Amendment of Altered Immune Response by Curcumin in *Drosophila* Model of Huntington’s Disease

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Abstract.

**Background:** Though primarily classified as a brain disorder, surplus studies direct Huntington’s disease (HD) to be a multi-system disorder affecting various tissues and organs, thus affecting overall physiology of host. Recently, we have reported that neuronal expression of mutant huntingtin induces immune dysregulation in *Drosophila* and may pose chronic threat to challenged individuals. Therefore, we tested the polyphenolic compound curcumin to circumvent the impact of immune dysregulation in *Drosophila* model of HD.

**Objective:** The present study examined the molecular basis underlying immune derangements and immunomodulatory potential of curcumin in HD.

**Methods:** UAS-GAL4 system was used to imitate the HD symptoms in *Drosophila*, and the desired female progenies (elav > Httx1pQ25; control and elav > Httx1pQ93; diseased) were cultured on food mixed without and with 10 μM concentration of curcumin since early development. Effect of curcumin supplementation was investigated by monitoring the hemocytes’ count and their functional abilities in diseased condition. Reactive oxygen species (ROS) level in cells was assessed by DHE staining and mitochondrial dysfunction was assessed by CMXros red dye. In addition, transcript levels of pro-inflammatory cytokines and anti-microbial peptides were monitored by qRT-PCR.

**Results:** We found that curcumin supplementation commendably reduced higher crystal cell count and phenoloxidase activity in diseased flies. Interestingly, curcumin significantly managed altered plasmatocytes count, improved their phagocytic activity by upregulating the expression of key phagocytic receptors in HD condition. Moreover, substantial alleviation of ROS levels and mitochondria dysfunction was observed in plasmatocytes of diseased flies upon curcumin supplementation. Furthermore, curcumin administration effectively attenuated transcriptional expression of pro-inflammatory cytokines and AMPs in diseased flies.

**Conclusions:** Our results indicate that curcumin efficiently attenuates immune derangements in HD flies and may prove beneficial in alleviating complexities associated with HD.

Keywords: Huntington’s disease, curcumin, hemocytes, reactive oxygen species, mitochondria dysfunction, cytokines, antimicrobial peptides

INTRODUCTION

Huntingtin (HTT) is a large 350 kDa protein which is expressed ubiquitously in human tissues, and normally harbors a repeat of ~8–25 glutamines in its N-terminal portion. Huntington’s disease (HD) is
chronic, progressive, dominantly inherited neurodegenerative disorder caused by an unstable expansion in the polyglutamine tract of HTT beyond 35, resulting in the formation of mutant HTT [1]. HD is characterized symptomatically by the triad of movement disorders, cognitive decline, and psychiatric changes and pathologically by death of specific neuronal subpopulations in striatal and cortical regions of brain [2, 3]. Contrary to popular belief, although being primarily classified as a neurodegenerative condition, recent research suggests it may also be a systemic disorder [4, 5]. Apart from the hallmarked manifestations, disease condition imposes a myriad of other health complications in the form of metabolic impairments, diabetes, sleep disturbances, endocrine dysfunction, cardiovascular diseases, gastrointestinal dysfunction, etc. [6–11].

Abnormalities related to immunological dysregulation, in particular, have been observed in clinical and experimental settings of HD [12, 13]. Neurodegenerative diseases are often linked to the activation of the innate immune system. The interactions between the brain and the immune system in healthy and diseased condition are diverse and complex. Neuroinflammation (activation of the brain’s innate immune cells and expression of inflammatory molecules) is a key characteristic of neurodegenerative disorders and is typically accompanied by parallel processes in periphery. HD encompass an array of immune dysregulation which include central microglia and astrocyte activation, systemic chronic inflammation mediated by upregulation of pro-inflammatory cytokines such IL-6, IL-10, TNF-α in cerebrospinal fluid, plasma as well as in vital peripheral organs such as liver, kidney, and spleen evidenced in HD patients and mouse models. Moreover, peripheral immune cells exhibit migration deficits, impaired phagocytic activity, and dampened immune cell activation in pre-manifest HD patients [14–16]. The multifaceted immunological fluctuation accompanied with neurodegeneration exacerbate medical condition and augment the likelihood of acute complications in HD patients and constraining their life expectancies.

Currently, despite of noteworthy development and understanding in the field of HD, there is no cure or effective treatment for HD. The current treatment options for HD include the use of target-specific synthetic medicines, which provide only modest alleviation by suppressing the neurological symptoms but cause an array of adverse effects [17]. In this context, testing natural compounds with accredited therapeutic potential and minimal adverse effects seems intriguing. Therefore, to overpower this constraint, we tested phytochemical “curcumin,” which has a broad range of pharmacological targets and has anti-inflammatory [18–20], neuroprotective [21], antioxidant [19, 22], and a myriad of immunomodulatory properties without any side effects in Drosophila model of HD.

Curcumin ((1E, 6E)-1, 7-bis (4-hydroxy-3-methoxyphenyl)-1, 6-heptadiene-3, 5-dione), a polyphenolic compound with an exceptional safety profile is the major bioactive component of turmeric (Curcuma longa Linn.), is a widely used spice and Ayurvedic medicine in Asian countries. Being a pleiotropic molecule, curcumin is a drug of interest in the treatment of several medical conditions like cancer, cystic fibrosis, gastric ulcers, liver diseases, and arthritis.

Several studies have showcased the efficacy of curcumin in animal models of neurodegeneration such as Alzheimer’s disease [23, 24], Parkinson’s disease [25], and HD [26–28]. In these disease models, curcumin is shown to limit aggregation of misfolded proteins [29–31], reduce inflammation by preventing the release of pro-inflammatory mediators [32, 33], suppress mitochondrial dysfunctions and scavenger reactive oxygen species (ROS) [34], ameliorate behavioral dysfunctions [27], and improve disease phenotype [28].

In the present study, we used Drosophila model of HD to assess curcumin’s efficacy to mitigate immune derangements associated with the disease. Drosophila harboring exon1 fragment of human huntingtin with expanded 93 glutamine residues (Httex1p Q93) in pan-neuronal population recapitulates most of the characteristic features of the disease condition, for instance, progressive accumulation of aggregates, locomotor dysfunction, degeneration of photoreceptor neurons, and short lifespan [35, 36]. The diseased flies also showed significant alteration in immune response with extensive modulations in major hemocytes count and their function with HD progression [37]. Therefore, we tested effective dose (10 μM) [27] of curcumin in HD flies and assessed its effectiveness to propitiate immune abnormalities in HD flies.

We found that an effective dose of curcumin significantly mitigated altered hemocytes’ count and their functions in diseased flies during the entire course of the disease. Quantification of crystal cells suggested that curcumin administration effectively managed crystal cell number and suppressed elevated
phenoloxidase activity in diseased flies. Further, curcumin administration effectively downregulated the mRNA levels of key phenoloxidase genes PPO1 and PPO2 in diseased flies. Additionally, quantification of plasmatocyte count revealed that dietary curcumin effectively curbed altered plasmatocyte number in diseased flies at progressive ages. Curcumin intake also resulted in an improvement of otherwise reduced phagocytic activity of plasmatocytes in diseased flies. Furthermore, expression profiling of key phagocytic receptors such as eater and NimC1 genes revealed considerable modulation in altered eater mRNA level in diseased flies at specific ages due to curcumin administration but no change in NimC1 levels. Interestingly, dietary intake of curcumin stifled mitochondrial dysfunction and elevated ROS levels in plasmatocytes of diseased flies. Furthermore, curcumin administration effectively downregulates elevated transcription expression of AMPs and pro-inflammatory cytokines in diseased condition, thereby attenuating inflammatory insult in HD flies. Taken together, these results indicate that curcumin is colossally beneficial in the resolving immune alterations in a Drosophila model of HD. We, therefore, propose that curcumin may be an effective therapeutic alternative for the management of HD complexities and its amelioration with no adverse side effects, when administered at disease onset.

