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Investigating the Role of Microglia and the Immune System in Huntington's Disease

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Investigating the Role of Microglia and the Immune System in Huntington's Disease

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Dedicated to

My Beloved Parents,

Sister,

& Min Min

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INVESTIGATING THE ROLE OF MICROGLIA AND THE IMMUNE SYSTEM IN HUNTINGTON'S DISEASE

Wanda Kwan

Huntington's disease (HD) is a fatal, incurable neurodegenerative disorder caused by an expanded polyglutamine (polyQ) tract in the protein huntingtin (htt). Recent studies have suggested the potential importance of immune cells in HD pathogenesis. Of particular interest, microglia have early activation and dystrophy in the brains of HD patients and mouse models. HD patients also have increased levels of plasma pro-inflammatory cytokines and chemokines before the onset of motor symptoms that correlate with disease progression. HD patients' monocytes also have increased cytokine levels in response to stimulation. Because htt is ubiquitously expressed, it may directly contribute to the dysfunction of microglia and peripheral immune cells as they have similar origins and functional similarities.

To investigate the effects of mutant htt in immune cells, we first established microglial cell lines that stably express this protein and obtained primary microglia from HD mouse models. We evaluated canonical activation responses and found that microglia expressing mutant htt selectively have increases in pro-inflammatory cytokines and an impairment in stimulus-dependent migration. This migration defect is also present in peripheral immune cells isolated from HD patients, such as monocytes and macrophages. We also observed defective migration of peripheral macrophages towards the site of inflammation upon induction of peritonitis in HD transgenic mice. In the brains of these

mice there was also abnormal microglial process extension and retraction, and a delayed response toward laser ablation injury.

Based on our results that mutant htt impairs some immune cell functions and previous evidence of abnormal immune activation in HD, we determined the effects of bone marrow transplantation (BMT) on pathogenesis *in vivo*. HD donated mice with wild-type (WT) bone marrow had modest but significant improvements of behavioral phenotypes, increased levels of synaptophysin, and normalized levels of peripheral inflammatory cytokines. Our results collectively indicate that mutant htt indeed impairs the function of peripheral and central immune cells in a manner that could be a contributing in a disease-modifying manner to pathogenesis in HD. The cell-autonomous effect of mutant htt in immune cells may also explain the early immune abnormalities in HD. This work has potential implications for HD patients, as there are drugs currently used to treat inflammation that could be tested in HD patients.

Contributions

Most of the work in chapter 2 will be submitted for publication to the journal ***PLoS Current (Huntington Disease)*** entitled *"Expression of Mutant Huntingtin Fragment in Microglia Leads to Neuronal Cell Death"* by Wanda Kwan, Elsa Raibon, Aurelio Silvestroni, Flaviano Giorgini, Christine Cheah, Sarah Swarts, Thomas Möller, and Paul J. Muchowski. Christine Cheah and Dr. Flaviano Giorgini generated the lentiviral constructs of htt exon 1. Dr. Elsa Raibon, Dr. Aurelio Silvestroni, and Sarah Swartz in Dr. Thomas Möller's laboratory performed the cytokines and IGF1 expression experiments in Figure 2 and 4.

The work and accompanying figures in chapter 3 are work in progress and will be submitted for publication to the journal ***Nature Neuroscience*** or ***Neuron*** entitled *"Mutant Huntingtin Profoundly Impairs Migration of Microglia and Peripheral Immune Cells"* by Wanda Kwan, Ralph Andre, Austin Chou, Aaron Miller, Dimitrios Davalos, Katerina Akassaglou, Nephi Stella, Sarah J. Tabrizi, Paul J. Muchowski. Dr. Ralph Andre in Dr. Sarah Tabrizi's laboratory performed the human monocyte migration assay (Fig. 4A). Austin Chou contributed the thioglycollate-induced peritonitis experiments (Fig. 4B,C). Dr. Aaron Miller in Dr. Nephi Stella's performed the initial Boyden migration assays in microglial cell lines (data not shown). In collaboration with Drs. Dimitrios Davalos and Katerina Akassalgou, we performed the *in vivo* two-photon microscopy to evaluate microglial response to tissue injury (Fig. 3).

