microglial model of HD (14); however, neither that study nor our current study revealed any increase in PU.1 expression in HD patient monocytes. Furthermore, activation of PU.1 in our dataset was not inferred by IPA. Despite this, we have shown that resting HD monocytes have a proinflammatory transcriptional profile which may be caused by a similar mHTT priming effect to that seen in the microglial cell line. Although it has been suggested that the separate developmental origins of microglia are responsible for the prominent role of PU.1 (14), we also suggest that the relative importance of intracellular signalling pathways in different cell types helps determine their varying contributions to HD pathogenesis. The mechanism of mHTT interaction with each signalling pathway seems unlikely to be drastically altered between cell types; this can be seen in the fact that NFκB binding motifs were also found to be enriched in the HD macroglial cell line (14). However, as the PU.1 and NFκB pathways are vital for microglial and monocyte/macrophage cell function, respectively, it is not surprising that their dysregulation by mHTT will cause them to have a central role in mediating mHTT-related phenotypic dysfunction in these cell types. Conversely, cells where these pathways have less important roles seem unlikely to experience comparable dysfunction as a result of the same mechanism, if indeed they are dysfunctional at all. Indeed, it has been shown that the intrinsinc function of HD T lymphocytes is not affected by mHTT expression (13); it is possible that this is due to the reduced importance of mHTT-affected signalling pathways in mediating T lymphocyte function.

Finally, we demonstrate the importance of studying specific cell populations using modern sequencing techniques and large cohorts in order to accurately characterize HD-associated transcriptional changes. Previous transcriptional studies of HD peripheral immune cells have investigated heterogeneous cell populations with varying transcriptional profiles, and it has often not been possible to validate differentially expressed genes between studies (21,25,26). The advent of high-throughput sequencing has provided dramatically improved coverage of the entire transcriptome when compared with traditional
excluded from the study. and those on immunomodulatory medications, were likewise
layered on Histopaque-1077 (Sigma-Aldrich) and centrifuged at

The study of individual cell populations is also particularly important given that a consistent transcriptional signature has not yet been established between different HD tissues (22), and observations made in one cell type may not hold true when studying another.

This study therefore provides the first evidence of significant basal proinflammatory changes in resting peripheral HD myeloid cells, with increased expression of proinflammatory cytokine mRNA in addition to the enrichment of functional gene sets associated with innate immunity, inflammation and cytokine production. This suggests that mHTT primes HD myeloid cells, resulting in an exaggerated proinflammatory response once a stimulus is encountered. We show that previously described NFKB dysfunction also extends to resting HD myeloid cells, and that abnormal activation of this pathway is likely to play a key role in mediating their basal proinflammatory transcriptional profile. However, the ERK and p38 MAPK pathways do not appear to play a comparable role. The existence of basal dysfunction further supports the possibility that modulation of the innate immune system may be used as a therapeutic strategy to modify HD progression, as the beneficial effects of immunomodulatory drugs are likely to extend beyond ameliorating the consequences of time-limited infectious or inflammatory events. Finally, this study reinforces the importance of systemic immunity in the wider study of neurodegeneration, as the existence of ex vivo myeloid cell abnormalities shows that peripheral tissues are in fact intrinsically abnormal, and are not merely responding to central pathology.

Materials and Methods

Collection and classification of human samples

All experiments were performed in accordance with the Declaration of Helsinki and approved by the University College London (UCL)/UCL Hospitals Joint Research Ethics Committee. Peripheral blood samples were donated by genetically diagnosed HD patients and control subjects, and all subjects provided informed written consent. HD subjects were classified by nHashed samples for gene expression analysis were non-smokers
to control for the effects of smoking on the monocyte transcrip-
tional analysis. RNA integrity was assessed using RNA 6000 Nano Chips on a 2100 Bioanalyzer (Agilent). Only samples with non-degraded RNA (RIN \( \geq 7 \)) were used for sequencing. Sequencing of RNA samples was performed by deCODE Genetics, Iceland. Preparation of indexed cDNA sequencing libraries was carried out using the TruSeq poly-A mRNA method (Illumina). Briefly, poly-A mRNA transcripts were captured from total RNA using poly-T beads, before cDNA was generated using random hexamer priming. Paired-end sequencing (2 \( \times 100 \) cycles) of indexed cDNA libraries was then carried out on a HiSeq 2500 machine (Illumina), generating at least 50 million reads (101 base pairs) per sample. Sequencing was performed using v4 SBS and Cluster Kits (Illumina). One sample failed the library generation step and was excluded from the study. After sequencing the indexed samples were demultiplexed before generation of FASTQ files for analysis. Raw data files are available from the European Nucleotide Archive (http://www.ebi.ac.uk/ena) under the study accession number PRJEB12995.

Data processing and expression analysis

Data quality control was performed using the RNA-SeQC package (40). Metrics including rRNA rate, mapping rate, concordance mapping rate and uniqueness rate were determined to confirm that they were within acceptable parameters. One sample failed quality control and was removed from further analysis. After quality control the remaining samples were aligned using TopHat2 (41), before the counts were summarized using HTSeq (keeping any read duplicates). Differential expression analysis was then performed using the R package DESeq2 (42). Outlier counts were removed using a Cook’s distance cut-off of 5 in DESeq2 packages and gender was used as a covariate in the

Human Molecular Genetics, 2016, Vol. 0, No. 0 | 9
analysis. Differential splicing analysis was carried out on the aligned data using the R package Ballgown (43).