MATERIALS AND METHODS

Drosophila stocks, crosses, and dietary conditions

The bipartite UAS-GAL4 system was used to express the transgenic human HTT exon1 fragment containing polyglutamine repeats. The transgenic stocks w; P w+mW.hs=GawB elavC155; a pan-neuronal driver (#8765; BDSC), w; P UAAS-Htex1p Q25, and w; P UAAS-Htex1p Q93(F) were used in the present study. The males of elav-GAL4 were mated with virgin females from UAAS-Htex1p Q25 (wild type) and UAAS-Htex1pQ93 (mutant), and the resulting female progenies elav>Htex1p Q25 (controls) and elav>Htex1p Q93 (diseased) were used for all the experiments. The desired progeny expressing polyQ peptides were cultured on food mixed without and with 10 μM concentration of curcumin (Sigma Aldrich) since early developmental stages. Dimethyl sulfoxide (DMSO, Sigma Aldrich) was added proportionally to each condition. Rearing conditions for Drosophila cultures included a consistent 12-h light: 12-h dark cycle at 25°C and 65% humidity.

Crystal cell count

To visualize crystal cells, 10 wandering 3rd instar larvae from each condition was collected in 1 ml of 1X PBS in glass vials and incubated at 65°C for 10 min. Immediately after heating, heat-shocked larvae from each condition were placed on a glass slide over a black backdrop and imaged using a Nikon SMZ 745 T stereozoom microscope. The number of melanized crystal cells were then determined in the three posterior most abdominal segments of larvae using Imagej software. The test employed five biological replicates (10 larvae/replicate) for each condition.

Estimation of phenoloxidase activity

For measuring enzymatic PO activity, ten individuals from each condition were homogenized in homogenization buffer with protease inhibitor (Sigma-Aldrich, S8820), centrifuged at 13,000 rpm. The supernatant was collected in pre-chilled eppendorf and the protein concentration of each sample was determined using Bradford assay. A reaction volume of 50μl containing 10μg of protein prepared by diluting supernatant in 1X PBS was plated in 96 well plate and 50μl of 3 mM L-3,4-dihydroxyphenylalanine (L-DOPA) (substrate for enzyme phenoloxidase) was added to it and then incubated at 25°C for 30 min. The absorbance was determined at 492 nm using a Biotek ELISA plate reader. The assay used a minimum of three biological replicates per condition and each biological sample was plated in duplicate.

Plasmatocyte count

For quantification, plasmatocytes were isolated from circulation of larvae and hematopoietic pockets of adults. We adopted the strategy mentioned by Dhankhar et al. (2022) [37] to isolate plasmatocytes for hematopoietic compartments and circulation in Drosophila. Following that, we prepared a 1:1 dilution of the hemolymph/plasmatocyte suspension with the 0.4% trypan blue solution (Himedia, TC193) and pipetted many times to ensure a homogenous cell suspension. 10 μL of trypan blue-cell suspension was loaded onto the hemocytometer by gently contacting the edge of the coverslip with the pipette tip. After
that, cells were counted in the four outer squares of the grid (each square comprises 16 smaller squares) of the hemocytometer under Nikon microscope. The average plasmatocyte count per individual was then determined using the formula provided. The assay used a minimum of 10 biological replicates (10 individuals/replicate) per condition and each biological sample was counted thrice.

Average cell count per larva/fly = \frac{(Average cell count per square \times Total volume of sample \times Dilution factor (D.F.))}{No. of larvae per sample}

**Phagocytic assay**

To access phagocytic activity, \( \sim 3 \times 10^4 \) cells were plated on pre-cleaned slides and incubated for an hour at 25°C in a humid chamber for adherence. Slides were then washed with 1X PBS to remove non-adherent cells. Following that, heat killed *E. coli* (100 \( \mu \)l of \(10^8 \) cells/ml) stained with propidium iodide was added on adhered cells to slide and incubated for 40 min at 25°C for phagocytosis. The slides were then rinsed with 1X PBS to eliminate any unphagocytosed bacteria, fixed with methanol, air-dried, and mounted with Vectashield media containing DAPI (Vector laboratories, H12000). The slides were observed under fluorescence microscope and percent phagocytosis was determined by counting number of cells showing bacterial uptake per total of 100 cells per sample slide. The representative images were acquired by confocal microscope. A minimum of five samples per condition were analyzed for this assay.

**Reactive oxygen species detection and quantification**

Dihydroethidium (DHE) staining was employed to detect superoxide radicals in plasmocytes of flies under various experimental conditions [38]. \( 1 \times 10^4 \) plasmocytes of each condition were seeded on the cleaned slides and allowed to adhere for 1 h at 25°C. Meanwhile, a stock solution of 30 mM DHE (Invitrogen Molecular Probes, D11347) was reconstituted in anhydrous DMSO (Sigma-Aldrich, 276855) just before use. The reconstituted dye was further diluted in 1X PBS to obtain a final working concentration of 25 \( \mu \)M for staining. Following incubation, slides were washed with 1X PBS to remove non-adhered cells. The plasmatocyte were incubated with DHE for 10 min in dark at 25°C. The slides were then washed with 1X PBS and mounted with Vectashield media containing DAPI. The images were acquired immediately under Nikon Eclipse (Ni-E) fluorescence microscope and fluorescence intensity was quantified using ImageJ software \((n = 5/\text{condition/age})\).

**Mitochondrial function assessment**

To evaluate mitochondrial activity in plasmocytes of diseased and control flies were labelled with MitoTracker red CMX Ros (Thermofisher scientific, M7512) dye. Plasmatocytes extracted from both diseased and control flies were cultured on pre-cleaned slides for 1 h at 25°C. A stock vial containing 50 g of lyophilized dye was reconstituted in anhydrous DMSO (Sigma-Aldrich, 276855) to make the stock concentration of 1 mM. The cells were washed three times with 1X PBS and then treated with a working solution of MitoTracker red (1 \( \mu \)M; diluted in 1X PBS) for 30 min in the dark at room temperature. The cells were washed thrice with 1X PBS and mounted in Vectashield mounting medium with DAPI (Vector Laboratories) for nuclei staining. The stained cells were immediately imaged under Nikon Eclipse (Ni-E) fluorescence microscope, and the images were analyzed for mean intensity using ImageJ software \((n = 5/\text{condition/age})\).

**qRT-PCR analysis**

For transcriptional analysis, control and experimental flies aged L3, 1, 7, and 13 days were collected in TRIZOL® Reagent (Thermo Fisher Scientific; 15596-026), snap frozen and stored at -80°C. Five biological replicates each with six flies were processed.

