

## Induction of chemokines, MCP-1, and KC in the mutant huntingtin expressing neuronal cells because of proteasomal dysfunction

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### Abstract

Huntington's disease is a hereditary neurodegenerative disorder caused by an aberrant polyglutamine expansion in the amino terminus of the huntingtin protein. The resultant mutant huntingtin form aggregates in neurons and causes neuronal dysfunction and degeneration in many ways including transcriptional dysregulation. Here, we report that the expression of mutant huntingtin in the mouse neuroblastoma cell results in massive transcriptional induction of several chemokines including monocyte chemoattractant protein-1 (MCP-1) and murine chemokine (KC). The mutant huntingtin expressing cells also exhibit proteasomal dysfunction and down-regulation of NF- $\kappa$ B activity in a time-dependent manner and both these phenomena regulate the expression of MCP-1 and KC.

The expression of MCP-1 and KC are increased in the mutant huntingtin expressing cells in response to mild proteasome inhibition. However, the expression of MCP-1 and KC and proteasome activity are not altered and inflammation is rarely observed in the brain of 12-week-old Huntington's disease transgenic mice in comparison with their age-matched controls. Our result suggests that the mutant huntingtin-induced proteasomal dysfunction can up-regulate the expression of MCP-1 and KC in the neuronal cells and therefore might trigger the inflammation process.

**Keywords:** chemokine, Huntington's disease, inflammation, monocyte chemoattractant protein-1, murine chemokine, proteasome.

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Huntington's disease (HD) is an autosomal, dominantly inherited, progressive neurodegenerative disorder characterized by motor, cognitive, and psychiatric symptoms. The disease is caused by an abnormal expansion of CAG repeats in the exon-1 of the IT15 gene. IT15 gene encodes a 350-kDa protein called huntingtin, which is expressed ubiquitously (The Huntington's Disease Collaborative Research Group 1993). Normal individuals carry repeat length varying from 6 to 35 glutamine, while the disease is associated with more than 40 repeats. The disease onset and severity are inversely related with the glutamine repeat length. Another eight neurodegenerative disorders also have been identified because of such polyglutamine expansions, including spinal and bulbar muscular atrophy, dentatorubral pallidolusian atrophy, and several spinocerebellar ataxias (SCA1, SCA2, SCA3, SCA6, SCA7, and SCA17) (Zoghbi and Orr 2000; Bates 2005; Di Prospero and Fischbeck 2005; Gatchel and Zoghbi 2005).

A common pathological hallmark of HD and other polyglutamine disorders is the accumulation of misfolded polyglutamine proteins as aggregates or neuronal intranuclear inclusions (Zoghbi and Orr 2000; Bates 2005; Di Prospero and Fischbeck 2005; Gatchel and Zoghbi 2005). The soluble expanded polyglutamine proteins or their aggregates can

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**Abbreviations used:** CBA, cytokine bead array; EGFP, enhanced green fluorescence protein; ERK, extracellular regulated kinase; HD, Huntington's disease; IL-6, interleukin-6; KC, murine chemokine; MCP-1, monocyte chemoattractant protein-1; NF- $\kappa$ B, nuclear factor kappaB; SCA, spinocerebellar ataxia; TBST, Tris-buffered saline containing 0.05% Tween; tNhtt, truncated N-terminal huntingtin.

aberrantly interact and associate with several proteins and disrupt the cellular function in many ways including transcriptional dysregulation (Cha 2000; Sugars and Rubinsztein 2003; Rubinsztein 2006) and impairment of proteasome function (Bence *et al.* 2001; Jana *et al.* 2001; Jana and Nukina 2003; Venkatraman *et al.* 2004; Bennett *et al.* 2005). The cAMP-response element binding protein, TATA box binding protein, tumor suppressor p53, Sp1, and other transcription factors have been demonstrated to associate with the polyglutamine aggregates and this may result in their sequestration and loss of function (Cha 2000; Shimohata *et al.* 2000; Nucifora *et al.* 2001; Sugars and Rubinsztein 2003; Bae *et al.* 2005). Various molecular chaperones and components of the ubiquitin-proteasome system also recruit to the polyglutamine aggregates and ubiquitin-proteasome system dysfunction have been observed in expanded polyglutamine protein expressing cells (Bence *et al.* 2001; Jana *et al.* 2001; Jana and Nukina 2003; Venkatraman *et al.* 2004; Bennett *et al.* 2005). However, there are conflicting reports regarding the proteasomal dysfunction in the HD transgenic mice brain (Diaz-Hernandez *et al.* 2006; Bett *et al.* 2006; Bennett *et al.* 2007; Ortega *et al.* 2007; Wang *et al.* 2008).

