Pan-neuronal expression of human mutant Huntingtin protein in *Drosophila* impairs immune response of hemocytes

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Abstract
Huntington’s disease (HD) is a late-onset; progressive, dominantly inherited neurological disorder marked by an abnormal expansion of polyglutamine (poly Q) repeats in Huntingtin (HTT) protein. The pathological effects of mutant Huntingtin (mHTT) are not restricted to the nervous system but systemic abnormalities including immune dysregulation have been evidenced in clinical and experimental settings of HD. Indeed, mutant huntingtin (mHTT) is ubiquitously expressed and could induce cellular toxicity by directly acting on immune cells. However, it is still unclear if selective expression of mHTT exon1 in neurons could induce immune responses and hemocyte function. In the present study, we intended to monitor perturbations in the hemocytes population and their physiological functions in Drosophila, caused by pan-neuronal expression of mHTT protein. We found that pan-neuronal expression of mHtt significantly alters crystal cells and plasmatocyte count in larvae and adults with disease progression. Interestingly, plasmatocytes isolated from diseased conditions exhibit a gradual decline in phagocytic activity ex vivo at progressive stages of the disease as compared to age-matched control groups. We also observed an increased production of reactive oxygen species (ROS) in plasmatocytes at advanced stages of the disease. In addition, diseased flies displayed elevated reactive oxygen species (ROS) in circulating plasmatocytes at the larval stage and in sessile plasmatocytes of hematopoietic pockets at of disease. All the parameters were monitored progressively, targeting the circulation at larvae stage and hematopoietic pockets in adults at different disease stages, and many alterations were documented in the early stage itself. These findings strongly implicate that neuronal expression of mHtt alone is sufficient to induce non-cell-autonomous immune dysregulation in vivo. Based on these findings, we propose that further insight into the mechanisms through which neuronal expression of mHtt might be inflicting the innate immune responses would facilitate therapeutic inventions aimed at amelioration of HD pathology and improving the quality of life of the patients.
1. Introduction

Huntington’s disease (HD) is a rare, dominantly inherited progressive neurodegenerative disorder caused by an unstable expansion in the polymorphic CAG trinucleotide repeat in exon 1 of Interesting Fragment 15 (IT15) gene on chromosome 4, which is ubiquitously expressed (Hoogeveen et al., 1993; MacDonald et al., 1993; Marques Sousa & Humbert, 2013). Mutant Huntington (mHTT) protein harbors an expanded polyglutamine tract beyond 35 repeats that confer toxic gain of function and causes severe neuronal degeneration in the striatum and cortical regions of brain; that results in motor impersistence, cognitive decline, psychiatric abnormalities leading to gradual loss of functional capacity and eventually death (Bates et al., 2002; Ho et al., 2001). This devastating disease has late-onset typically at 40s and 50 years of life and is inversely related to the number of CAG repeats (Andrew et al., 1993). The traditional research in HD focuses on preferential neuronal dysfunction in the specific brain regions resulting in characteristic clinical symptoms; however, emerging evidence indicates that mHTT might affect the region beyond the central nervous system (CNS). Besides neuronal deposition of mHTT, other mechanisms such as oxidative stress, free radicals, dysregulated immune response including inflammation seem to contribute to HD pathogenesis and progression (Kotrcova et al., 2015; Tai et al., 2007). Abnormal phenotypic effects caused by the expression of mHTT in various non-neuronal, peripheral cells and tissues have been described in skeletal muscles, cardiac muscles, adipose tissues, fibroblasts, and immune cells of HD animal models and patients (Björkqvist et al., 2008; Moffitt et al., 2009; Sassone et al., 2009; Sathasivam et al., 1999). Abnormalities related to immune system in particular, have been reported in several animal HD models and HD patients (Andre et al., 2016; Leblhuber et al., 1998). Expression of...
mHTT in astrocytes of transgenic R6/2 mice model induces age-dependent neurological phenotypes such as weight loss, motor deficits, and early death (Bradford et al., 2009). In healthy state, microglia the key player of cerebral innate immune system remains quiescent and regulates immune response by producing anti-inflammatory and neurotrophic factors (Streit., 2002). Activation of microglia can induce neuronal damage via several mechanisms such as production of free radicals, caspase activation, and excitotoxicity (Crotti et al., 2014; Hanisch, 2002; Kim & de Vellis, 2005; Wang et al., 2004). In this regard, a set of studies have shown that increased expression of cannabinoid receptor 2 (CB2) attenuates activation of microglia and peripheral immune cells and thus suppresses disease pathogenesis in HD mice model (Bouchard et al., 2012; Palazuelos et al., 2008). The expression of mutant protein induces activation of both brain and peripheral immune cells in HD patients and in mouse models. In a report, upregulation of pro-inflammatory cytokines such as IL-6, IL-12, tumor necrosis factor (TNFα), and acute-phase protein α2-microglobulin clusterin (involved in clearance of cellular debris in cerebrospinal fluid (CSF) and plasma of HD patients was observed (Björkqvist et al., 2008). A recent study also showed increased levels of pro-inflammatory and regulatory cytokines such as IL-4, IL-6 IL-12, and TNFα in vital peripheral organs i.e. liver, heart, spleen, and kidney of BACHD mice model (Valadão et al., 2019). Besides, microglia and peripheral immune cells, human HD patients exhibited impaired migration and recruitment of chemotactic stimuli (Kwan et al., 2012). Taken together, these reports suggest that innate immune dysfunction plays an important role in HD pathogenesis. However, the interaction between CNS pathology and changes in peripheral immune cells in HD patients is poorly understood but can be of utmost importance in suppressing disease progression. Additionally, few recent studies reported that ectopic expression of mHTT expression in immune cells leads to altered immune responses, higher susceptibility against
infectious agents, and premature mortality in animal HD models (Donley et al., 2016; Lin et al., 2019).