**RNA extraction**

The standard trizol method was used to extract total RNA. Six individuals per sample were kept in an Eppendorf tube with 300 \( \mu \)l of trizol Reagent. The samples were homogenized for 30-60 s and then kept at room temperature for 10 min. After that, samples were centrifuged at 13,000 rpm for 10 min at 4°C to pellet debris and transfer the supernatant to a fresh Eppendorf. 60 \( \mu \)l (0.2 volume) of chloroform was added and vigorously shaken for 15 s. Samples were centrifuged for 15 min at 4°C at 13,000 rpm. Upper phase was carefully collected to a fresh RNase-free Eppendorf without contacting the interphase. Finally, 210 \( \mu \)l (0.7 volume) isopropanol was added to precipitate the RNA and incubated at -20°C for 2 h. The
samples were centrifuged again at 13,000 rpm for 15 min, with the supernatant discarded and the pellet rinsed with 1 ml 70% ethanol. The pellet was air dried and resuspended in 20 μl of DEPC MilliQ water. The quality was checked with a NanoDrop 2000 spectrophotometer; 260/280 nm absorbance ratio close to 2.0 was accepted as ‘pure’ RNA.

Reverse transcription and qRT-PCR

To remove any genomic DNA contamination, RNA aliquots were treated with RQ1 DNase (Promega, USA; M6101) prior to the reverse transcription procedure. 1 μg of RNA from each sample was then reverse transcribed using RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific; K1622). A total reaction volume of 20 μl contained 8 μl of RNA sample having 1 μg of RNA, 1 μl of each DNase enzyme and DNase buffer, 1 μl stop solution, 1 μl random primers, 1 μl of each enzyme reverse transcriptase and RNase inhibitor, 2 μl of dNTPs solution, and 4 μl of reaction buffer. The cDNA synthesis was validated by PCR amplification of the β-actin gene. The cDNA is assigned a concentration unit relative to the original concentration of RNA in the RT reaction. As we loaded 1 μl of RNA in 20 μl RT reaction, the resultant cDNA concentration would be 50 ng/μl. Prior to qRT-PCR, cDNA samples were diluted 5 times to a final concentration of 10 ng/μl. To measure the mRNA expression level of the target gene, a real-time quantitative polymerase chain reaction (qPCR) amplification was performed using the QuantiNova SYBR green PCR Kit (Qiagen; 208052). A total reaction volume of 6 μl contained 3 μl SYBR Green PCR MasterMix, 1 μl each of forward and reverse primers (1 μM), and 1 μl cDNA (10 ng/μl). The ΔΔCt method was used to calculate the fold change in expression levels of selected genes relative to the normalisation control (RP49), and the results were expressed as arbitrary units. Primer3 was used to design the primers, which were then validated in silico using NCBI BLAST. The sequence of primer used are as listed below:

**RP49**
Forward 5′-CCGCTTCAAGGGACAGTATC-3′
Reverse 5′-ATCTCGCCAGTAAACGAAA-3′

**PPO1**
Forward 5′-TTGCCCAGATCCTGATTACC-3′
Reverse 5′-TGTCGATGAATCCCGTGACAACC-3′

**PPO2**
Forward 5′-TGACCTGCACAAACGGACAC-3′

Reverse 5′-TCACCCATCAGCAGCAGGGGCA-3′

**Eedin**
Forward 5′-GTCGTAACCGCAGCAAGGAC-3′
Reverse 5′-TGCCACCGACCTGCCACATAT-3′

**NimC1**
Forward 5′-AGGTTGTGCGAAGTGTAAA-3′
Reverse 5′-CATAATCCGTTCTCAGGGCGG-3′

**Eater**
Forward 5′-AATTCCTCTGCAGGCGTGATC-3′
Reverse 5′-GGGGTATGCGAAGTCCGAA-3′

**Unpaired3**
Forward 5′-TCCGACGATGGGGGAAGATGA-3′
Reverse 5′-CGCTGCTTGTTGGGTTGTA-3′

**Drosomycin**
Forward 5′-TGGGACACGAGGACCTGT-3′
Reverse 5′-ATCCTTGCCGACCAGCATT-3′

**Defensin**
Forward 5′-GTACATGCATTGCTCAGCT-3′
Reverse 5′-TCTCTGGGTGGCATCCTCAT-3′

**Diptericin**
Forward 5′-TTGAAACTGGGTGGCGGTGAA-3′
Reverse 5′-AGGTTGCTGGCCATGACGCT-3′

**Metchakowin**
Forward 5′-GTACATCGTTGCTGACCAGA-3′
Reverse 5′-CCCGTTCTGGTGTAGGGA-3′

**Drosocin**
Forward 5′-GTCTCTTGTTGGTGTGGCG-3′
Reverse 5′-TGATCTCGAGTGGCATGCTC-3′

**Attacin**
Forward 5′-GCAGTCTCCAGTGCGACATT-3′
Reverse 5′-ATTCAACGGGCATCGCCCTC-3′

**CecropinA**
Forward 5′-TTGGACACGAGCAGAATCAGCT-3′
Reverse 5′-GCCAGAATGAGACGGCAGAAA-3′

Statistical analysis

All graphs represent the mean value ± S.E.M. The Shapiro-Wilk test was used to determine the data’s normality. Statistical significance was determined by one-way analysis of variance (ANOVA) followed by a Tukey post hoc test for multiple comparisons. Data were analyzed using GraphPad Prism (version 9.5.1, GraphPad Software). A p value of 0.05 or less was considered to represent a statistically significant difference. The p value ≤ 0.05 marked as */*, p value ≤ 0.01 marked as **/**, p value ≤ 0.001 marked as ***/**##. All experiments were repeated 3–4 times as per requirement.
RESULTS

Curcumin controls crystal cell count and phenoloxidase activity in HD condition

Dhankhar et al. recently showed substantial immunological changes in HD flies as a consequence of neuronal expression of a mutant huntingtin exon1 fragment (Httex1pQ93) [37]. HD flies exhibited significant increase in crystal cell number at larval stage and higher phenoloxidase activity during entire course of disease. Higher phenoloxidase activity in diseased larvae was strongly correlated with their increased crystal cell count. Based on our observation, regulation of immune amendments may obscure the HD symptoms, particularly at early and advanced stages of the disease. Therefore, in the present study, we investigated if curcumin, a potent immunomodulatory phytochemical, can regulate immune dysregulation in HD flies. Intriguingly, our research group has previously reported that administration of 10 μM curcumin from early developmental stage in diseased flies substantially alleviates behavioral dysfunction as well as metabolic abnormalities [27, 39].

To further investigate the effect of curcumin on crystal cell number and function, control and diseased flies were raised on food supplemented without and with 10 μM concentration of curcumin from the early embryonic stage until 13 days post-eclosion. Concerning survivorship, motor dysfunction, and photoreceptor neuronal loss, we referred to 1 day post eclosion (1dpe) as early stage, 7dpe as advanced stage, and 11dpe and 13dpe as terminal stages of the disease throughout the study. Quantification of crystal cells showed that curcumin considerably modulates altered crystal cell count observed in HD flies that were not fed with curcumin. Diseased larvae fed with 10 μM curcumin exhibited a significant decline in abnormally higher number of crystal cells (L3; n = 5; p = 0.003407) as compared to the age matched diseased larvae (Fig. 1a, b). Control larvae with unexpanded glutamines did not show any variation in their crystal cell count following administration of the same dose of curcumin since early developmental stages. Assessment of phenoloxidase activity in HD flies with disease progression revealed that diseased flies exhibited significantly higher PO activity throughout the course of the disease, i.e., in larvae (L3; n = 3; p = 0.0093) as well as at advanced (day 7; n = 3; p = 0.007785), and terminal (day 13; n = 3; p = 0.00485) stages of the disease as compared to age matched control flies, as reported previously [37]. Interestingly, diseased flies fed with 10 μM curcumin displayed substantial reduction in their abnormally high phenoloxidase activity in 3rd instar larvae (n = 3; p = 0.03946), as well as in adults at advanced (day 7; n = 3; p = 0.0474), and terminal (day 13; n = 3; p = non-significant) stages as compared to age matched control flies (Fig. 1c). Intriguingly, we observed that phenoloxidase activity in diseased flies reared on curcumin-supplemented food was comparable to age-matched control flies at the third instar stage as well as day 1 (n = 3) and 7 post-eclosion.