The work and accompany figures in chapter 4 will be submitted for publication in May 2011 to the ***Journal of Neuroscience*** entitled *"Bone Marrow Transplantation Confers Modest Benefits in Mouse Models of Huntington's Disease"* by Wanda Kwan,

Anna Magnussen, Austin Chou, Anthony Adame, Thomas Möller, Monica Carson, Richard Ransohoff, Eliezer Masliah, Sarah J. Tabrizi, Maria Bjorkqvist, Paul J. Muchowski. Anna Magnussen in Dr. Maria Bjorkqvist's laboratory measured the cytokine levels in the mouse serum samples. Austin Chou contributed to the sectioning and the immunochemistry for the neuropathology. Anthony Adame and Dr. Eliezer Masliah provided training in synaptophysin evaluation and quantification. Dr. Monica Carson provided training and scientific input on the bone marrow transplantation procedures. Drs. Thomas Möller, Richard Ransohoff & Sarah J. Tabrizi provided intellectual support.

All other data presented in this thesis were obtained by myself under the supervision of Dr. Paul J. Muchowski.

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Chapter 1

Introduction

Huntington's Disease

Huntington's disease (HD) is an autosomal dominant neurodegenerative disorder caused by an expansion of CAG triplet repeat codons in the *IT-15* gene, which results in a polyglutamine expansion in the protein huntingtin (htt). The disease is characterized by selective neuronal death primarily in the cerebral cortex and striatum. A major pathological feature is the selective loss of medium-spiny projection neurons (MSN) in the striatum that are involved in the inhibitory circuitry of movements[1,2]. The phenotype of HD patients includes progressive chorea, cognitive impairment, emotional disturbances, and ultimately, death[3]. The length of polyQ expansion in htt determines disease severity and is inversely proportional to age of onset[4]. The threshold for HD development is >37 polyQ residues, while individuals with 11–34 glutamine residues are unaffected[5]. Htt is ubiquitously expressed in various tissue types, but it is the most abundant in neurons[6]. Htt interacts with many different proteins, such as transcription factors, heat shock proteins, and actin cytoskeleton[5]. Expanded htt is thought to undergo a conformational change to form insoluble protein aggregates or inclusion bodies (IBs) in neuronal cell nuclei and cytoplasm, a pathological hallmark for HD[7,8]. IBs may function as a protective cellular coping response[9]. Despite a plethora of research, the exact role of htt in the pathogenesis of HD remains unclear.

Several transgenic mouse models have been developed for HD. These include the R6/2 (that expresses a mutant htt fragment exon 1), and the YAC128 and BACHD mouse models (that express mutant full-length htt). The R6/2 mouse was the first transgenic mouse model of HD and has been extensively characterized[10]. The mouse has a transgene that expresses ~150 CAG repeats in the *IT-15* gene (exon 1) under the control of the endogenous human *IT-15* promoter. R6/2 mice develop a progressive neurological

phenotype with a movement disorder and weight loss similar to that in HD[10]. These mice also have neuronal inclusions in the brain. The advantages of the R6/2 mice are that they develop a homogenous phenotype and disease onset occurs as early as 5 weeks. However, they have a more extensive distribution of neuronal inclusions than in HD patients. The YAC128 mouse model expresses full-length htt with 128 CAG repeats from a yeast artificial chromosome transgene while the BACHD model expresses full-length htt from a bacterial artificial chromosome[11,12]. In both models, disease progression is much slower than in the R6/2 mouse. These mice develop age-related motor dysfunction, behavioral abnormalities, and selective neurodegeneration of medium spiny neurons of the striatum[13]. Although there has been some controversy regarding which model is the most relevant for HD, the consensus is that each these models recapitulate at least some important aspects of the disease phenotype and are widely used for studies of HD[14].

HD Involves Non-Neuronal Cell Types

HD is widely recognized as a movement disorder that is associated with the massive loss of MSN in the striatum. However, many studies have indicated that pathology is also observed in other brain regions and in non-neuronal tissues[15-20]. In fact, pathological phenotypes in peripheral tissues, such as weight loss and altered glucose homeostasis, are robust in HD patients and are thought to be caused in part by mutant htt expression in peripheral cells[15-17,19]. As well, other peripheral cell types such as fibroblasts, lymphocytes, and erythrocytes from HD patients and/or HD mouse models also appear to be abnormal, most likely due to expression of mutant htt[17,21]. These changes in HD

peripheral tissues emphasize that the brain may not be the only contributing system to HD pathogenesis.