_Drosophila melanogaster_ is a well-established animal model for HD studies. Despite apparent differences between humans and flies, several biological mechanisms are highly conserved across evolution such as networking of complex nervous system, genetic mutations, innate immunity control, and biological rhythms (Jennings, 2011; Lemaitre & Hoffmann, 2007; Zehring et al., 1984). Moreover, key aspects of _Drosophila_ immunity possess striking similarities with mammalian innate immunity (Dhankhar et al., 2020). Innate immunity of fruit fly constitutes humoral and cellular defense mechanisms. Humoral immunity involves the production of antimicrobial peptides (AMPs) from the fat body; however, cellular immunity is mediated by three types of hemocytes: plasmatocytes, crystal cells, and lamellocytes. Plasmatocytes are responsible for phagocytosing invading pathogens and apoptotic debris and represent the functional equivalent of mammalian macrophages (Elrod-Erickson et al., 2000). Crystal cells contain crystalline inclusions of phenoloxidase enzyme which is released during melanization, a process required for successful wound healing. Lamellocytes are the cryptic, stress-induced cell type that is responsible for encapsulation of objects that are too large to be phagocytosed such as eggs of parasitic wasps (Meister & Lagueux, 2003). These hemocytes reside in three major compartments: in the circulation, lymph gland, and the hematopoietic pockets segmentally distributed on both sides of dorsal vessel.

The expression of human mHTT exon 1 fragment with expanded CAG repeats in the neuronal tissue of transgenic flies causes neurodegeneration _in vivo_ and recapitulate the characteristic feature of HD including gradual accumulation of mHTT aggregates in neural cells and subsequent apoptosis, transcriptional dysregulation, motor abnormalities, and ultimately death similar to those in human patients (Steffan et al., 2001; Taylor et al., 2003).
Furthermore, *Drosophila* provides an excellent system for spatiotemporal expression of transgenes using the bipartite UAS-GAL4 system and evaluating direct effects of transgene in an *in vivo* condition (Brand & Perrimon, 1993; Marsh et al., 2000). The present study aimed to elucidate major changes occurring in the hemocyte population and their physiological functions in the *Drosophila* model of HD when exon 1 fragment of human mHTT selectively expressed in neuronal cells. To experiment, UAS-GAL4 system was used to express mHTT with pan-neuronal elav-Gal4 driver and investigated possible repercussions of neuronal mHTT on hemocytes of transgenic *Drosophila*. Interestingly we found that the expression of mHTT in neural cells affects hemocyte population and alters their functional abilities which include melanization, phagocytosis of infectious agent, and production of reactive oxygen species. Altogether, our findings suggest that amendment in innate immune response in HD flies might be due to an indirect effect of mHTT on the integrated process of immune regulation, which may occur due to extensive neuronal impairment mimicking disease condition. Therefore, identification of new therapeutic targets aimed towards immune regulation may be effective in delaying disease progression and improving the quality of life of the patient’s.

2. **Materials and Methods**

2.1. *Drosophila* stocks and crosses. *Drosophila* cultures were grown at 25°C and 65% humidity on standard cornmeal under a constant 12h light: 12h dark cycle. Expression of transgene human HTT exon1 fragment containing polyglutamine repeats carried out using the bipartite UAS-GAL4 expression system. Transgenic stocks used in this study include *w*; P{w+mW.hs=GawB}elavC155: a pan-neuronal driver (#8765; BDSC), *w*; P{UAS-Httex1p Q25}, and *w*; P{UAS-Httex1p Q93}4F1; generously gifted by Prof. J. Lawrence Marsh, UCI, Irvine, California. Virgin females from UAS-Httex1p Q25 (wild type) and UAS-Httex1pQ93 (mutant) were mated with the males of elav-GAL4 and resulting female
progenies elav>Httex1p Q25 (controls) and elav>Httex1p Q93 (diseased) were used for all the assays. All animal experiments were conducted following the national and international guidelines and the relevant national laws on the protection of animals. All experiments of this study were conducted using Drosophila and it remains out of bioethical considerations.

2.2. Crystal cell melanization assay. For crystal cell visualization, at least 10 wandering 3\textsuperscript{rd} instar larvae of both control and diseased conditions were kept in 1ml of 1X PBS in glass vials and incubated at 65°C for 10 mins. Heat-shocked larvae from each condition were then arranged on a glass slide over a black background and imaged using Nikon SMZ 745T stereozoom microscope right after heating. Melanized crystal cells appeared as black puncta on larval cuticle in three posterior-most abdominal segments A6, A7 and A8 were quantified using ImageJ software. Minimum three replicates (10 larvae/replicate) per condition were used for the assay.

2.3. Estimation of phenoloxidase activity. For estimation of phenoloxidase (PO) activity, 10 individuals of each condition were homogenized in homogenization buffer (containing protease inhibitor (Sigma-Aldrich, S8820) and then centrifuged at 13000 rpm for 10 mins. The supernatant was collected in fresh pre-chilled eppendorf and the protein concentration of each sample was estimated using Bradford assay (Bradford, 1976). PO activity in the supernatant was quantified using a microplate enzyme assay. A reaction mixture having protein concentration of 10μg/μl was prepared by diluting the supernatant in 1X PBS containing protease inhibitors. 50 μl of each sample was then plated in 96 well plate and added 50 μl of 3mM L-3-4-dihydroxyphenylalanine (L-DOPA) (substrate for enzyme phenoloxidase) to each well and incubated at 25°C for 30 min. Absorbance was measured at 492nm using an ELISA plate reader (Biotek). Minimum three replicates per condition were used for the assay.