Regulation of transcription expression of PPO1 and PPO2 by curcumin in diseased flies

To decipher the basis of curcumin action in regulating phenoloxidase activity in diseased condition, we monitored transcriptional levels of PPO1 and PPO2 genes in control and diseased flies reared on food supplemented without and with curcumin. We observed that PPO1 expressed in the control flies (elav > Httex1pQ25) in a regulated manner throughout the span of 13 days from emergence to adulthood. Interestingly, high transcript level of PPO1 was observed at larval stage in diseased, (elav > Httex1pQ93) condition (n = 5, p = 0.005835) as compared to the age-matched control (Fig. 2a). However, diseased flies had no comparable change in PPO1 transcription levels at day 1 (n = 5, p = 0.2947), day 7 (n = 5, p = 0.2218), and day 13, (n = 5, p = 0.7052) when compared to control cohorts. Interestingly, significant decline in elevated mRNA expression of PPO1 in diseased larvae reared on 10 μM curcumin was found (n = 5, p = 0.0081) as compared to those reared without curcumin (Fig. 2a).

Similarly, PPO2 transcript levels were tightly regulated in control condition at larval stage and throughout the period of 13 days from emergence to adulthood. We found that transcript levels of PPO2 in diseased condition was comparable to control at larval stage of disease but were significantly elevated in HD flies throughout the course of the disease i.e., at early (day 1; n = 5, p = 0.87), advanced (day 7; n = 5, p = 0.0049), and terminal (day 13; n = 5, p = 0.00675) stages as compared to control cohorts (Fig. 2b). Curcumin supplementation showed considerable decline in PPO2 transcript levels in diseased flies (day 1; n = 5, p = 0.03993) at early and advanced (day 7; n = 5, p = not significant) stages as compared to age-matched diseased flies without curcumin. But
Fig. 1. **Dietary curcumin manages altered crystal cell count and phenoloxidase activity.** (a) Crystal cells in the posterior abdominal segments of control (elav > Httx1pQ25) and diseased (elav > Httx1pQ93) larvae reared without and with curcumin supplementation. Quantification revealed that curcumin administration significantly regulates (b) abnormally high crystal cell number in diseased larvae, and (c) phenoloxidase activity at larval stage as well as at day 7 in diseased flies. The Shapiro-Wilk test was used to determine the data’s normality. Statistical significance was determined by one-way analysis of variance (ANOVA) followed by a Tukey post hoc test. Data is represented as mean ± S.E.M. * represents significance against age-matched control; # represents significant difference between diseased flies supplemented without and with Curcumin.

Fig. 2. **Curcumin regulates transcript levels of PPO1 and PPO2 in HD.** Transcript levels of PPO1 and PPO2 remained tightly regulated in control flies reared without and with curcumin supplementation. (a) Diseased flies exhibited significant increase in PPO1 transcript levels at 3rd instar stage but remain unchanged in day 1, day 7, and day 13 old flies. A significant decrease in transcript level of PPO1 at 3rd instar stage upon curcumin supplementation; (b) PPO2 transcript levels were significantly higher at day 1, 7, and 13 in diseased flies as compared to control cohorts. However, curcumin administration resulted in considerable decrease in PPO2 transcript levels at day 1 and day 7 old diseased flies. RP49 was used as normalization control. The data’s normality was determined using the Shapiro-Wilk test. Statistical significance was determined by one-way analysis of variance (ANOVA) followed by a Tukey post hoc test. Sample size: 6 flies/replicate, 5 replicates/condition. Data is represented as mean ± S.E.M. * represents significance against age-matched control; # represents significance between diseased flies supplemented without and with Curcumin. α=0.05; p-value: ***p<0.001; **p<0.01; #p<0.01; *p<0.05.
no discernible effect was observed at terminal stage of the disease with curcumin feeding (Fig. 2b).

**Curcumin superintends plasmatocyte count in HD flies with disease progression**

In addition to crystal cells, plasmatocytes make up about 95% of the total number of circulating hemocytes in *Drosophila*. Importantly, these cells act as professional phagocytes and are actively involved in release of cytokines as well anti-microbial peptides in response to infections or other immunological challenges. Previously, we reported significantly higher number of circulating plasmatocytes in diseased larvae as compared to age-matched control larvae. Moreover, plasmatocyte count remained obstante in hematopoietic pockets of diseased flies over the course of disease i.e., from day 1 to day 13 post eclosion, but there was a steady drop in plasmatocyte number in control’s hematopoietic pockets with aging [37]. Therefore, we looked at the modulatory effect of curcumin on plasmatocyte count with disease progression. We found that diseased larvae fed with curcumin did not show any significant modulation (L3; n = 10; p = 0.132) in circulatory plasmatocyte count as against diseased larvae fed without curcumin (Fig. 3a). However, a significant decline in plasmatocyte count was seen in hematopoietic pockets of curcumin-fed diseased flies at advanced (day 7; n = 10; p = 0.0145) and terminal (day 11; n = 10; p = 0.02324, day 13; n = 10; p = 0.039210932) stages, which otherwise remained persistent in diseased flies reared without curcumin (Fig. 3b). Interestingly, at day 11, plasmatocyte count in hematopoietic pockets of diseased flies fed with curcumin became comparable to age-matched control flies. Additionally, no discernible effect of curcumin was seen on the plasmatocyte count in control flies from day 1 till day 13 post eclosion.

To investigate the molecular basis underlying the altered plasmatocyte count in diseased flies, we measured transcriptional levels of *edin*, a critical gene responsible for the release of sessile plasmatocytes from hematopoietic compartments into circulation [40]. In diseased (elav > Htx1PQ93) condition, though, we observed a steep increase in *edin* expression at 3rd larval instar stage (n = 5; p = 0.0030) (Fig. 3c), which may contribute to higher number of plasmatocytes in circulation as reported earlier. However, *edin* expression in diseased flies was significantly downregulated at advance (day 7; n = 5; p = 0.03975) and terminal (day 13; n = 5; p = 0.022) stages as compared to age matched controls but remained comparable to control at an early stage (day 1; n = 5; p = 0.815) (Fig. 3c). Downregulation of *edin* at advanced and terminal stages of disease was coinciding with an obstante plasmatocyte count in hematopoietic pockets at these ages. In addition, we found significantly lower *edin* transcript levels in curcumin fed diseased larvae as compared to diseased larvae not fed with curcumin (L3; n = 5; p = 0.0326). While curcumin supplementation results in significant increase in *edin* transcript levels in diseased flies at advanced (day 7; n = 5; p = 0.427) and terminal (day 13; n = 5; p = 0.001056573) stages relative to diseased flies reared without curcumin, becoming comparable to control flies (Fig. 3c). Collectively, these results indicate that administration of curcumin effectively regulates altered transcription expression of *edin* at progressive ages, which may ultimately reinstates the plasmatocyte count in diseased flies.