Chemokines (chemoattractant cytokines) constitute a superfamily of small proteins that are instrumental in leucocyte trafficking during inflammatory response (Biber *et al.* 2002). In the brain, chemokines are not only expressed by microglia, astrocytes, oligodendrocytes in response to inflammatory stimuli, but are also inducibly expressed in neurons during injury (Flugel *et al.* 2001; Cartier *et al.* 2005). Chemokines receptors are expressed in both glial cells and neurons. Increasing lines of evidence have implicated an involvement of chemokines in neuroinflammation of several chronic neurodegenerative disorders including Alzheimer's disease, Parkinson's disease, and amyotrophic lateral sclerosis and in acute neurodegenerative conditions such as stroke and ischemic brain injury (Hensley *et al.* 2006; Heneka and O'Banion 2007; Wang *et al.* 2007; Mines *et al.* 2007). In HD brain, there are also reports of profound inflammation as evident from the marked increase in reactive astrocytes and activated microglia (Myers *et al.* 1991; Sotrel *et al.* 1991; Sapp *et al.* 2001). However, the precise role of chemokines in the inflammation in HD brain is not known. Interestingly, the HD model mice brain rarely shows gliosis and neurodegeneration at a stage when the mice exhibit severe symptoms (Turmaine *et al.* 2000; Ma *et al.* 2003; Simmons *et al.* 2007).

In the present investigation, we have studied the expression of various inflammatory genes in the normal and mutant huntingtin expressing neuronal cells and found that several chemokines, like monocyte chemoattractant protein-1 (MCP-1) and murine chemokine (KC) related to human chemokine interleukin-8 (IL-8) are dramatically induced in the mutant huntingtin expressing cells. We have also observed that the mild proteasomal dysfunction is associated with increased expression of these chemokines.

## Materials and methods

### Materials

MG132, curcumin, dexamethasone, proteasome substrates, and all cell culture reagents were obtained from Sigma (St Louis, MO, USA). LipofectAMINE 2000, Zeocin, G418, ponasterone A, and RT-PCR kit were purchased from Invitrogen (Carlsbad, CA, USA). Goat polyclonal anti-MCP-1 and mouse monoclonal anti- $\beta$ -tubulin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), rabbit polyclonal anti-p38, anti-c-Jun, phosphorylated anti-p38, phosphorylated anti-c-Jun, and phosphorylated anti-extracellular regulated kinase (ERK)1/2 were from New England Biolab (Ipswich, MA, USA). Rabbit polyclonal anti-ERK1/2 antibody was from Sigma and mouse monoclonal anti-KC was from R&D system (Minneapolis, MN, USA). Horseradish peroxidase-conjugated anti-mouse, anti-rabbit IgG, and ABC Elite kits were purchased from Vector Laboratories (Burlingame, CA, USA).

The truncated N-terminal huntingtin (tNhtt) expression constructs fused with enhanced green fluorescence protein (EGFP) (pIND-tNhtt-EGFP-16CAG, pIND-tNhtt-EGFP-60CAG, and pIND-tNhtt-EGFP-150CAG) and the generation of the stable cell lines in an ecdysone-inducible system of these constructs has been described previously (Jana *et al.* 2001). These stable cell lines can be induced with ponasterone A (an insect steroid hormone with strong molting activity) to express the desired proteins. The generation of stable neuro 2a cell line expressing retinoic acid X receptor also have been described earlier (Jana *et al.* 2001). The plasmid nuclear factor kappaB (NF- $\kappa$ B)-luciferase (contains multiple copies of NF- $\kappa$ B response elements) was obtained from Clontech (Mountain view, CA, USA). The plasmid PRL-SV40 and the dual luciferase reporter assay system were obtained from Promega (Madison, WI, USA).

Huntington's disease exon 1 transgenic mice of R6/2 line (containing 145 CAG repeats) were maintained and genotyped as described earlier (Jana *et al.* 2000). The transgenic mice along with their age-matched wild-type controls were killed using ether anesthesia, their brains were carefully removed, and different parts were dissected and stored at  $-80^{\circ}\text{C}$ .

### Cell culture, treatment, transfection, reporter gene, and proteasome activity assays

The wild-type mouse neuro 2a cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum and antibiotics penicillin/streptomycin. The stable neuro 2a cell lines, HD 16Q, HD 60Q, and HD 150Q were maintained in the same medium containing 0.4 mg/mL Zeocin and 0.4 mg/mL G418. For routine experiments, cells were plated into six-well tissue cultured plates, induced for different days with ponasterone A and then processed for immunoblot analysis or proteasome activity assay. For reporter gene assay, cells were transiently transfected with NF- $\kappa$ B-luciferase and PRL-SV40 plasmids together using LipofectAMINE 2000 reagent according to the manufacturer's instructions. Transfection efficiency was about 80–90%. The cells were left untreated or induced with ponasterone A (1  $\mu\text{M}$ ) for different time periods and then processed for luciferase assay. Luciferase activity was measured using dual luciferase reporter assay system as per the manufacturer instructions. PRL-SV40 was used for co-transfection to normalize the data and was transfected at very low concentration (150-fold

lower than NF- $\kappa$ B luciferase plasmid). Values were represented as relative luciferase activity (the ratio of firefly to Renilla values). Proteasome assay was performed as described earlier (Jana *et al.* 2001).