2.4. Plasmatocytes Isolation
a) **Isolation of circulating plasmatocytes from larvae:** Wandering 3\textsuperscript{rd} instar larvae were collected from the wall of food vials in a petri-plate having wet filter paper to remove the food debris present on the larval cuticle. The larvae were then placed on ice to slow down their movement. Seven larvae of each condition were placed in 100µL of 1X PBS in a cavity block. Using a pair of fine tweezers, the cuticle of the larvae was pinched very slightly to release the hemolymph into the media while avoiding gut breaking. Hemolymph samples were collected and placed on ice for further analysis. Analysis was performed just after the dissection to avoid melanization reactions in the hemolymph.

b) **Isolation of plasmatocytes from hematopoietic pockets of adult flies:** Anesthetized flies were positioned dorsal side down in a drop of PBS on a clean glass slide. Using the needles, the wings stretched apart to ensure that the dorsal abdomen submerged in 1X PBS. A fine incision is then made from the posterior tip of the fly abdomen ventrally and continued up to the head. The dissected fly body part consists of the dorsal side of the thorax and abdomen. The tissues present inside the abdominal cavity like gut, ovaries, Malpighian tubules were gently removed while the dorsal abdominal diaphragm is kept untouched. Ten dissected abdomen cuticles were placed in 100µl of 1X PBS in a cavity block and disturbed with the help of a needle to release hemocytes from the pockets. The resulting hemocytes suspension was collected in pre-chilled eppendorf for further analysis.

2.5. **Screening of plasmatocytes.** Isolated hemolymph/plasmatocytes suspension was plated on the pre-cleaned slides and placed in a humified chamber for adherence of plasmatocytes for an hour at 25°C. After incubation, slides were washed with 1X PBS to remove non-adherent cells. Adhered plasmatocytes were fixed in 100% methanol for 30 seconds; air-dried and stained with 0.2% crystal violet (HiMedia, RM114) stain for 90 seconds. Preparations
were mounted in DPX and images were taken at different magnifications under the Nikon Eclipse E-100 microscope.

2.6. Plasmatocytes counting. To evaluate plasmatocyte count, we prepared a 1:1 dilution of hemolymph/plasmatocytes suspension with the 0.4% trypan blue solution (Himedia, TC193) and pipetted several times to ensure a uniform cell suspension. 10μL of trypan blue-cell suspension was loaded on a hemocytometer carefully by touching the coverslip at its edge with the pipette tip. Hemocytometer was then observed under the Nikon microscope at 10x magnification and cells were counted in four outer squares in the grid (each square contains 16 smaller squares). The average plasmatocyte count per individual was then calculated using the given formula.

Average cell count per larva/fly = Average cell count per square x Total volume of sample x D.F. / No. of larvae per sample

2.7. Staining of bacteria. Overnight grown *E. coli* culture was harvested by centrifugation at 10,000 rpm for 10 minutes and washed three times with 1X PBS to remove LB. Subsequently, bacteria were heat-killed at 90°C for an hour. Heat killed bacteria again washed three times with 1X PBS and incubated with 10 μg/ml propidium iodide (PI) (Sigma-Aldrich, P4170) stain for an hour on a shaker at room temperature. Finally, bacteria were washed with 1X PBS and diluted to a concentration of $10^8$ bacterial cells/ml. Bacterial concentration was checked by measuring optical density at 600 nm (OD$_{600}$) (modified from Neyen et al., 2014).

2.8. Phagocytic assay. To assess phagocytosis, 100μL volume of cell suspension (~3 x $10^4$ cells) was plated on pre-cleaned slides and incubated in a humified chamber at 25°C for an hour for adherence of plasmatocytes. The number of larvae/adults to sacrifice depends on the genotype. After incubation, slides were washed with 1X PBS to remove non-adherent cells. After that heat-killed PI-stained *E. coli* (100μl of $10^8$ cells/ml of suspension) was added to the
slide and again incubated for 40 mins at 25°C to enable phagocytosis. Slides were then washed to remove un-phagocytosed bacteria, methanol fixed and air-dried and mounted with Vectashield media containing DAPI (Vector laboratories, H1200). The slides were observed under confocal microscope and percent phagocytosis was calculated by counting the number of plasmatocytes showing phagocytosis per 100 plasmatocytes. Minimum five replicates per condition were used for the assay.

2.9. Estimation of nitric oxide production. The production of nitric oxide (NO) was estimated from the accumulation of nitrite, a stable end product of nitric oxide, by the activated plasmatocytes using Griess reagent (Ding et al., 1988). Briefly, 1 x10^5 cells of each genotype were plated in 96 well plate and left for an hour at 25°C for adherence. The adhered cells were then treated with LPS (10µg/ml) and heat-killed E. coli (100µl of 10^8 cells/ml of suspension) and further incubated at 25°C. After 48 hrs of treatment, 25µL of culture soup from each well was taken and centrifuged at 12000 rpm for 5 mins to remove cells from the soup. 20µL of supernatant was taken, added an equal volume of freshly prepared Griess reagent (1:1 of 1% sulphanilamide in 2.5% H3PO4 and 0.1% ND in distilled water) and incubated at room temperature for 10 mins. Absorbance was measured at 540 nm on an ELISA plate reader (Biotek). Nitrite concentrations were estimated by extrapolation from the sodium nitrite standard curve. Minimum three replicates per condition were used for the assay.