The Immune System is Abnormal in Pre-manifest HD Patients

While abnormal inflammation in the CNS might contribute to neurodegeneration, a number of recent studies indicate that the peripheral immune system may also play an important role in several neurodegenerative diseases. For example, plasma levels of interleukin (IL)-1β and IL-6, cytokines that can initiate an inflammatory response, are elevated in the plasma of Alzheimer's disease (AD) patients[22,23]. In multiple sclerosis (MS), the adaptive immune cells (T and B cells) clearly contribute to the disease manifestation[24], and innate immune responses also likely contribute to neurodegeneration[24]. In Parkinson's disease (PD), high concentrations of IL-6 may predict an increased risk of developing the disease[25]. In ALS, levels of IL-6, monocyte chemoattractant protein-1α (MCP-1α), plasma transforming growth factor-β1 all increase with disease progression[26]; these are chemokine or cytokines that participate in immune cell signaling or recruitment.

The immune system is also be abnormal in HD. Levels of serum soluble immune markers such as tumor necrosis factor (TNF) receptor, IL-2-receptor, and immunoglobulins are elevated in HD patients[27]. Plasma samples from HD patients also have increased levels of pro-inflammatory cytokines (i.e., IL-6, IL-8, and TNF-α that correlate with disease progression[28]. These increases are significant 16 years before the onset of other HD symptoms, like chorea. It was further demonstrated that monocytes, macrophages, and microglia isolated from HD patients and/or mouse models are

hyperactive in the production of these pro-inflammatory cytokines in response to stimuli. IL-6 can lead to activation of the complement cascade. In fact, a key modulator of the complement cascade, clusterin, is found at increased levels in the blood of HD patients[29]. In addition, chemokines are central to processes related to infection, migration of leukocytes into the CNS, and modulation of the function of blood brain barrier; five peripheral chemokines (eotaxin-3, macrophage inflammatory protein-1β, eotaxin, MCP-1,-4) are increased in HD (Wild et al., *PLOS Currents HD*, in press). The transcription of genes in HD blood is deregulated and associated with disease progression[17,30,31]. For example, immediate early response 3 (IER3), a gene that functions to protect cells from TNF-α-induced apoptosis, is upregulated in HD blood cells[32]. Mitochondrial dysfunction and the apoptotic protein, Bax, are also increased in lymphocytes and monocytes from HD patients[33]. Together, these studies provide strong evidence that the peripheral immune system might be abnormal in HD.

Microglia are the Immune Cells in the Brain

Microglia are the resident immune cells of the CNS[34]. Their developmental origin is controversial; however increasing evidence indicates that these cells are of myeloid origin and are the mononuclear phagocytes in the brain[35-37]. In animals that lack the transcription factor PU.1 (*Pu.1*$^{-/-}$ mice), in which cells in the myeloid lineage cannot differentiate, microglia are also undetected in the CNS of these mice[35]. The myeloid progenitors of the yolk sac are the source for the embryonic wave of myeloid cells that colonize the developing brain; adult microglia are derived from these precursors in a restricted time frame (~E8.0)[37].

Microglia have the same myeloid lineage as the peripheral immune cells that include monocytes and macrophages; they also have many similar functions as their peripheral counterparts, along with some important functions in the brain-specific functions. They display many cell-surface antigens found for macrophages, including CD11b, CD11c, CD40, CD45, CD64, CD68 and MHCI/II[34,38]. In the inactivated state, microglia extend and retract their processes to actively survey the microenvironment[39]. They also provide trophic support for neurons, such as producing trophic factors and physically interacting with neuronal synapses[40]. When the brain is exposed to pathological insults, microglia become activated, transform from their ramified morphology to amoeboid/phagocytic, release cytotoxic substances (i.e., oxygen radicals, nitric oxide) or cell-signaling molecules (i.e., pro-inflammatory cytokines, tumor necrosis factor-α), and migrate or extend their processes toward the site of injury. Microglia may also increase expression of surface antigens as part of their normal function as antigen-presenting cells (APCs) to further elicit immune responses[41]. These changes in activated microglia may be protective or toxic to the normal or disease brain. Ultimately, their dynamic functions are likely to play an important role in the CNS during normal and pathological states.

Microglia and HD

Microglia have been long overlooked in HD; however, recent studies increasingly implicate their potential role in the disease. Coincident with selective neuronal loss in the cortex and striatum, reactive microglia are found in these regions in HD brains[42,43]. Reactive gliosis also occurs in early stages of neuronal dysfunction in HD mouse models[44]. Recently, a study demonstrated that, beginning at 2–4 weeks of age, microglia

in the R6/2 mouse model show more ferritin immunostaining than their wild-type littermates. Ferritin accumulation is often indicative of cellular dysfunction and thought to contribute to pathology in various neurodegenerative diseases[45]. These changes also progressed with disease severity[46]. Microglia showed morphological changes, such as thickening and dystrophy of their processes, which began at 8–10 weeks of age. Brains from mid-stage HD patients also had increased ferritin immunostaining in microglia, many of which are dystrophic[46]. A second study also showed that R6/2 brains have microglia with condensed nuclei and fragmentation of their processes at 14.5 weeks of age[47].