#### Cytokine bead array and mouse inflammation antibody array

Cytokine bead array (CBA; mouse inflammation kit, BD Biosciences, Singapore) was used to quantitatively measure the inflammatory cytokines expression in the ponasterone-induced HD 16Q and HD 150Q cells and also in the control and HD transgenic mice brain. The assay was performed according to the manufacturer instruction using FACS calibur (BD Biosciences) and data were analyzed using CBA software. Mouse inflammation antibody array membranes (containing 40 different antibodies against various inflammatory molecules) were purchased from RayBiotech (Norcross, GA, USA). The membranes were blocked with blocking buffer, incubated with induced HD 16Q and HD 150Q cell lysates (100  $\mu$ g/mL) for overnight, and then subjected to the enhanced chemiluminescence detection according to the manufacturer instruction.

#### Immunoblotting experiment

HD 16Q and HD 150Q cells were plated onto six-well plates and induced with ponasterone A for different time periods. In some experiments, cells were treated with different doses of MG132, curcumin, and dexamethasone for different time periods. Cells were then washed with cold phosphate-buffered saline, scraped, pelleted by centrifugation (3000 *g* for 10 min), and lysed with sodium dodecyl sulfate sample buffer. The samples were then separated through sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes. The membranes were successively incubated in blocking buffer [5% skim milk in Tris-buffered saline containing 0.05% Tween (TBST; 50 mM Tris, pH 7.5, 0.15 M NaCl, and 0.05% Tween)], with primary antibody in TBST, and then with secondary antibody conjugated with horseradish peroxidase in TBST. Detection was carried out using enhanced chemiluminescence method.

#### Semi-quantitative and quantitative real-time RT-PCR analysis

The total RNA was extracted using TRIzol reagent and semi-quantitative RT-PCR was carried out with a RT-PCR kit. The quantitative real-time PCR for MCP-1 and KC were carried out using iQ SYBR green super mix (BioRad, Hercules, CA, USA) after cDNA synthesis from total RNA. The real-time PCR was performed using an ABI Prism 7500 system and results were analyzed using the sequence detection software (Applied Biosystems, Foster City, CA, USA). All reactions were normalized with 18S rRNA as internal control. The primer sequences for MCP-1, KC, and  $\beta$ -actin were as follows: MCP-1: F, 5'-AGGTCCTGTCATGCTTCT-3' and R, 5'-GCTGAAGACCTTAGGGCAGA-3'; KC: F, 5'-GCTGGGATTCACCTCAAGAA-3' and R, 5'-TGGGGACACCTTTAGCATC-3'; and actin: F, 5'-TACAGCTTACCACC-3' and R, 5'-ATGCCACAGGATTTC-3'. PCR conditions for MCP-1, KC and  $\beta$ -actin were same: an initial denaturation step at 94°C for 4 min and then cycling through 94°C for 30 s denaturation, 60°C for 30 s annealing, 72°C for 45 s extension, and a final extension step at 72°C for 5 min. The cycle numbers for MCP-1, KC was 25 and for  $\beta$ -actin was 23.

#### Immunohistochemical staining

The frozen brains, mounted on Tissue-Tek were sectioned in freezing microtome to 20  $\mu$ m thickness. Sections were fixed with 4% *p*-formaldehyde in phosphate-buffered saline for 20 min, washed several times, blocked with 5% non-fat dried milk for 2 h, and then incubated overnight with Iba-1 antibody. Staining was carried out using ABC Elite kit as described earlier (Jana *et al.* 2000).

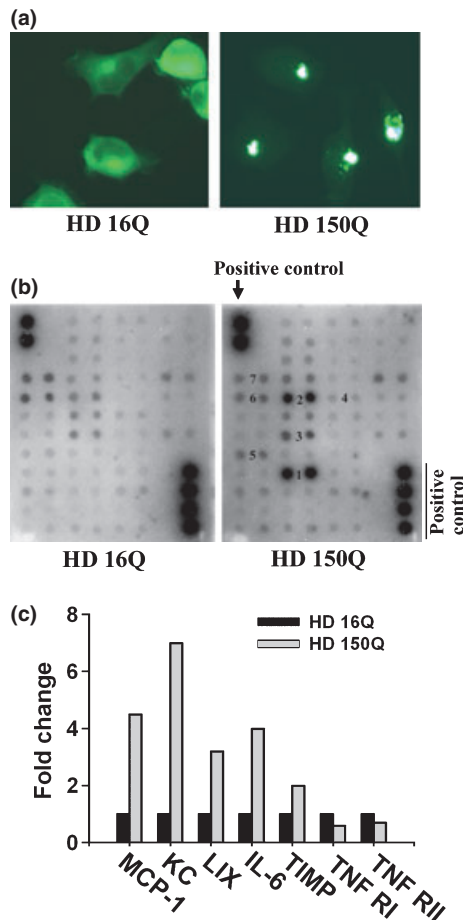
#### Statistical analysis

Statistical analysis was carried out using Microsoft Excel software (Microsoft India, Bangalore, India). All data are presented as mean  $\pm$  SD. Inter group comparisons were performed by two-tailed Student's *t*-test; *p* < 0.05 was considered statistical significant.