2.10. Reactive oxygen species (ROS) estimation. Dihydroethidium (DHE) staining was used to monitor superoxide radicals in the plasmatocyte isolated from healthy and diseased conditions. 1 x 10^4 plasmatocytes of each condition were plated on the pre-cleaned coverslips and left for adherence for an hour at 25°C. Cells were washed with 1X PBS to remove excess of bacteria. A stock solution of 30mM DHE (Invitrogen Molecular Probes, D11347) was reconstituted in anhydrous dimethyl sulfoxide (DMSO, Sigma-Aldrich, 276855). For
staining, the reconstituted dye was further diluted in 1X PBS to obtain a final working concentration of 25µM. The cells were incubated with dye for 10 mins in dark. This was followed by rinsing of cells with 1X PBS and mounting with Vectashield media containing DAPI. Images were acquired immediately under the Nikon Eclipse (Ni-E) fluorescence microscope and fluorescence intensity quantification was performed using ImageJ software. Minimum five samples per condition were analyzed for ROS quantification.

2.11. Statistical analysis. All the graphically presented values represent the mean value. Normality of data sets was determined using the Shapiro-Wilk test. Data analysis of normally distributed data was done by Student’s t-test for pair-wise comparison. All statistical analysis was performed using the IBM SPSS package. P-value 0.05 was considered statistically significant. 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.

3. Results

3.1. Experimental scheme

In present study, expression of transgene human HTT exon1 fragment containing unexpanded and expanded polyglutamine repeats was carried out using the bipartite UAS-GAL4 expression system so that subsequent progeny mimic the disease symptoms. All the experiments were performed at two developmental stages of Drosophila i.e. late 3rd instar larvae indicating the early manifestation stage of disease and adult flies at indicated ages representing the disease’s progressive stages. We targeted the circulating system at the larval stage and hematopoietic pockets in adults for quantification of plasmatocytes and their functional activities. However, crystal cell count and phenoloxidase activity was appraised in the whole body of larvae and adults. In addition, to ensure that elav-Gal4 driver is exclusively expressed in pan neurons but not in immune cells, we examined elav-GAL4 (elav-GAL4>UAS-GFP) expression by GFP reporter throughout different developmental stages.
By microscopic observation, it was evident that elav is neither expressed in plasmatocytes at larval and adult stage (fig.1b). Hence, it can be concluded that immune cells are completely devoid of elav-Gal4 expression.

Figure 1. Outline of experimental scheme and visualization of elav driven GFP expression in plasmatocytes of larvae and adult flies. (a) Schematic representation of research design and parameters studied, (b) Microscopic visualization of plasmatocytes...
isolated from 3\textsuperscript{rd} instar larvae and adults confirmed lack elav driven GFP expression in immune cells.

3.2. Neuronal expression of Httex1p Q93 causes alteration in crystal cell count and phenoloxidase activity in HD condition. Abnormalities related to the immune system were observed in several studies of HD patients. In an attempt to delineate the immune response anomalies associated with HD, we used transgenic flies expressing human HTT exon1 with unexpanded polyQ tract (Httex1p Q25, wild type) and expanded polyQ tract (Httex1p Q93, mutant) under the control of elav-GAL4 driver since the embryonic stage.

Crystal cells serve as a critical indicator of Drosophila’s stress status and any abnormal change in crystal cell count and their activity can be the first crucial sign for outlining the alteration in immune response. To better understand the involvement of immune system in Huntington’s disease and its progression, we primarily sought to discern the alterations, if any, in crystal cell number in elav>Httex1p Q25 (control, referred hereafter 25QHtt\textsuperscript{ex1}) and elav>Httex1p Q93 (diseased, referred hereafter 93QHtt\textsuperscript{ex1}) larvae. Heat shock to larvae in PBS at 67°C causes spontaneous activation of the prophenoloxidase zymogen within crystal cells, leading to their blackening, and consequently making them visible through the cuticle as black puncta (Rizki et al., 1980). Surprisingly, a higher number of black puncta were seen in three posterior abdominal segments (A6, A7, and A8) of diseased larvae (Fig.2a).

Numerical variation in crystal cell count was quantified using ImageJ software. Quantification result confirmed that 93QHtt\textsuperscript{ex1} larvae show significant increase in crystal cell number (n = 10, p = 5.66397E-12) (Fig. 2b) than age-matched controls. This prominent change in crystal cell population could be a reflection of amended/altered immune response in HD pathogenesis.

As more melanization dots were observed on the cuticle of diseased larvae, we further measured enzymatic phenoloxidase (PO) activity with an L-DOPA assay in the hemolymph...
of larvae and adults with disease progression. Phenoloxidase is a key enzyme in the melanization process that catalyzes the oxidation of phenols to quinones, which subsequently polymerize into melanin. Diseased larvae showed significantly higher PO activity (n = 3; \( p = 0.009782 \)) (Fig. 2c) as compared to age-matched controls. Similarly, diseased flies had significantly higher PO activity at the indicated ages compared to controls (n = 3; day 1, \( p = 0.009782 \); day 7, \( p = 0.006137 \); day 11, \( p = 0.006178 \); day 13, \( p = 0.002072 \)) (Fig. 2d). These results together support that crystal cell population and function remain altered in HD flies.