Gene set enrichment analysis
Enrichment of differential expression among gene sets corresponding to biological hypotheses (pathways) was tested using the GSEA method (44). Rather than defining a list of significant genes, GSEA ranks all genes in order of their differential expression statistic, and tests whether the genes in a particular gene set have a higher rank overall than would be expected by chance. The analysis is weighted by the differential expression statistic, thus giving more weight to more significant genes. Significance of enrichment was obtained by randomly permuting gene-wide association statistics among genes. One-sided p-values were calculated separately for differential upregulation and downregulation of expression in HD, and these were then converted into the corresponding chi-square statistic for use in the GSEA analysis. To avoid making a priori assumptions, we chose to use a large pathway set comprising Gene Ontology (GO) (45), Kyoto Encyclopedia of Genes and Genomes (46), PANTHER v8.1 (47), Mouse Genome Informatics (MGI) (48), Reactome (49), Biocarta and NCIPathway interaction database (50) pathways. This resulted in a total of 14 243 functional gene sets, many with overlapping members, containing between 3 and 500 genes. To correct for multiple testing, we converted the GSEA P-values into q-values (51), which can be interpreted as the minimum false discovery rate (FDR) at which a q-value would be counted as significant.

Upstream regulator analysis
Upstream regulator analysis was carried out using QIAGEN’s IPA. IPA identifies potential upstream regulators of transcriptional change using the P-value of overlap and activation z-score statistics. The P-value of overlap uses Fisher’s exact test to determine whether there is statistically significant overlap between gene expression changes in a dataset and the genes which are affected by an upstream regulator; significance is attributed to molecules with a P-value of < 0.01. This does not take the direction of expression changes into account, so the activation z-score is used to predict whether potential upstream regulators are activated or inhibited in the dataset. A z-score of > 2 or < -2 is generally considered to be significant. Further information on how the activation z-score is calculated is available on the IPA website (http://www.qiagen.com/, Last accessed March 29, 2016). A P-value cut-off of 0.01 was used to determine which genes were included in the analysis.

Western blotting
Cells were lysed in RIPA buffer (25 mM Tris-HCl (pH 7.6), 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS and 1X complete protease inhibitor cocktail (Roche)), mixed with Laemmli buffer and denatured at 95 °C for 10 min. Lysates were run on 12% Tris-Glycine gels (Invitrogen) at 120 V, before transfer to 0.2 μm nitrocellulose membranes at 35 V for 2 h. Membranes were blocked in 1:1 PBS/Odyssey Blocking Buffer (LI-COR) for 1 h before incubation with primary antibody diluted in 1:1 PBS/Odyssey Blocking Buffer at 4 °C overnight. Primary antibodies used were to iκBα (Santa Cruz Biotech; 1:500), p38, phospho-p38, ERK (p42/44), phospho-ERK (p42/44) (all Cell Signalling Technology; 1:1000) and β-actin (Abcam; 1:10000). After washing with PBS the membranes were incubated with secondary antibody diluted in 1:1 PBS/Odyssey Blocking Buffer for 1 h. Secondary antibodies used were IRDye 680RD Goat Anti-Mouse IgG and IRDye 800CW Goat Anti-Rabbit IgG (LI-COR; 1:5000). After washing the signal was visualized using an Odyssey Infrared Imager (LI-COR) and quantified using TL100 software (TotalLab).

Statistical analysis
Statistical analysis of differential expression, splicing, GSEA and IPA data was carried out using the software packages outlined above. iκBα Western blotting was analysed using unpaired two-tailed Student’s t-tests, while ERK and p38 MAPK Western blotting experiments were analysed by two-way ANOVAs with Bonferroni post-tests. All error bars represent SEM.

Supplementary Material
Supplementary Material is available at HMG online.

Acknowledgements
We would like to thank Lucy Carty and Salman Haider for their help in collecting the samples, and Cardiff University Central Biotechnology Services for their analytical support. We would also like to thank the patients and control subjects who donated samples and the staff of the multidisciplinary HD Clinic at the National Hospital for Neurology and Neurosurgery, Queen Square, London. This work was undertaken at UCLH/UCL who acknowledge support from the Department of Health’s National Institute for Health Biomedical Research Centre.

Conflict of Interest statement. None declared.

Funding
This study was funded by the EU FP7 Health Call (261358 and 305121 to S.J.T.); the Medical Research Council (MR/L003832/1 to S.J.T., MR/L010305/1 to L.J. and PH, MR/L02053X/1 to R.A. and S.J.T.); Rosetrees Trust (JS16/M220 to J.R.C.M.); CHDI (A-5053 to T.C.S., A-9412 to S.J.T.); the National Institute for Health Research University College London Hospitals Biomedical Research Centre (BRC54/NS/RA and BRC230/NS/RA/101410 to R.A.); and the Brain Research Trust (Di,H.M.). Funding to pay the Open Access publication charges for this article was provided by the Medical Research Council.

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