## Results

### Increased expression of several inflammatory molecules in the mutant huntingtin expressing cells

To identify the inflammatory genes that are increased or decreased in the mutant huntingtin expressing cells, we used two stable and inducible neuro 2a cell lines expressing tNhtt fused with EGFP containing 16Q (normal) and 150Q (mutant) repeats. The cell lines were named as HD 16Q and HD 150Q and their corresponding expressed protein were named as tNhtt-EGFP-16Q and tNhtt-EGFP-150Q. The cell lines were induced with ponasterone A (1  $\mu$ M) for 2 days and observed under fluorescence microscope. The treatment of ponasterone A to HD 16Q and HD 150Q cells resulted massive expression of tNhtt-EGFP-16Q and tNhtt-EGFP-150Q proteins, respectively (Fig. 1a). The 2 days induced HD 16Q and HD 150Q cell lyaste were then subjected to analysis of inflammation antibodies array. As shown in the Fig. 1b and c, the expressions of several inflammatory genes were dramatically increased in the HD 150Q cells in comparison with HD 16Q. The inducible genes include KC, MCP-1, lipopolysaccharide-induced chemokine (LIX), IL-6, and tissue inhibitor of matrix metalloproteinase. Many of the inducible genes were chemokines and maximum levels of induction were observed in case of chemokines, KC, and MCP-1. The expressions of tumor necrosis factor receptors were significantly decreased in the HD 150Q cells. We also performed CBA to check and quantify the expression levels of various cytokines in the HD 16Q and HD 150Q cells. As expected the expression of MCP-1 and IL-6 were increased several fold in the HD 150Q cells (Table 1). The expression of MCP-1 was about 160-fold higher in the uninduced HD 150Q cells when compared with uninduced or induced HD 16Q cells. Induction of expression of mutant huntingtin in HD 150Q cells for 2 days resulted decrease in the expression of MCP-1 (expression was about 90-fold). Similarly, the IL-6 showed about 70-fold increase in the expression in uninduced HD 150Q cells which decreased to



**Fig. 1** Up-regulation of several inflammatory genes in the mutant huntingtin expressing neuro 2a cells. (a) The HD 16Q and HD 150Q cells were induced with 1  $\mu$ M of ponasterone A for 2 days and photographed under fluorescence microscope. (b) The cells were collected; lysates (100  $\mu$ g/mL) were made and then added to the mouse inflammation antibody array membranes and incubated overnight. The membranes were sequentially washed, incubated with biotin-conjugated antibodies followed by horseradish peroxidase-conjugated streptavidin, and detected with enhanced chemiluminescence as described in the Materials and methods. (c) Various spots of the scanned image (shown in b) were quantitated using NIH Image analysis software (Bethesda, MD, USA). Spots 1–7 are as follows: 1, KC; 2, MCP-1; 3, LIX; 4, IL-6; 5, TIMP; 6, TNF-RI; 7, TNF-RII. LIX, lipopolysaccharide-induced CXC-chemokine; TIMP, tissue inhibitor of matrix metalloproteinase; TNF, tumor necrosis factor.

about 50-fold after 2 days of induction. We also detected a significant decrease in the expression of IL-10 in the HD 150Q cells (Table 1).

As, KC and MCP-1 showed highest levels of induction in the HD 150Q cells, we further characterized their expression and studied functional significance with respect to the HD pathogenesis. First we analyzed the mRNA and protein levels of MCP-1 and KC in both HD 16Q and HD 150Q cells in uninduced and induced conditions. The mRNA and protein levels of both MCP-1 and KC were undetectable in the

**Table 1** Up-regulation of various inflammatory cytokines in the mutant huntingtin expressing neuro 2a cells

Inflammatory molecules (pg/mL)	HD 16Q		HD 150Q	
	Control	Induced	Control	Induced
MCP-1	30 $\pm$ 4	33 $\pm$ 6	5010 $\pm$ 203*	3027 $\pm$ 103*
TNF- $\alpha$	12 $\pm$ 2	10 $\pm$ 0.8	9.9 $\pm$ 1	7.8 $\pm$ 0.9
IFN- $\gamma$	ND	ND	ND	ND
IL-6	1.9 $\pm$ 0.4	1.8 $\pm$ 0.2	137 $\pm$ 11*	90 $\pm$ 8*
IL-10	28 $\pm$ 5	38 $\pm$ 7	ND	ND
IL-12p70	90 $\pm$ 8	113 $\pm$ 12	93 $\pm$ 9	83 $\pm$ 6

HD 16Q and HD 150Q cells were plated onto six-well tissue cultured plates and induced for 2 days with ponasterone A (1  $\mu$ M). Cells were collected; lysates were made (100  $\mu$ g/mL) and subjected to cytokine bead array in FACS calibur as described in the Materials and methods. Results are mean  $\pm$  SD of two independent experiments each performed triplicate; \* $p$  < 0.001 when compared with control and induced HD 16Q cells. HD, Huntington's disease; IFN- $\gamma$ , interferon- $\gamma$ ; MCP-1, monocyte chemoattractant protein-1; TNF- $\alpha$ , tumor necrosis factor- $\alpha$ ; IL-6, interleukin-6.