**Figure 2. Increased crystal cell count and phenoloxidase activity in diseased condition.**

(a) Crystal cells in the posterior abdominal segments of 93QHt\textsuperscript{ex1} (diseased, right panel) and 25QHt\textsuperscript{ex1} expressing (control, left panel) 3\textsuperscript{rd} instar larvae. (b) Circumferential crystal cells were counted from the three posterior segments A6, A7, and A8 of the heat-shocked control and diseased third instar larvae. Diseased larvae (black bar) displayed a significant increase in crystal cell number as compared to age-matched control (grey bar). Crystal cells were
quantified using imageJ software. Error bars represent ± SEM (n=10/genotype); P-value:

***$P < 0.001$. Diseased larvae (black bar) (c) and (d) flies exhibit increased phenoloxidase
(PO) activity with disease progression as compared to age-matched controls (grey bar). Error
bars represent ± SEM (n=3); P-value: ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$.

3.3. Httex1pQ93 expression causes alteration in plasmatocyte number with disease
progression. To further extend our observation to decipher the relationship between impaired
immune response and Huntington’s disease, we evaluated major changes occurring in the
plasmatocyte population, major circulating hemocytes of Drosophila, and their functional
activities induced by neuronal expression of mHTT in larvae and adults at indicated ages. For
morphological observations, plasmatocytes adhered to glass slides were stained with 0.2%
crystal violet and imaged the Nikon Eclipse E-100 microscope. We observed that
plasmatocytes isolated from diseased larvae display heightened cell spreading with extended
lamellipodial protrusions and form large vacuoles in the cytoplasm, whereas plasmatocytes
isolated from control larvae appeared smaller and round on glass surface (Fig. 3a). The
morphological changes of plasmatocytes in larvae prompted us to count their numbers in
diseased condition with disease progression. Interestingly, we found that diseased larvae
displayed significantly higher number of circulating plasmatocytes ($n = 10; p = 1.89865E-06$)
as compared to age-matched control larvae (Fig. 3b). Moreover, an obstinate plasmatocyte
count was observed in hematopoietic pockets of diseased flies throughout disease progression
from day 1 post eclosion (dpe) to 13dpe, while there was a gradual decline in plasmatocyte
number of hematopoietic pockets of controls with aging. During early stage (1dpe and 7dpe),
diseased flies had significant lesser number of sessile plasmatocytes ($n = 10; 1dpe, p = 6.14571E-06; 7dpe, p = 0.002965139$) compared to control condition. However, at the
advanced ages (11dpe and 13dpe), plasmatocyte count remains persistent in diseased flies
and becomes comparable to age-matched controls that showed a significant decline in
plasmatocyte count with aging (Fig. 3c). These results indicate that the expression of mHTT under control of pan-neuronal elav-GAL4 driver alters plasmatocyte population and their morphological characteristics.

Figure 3. Alteration of plasmatocyte morphology and number in HD condition with disease progression (a) Images showing characteristic morphology of plasmatocytes isolated from 25QHtt<sup>ex1</sup> (control; left panel) and 93QHtt<sup>ex1</sup> expressing larvae (diseased; right panel). Plasmatocytes isolated from diseased larvae appear more flattened and more dispersed as compared to the plasmatocytes isolated from control larvae. (b) Quantification of plasmatocytes from the diseased larvae (black bar) showed a significant increase in circulating plasmatocyte number as compared to age-matched controls (grey bars). (c) Diseased flies exhibit obstinate plasmatocyte count in hematopoietic pockets with disease progression. Interestingly on day 1 and day 7 diseased flies (black bars) have significant
lesser number of sessile plasmatocytes number in hematopoietic pockets that becomes comparable to age-matched controls (grey bars) at later stages (day 11 and 13). Error bars represent ± SEM. (n = 5/genotype/age) P-value: ***P < 0.001; **P < 0.01; *P < 0.05.

3.4. Plasmatocytes display impaired phagocytic activity in HD condition. We found that the Drosophila model of HD displayed an altered hemocytes population. Even though mutant HTT protein is expressed only in the neuronal population, peripheral immune cells appear inflected at larval stage itself. To test whether the expression of mHTT under the control of pan-neuronal elav-GAL4 driver could cause any detrimental effect on hemocyte functions, we examined the phagocytic activity of plasmatocytes isolated from 3rd instar larvae and adult at different ages post eclosion from control and diseased conditions. We quantified the ability of plasmatocytes to engulf heat-killed propidium iodide (PI)-stained E. coli by counting the number of cells capable of active phagocytosis. We found that plasmatocytes isolated from diseased larvae showed a significant decline in percentage phagocytosis (n = 5; p = 0.006178) compared to control condition (Fig. 4a & b). Moreover, diseased flies exhibited a gradual decline in the phagocytic ability of plasmatocytes at indicated ages compared to age-matched controls as disease progresses. In diseased adults, percentage phagocytosis was comparable to normal at early age i.e. 1dpe that significantly reduced from 7dpe to 13dpe (n = 5; 7dpe, p = 0.003398607; day 11, p = 3.92116E-06; 13dpe, p = 0.000513315) (Fig. 4d). Instances can also be documented where a lesser number of bacteria were phagocytized by a single plasmatocyte in diseased flies as compared to the age-matched controls (Fig. 4c). These results confirmed that impaired phagocytic activity of plasmatocytes in diseased condition without targeting the expression of mHTT in immune cells is solely a consequence of the diseased condition.
Figure 4. Reduction in phagocytic activity of plasmatocytes in HD condition with disease progression. (a & c) Confocal microscope images showing the engulfment of heat-killed PI-stained *E. coli* DH5α by plasmatocytes isolated from 25QHtt<sup>ex1</sup> (control; left panel) and 93QHtt<sup>ex1</sup> (diseased; right panel) expressing individuals at indicated ages. Scale bars represent 20µm. Phagocytic ability was calculated by counting the number of plasmatocytes showing phagocytosis per 100 plasmatocytes i.e., percentage phagocytosis. (b) Diseased larvae displayed a significant decline in phagocytic ability as compared to age-matched
controls. (d) In diseased adults, the phagocytic ability of plasmatocytes was comparable to control condition at initial stage i.e. day1. At later stages of disease (7dpe, 11dpe, and 13dpe), the phagocytic ability of plasmatocytes was significantly reduced as compared to age-matched controls. Error bars represent ± SEM. (n = 5/genotype/age) P-value: ***P < 0.001; **P < 0.01; *P < 0.05.