**Curcumin restored altered phagocytic activity in diseased condition**

To comprehend the impact of curcumin on functional activities of plasmatocytes, we monitored phagocytic ability of plasmatocytes isolated from control and diseased flies reared without and with curcumin up to 13 days post eclosion. We have earlier reported that plasmatocytes isolated from diseased larvae (L3; n = 5; p = 0.00550296) have significantly less phagocytic activity than age-matched control larvae. Additionally, there is a progressive decline in phagocytic activity in diseased flies over the course of the disease (n = 5; day 7; p = 0.01289; day 11; p = 0.0008658; day 13; p = 0.000408) that initially becomes comparable and then significantly drops in comparison to age-matched control flies [37]. Interestingly, in the present study with curcumin supplementation, significant restoration in phagocytic activity of plasmatocytes in diseased condition was observed at 3rd instar stage (n = 5; p = 0.027739172) (Fig. 4a, b), and upon progression at day 7 (n = 5; p = 0.01530279), day 11(n = 5; p = 0.00876224), and day 13 (n = 5; p = 0.03294621) (Fig. 4c, d). However, at terminal stage of disease at day 13, curcumin could partially restore phagocytic activity of plasmatocytes.

In order to apprehend the molecular basis underlying reduced phagocytic activity in diseased condition, we monitored transcription profile of key genes responsible for effective phagocytosis. The key genes selected were - *NimC1* and *eater* - encodes for transmembrane receptors involved in engulfment process.
Fig. 3. Curcumin modulates plasmatocyte count in HD condition. Curcumin administration significantly modulates (a) plasmatocyte number in circulation of 3rd instar larvae and (b) plasmatocyte count in hematopoietic pockets of 7, 11, and 13 days old diseased flies. (c) Edin mRNA levels were significantly higher in diseased larvae and then dramatically decreased in diseased flies at day 7 and 13 as compared to control cohorts. Curcumin-fed diseased flies exhibited significant reduction in their otherwise elevated edin mRNA levels at 3rd instar stage and increase in their otherwise decreased edin mRNA levels at day 7 and day 13.

We observed that control flies maintain constant transcription expression of both these genes throughout the period of 13 days of adulthood. In the HD condition, flies did not exhibit any significant alteration in transcript levels of NimC1 at larval stage (L3; n = 5; p = 0.7793) as well as at early (day 1; n = 5; P = 0.1504) and advanced (day 7; n = 5; p = 0.9577) stages, but considerably downregulated at terminal (day 13, n = 5; p = 0.0162) stage of disease, as compared to age-matched controls (Fig. 4e). However, no change in the transcript levels of NimC1 was observed in diseased flies upon curcumin supplementation. Additionally, we found that transcript level of eater was significantly downregulated at advanced (day 7; n = 5; p = 0.0183) and terminal (day 13; n = 5; p = 0.0042) stages in the diseased flies as against control cohorts (Fig. 4f). Interestingly, significant increase in transcript levels of eater was seen in curcumin-fed diseased flies at early (day 1, n = 5, p = 0.027000978), advanced (day 7, n = 5, p = 0.047), and terminal (day 1, n = 5, p = 0.03278) stages as compared to age-matched diseased flies not fed with curcumin. Altogether, these results indicate that curcumin supplementation effectively modifies altered transcriptional expression of eater in diseased flies at different ages which may be associated with improved phagocytic activity of plasmatocytes by curcumin.

Management of oxidative stress by curcumin in plasmatocytes of HD flies

Phagocytes release ROS during activation and phagocytosis of microorganisms and cellular debris. It has been established that ROS production affects phagocytic activity of macrophage and disorders linked to chronic inflammation are characterized by reduced phagocytic activity of the macrophage, which is linked to elevated ROS signaling. We have previously reported that plasmatocytes isolated from diseased condition exhibited significantly higher intracellular ROS levels at larval stage (n = 5;
Fig. 4. Dietary curcumin improves reduced phagocytic activity of plasmatocytes in diseased condition. (a, c) Confocal microscopic images representing engulfment of heat-killed PI-stained *E. coli* by plasmatocytes isolated from control and diseased individuals supplemented without and with curcumin at indicated ages. Scale bars represent 20 μm. The percentage phagocytosis was calculated by counting the number of plasmatocytes showing bacterial engulfment per 100 plasmatocytes. Bacteria stained with propidium iodide are colored red, and plasmatocyte nuclei are blue. (b, d) Quantification of percentage phagocytosis revealed significant decline in phagocytic activity of plasmatocytes isolated from diseased 3rd instar larvae (b) and gradual decline in phagocytic activity of diseased flies (d) from day 7 to 13 as compared to control cohorts. Curcumin supplementation substantially improved otherwise reduced phagocytic activity of plasmatocytes in 3rd instar larvae and day 7, 11, and 13 old flies in disease condition. (e) Nim C1 mRNA levels were significantly reduced in 13 days old diseased flies as compared to age-matched control group, whereas no change in Nim C1 mRNA level was seen in diseased flies upon curcumin supplementation. (f) Diseased flies showed significant decrease in *eater* mRNA levels at day 7 and 13. Curcumin feeding resulted in substantial increase in *eater* mRNA levels in 7 and 13 days old diseased flies. RP49 was used as normalization control. The normality of data was analysed using the Shapiro-Wilk test. Statistical significance was determined by one-way analysis of variance (ANOVA) followed by a Tukey post hoc test. Data are represented as mean ± S.E.M. * represents significance against age-matched control; # represents significance between diseased flies supplemented without and with curcumin. α=0.05; p-value: ***p<0.001; **p<0.01; *p<0.05; ##p<0.01; #p<0.05.
Collectively, these results indicate that high ROS levels detected in plasmatocytes of diseased flies at day 11 and 13 might be one of the contributing factors of their decreased phagocytic activity seen in HD flies. Dietary administration of curcumin reduces ROS level in plasmatocytes at terminal stages of disease thereby providing protection against increased oxidative insult and ultimately results in improving phagocytic activity of plasmatocytes in HD flies.

Curcumin averts mitochondrial dysfunction in HD condition

Mitochondria are extremely sensitive to changes in the cellular environment, such as pH, redox potential, and ionic concentration and are crucial for the effective operation of multiple pathways. Although neuronal mitochondrial dysfunction has been widely identified as the pathological characteristic of HD, various investigations have also reported peripheral mitochondrial abnormalities in HD models and patients [41, 42]. In this study, we monitored the mitochondrial function in the plasmatocytes of diseased flies to check energy homeostasis of the cells. Plasmatocytes isolated from healthy and diseased individuals were stained with MitoTracker red CMXros, a cell-permeant mitochondria specific dye. In healthy cells, MitoTracker probes passively diffuse across the plasma membrane, accumulate in active mitochondria in a potential-dependent manner and fluoresce brightly. However, fluorescence is lost when the mitochondrial transition pore opens and its membrane potential changes (representing dysfunctional mitochondria) (Fig. 6a, c). Intriguingly, we observed a significant reduction of active mitochondrial staining at the larval (L3; n = 5; p = 0.0036) control (Fig. 6b) and at terminal stages (day 11; n = 5; p = 0.0393 and day 13; n = 5; p = 0.0039) (Fig. 6d) of adult flies in diseased condition. As mitochondrial functioning is crucial to provide energy for plasmatocytes to execute phagocytosis, decreased phagocytic activity in diseased flies may be a consequence of its dysfunction. Curcumin supplementation, on the other hand, resulted in a substantial reduction in the mitochondrial dysfunction of plasmatocytes in diseased larvae and 11 and 13 days old diseased flies (Fig. 6b, d). These results indicate that administration of 10 μM curcumin exerts mitochondria-protective effects on plasmatocytes of HD flies.