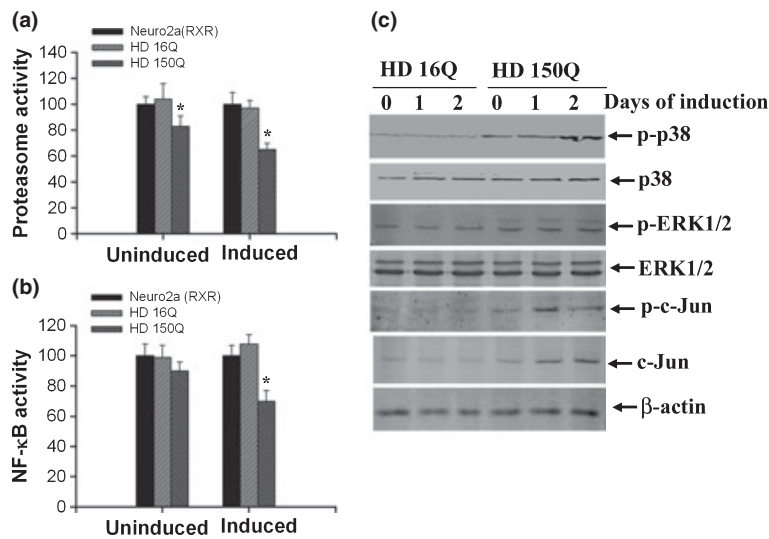
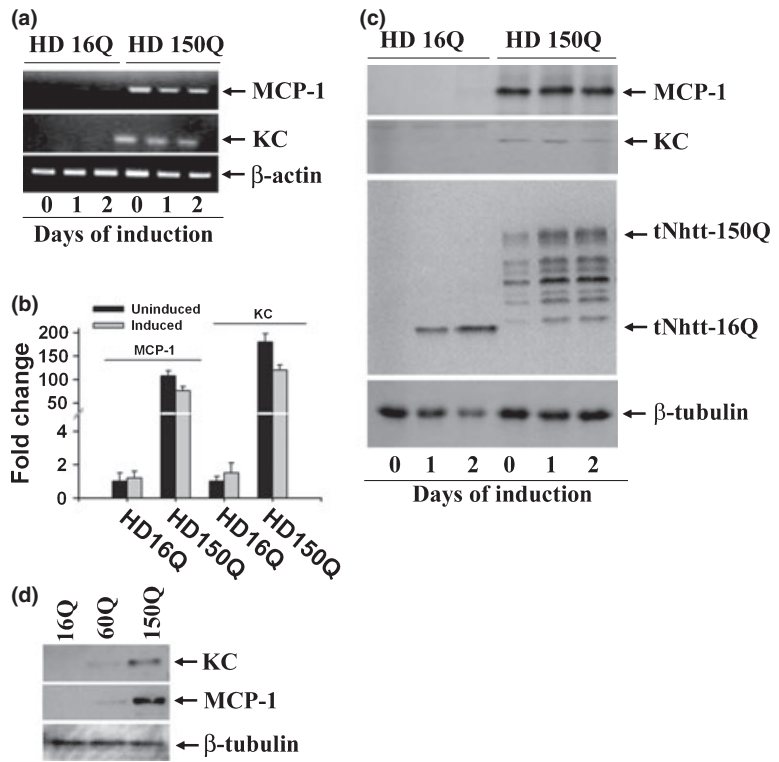
uninduced and induced HD 16Q cells, but increased in uninduced and induced HD 150Q cells (Fig. 2). Real-time RT-PCR results showed approximately 200- and 120-fold increase in the mRNA levels of KC and MCP-1, respectively, in the uninduced HD 150Q cells (Fig. 2b). Induction of expression of mutant huntingtin in HD 150Q cells caused gradual decrease in the mRNA levels of both MCP-1 and KC. The uninduced HD 150Q cells showed very high levels of MCP-1 and KC most likely because of low levels of expression and aggregation of mutant huntingtin without any induction. The uninduced HD 150Q cells also showed proteasomal dysfunction and high levels of heat-shock protein 70 (Jana *et al.* 2001). To further confirm our findings, we transiently transfected the pIND-tNhtt-EGFP-16CAG, pIND-tNhtt-EGFP-60CAG, and pIND-tNhtt-EGFP-150CAG constructs into the neuro 2a (retinoic acid X receptor) cells and induced with ponasterone A for 3 days. As shown in Fig. 2d, the expression of MCP-1 and KC were induced in polyglutamine length-dependent manner.

#### Expression of mutant huntingtin causes proteasomal dysfunction, down-regulation of NF- $\kappa$ B activity, and activation of mitogen-activated protein kinases

Next, we investigated the probable cause of increased expression of chemokines, MCP-1, and KC. Earlier, we have demonstrated proteasomal dysfunction and down-regulation of NF- $\kappa$ B activity in the induced HD 150Q cells (Jana *et al.* 2001; Goswami *et al.* 2006). But, we were not sure whether uninduced HD 150Q cells also exhibit these abnormalities because of the low levels of mutant huntingtin expression. As shown in Fig. 3a, the proteasome activity was significantly decreased in the uninduced HD 150Q cells and the effect was more pronounced upon induction for 2 days.