Abnormal microglia have also been described in the brains of pre-manifest HD patients. Positron emission tomography from HD patients also showed an increase in binding of ^{11}C-(R)-PK11195, a surrogate marker for microglial activation *in vivo*, in the striatum and cortex that correlates to HD severity[48,49]. Furthermore, microglial activation in brain regions required for cognitive function can predict disease onset[50]. Although there is strong evidence demonstrating that microglia and their dysfunction may be an important contributor to HD pathogenesis, these previous studies are merely descriptive and do not establish a causal link between any of these abnormalities and neurodegeneration.

Bone Marrow Transplantation As a Method to Study Immune Cells in Neurological Diseases

Several studies have addressed the effects of bone marrow transplantation (BMT) ostensibly as means to understand the role of microglia in mouse models of

neurodegeneration; however, these studies have some important caveats. The bone marrow derived cells (BMDCs) that enter the brain, though often referred to as microglia, are really bone marrow (BM)-derived brain macrophages which are not identical to parenchymal microglia, which populate the CNS in development. In addition, total body irradiation causes breakdown of the blood brain barrier and leads to infiltration of peripheral cells into the CNS[51]. Nonetheless, these studies still provide insights on how replacement of the peripheral immune system, and introduction of BMDCs in the brain may play a role in neurodegenerative diseases.

In AD, introduction of BM-derived brain macrophages may be neuroprotective. AD patients have reactive microglia that reside by senile plaques; BMT study in mouse models further showed that BMDCs can be recruited to these plaques[52,53]. Cells are recruited to the brain in young AD mice (before disease onset) than in old AD mice (after disease onset); this was associated with a greater decrease in hippocampal Aβ burden[53]. Another BMT study also demonstrated that amyloid deposits were eliminated by phagocytosis by bone marrow derived brain macrophages[54].

In ALS, strong activation and proliferation of microglia occur in regions of neuronal loss; as such, BMT and selective reduction of the mutant protein in microglia were used to evaluate the role of microglia[55]. The most common inherited form of ALS is caused by the ubiquitously expressed mutant superoxide dismutase ($mSOD1^{G93A}$). $mSOD1^{G93A}$ mice were genetically modified to lack cells of myeloid lineage, including microglia (breeding with $Pu.1^{-/-}$) to determine whether expression of $mSOD1^{G93A}$ in microglia was required for disease[55]. Wild-type donor-derived brain macrophages slowed neuronal loss and prolonged disease duration and survival, in comparison to $mSOD1^{G93A}$ mice or

mSOD1^{G93A}/*Pu.1*$^{-/-}$ mice that received mSOD1^{G93A}-expressing cells[55]. In a separate study, mSOD1^{G93A} reduction in microglia had little effect on the early disease phase but sharply slowed later disease progression[56]. These studies, however, did not account for the possibility that the improvements may also be contributed by the absence/reduction of mSOD1 in peripheral immune cells. Nevertheless, these past studies open the question if BMT and wild-type BMDCs could also influence pathogenesis in mouse models of HD.

Based on the evidence that microglia and the peripheral immune cells are affected in HD, and that mutant htt is ubiquitously expressed, in this thesis we test the hypothesis that the expression of mutant htt in microglia and peripheral immune cells, such as monocytes and macrophages, leads directly to changes in their cellular functions. We first focused on how mutant htt affects the normal functions of microglia and peripheral monocytes and macrophages. We also evaluated if mutant htt in immune cells could be a contributing factor to HD pathogenesis by performing bone marrow transplants in HD mouse models. In **Chapter 2**, we show that expression of mutant htt lead to cell autonomous changes in microglial cell lines and primary microglia. These changes include increased production of pro-inflammatory cytokines IL-1β and IL-6. We also observed a mutant htt-mediated decrease in insulin growth factor 1 (IGF-1) production. However, despite these changes, other canonical activation phenotypes such as increased ROS levels and expression of cell surface antigen markers were unaffected by mutant htt expression in these cells. In **Chapter 3**, we show that mutant htt causes profound migration impairment in both microglia and peripheral immune cells. We also show that microglia have delayed process extension in response to laser ablation injury in brains of living HD mice, and that peripheral macrophages also have defective recruitment toward

the inflammation site upon peritonitis induction. Importantly, migration deficits were observed in monocytes isolated from the blood of HD patients even prior to the onset of neurological and motor symptoms. We found that migration defects might be due to impairment of actin remodeling by mutant htt. In **Chapter 4**, we show the effects of BMT on pathogenesis *in vivo* in two mouse models of HD. We show that HD mice with BMT have modest but significant improvements of behavioral phenotypes, increased levels of synaptophysin, and normalized levels of peripheral inflammatory cytokines and chemokines. Our results collectively indicate that mutant htt indeed impairs the function of immune cells in a manner that may contribute to HD pathogenesis.