Curcumin detains expression of pro-inflammatory cytokines in HD flies

It has been reported that the level of cytokines and chemokines increases significantly in the plasma of HD patients and mouse models. Consistently, the production of cytokines from monocytes and macrophages of HD patients have been evidenced after lipopolysaccharide stimulation. As mHTT is ubiquitously expressed in patients and mouse models, it is possible that autonomous mHTT expression in immune cells could result in a higher level of proinflammatory cytokine expression in the plasma. To test whether neuronal expression of mHTT has similar effects, we measured the transcription expression of Drosophila cytokines eiger (homolog of TNF) and unpaired3 (homolog of IL-6). Interestingly, we found that eiger transcript levels in diseased flies were comparable to control cohorts at the early stage (day1; n = 5; p = 0.9550) but significantly higher at the advanced (day 7; n = 5; p = 0.030026) and terminal (day 13; n = 5; p = 0.04698) stages of the disease (Fig. 7a). However, upd3 transcript levels in diseased flies were higher throughout the entire course of the disease, including early (day 1; n = 5; p = 0.00835), advanced (day 7; n = 5; p = 0.0106) and terminal (day 13; n = 5; p = 0.0042) stages (Fig. 7b). These findings indicate that pan-neuronal mHTT expression induces production of pro-inflammatory cytokines in flies. Further, we explored the effect of curcumin supplementation on the transcription expression of the examined cytokines and interestingly, did not find significant change in the transcript levels of eiger in all stages of disease (Fig. 7a). However, upd3 expression was significantly downregulated in diseased flies rear on curcumin supplemented food at early (day 1; n = 5, p = 0.00486) and advanced (day7; n = 5, p = 0.0316) stages, but unexpectedly increased at terminal (day 13; n = 5, p = 0.0345) stage as compared to age-matched diseased flies reared without curcumin (Fig. 7b).

Curcumin regulates transcriptional expression of AMPs in disease flies

In Drosophila, the Toll and Imd pathways regulate the induction of downstream AMP genes in both hemocytes and the fat body, which are the key mediators of humoral immune response [43]. AMPs in Drosophila provide defense upon systemic infection and along with the lysozymes, many digestive enzymes with antimicrobial prop-
Fig. 5. Curcumin reduces elevated ROS level in plasmatocytes of diseased flies. (a, c) Evaluation of ROS production in plasmatocytes of control and diseased flies reared without and with curcumin supplementation at progressive ages. Red = dihydroethidium (DHE) and, Blue = DAPI, scale bars represent 20 μm. (b, d) Quantification of Corrected Total Cell Fluorescence (CTCF) intensity of DHE staining revealed significant higher ROS levels in plasmatocytes of (b) diseased larvae and (d) in day 11 and 13 old flies as compared to control groups which was compensated by curcumin supplementation Statistical significance was determined by one-way analysis of variance (ANOVA) followed by a Tukey post hoc test. Data are represented as mean ± S.E.M. * represents significance against age-matched control; # represents significance between diseased flies supplemented without and with Curcumin. α=0.05; p-value: *** p<0.001; ### p<0.001; ## p<0.01.

properties manages microbiota in the gut as well as involved in cancer, aging, brain function, and neurodegeneration. To investigate whether pan-neuronal expression of mHTT had any impact on the induction AMPs, we measured relative mRNA levels of seven antimicrobial peptide genes, including Drasomycin (Drs), Defensin (Def), Diptericin (Dpt), Metchnikowin (Mek), Attractin (Att), Drosocin (Dro), and Cecropin A (CecA), and further monitored effect of curcumin supplementation on AMPs induction in HD flies with disease progression. Importantly, we found that transcript levels of all seven AMPs were tightly regulated in the control condition from emergence to day 13. In diseased condition, the transcript levels of the AMPs Drs, Dro, and Att were markedly increased from early (day 1; n=5; p(Drs) =0.29; p(Dro) =0.120226; p(Att) =0.0049) stage and continued to rise throughout the advanced (day 7; n=5; p(Drs) =0.0007; p(Dro) =0.0114; p(Att) =0.0022) and terminal (day 13; n=5; p(Drs) =0.0099; p(Dro) <0.0001; p(Att) =0.0033) stages. However, we found no change in the transcript levels of Drs and Att in diseased flies upon curcumin supplementation; whereas Dro transcript levels significantly reduced in diseased flies reared with curcumin at advanced (day 7; n=5; p(Drs) =0.0339) and terminal (day 13; n=5; p(Drs) =0.0007) stages as compared to diseased flies reared without curcumin supplementation (Fig. 8a-c). The relative transcript levels of AMPs Dpt and Mek were significantly higher at the early (day 1; n=5; p(Dpt) <0.0001; p(Mtk) <0.0001) stage, followed by a rapid drop at the advanced and terminal stages of disease, becoming comparable to control cohorts. Interestingly, curcumin supplementation significantly reduced transcript levels of Dpt and Mek in diseased flies at early (day 1; n=5; p(Dpt) =0.00045; p(Mtk) =0.0023) and advanced (day 1; n=5; p(Dpt) =0.0354) stages but could not match transcript levels of control cohort (Fig. 8d, e). Furthermore, Def mRNA levels in diseased flies were higher at terminal stage (n=5; p(Def) <0.0001) as compared to controls; however, curcumin supplementation could not make any change (Fig. 8e). Lastly, diseased flies reared without and with curcumin showed no com-
Fig. 6. **Curcumin defers mitochondrial dysfunction in plasmatocytes.** (a, c) Mitochondrial membrane potential (as visualised by staining with mitoTracker red CMX ros) was significantly reduced in plasmatocytes isolated from diseased (elav>Hettx1pQ93) flies compared to control (elav>Hettx1pQ25) at indicated ages, representing mitochondrial dysfunction. Scale bar represents 20 μm. (b) Quantification of images using ImageJ software revealed significant decrease in fluorescence intensity of MitoTracker stained plasmatocytes of diseased larvae as compared to the age-matched controls. However, curcumin administration significantly reduced active mitochondrial staining in plasmatocytes of diseased larvae. (d) In diseased flies, fluorescence intensity remained comparable to control cohorts at early (day1) and advanced (day7) stages but increased significantly at terminal stages (day11 & day13). Curcumin feeding results in significant reduction in mitochondrial dysfunction in plasmatocytes of 7 and 13 days old diseased flies. Statistical significance was determined by one-way analysis of variance (ANOVA) followed by a Tukey post hoc test. Data are represented as mean ± S.E.M. * represents significance against age-matched control; # represents significance between diseased flies supplemented without and with Curcumin. $p$-value: **$p<0.01$; ***$p<0.001$; ##$p<0.01$; # $p<0.05$.