**Fig. 2** Increased mRNA and protein levels of MCP-1 and KC in the HD 150Q cells. HD 16Q and HD 150Q cells were either left untreated or induced with ponasterone A for different time periods. Cells were collected and subjected to RNA extraction followed by semi-quantitative (a) and quantitative real-time (b) RT-PCR analysis of MCP-1 and KC. (c) Immunoblot analysis of MCP-1, KC, truncated N-terminal huntingtin, and  $\beta$ -tubulin in HD 16Q and HD 150Q cell lysates. (d) Neuro 2a cells stably expressing retinoic acid X receptor (RXR) were transiently transfected with truncated huntingtin constructs containing different CAG repeats and then induced with ponasterone A for 3 days. The cell lysates were then processed for immunoblot analysis using antibodies against MCP-1, KC, and  $\beta$ -tubulin.



**Fig. 3** Analysis of proteasome and NF- $\kappa$ B activity as well as expression and activation of various stress kinases in the HD 16Q and HD 150Q cells. (a) The Neuro 2a (RXR), HD 16Q, and HD 150Q cells were either left untreated or induced with ponasterone A for 2 days as described in the Fig. 2. Cell lysates were then processed for proteasome activity assay. (b) The cells were transiently transfected NF- $\kappa$ B luciferase and PRL-SV40 plasmids as describe in the Materials and methods. The cells were left uninduced or induced with 1  $\mu$ M of

ponasterone A for 2 days as described in the Fig. 2. Cells were then collected and processed for dual luciferase reporter gene assay. Results are mean  $\pm$  SD of three independent experiments each performed triplicate; \* $p$  < 0.01 when compared with neuro 2a (RXR) or HD 16Q cells. (c) The lysates of 2 days induced HD 16Q and HD 150Q cells were subjected to immunoblot analysis of c-Jun, ERK1/2, and p38 mitogen-activated protein kinases as well as their phosphorylated forms.

The NF- $\kappa$ B activity was not affected in the uninduced HD 150Q cells, but decreased significantly after 2 days of induction (Fig. 3b). The uninduced and induced HD 150Q

cells also showed increased levels of c-jun and phosphorylated c-jun, ERK1/2, and p38 (Fig. 3c). These effects are most likely because of proteasomal dysfunction. The down-

regulation of NF- $\kappa$ B activity in the induced HD 150Q cells is most likely involved in the decreased expression of MCP-1 and KC as shown in Fig. 2b.

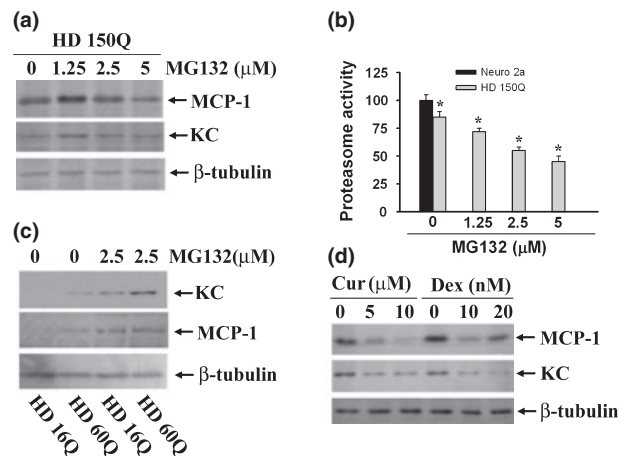
### Regulation of the expression of MCP-1 and KC by proteasome and NF- $\kappa$ B activity

NF- $\kappa$ B is known to negatively regulate the expression of various cytokines and chemokines including MCP-1 and KC (Baeuerle and Henkel 1994; Ueda *et al.* 1994), and proteasome inhibitors are also known to induce the expression of MCP-1 (Nakayama *et al.* 2001). Therefore, we tested the possibility of proteasomal dysfunction on the up-regulation of MCP-1 and KC in the HD 150Q cells. The HD 150Q cells were treated with different doses of proteasome inhibitor, MG132 for 12 h and then cells lysates were subjected to immunoblot analysis using MCP-1 and KC antibodies. The MG132 at a dose of 1.25  $\mu$ M significantly increased the expression of both MCP-1 and KC, while higher doses of MG132 decreased the expression of these chemokines (Fig. 4a). The uninduced HD 150Q cells showed about 20% decrease in proteasome activity in comparison with wild-type neuro 2a cells, which was further reduced to 30% upon treatment of 1.25  $\mu$ M of MG132 for 12 h. Therefore, approximately 15–30% inhibition of proteasome activity might be associated with increased expression of MCP-1 and

KC. The treatment of MG132 (2.5  $\mu$ M) for 12 h to the induced HD 16Q cells also lead to increased expression of MCP-1 and KC (Fig. 4c). The expression of MCP-1 and KC in the induced HD 60Q cells were also sensitized upon MG132 treatment (Fig. 4c). The inhibition of NF- $\kappa$ B activity by either curcumin or dexamethasone decreased the expression of MCP-1 and KC in the HD 150Q cells (Fig. 4d).