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Chapter 2

Mutant Huntingtin Expression Induces Cell Autonomous Effects in Microglia

Abstract

Recent studies suggest Huntington's disease (HD) may involve non-neuronal cells including astrocytes and microglia. Although mutant huntingtin (htt) is ubiquitously expressed, functional consequences of its expression in microglia have not been explored. Here, we report on a cellular model that we developed to study the functional consequences of expression of a mutant htt fragment in microglia. A series of lentiviral constructs were used to generate immortalized microglial cell lines that express a mutant htt fragment with 25, 46, 72, and 103 polyglutamine (polyQ) repeats fused to the green fluorescent protein (GFP). We found that mutant htt expression in these cell lines leads to increased production of pro-inflammatory cytokines and decreased nitric oxide (NO) production in response to lipopolysaccharide (LPS) and interferon-γ (IFN-γ). These cells also had a basal reduction in production of insulin growth factor 1 (IGF-1). Similar changes were obtained with primary microglia isolated from transgenic mouse models of HD. Furthermore, conditioned media from microglia that express a mutant htt fragment with 72Q, but not 25Q, is toxic to primary neurons. These results indicate mutant htt may impair microglial functions in a manner that might contribute to pathogenesis in HD.

Introduction

Huntington's disease (HD) is an autosomal dominant neurodegenerative disorder caused by an expansion of CAG triplet repeat codons in the *IT-15* gene, which results in a polyglutamine expansion in the protein huntingtin (htt). The disease is characterized by selective neuronal death primarily in the cerebral cortex and striatum. A major pathological feature is the selective loss of medium-spiny projection neurons (MSN) in the striatum that are involved in the inhibitory circuitry of movements[1,2]. Htt is ubiquitously expressed in various tissue types, but is most abundant in neurons[3].

Historically, much attention has been focused on MSN since they are the most vulnerable cell type in HD; however, recent studies support the hypothesis that neurodegeneration in HD likely involves other neuronal populations and also non-neuronal cells. For example, transgenic HD mouse models that were generated to express mutant htt only in cortical or striatal neurons showed no obvious gliosis, motor deficits and neuropathology. This study suggests that cell-cell interactions play a critical role in HD[4]. Furthermore, expression of mutant htt in astrocytes in mice leads to gliosis in the brain and cognitive impairment. This study supports the hypothesis that mutant htt in glial cells contributes to pathogenesis in HD[5].

A number of studies have shown that microglia, the immune cells in the brain, are abnormal in HD and may play a role in the disease. Coincident with selective neuronal loss in the cortex and striatum, reactive microglia are found in these regions in HD brains[6,7]. Reactive gliosis also occurs in early stages of neuronal dysfunction in HD mouse models[8]. Microglia in postmortem HD brains and brains from a HD mouse model show more ferritin immunostaining than control mice, in a manner that correlates with

disease progression[9]. Positron emission tomography from HD patients also show an increase in binding of 11C-(R)-PK11195, a surrogate marker for microglial activation *in vivo*, in the striatum and cortex that correlates to HD severity[10,11]. Furthermore, microglial activation in brain regions required for cognitive function can predict disease onset[12]. However, these previous studies are merely descriptive and do not establish a causal link between these abnormalities and neurodegeneration. It is unclear whether these changes are a response to neuronal degeneration, or rather, are due to a cell autonomous effect of mutant htt expression, or both.

Microglia are the mononuclear phagocytes in the brain. In the inactivated state, microglia extend and retract their processes to actively survey the microenvironment[13]. They also provide trophic support for neurons and physically interact with synapses[14]. These cells are activated upon stimulation such as CNS injury[15]. Their rapid responses include cell migration, proliferation, and release of soluble factors such as nitric oxide (NO), and cytokines[15]. They may also increase the expression of surface antigens like MHC-II to function as antigen-presenting cells to elicit further immune responses.