Fig. 7. **Curcumin regulates expression of pro-inflammatory cytokines in HD flies.** In diseased flies, (a) eiger transcript levels were comparable to control condition at day 1, upregulated at day 7 and day 13 as compared to control cohorts. No change in eiger transcript levels was seen in 7 or 13 days old diseased flies reared on 10 μM curcumin diet. (b) unpaired 3 transcript levels were significantly higher throughout disease progression as compared to control cohorts. Curcumin feeding results in significant decrease in unpaired 3 transcript levels at day 1 and day 7 of diseased flies but unexpectedly increase in 13 days old diseased flies. All the expressions were normalized to RP49 expression. Data’s normality was analyzed using the Shapiro-Wilk test. Statistical significance was determined by one-way analysis of variance (ANOVA) followed by a Tukey post hoc test. Sample size: 6 flies/replicate, 5 replicates/condition. Data are represented as mean ± S.E.M. * represents significance against age-matched control; # represents significance between diseased flies supplemented without and with Curcumin. $p$-value: **$p<0.01$; *$p<0.05$; ##$p<0.01$; # $p<0.05$. 
Fig. 8. Curcumin differentially regulates transcriptional expression of AMPs in disease. mRNA expression levels of antimicrobial peptides (a) Drosomycin (Drs), (b) Drosocin (Dro), (c) Attacin (Att) in diseased (elav > Htx1pQ93) flies were upregulated at all stages as against control (elav > Htx1pQ25) cohorts. Curcumin administration effectively downregulated Dro mRNA levels in day 7 and 13 old diseased flies upon curcumin feeding (d) Dipterocin (Dpt) and (e) Metchnikowin (Mek), transcript levels were significantly higher in diseased flies at day 1 and remains comparable to the control group thereafter. Diseased flies showed significant decrease in transcript levels of Dpt at day 1 and 7, and Mtk at day 7 upon curcumin feeding (f) Defensin (Def) mRNA levels were significantly upregulated in 13 days old diseased flies, as against the control cohort. No change was seen in Def transcript levels in diseased flies upon curcumin feeding (g) CecropinA (CecA) levels remained unchanged in control and diseased flies reared without and with 10 μM curcumin at all ages. All the expressions were normalized to RP49 expression. Data’s normality was analyzed using the Shapiro-Wilk test. The significance was analyzed by one-way analysis of variance (ANOVA) followed by a Tukey post hoc test. Sample size: 6 flies/replicate, 5 replicates/condition. Data are represented as mean ± S.E.M. * represents significance against age-matched control; # represents significance between diseased flies supplemented without and with Curcumin. α=0.05; p-value: ***p<0.001; **p<0.01; *p<0.05; ###p<0.001; ##p<0.01; #p<0.05.

parable change in the transcript levels of CecA at any disease stage when compared to age-matched control flies (Fig. 8f). Altogether, from these results it is evident that HD flies exhibit higher levels of antimicrobial peptides during the entire course of the disease and curcumin supplementation effectively manages transcriptional expression of certain AMPs.

**DISCUSSION**

HD results from progressive deterioration of specific neuronal subpopulation in the striatum and cortical regions of CNS. Although primary clinical manifestations of the disease include loss in the triad of motor, cognitive, and behavioral capabilities, a number of systemic co-morbidities accompanying grave neuropathology are progressively recognized in HD [4]. Systemic immune dysregulation is considered as one of the major critical contributors to HD pathology and imposes additional burden on the health of already challenged individuals and gradually disables them. Despite of several therapeutic strategies employed to slow down the disease progression, there is currently no effective cure. In addition, target-specific synthetic drugs can only provide symptomatic alleviation with serious side effects in challenged individuals. Interestingly, phytochemicals that retains a broad spectrum of pharmacological
targets with least side effects are extremely beneficial in the treatment of neurodegenerative diseases [44].

Due to broad range of therapeutic properties and excellent safety profile, ‘curcumin’, a polyphenolic molecule found in turmeric, is widely acknowledged as one of the most divine phytochemicals in treatment of multiple neurodegenerative diseases [45, 46]. Turmeric has a long history of traditional use as dietary spice and Ayurvedic medicine in Asian subcontinent [18, 19]. Noticeably, the Asian subcontinent, where spices are widely consumed, has a lower prevalence (0.42/100,000) of HD compared to a high prevalence of 2.71/100,000 worldwide [47, 48].

We had reported immunological derangements in in vivo transgenic HD Drosophila expressing mutant Htt exon 1 fragment with expanded polyQ peptide (Htt1p Q93) [37]. Recently, as a treatment regimen, an effective concentration of 10 μM curcumin administered from early developmental stages mitigated their motor abnormalities and improved survival and metabolic abnormalities otherwise observed in HD condition [27, 39, 49]. In the current study, we aimed to examine the effect of curcumin in regulation of altered immune response in diseased flies. For this we reared control and diseased flies on curcumin-supplemented diet from early development stages until 13 days post eclosion. Interestingly, curcumin administration displayed a significant reduction in crystal cell count which is higher in diseased larvae. Additionally, curcumin administration reduced otherwise elevated phenoloxidase activity in at larval and advanced stages of disease while reduction was insignificant at terminal stage. In Drosophila, the phenoloxidase enzyme produced by crystal cells (functional equivalent to mammalian melanocytes) is responsible for melanin synthesis. Consistent with our observation, Curcumin has been shown to reduce melanin formation in both healthy and melanoma cells, supporting effectiveness of curcumin as an anti-melanogenic therapeutic agent [50–52]. PPO1 and PPO2 genes encode for the phenoloxidase enzyme in Drosophila. We monitored transcription expression of these two genes in HD flies without and with curcumin supplementation. PPO1 mRNA levels were upregulated in at larval stage while PPO2 levels were upregulated at advanced (day 7) and terminal (day 13) stages of the disease. Binggeli et al. (2014) [53] has reported that PPO1 is engaged in the quick early delivery of PO activity when required, but PPO2 accumulates in the crystal cells and serves as storage that can be deployed in a later phase. In line with their findings, elevated PPO1 transcript levels during the larval stage in our study may be responsible for a steep increase in PO activity against abrupt mHTT aggregates load in neural cells. However, as the disease progresses, PPO2 expression takes over in response to a chronic load of mHTT aggregates. Conversely, curcumin administration significantly downregulated PPO1 expression at larval stage and PPO2 expression at early stages in diseased flies but not at advanced and terminal stages of the disease.

We further examined the efficacy of curcumin on plasmatocyte count in HD flies with disease progression. Interestingly, administrated dose of curcumin effectively regulated altered plasmatocyte count in hematopoietic pockets of diseased flies at advanced and terminal (partially) stages. Previous studies have also shown beneficial effect of curcumin on blood metrics such the total cell count, WBC count, macrophage count, and neutrophil count in several diseases [54–56], however, there are no direct studies reporting curcumin’s impact on plasmatocyte count in a Drosophila model. Plasmatocytes in Drosophila are characterized by a dynamic behavior of continuously exchanging between the sessile hematopoietic pockets and circulating state [57]. Following that, we observed a significant increase in edin transcript levels (a fat body secreted peptide that is necessary for the release of sessile plasmatocytes from hematopoietic pockets) during the third instar stage in diseased conditions when compared to age-matched controls, which may be the cause of the release of cells from pockets and maintenance of a high plasmatocyte count in circulation as previously reported [37, 40, 58, 59]. Furthermore, edin mRNA levels in diseased flies were considerably lower at advanced (day 7) and terminal (day 13) stages compared to control cohorts, which is on par with the persistent plasmatocyte count isolated from hematopoietic pockets of diseased flies. However, curcumin administration markedly reduced otherwise elevated transcription of edin in diseased larvae and increased at advanced and terminal stages in diseased flies, rendering it comparable to control flies. From these results, we apprehend that possibly the modulation in edin’s expression in diseased condition at different ages in response to curcumin supplementation further resulted in reinstatement of the plasmatocyte count in diseased flies, to a certain extent.