### The expression of KC and MCP-1 as well as proteasome activity is not altered in the brain of HD transgenic mice

As we found dramatic increase in the expression of MCP-1 and KC in the mutant huntingtin expressing cells, we next checked the expression of these chemokines in the HD transgenic mice brain (R6/2 line). We used 12-week-old transgenic mice brains along with their age-matched controls. The HD transgenic mice show severe symptoms at 12 weeks age. The expressions of various inflammatory molecules were first analyzed using cytokine beads array. To our surprise, we did not find any significant changes in the expression of MCP-1, IL-6, IL-10, tumor necrosis factor- $\alpha$ , interferon- $\gamma$ , and IL-12 in the brain samples of HD transgenic mice compared with the age-matched control (Table 2). We were also unable to detect any expression of MCP-1 and KC in these brain samples using immunoblot analysis (data not shown). The proteasome activity was also unaltered in the different parts of HD transgenic mice brains in comparison with their age-matched controls (Fig. 5). Finally, we performed immunohistochemical staining of glial fibrillary acidic protein and Iba-1 in the brain sections collected from HD transgenic and control mice. The glial fibrillary acidic protein-positive reactive astrocytes were rarely observed in the cortex and striatum of HD mice (data not shown). Iba-1 immunostaining also did not reveal any significant increase in the number of microglia in the cortex and striatum of HD mice brain (Fig. 6). However, the microglia in the HD mice brain sometimes had larger soma and had processes with more number of branches. These results indicate that there was apparently no inflammation in the 12-week-old HD mice brain when the mice show severe symptoms.



**Fig. 4** Regulation of expression of MCP-1 and KC in the mutant huntingtin-expressing cell by proteasome and NF- $\kappa$ B inhibitors. (a and b) HD 150Q cells were treated with different doses of proteasome inhibitor, MG132 for 12 h, and then the cell lysate was subjected to immunoblot analysis of MCP-1, KC, and  $\beta$ -tubulin and proteasome activity assay. Results are mean  $\pm$  SD of three independent experiments each performed triplicate; \* $p < 0.01$  when compared with neuro 2a cells. (c) HD 16Q and HD 60Q cells induced for 60 h and then the cells were left untreated or treated with MG132 for 12 h. The cells were collected and processed for immunoblot analysis using antibodies against MCP-1, KC, and  $\beta$ -tubulin. (d) HD 150Q cells were treated with different doses of curcumin (Cur) and dexamethasone (Dex) for 12 h and then processed for immunoblotting using MCP-1 and KC antibodies.

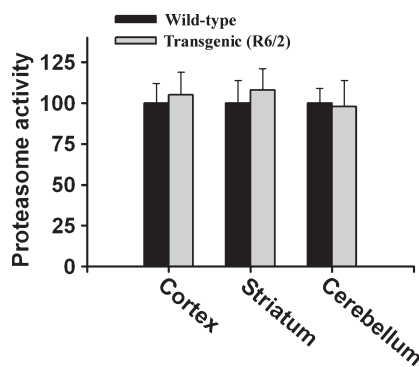
## Discussion

In the present investigation, we have shown for the first time a very unusual transcriptional induction of chemokines, MCP-1, and KC in the mutant huntingtin expressing neuronal cells. In exploring the mechanisms involved, we found that mild proteasomal dysfunction is associated with the increased expression of these chemokines. The functions of both proteasome and NF- $\kappa$ B are inhibited in the mutant huntingtin expressing cells and down-regulation of NF- $\kappa$ B activity by curcumin or dexamethasone inhibits the expression of MCP-1 and KC. On the other hand, 20–30% inhibition of proteasome function increases, while more than 50% inhibition of proteasome activity decreases the expression of these chemokines.

**Table 2** Analysis of the expression of various inflammatory cytokines in the brain samples collected from HD transgenic mice (R6/2) along with their age-matched control

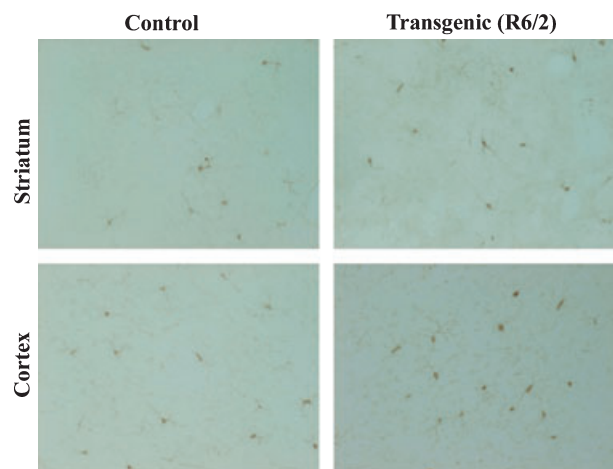
Inflammatory molecules (pg/mL)	Striatum		Cortex		Cerebellum	
	Wild-type	R6/2	Wild-type	R6/2	Wild-type	R6/2
MCP-1	255 ± 20	284 ± 40	256 ± 23	281 ± 10	150 ± 12	130 ± 16
TNF- $\alpha$	8.2 ± 2	13 ± 0.8	8.8 ± 1	7.3 ± 0.9	5.7 ± 0.8	6.2 ± 0.6
IFN- $\gamma$	3.8 ± 0.2	3.9 ± 0.3	3.7 ± 0.2	4.1 ± 0.1	3.4 ± 0.1	3.1 ± 0.3
IL-6	ND	ND	ND	ND	ND	ND
IL-10	186 ± 13	203 ± 16	206 ± 18	197 ± 12	198 ± 10	214 ± 19
IL-12p70	84 ± 6	89 ± 12	132 ± 15	162 ± 13	141 ± 15	167 ± 18