3.5. Enhanced ROS production in plasmatocytes of diseased larvae and flies at the terminal stage of the disease. Although HD may arise through several mechanisms, oxidative stress, the result of altered cellular redox status, has emerged as a potential factor involved in neurodegeneration and cell death in the etiology of HD. Phagocytes are the major producers of reactive oxygen species during their activation and phagocytosis of microbes and cellular debris. ROS are generally considered deleterious to cells, but ROS also has an important role in regulating signal transduction pathways. There is a possibility that reactive oxygen species generated by plasmatocytes could play a role in HD pathogenesis. Therefore, to unravel possible mechanisms underlying the impaired phagocytosis activity of plasmatocytes, we monitored levels of intracellular ROS in plasmatocytes in normal and diseased conditions at the indicated ages with disease progression. We measured levels of intracellular ROS in plasmatocytes of normal and diseased larvae. Cell permeable dye DHE with high permeability and specificity for superoxide radicals generated upon oxidative stress was used for ROS quantification. The intensity of DHE staining in cells/tissues is proportionate to the ROS levels. Basal ROS levels were detected in the plasmatocytes of both 25QHet and 93QHet larvae. However, ROS signals in the plasmatocytes of diseased
Figure 5. Increased levels of ROS in plasmatocytes of HD condition. (a & b) Estimation of ROS levels in circulating plasmatocytes isolated from 25QHtt<sup>ex1</sup> (control; left panel) and 93QHtt<sup>ex1</sup> (diseased; right panel) expressing individuals at indicated ages. Red = dihydroethidium (DHE) and, Blue = DAPI, scale bars represent 50µm. (c) Quantification of the images done using ImageJ software (n=5/genotype/age) revealed that plasmatocytes of 93QHtt<sup>ex1</sup> expressing larvae exhibit a significant increase in ROS intensity as compared to the age-matched controls and in adults (d) ROS levels remained comparable between diseased and control groups at 1dpe and 7dpe but were remarkably higher at 11dpe and 13dpe in the diseased group, as compared to the control group. Statistical analysis was done using
Student’s t-test. * represents significance against age-matched control. Error bars represent ± SEM. P-value: ***P < 0.001; **P < 0.01; *P < 0.05.

larvae were considerably higher as compared to age-matched controls (Fig. 5a). Further quantification of DHE intensity as a representative of ROS levels confirmed that plasmatocytes of diseased larvae exhibit significant elevation in ROS levels as compared to age-matched controls (Fig. 5b). In adults, ROS level in diseased condition remains comparable to the control condition during initial (1dpe) and symptomatic stages (7dpe), whereas it increases significantly at later ages (11dpe; p= 0.002733194 and 13dpe; p= 0.000971124) (Fig. 5c & d). Further, diseased flies showed significantly high ROS at day13 in comparison to day11 counterparts which suggests that ROS levels increase with disease progression. Taken together, we can establish that neuronal expression of mHTT results in altered immune response, in the form of impaired phagocytosis, and increased ROS levels at terminal age may be contributing to the surge in deaths observed.

3.6. Increased nitrite production by plasmatocytes in HD larvae.

Nitric oxide (NO) is involved in several physiological functions of the CNS, including neurotransmission, memory, and synaptic plasticity. Excessive NO production, as elicited by inflammatory signals, plays a key role in diverse neurodegenerative-associated processes such as neuronal death, necrosis, apoptosis, and autophagy (Calabrese et al., 2007). Thereby we further extended our study by evaluating the levels of nitrite production by plasmatocytes in vitro. To monitor NO release by plasmatocytes we stimulated plasmatocytes with LPS and bacterial challenge. We observed that basal levels (without stimulation) of nitrite release by plasmatocytes isolated from diseased larvae is comparable to the control condition. However, a significant increase in nitrite release by plasmatocytes of diseased larvae is seen at 48 hours after LPS and bacterial treatment (n = 3, p = 0.02915143) as
compared to control condition (Fig. 6). As NO is principally involved in many immune signaling pathways, a higher concentration of NO in plasmatocytes of diseased condition may also be a reason for immune dysfunction.

**Figure 6. Increased nitrite production by E. coli treated plasmatocytes of diseased larvae.** The nitrite production was measured in the supernatant after 48 hours of LPS and bacterial challenge using the Griess reagent. Quantification of NO levels revealed a significant increase in NO production in diseased condition after 48 hrs of bacterial treatment. Statistical analysis was done using Student’s t-test. Values are represented as ± SEM; (n=3/genotype). P-value: ***P < 0.001; **P < 0.01; *P < 0.05.