We previously demonstrated that plasmatocytes from HD flies gradually lose their ability to phagocytose as the condition worsens [37]. In the present study, we found that administering curcumin significantly improved the decreased phagocytic activity...
of plasmatocytes in diseased conditions at larval as well as advanced and terminal stages. Several previous studies have also shown positive effect of curcumin on microglia’s phagocytic ability in AD and other neurodegenerative diseases [60–62], that collaborates with our results too.

Further, to apprehend the molecular basis underlying reduced phagocytic activity in diseased condition, we monitored the transcription profile of key genes-

*NimC1* and *eater* - encode for transmembrane receptors involved in engulfment process and effect of curcumin on their expression. We observed reduced expression of *NimC1* in the terminal stage and *eater* at the advanced and terminal stages of diseased flies, providing the possible molecular basis for the decreased phagocytic activity in HD. Interestingly, curcumin administration resulted in significant increase in *eater* expression in diseased flies at both advanced and terminal disease stages, which may have aided in improving phagocytic activity of plasmatocytes. In line with our findings, Teter et al. reported that transcription expression of TREM2 (Triggering Receptor Expressed on Myeloid Cells) and CD68 (a marker of microglial phagocytosis) were significantly reduced in AD mice model, but substantially elevated after curcumin treatment, leading to increased phagocytic activity [62].

Mitochondrial dysfunction in HD is not restricted to the brain; they are also detected in peripheral tissues such as muscles, immune cells, adipose tissue, testis, bones, etc. of patients and mouse models [63–68].

Oxidative stress and mitochondrial dysfunction are linked to the pathogenesis of a variety of human diseases. Mitochondria are essential for cellular energy production and also play a crucial role in regulation of immune cell activation, differentiation, transcription, and survival via the release of signals such as mtDNA or mitochondria-derived reactive oxygen species (mtROS) [69]. Hence, assessing the mitochondrial function in diseased condition was crucial in order to comprehend the cellular mechanisms leading to decreased phagocytic activity of plasmatocytes. Interestingly, we observed substantial depolarization of the mitochondrial membrane potential in plasmatocytes isolated from HD flies at the advanced stage, which exacerbated in terminal stages. Mitochondrial membrane potential disruption results in defective calcium influx into the mitochondria, followed by the opening of mitochondrial permeability transition pores, which inevitably leads to decreased cellular respiration, increased oxidative damage, cytochrome c release, and, eventually, cell death. Recent reports suggest that phagocytes associated with mitochondrial dysfunction have lower phagocytic activity and are unable to regulate mtROS [70]. Mitochondria impairment in the pathogenesis of several neurodegenerative diseases along with the mitochondrial dysfunction in the fat body of the HD flies also has been reported [41, 42]. Additionally, curcumin administration effectively alleviated mitochondrial dysfunction in the plasmatocytes of diseased larvae and flies at terminal stages. Other investigations have also reported effectiveness of curcumin in ameliorating mitochondrial dysfunction in several neurodegenerative diseases such as Alzheimer’s disease, Parkinson’s disease, and Spinocerebellar Ataxia (a polyQ disorder) [71–73].

The significance of ROS in mediating physiological and pathophysiological signal transduction is well understood. Previously, we have shown that plasmatocytes from diseased flies had elevated levels of intracellular ROS, which may be linked to their decreased phagocytic activity and, thus, may be one of the contributing factors to HD pathogenesis. Upon further investigation, we found that curcumin effectively abended abnormally high ROS levels at both larval and terminal stage of diseased flies. A comprehensive view on curcumin’s antioxidant mechanism and significance in reducing oxidative stress and suppressing mitochondrial dysfunction has been reported recently [74].

Neurodegenerative diseases are often accompanied by chronic inflammatory conditions. Increased levels of proinflammatory cytokines in afflicted brain regions, advocating neuroinflammation, had been observed in various neurodegenerative diseases. Moreover, HD has been associated with alterations in cytokine levels in the bloodstream. For instance, elevated serum levels of IL-6, TNF-α, IL-10, and IL-4 were detected in HD patients at moderate stage or premanifest stage [75, 76]. To test whether pan-neuronal expression of mHTT results in similar fashion in *Drosophila*, we measured expression levels of cytokines eiger (homologue of TNF-α) and upd3 (homologue of IL-6). Consistent with previous findings in HD mice and patients, we also observed that transcript levels of *eiger* and *upd3* were significantly upregulated in diseased flies from the onset of the disease, with the increase being more pronounced towards the terminal stage, which may result in the systematic inflammation and mortality. Interestingly, increased *upd3* transcript levels were considerably downregulated upon curcumin supple-
mentation in diseased flies at early and advanced stages but unexpectedly upregulated at terminal stage. Consistent with our observation, curcumin has been shown to suppress the expression of pro-inflammatory cytokines, like IL-6 and TNF-α, as well as ROS in PD and AD animal models [77].

As antimicrobial peptides are key mediators of humoral response in *Drosophila* immunity, recent studies have discovered role of *Drosophila* antimicrobial peptides in the gut, tumor, aging, and neurodegeneration in addition to their anti-bacterial and anti-fungal activities [78]. The transcription levels of six of the seven AMPs (except CecA) were shown to be differentially upregulated in HD flies at different stages of disease progression. Consistent with our findings, a study from *Drosophila* model of AD has demonstrated considerably elevated expression levels of the AMPs Mek and Att [79]. However, contrary to our findings, a prior investigation had found that mHTT-expressing cells significantly suppressed the induction of antimicrobial peptides upon bacterial infection *in vitro* [80].

Our results apparently differ as we targeted mHTT expression in the pan-neuronal population and evaluated systemic AMPs expression, which has not yet been observed in any other HD model. In addition, curcumin administration effectively downregulated transcriptional expression of Mek in HD flies at early stage, Dro at advanced and terminal stages, and Dpt at early and advanced stages. However, curcumin-fed HD flies did not show any modulation in Drs, Def, and Att expression at any age. Considering these results, we may conclude that the potent anti-oxidant and anti-inflammatory properties of curcumin proved immensely beneficial in attenuation of inflammatory and oxidative insult in HD and thereby attributed to amelioration of the disease state. Additionally, there was no difference in accumulation of mHTT aggregates in the eye-antennal discs of diseased larvae fed without and with curcumin supplemented diet (Chongtham and Agrawal, unpublished data), indicating that curcumin may be beneficial in modulating downstream pathology.

**Conclusion**

HD pathophysiology is complicated and encompasses various systemic problems in addition to severe loss of neuronal population in specific brain areas. In this view, administration of therapeutic agents with diverse targets and little or no side effects are thought to provide better pathological alleviation than mono-targeted treatments. Recently, we have reported extensive immune alterations in HD flies expressing mutant huntingtin exon 1 fragment with expanded polyQ peptide pan-neuronally. In the present study, we explored the effect of potent immunomodulator, curcumin in alleviating immune dysregulation associated with HD. Our findings convincingly show that administration of curcumin since early embryonic stages effectively regulates altered immune response in HD condition. Though curcumin may not completely prevent neurodegeneration or immune abnormalities until terminal stages, it can effectively suppress or postpone immune amendments at early and advanced stages. We, therefore, propose that curcumin can serve to be a safe and effective therapeutic regime for alleviating systemic immunological abnormalities associated with HD. However, additional emphasis will be required to explicate the intricate mechanism of curcumin actions.

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**CONFLICT OF INTEREST**

The authors have no conflict of interest to report.

**DATA AVAILABILITY**

The data supporting the findings of this study are available on request from the corresponding author.
SUPPLEMENTARY MATERIAL

The supplementary material is available in the electronic version of this article: https://dx.doi.org/10.3233/JHD-230595.

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