To determine if mutant htt expression may influence the function of microglia, we generated and characterized immortalized cell lines that express a mutant htt fragment. We found that a mutant htt fragment caused decreased production in nitric oxide (NO) and increased levels of proinflammatory cytokines upon stimulation. Production of insulin growth factor 1 (IGF-1) was also decreased at the basal level. Primary microglia isolated from HD mouse models also had similar changes in cytokines and IGF-1 levels. We also found that conditioned media from microglia expressing a mutant htt fragment

led to toxicity in neurons. Our results suggest that abnormalities in microglia may be due in part to cell autonomous effects of mutant htt in these cells.

Methods

Animals and breeding strategy. The University of California San Francisco and the University of Washington IACUC Committee approved all experiments and procedures involving mice. Mice were maintained and bred in accordance with National Institutes of Health guidelines. YAC128 founder mice (FVB/NJ background) were kindly provided by Dr. Blair Leavitt (University of British Columbia). R6/2 found mice (C57BL/6) have been described[16], with 150-207 CAG repeats in these experiments.

Generation of lentiviral constructs to express htt fragments in microglia. We generated a transfer vector with exon 1 htt fused to GFP based on pRRL-cPPT-CMV-X-PRE-SIN. The VSV-G-pseudotyped lentiviruses were produced by cotransfecting 293T cells with the transfer vector and three packaging vectors and purified by ultracentrifugation. The viral particle concentration was determined by measuring viral p24gag antigen. Primary microglia and the microglial cell lines N9 and BV2 were plated in 24-well plates at a density of 1 x 10^4 cells per well and cultured for 72 h. Viral particles (1–10 μl) were added to the wells, and the cells were cultured for 72 h. For N9 and BV2 cells, viral particles (1–10 μl) were added to the wells 1 day after plating. GFP expression was determined by fluorescence microscopy (LSM 510 Zeiss). Clonal cell lines from N9 cells that stably expressed 25Q and 72Q htt were generated by sorting transduced cells with similar fluorescent intensities into 96-well plates by flow cytometry (FACS-DIVA, Becton Dickinson), monitored for clonal populations, and expanded into clonal cell lines. Expression of htt in each clonal line was analyzed by qRT-PCR and immunoblotting. Viral copy numbers were also determined by qPCR. Pairs of 25Q and 72Q htt expressing

clones with similar htt expression and low viral copy numbers were selected for subsequent experimentation (data not shown).

Immunoblots of transduced cell lines. Microglial cell lines were lysed in RIPA buffer (150 mM NaCl, 1% NP-40, 0.1% SDS, 0.5% sodium deoxycholate, 50 mM Tris, pH 7.6) with complete protease inhibitors (Roche). Soluble proteins (25 μg) were fractionated on 4-20% SDS-PAGE gels (Pierce) and blotted onto nitrocellulouse membranes (Schleicher and Schuell) with a transfer apparatus (Bio-Rad) submerged in transfer buffer (25 mM Tris, 192 mM glycine, 20% v/v methanol). Membranes were blocked for 1 h in 4% nonfat dried milk in PBS, 0.1% Tween-20 (PBST) and incubated with gentle agitation for 1 h at room temperature with anti-EM48 (1:500, Chemicon), anti-GFP (1:1000, Chemicon), or anti-GAPDH (1:1000, Chemicon). Blots were washed three times in PBST, probed with horseradish peroxidase–linked anti-mouse secondary antibody (1:10000, Jackson Immunoresearch) in PBST with 0.5% nonfat dried milk for 1 h at room temperature, and washed three times in PBST. Protein was detected by chemiluminescence (ECL kit, Amersham Biosciences), according to the manufacturer's instructions.

Cell culture. Primary microglia were isolated from P1-3 mice as described[17]. Briefly, brains were dissected, trypsinized and triturated into single cells and cultured in Dulbecco's modified Eagle's medium with high glucose (DME-H21) with 10% heat-inactivated FBS and penicillin and streptomycin. After 10-20 d, cultures were gently shaken by hand, and microglia collected as floaters, resulting in >99% purity. Granulocyte macrophage-colony stimulating factor (GM-CSF, 2 ng/ml) was used to help stimulate microglial yield. Isolated cells were incubated with macrophage serum-free

medium (MSFM, Invitrogen) for 24 h, unless otherwise specified, and used as required. Microglial cell lines were culture in DME-H21 with 5% heat-inactivated FBS. Before use, cells were incubated with MSFM for 24 h.