4. **Discussion**

The most predominant neuropathological feature in HD involves degeneration of a specific set of neurons in the striatum and cortical regions of brain that might give rise to characteristic clinical symptoms of the disease. In addition to neurobiological anomalies, immune dysregulation is considered one of the clinical challenges and may critically contribute to the pathology of HD. Previous studies from HD patients and mouse models have suggested that mHTT expression persuades both microglial activation in the HD brain and elevated levels of pro-inflammatory molecules released by peripheral immune cells such as monocytes and macrophages in the blood, supporting the idea that inflammation is not just
a repercussion of disease but an active contributor too (Björkqvist et al., 2008; Crotti et al., 2014; Kwan et al., 2012). Since mHTT expresses ubiquitously in human patients and HD mice, it is still unclear whether mutant protein contributes to immune dysregulation in the cell-autonomous or non-cell-autonomous manner (Hooigeveen et al., 1993). In the present study, we expressed the mHTT exon 1 fragment specifically in the neuronal cells of *Drosophila* since the embryonic stage and assessed its detrimental non-cell-autonomous *in vivo* effects on *Drosophila* hemocytes. Melanization reaction carried out by crystal cells of *Drosophila* involves both humoral and cellular components of the innate immune system can be used as a sensor of stress and environmental changes to elect the necessary responses. A phenotypic and quantitative examination of crystal cells demonstrated a significant increase in crystal cell count in late 3rd instar larvae. Melanization has been considered an important tool to minimize damage from physical and pathogenic stresses. Crystal cells possess crystalline inclusions of prophenoloxidase (proPO) in their cytoplasm which gives them their characteristic appearance. Under stressed conditions, the serine protease cascade may become activated, ultimately cleaving proPO to their active form of phenoloxidase (PO). PO in turn oxidizes phenols into quinones which subsequently polymerize to produce melanin (Meister & Lagueux, 2003). Estimation of prophenoloxidase (PO) activity also showed that enzyme is present in significantly high concentration in the hemolymph of diseased larvae. As crystal cells are directly linked with the melanization process, their proliferation increases in response to pathogenic stress to synthesize a more abundant amount of melanin to minimize the damage caused by mutant protein. Moreover, activated PO results in the production of reactive oxygen species during melanin biosynthesis that acts as a signaling molecule for activation of other crucial immune responses such as encapsulation, nodule formation, phagocytosis, and AMP production (Cerenius et al., 2008). Likewise, a recent study demonstrated that during trauma, PO-
induced ROS activates JNK-dependent cytoprotective programs in neuronal tissue required for systemic host protection (Nam et al., 2012).

We further investigated the number of circulating plasmatocytes in 3rd instar larvae of both healthy and diseased conditions that exhibited a significant increase in circulating plasmatocyte count in diseased condition. In line with these results, other studies have shown the high plasmatocyte count in circulation in response to parasitoid infections is associated with increased resistance against parasitoid wasps in several Drosophila species, although the molecular mechanism behind this phenomenon is not inferred (Eslin and Prevost., 1998; Kacsoh and Schlenke., 2012). Studies from several groups have extensively elucidated the process of hematopoiesis in Drosophila during larval stage. Drosophila hematopoiesis at larval stages occurs in two separate waves during development. The first wave of Drosophila larval hematopoiesis is founded by differentiated hemocytes of the embryo, which colonize segmentally repeated epidermal-muscular pockets and proliferate in these locations (Makhijani et al., 2011; Makhijani and Bruckner, 2012). During larval hematopoiesis, sessile plasmatocyte population undergoes a significant proliferation, expanding by self-renewal. Moreover, during these developmental stages, plasmatocytes are characterized by a dynamic behaviour, continuously exchanging between the sessile and circulating state. Like plasmatocytes, crystal cells increase in number during larval stages. However, crystal cell proliferation is not due to a self-renewal mechanism because mature crystal cells do not divide. Instead, a recent study has shown that new crystal cells originate from trans-differentiation of sessile plasmatocytes via a Notch–Serrate dependent process (Leitao and Sucena., 2015). Second wave of hematopoiesis occurs late in larval development within the lymph gland, a dedicated hematopoietic organ of mesoderm origin. Hematopoiesis in lymph gland only occurs in response to stress or immune challenge by synchronous differentiation of lymph gland prohemocytes (Krzemień et al., 2007; Grigorian et al., 2011; Gold and
Brückner, 2015; Banerjee et al., 2019). In our study, a high plasmatocyte number of diseased larvae might be an outcome of release of plasmatocytes either from lymph gland or hematopoietic pockets into the circulation. We further evaluated the propensity of neuronal-specific expression of mHTT on plasmatocytes of hematopoietic pockets in HD flies at different ages and found an obstinate plasmatocyte count in hematopoietic pockets/hubs of diseased flies throughout disease progression whereas there was a gradual decline in plasmatocyte number in hematopoietic pockets of controls with aging. In the course of metamorphosis, hemocytes from both the hematopoietic pockets or the lymph gland enter into the circulation system and persist to adulthood as mixed population Lanot et al., 2001; Gold and Brückner, 2015; Banerjee et al., 2019). Additionally, most studies claimed the lack of new hemocyte production in adult Drosophila; that is consistent with increased immunosenescence observed in aged adult flies Banerjee et al., 2019; Bosch et al., 2019; Mackenzie et al., 2011), while one study claimed active hematopoietic activity in adult Drosophila (Ghosh et al., 2015). Also, it has been reported that plasmatocyte count in hematopoietic hubs does not remain the same during adult life. There is a gradual increase in the number after eclosion till 5 days post eclosion (dpe); thereafter remains relatively constant up to 8 dpe and then declines as the age progress. This gradual loss of resident plasmatocytes suggests that adult employs plasmatocytes from the hubs at it ages (Ghosh et al., 2015). Additionally, it has been reported that sessile hematopoietic pockets are in close contact with the peripheral nervous system (PNS) that provides an attractive and tropic microenvironment for localization, survival, and proliferation to these resident hemocytes (Makhijani et al., 2011). More recently, it has been shown that sensory neurons of the peripheral nervous system produces Activin-β, which turned out to be an important factor in the regulation of haemocyte proliferation and adhesion (Makhijani et al., 2017). Based on these reports we assume that possibly mHTT expression in neurons is affecting production
and release of Activin-β or some unknown tropic factor from peripheral neurons which 
subsequently maybe responsible for proliferation and release of plasmatocytes in circulation 
as well as in trans-differentiation into crystal cells. However, further insight into the blood 
cell differentiation and neuronal regulation of *Drosophila* hematopoietic sites, providing a 
link between neuronal sensing and adaptive responses of local blood cell populations would 
expand understanding of present work. In contradiction to our result, a previous study 
showed that cell-autonomous expression of mHTT with hemocyte-specific driver causes a 
significant reduction in the circulating plasmatocyte count in diseased larvae (Lin et al., 
2019). Our results ostensibly differ as we targeted mHTT expression specifically in neuronal 
cells using pan-neuronal driver, elav-*Gal4* and assessed plasmatocyte number in a cell-non-
autonomous manner. However, similar to their results, we observed that despite a significant 
increase in plasmatocyte count mHTT expression in neuronal cells results in decreased 
phagocytic activity towards bacterial particles at both larval and later stages of disease 
progression i.e., from 7dpe to 13 dpe. We found that a lesser number of plasmatocytes of 
mHTT expressing flies were able to initiate phagocytosis and also passively engaged in 
engulfment of bacterial particles compared to the healthy controls. Subsidiary our results, 
studies from human patients also reported the compromised phagocytic activity of peripheral 
monocytes in other neurodegenerative diseases such as AD and PD (Grozdanov et al., 2014; 
Gu et al., 2016). In addition, defective actin remodeling in HD mouse immune cells leads to 
failure of membrane ruffling, which supports our result since actin assembly is required to 
trigger engulfment and phagolysosome maturation for successful phagocytosis (Kwan et al., 
2012; Swanson, 2008).