The lysates (200  $\mu$ g/mL) made from the different parts of the brain collected from both HD transgenic and control mice (12-week old) were subjected to cytokine bead array in FACS caliber as described in the Materials and methods. Values are mean  $\pm$  SD of four independent animals each performed triplicate. HD, Huntington's disease; IFN- $\gamma$ , interferon- $\gamma$ ; MCP-1, monocyte chemoattractant protein-1; TNF- $\alpha$ , tumor necrosis factor- $\alpha$ ; IL-6, interleukin-6.



**Fig. 5** Analysis of proteasome activity in the HD transgenic mice brain along with their age-matched wild-type control (12-week old). The different parts of brain lysates made from HD transgenic (R6/2) and wild-type control mice were subjected to proteasome activity assay. Values are mean  $\pm$  SD of four independent animals each performed triplicate.

The NF- $\kappa$ B pathway is well known to regulate the expression of various chemokines and cytokines (Bauerle and Henkel 1994; Ueda *et al.* 1994). Therefore, it is not surprising that the down-regulation of this pathway inhibits the expression of MCP-1 and KC. However, the role of proteasome in the biphasic regulation of expression of these chemokines is very unusual and interesting. The proteasome has multiple cellular targets, and different proteins might be degraded and various signaling pathways might be activated/inhibited depending on the degree of proteasome inhibition. An earlier report has demonstrated that the inhibition of proteasome function leads to the increased expression of MCP-1 via the activation of c-Jun/activator protein-1 pathway (Nakayama *et al.* 2001). Others and we also have observed activation c-Jun and several mitogen-activated protein kinases in the mutant huntingtin expressing cells (Apostol *et al.* 2006; Morfini *et al.* 2006; Merienne *et al.* 2007; Tsirigotis *et al.* 2008). It is possible that mild proteasomal dysfunction for prolonged time might induce the expression of MCP-1 and



**Fig. 6** Microglial activation was rarely observed in HD transgenic mice brain. The brain sections made from 12-week-old HD transgenic mice (R6/2) along with their age-matched wild-type control were processed for immunohistochemical staining using antibody against Iba-1 (a microglial marker).

KC via the activation c-jun/activator protein-1 pathway, while moderate to strong proteasome inhibition could suppress the expression of these chemokines through the down-regulation of NF- $\kappa$ B pathway.

Another interesting aspect of our findings is that these chemokines are produced from the neuronal cells. Various cytokines and chemokines are normally produced by the glial cells in the brain in response to inflammatory stimuli. However, there are reports that have shown increased expression of MCP-1 from the neurons during injury or focal ischemia suggesting the role of neurons to initiate the inflammation process. The massive induction of MCP-1 and KC from the mutant huntingtin expressing cells indicate that the mutant huntingtin expressing neurons might also play an important role to trigger inflammation. Additionally, these chemokines might be produced as a compensatory response

in maintaining cellular function. In fact, a recent study showed increased expression of several inflammatory genes (the matrix metalloproteinase-2, the stromal cell-derived factor-1 $\alpha$ , and IL-1 receptor related Fos-inducible transcript) in the mutant ataxin-3 expressing neuronal cell (Evert *et al.* 2001). However, the role of these inflammatory molecules in the disease pathogenesis is not known.

Surprisingly, the expression of MCP-1 and KC has not been found to increase in the HD transgenic mice (R6/2 line) brains when compared with age-matched controls. We have used 12-week-old transgenic mice for our experiments, when they showed severe behavioral and motor symptoms. The proteasome activity is also not significantly disturbed in the different parts of the HD transgenic mice brain indicating that the proteasomal dysfunction might be linked with the increased expression of MCP-1 and KC. Moreover, decreased NF- $\kappa$ B activity in the astrocytes of the HD mice brain also could be involved in the suppression of chemokines expression (Chou *et al.* 2008). The reactive astrocytes and activated microglia were also rarely observed in the 12-week-old transgenic mice brain indicating no major sign of inflammation (Turmaine *et al.* 2000; Ma *et al.* 2003). In fact, there are reports of decrease in microglial density and their abnormal morphology as well as decrease in the production of chemokine, regulated upon activation, normal T cell expressed and secreted (RANTES) by astrocytes in the 12- to 14-week-old R6/2 mice brain (Ma *et al.* 2003; Chou *et al.* 2008). However, the reactive astrocytes and activated microglia have been observed in the HD patient brain (Myers *et al.* 1991; Sotrel *et al.* 1991; Sapp *et al.* 2001). At present, it is not clear why the symptomatic HD mice brain do not show much inflammation. Possibly the interplay between proteasome dysfunction and NF- $\kappa$ B activity regulates the magnitude of inflammation, which needs further investigation. Taken together, our results suggest that the mild proteasomal dysfunction for prolonged periods might be involved in the massive induction of chemokines, MCP-1, and KC in the mutant huntingtin expressing neuronal cells.

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