Flow cytometry, nitric oxide (NO), TNF-α, reactive oxygen species (ROS) detection. Single-cell suspensions were pretreated for 20 min at RT with Fc-block (anti-CD16/CD32; Caltag) and then incubated for 30 min at RT with anti-MHCII conjugated with APC (1:1000, eBioscience). Cells were analyzed on a FACSCalibur (Becton Dickinson). NO and TNF-α levels were assessed in the supernatants at 24 h poststimulation. NO production was assessed using the Griess reagent in accordance with the manufacturer's instructions (Biovision). TNF-α concentrations were determined by using Duo set enzyme-linked immunoassay (ELISA) kit (R&D Systems). Cells were stained with dihydrorhomdamine 123 for ROS detection according to manufacturer's instructions (Invitrogen).

qPCR. Total RNA was isolated from mouse and human samples with the RNeasy mini kit (Qiagen). mRNA was reverse-transcribed with SuperScript III (Invitrogen), according to the manufacturer's instructions. Probe-based qRT-PCR was performed in triplicate with Roche Universal Probe Library primers and probes as suggested by https://www.roche-applied-science.com/sis/rtpcr/upl/acenter.jsp?id=030000 (Roche Diagnostics) and FastStart TaqMan Probe Master (ROX) mix (Roche) or Sybr Green mix (Applied Biosystems) with a PRISM® 7500 qPCR System (Applied Biosystems). Purity of mRNA was checked by performing qPCR without prior RT. qPCR on mouse tissue and microglial samples were normalized against the geometric mean of the housekeeping genes ATP5, eIF4A2 and/or HPRT[18].

Neuronal cocultures in microglia-conditioned medium. Neuronal cultures were prepared from dissected cerebral cortices of embryonic day 16-18 mice. Viable cells were plated at 5 x 10^5 cells/ml on poly-D-lysine/laminin-coated coverslips in B27-supplemented neurobasal medium (Invitrogen). For microglia conditioned-medium neuronal cultures, microglia expressing empty vector or vector encoding 25Q or 72Q htt exon 1 were grown at 10,000 cells/ml on poly-L-lysine coated coverslips, cultured in macrophage serum-free medium for 24 h in the presence or absence of lipopolysaccharide (LPS, 100 μg/ml) and interferon-gamma (IFN-γ, 10 units/ml), and cocultured with DIV7 neurons for 24 h. For direct microglia-neuron cocultures, we placed 50,000 cells/ml directly onto DIV 7 neurons. Coverslips with neurons were fixed in 4% paraformaldehyde in PBS for 20 min at room temperature. After permeabilization in PBS with 0.1% Triton X-100 for 10 min, cells were placed in blocking buffer (PBS with 10% FBS and 0.01% Triton X-100) for 30 min. The primary antibody to MAP2 (1:500, Sigma) was applied in this blocking buffer for 2 h at room temperature or overnight at 4°C and visualized with anti-mouse IgG conjugated with Texas Red (1:500, Vector Laboratories) in blocking buffer. Fluorescence images were captured at 20x using fluorescent microscopy, and neurons with normal morphology were counted in 6–10 random fields in triplicates for each experimental group. Four independent experiments were performed.

Results

Stable expression of 25Q and 72Q htt in microglia

Although mutant htt is ubiquitously expressed, its effects on the function of microglia are unknown. We generated lentiviral constructs that express a htt fragment (exon 1) with 25, 46, 72, and 103 polyQ repeats fused to GFP (Fig. 1A). N9 and BV2 are murine microglial cell lines widely used as microglial cell models, both of which retain most of the morphological, phenotypical and functional properties as primary microglial cells[19,20]. BV2[20] cell line particularly express phagocytic properties, while N9[19] cells have robust secretory properties, such as cytokine production. The expression of htt fragments after transduction in N9 and BV2 microglial cell lines was confirmed by western blotting with antibodies against htt and GFP (Fig. 1B). The apparent molecular mass of protein bands was as predicted, and the level of expression across different polyQ repeats was similar, with the exception of 103Q htt, which was expressed at a slightly lower level. No insoluble fraction was observed in the stacking gel (data not shown). We further characterized the expression of mutant htt in transduced microglial cell lines and primary microglia by fluorescence microscopy (Fig. 1C). As observed in neurons, a htt fragment with 25Q displayed a diffuse cytoplasmic expression pattern in microglial cells. In contrast, fluorescence in microglial cells expressing 46, 72, and 103Q htt formed perinuclear or intranuclear inclusion bodies (IBs). These IBs were found only in 3–5% of transduced cells (data not shown) and are consistent with a previous study in the R6/2 transgenic mouse model of HD[16]. That study showed that IBs were found in a low percentage of microglia by electron microscopy[21].