Further, we measured the levels of nitrite production by plasmatocytes because NO, a 
fundamental signaling agent, participates in several physiological and pathological processes, 
including immune response to microbes, neurodegenerative diseases, and asthma (Coleman,
2001; Nappi et al., 2000; Pacher et al., 2007). Increased nitrite release by LPS and bacterial
stimulated plasmatocytes of mHTT expressing larvae indicates NO is a crucial factor that
regulates immune sensitivity against challenges. NO, a volatile signaling agent, released from
phagocytic cells may easily diffuse through BBB and further exacerbate cytotoxicity by NO
mediating inflammatory response in Drosophila nervous tissues (Ellrichmann et al., 2013;
Inamdar & Bennett, 2013; Parathath et al., 2006). Indeed, previous studies have shown that
induced NO release from the hemocytes leads to the activation of antimicrobial peptides in
the fat body (Foley and O’Farrell, 2003). To gain a mechanistic understanding of
dysregulated peripheral immune response in diseased condition, we investigated the status of
reactive oxygen species (ROS) in plasmatocytes of mHTT expressing larvae and flies with
disease progression. Consistent with previous studies in HD patients, we observed
significantly high ROS levels in circulating plasmatocytes of 3rd instar larvae and resident
plasmatocytes at advanced disease stages i.e. 11dpe and 13 dpe as compared to age-matched
controls which might be the outcome of aggravated inflammatory environment and metabolic
dysregulation in immune cells. Earlier Chen et al. and Hersch et al. reported increased
oxidative damage and suppressed anti-oxidant capacity of peripheral immune cells in HD
patients (Chen et al., 2007; Hersch et al., 2006). It is well documented that ROS is critical to
macrophage phagocytic activity. Notably, diseases associated with chronic inflammation
display impaired macrophage phagocytic activity that is correlated with changes in the ROS
signalling (Forrester et al., 2018). The available evidence, however, indicates that non-cell-
autonomous expression of mHTT dysregulates peripheral immune response, although the
underlying mechanisms are yet to be elucidated.

To our knowledge, this is the first in vivo report showing that expression of mHTT exon1
fragment exclusively in neuronal cells is sufficient to cause immune dysregulation in
Drosophila hemocytes in a non-cell-autonomous manner. It might also possible that the
recognition and clearance of mutant protein aggregates by microglia and astrocytes in the brain triggers innate immune response including the release of pro-inflammatory cytokines, which subsequently distress circulating immune cells (Heneka et al., 2014; Tian et al., 2012). The dysregulated immune response of peripheral immune cells may be, at least in part, responsible for the toxicity of mutant Huntingtin proteins on peripheral tissues and the progression of HD. Further insights into the molecular mechanism of phagocytosis suppression and molecular interactions between mHTT and immune signaling pathways would facilitate the quest for novel therapeutic interventions aimed to alleviate HD pathology and improving the quality of life of the patients.

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Disclosure of Ethical Statements

Approval of research protocol: No human participant was involved in this study.

Informed Consent: N/A

Registry and the Registration No. of the study/trial: N/A

Animal studies: All animal experiments were conducted following the national and international guidelines and the relevant national laws on the protection of animals. All experiments of this study were conducted using Drosophila and it remains out of bioethical considerations.

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**Author's contributions**

J.D., N.A., and A.S. conceptualized, designed, and analyzed the experiments. J.D. performed the experiments and wrote the original draft. N.A. and A.S. reviewed and edited the manuscript. All authors read the manuscript carefully.

**Conflict of Interest**

The authors declare no conflict of interest.

**References**