Expression of mutant htt in microglia leads to increased production of cytokine interleukin (IL)-1β and IL-6 and decreased production of NO

Microglia are macrophage-like cells that produce toxic factors, such as cytokines and NO, as part of their normal function upon stimulation. Cytokines like IL-1β and IL-6 are important mediators of the inflammatory response that are increased in HD and/or other neurodegenerative diseases[22-25]; NO can lead to neuronal cell death by production of reactive radicals[26].

Levels of proinflammatory cytokines IL-1β and IL-6 were increased in microglia expressing mutant htt. Microglia expressing 72Q htt produced higher protein levels of IL-6 ($P<0.05$) and a strong trend for an increase in IL-1β ($P=0.0553$, Fig. 2A, B). Because cell line models may not always reflect the biology of primary cells from disease mouse models, we confirmed this effect on primary microglia cultured from R6/2 mice, which also displayed increased levels of IL-1β and IL-6 ($P<0.05$, Fig. 2C, D). Furthermore, we obtained striatal tissues from brains of 14 week old R6/2 mice and observed similar results ($P<0.05$, Fig. 2E, F). In addition, we wanted to determine if this increase in IL-1β and IL-6 levels is also found in microglia that express full-length htt. We isolated primary microglia from a full-length htt model, YAC128, which develops phenotypes more slowly than the R6/2 model and has been used in numerous HD studies[27]. We found increased gene expression levels of both cytokines in primary microglia from these mice (Fig. 2G, H), and are in the process of determining if similar changes are observed at the protein level. These results together indicate that the expression of mutant htt in microglia is sufficient to cause an increase of some pro-inflammatory cytokines known to be increased in brains from HD patients.

To our surprise, microglial cell lines N9 and BV2 that expressed 72Q htt released less NO than 25Q cells upon stimulation with LPS/IFN-γ (Fig. 3A, B). We also cultured primary microglia from the fragment mutant htt model, R6/2, and the full-length htt model, YAC128, to evaluate NO production levels. However, no changes were found (Fig. 2C, D).

We also evaluated ROS levels and expression of the cell surface antigen marker MHC-II in primary microglia from YAC128 mice in response to LPS/IFN-γ stimulation. Normally, when microglia are activated, they increase ROS production; they may also increase MHC-II to act as antigen presenting cells to elicit further immune responses. However, there were no changes in ROS levels or MHC-II expression in primary microglia from YAC128 mice compared to the controls after LPS/IFN-γ stimulation (Supplementary Fig. 1A,B). Our preliminary data with microglial cell lines expressing a mutant htt fragment also suggest the same result.

Expression of mutant htt impairs IGF1 production in microglia

A normal function for microglia is to provide trophic support for neurons; thus we evaluated the level of IGF-1 in microglia expressing mutant htt. IGF-1 is a neurotrophic factor with an important role in ageing. Over-expression of IGF-1 in mouse models of Parkinson's disease and amyotrophic lateral sclerosis (ALS) restored neuronal functions and prolonged survival, respectively[28,29]. Microglial cells are the predominant source of IGF-1 in the adult CNS[30]. We found that in microglial cell lines expressing 72Q htt, there is a severe reduction in IGF-1 protein levels relative to the control ($P<0.001$, Fig. 4A). This decrease in IGF-1 levels was also found in primary microglia from R6/2 mice

(P<0.01, Fig. 4B). We also determined the gene expression level of IGF-1 in striatal brain tissues obtained from R6/2 and YAC128 mice. In the R6/2 striatum, IGF-1 expression level is significantly lower than the control (P<0.001, Fig. 4C), while there may be a trend for decrease in the striatum of YAC128 mice (P<0.1412, Fig. 4D). These results suggest that decreased IGF-1 levels that are found in brains of HD mice might be due to cell autonomous effects of mutant htt in microglia.

Conditioned media from microglia that express a mutant htt fragment induce toxicity in cortical neurons

We next determined if conditioned-medium from microglia that express 72Q mutant htt is neurotoxic. In the absence of stimuli, conditioned medium from 72Q htt cells resulted in 40% fewer (P < 0.01) viable neurons compared to 25Q htt (Fig. 5A, B). Interestingly, this reduction in viable neurons was similar to a positive control in which conditioned media was collected from 72Q htt cells that were pretreated with LPS and IFN-γ. These results indicate that microglia that express mutant htt with expanded polyQ, but not htt with normal polyQ length, secrete substances that are toxic to cortical neurons.

We also evaluated the effect of primary microglia from YAC128 mice on cortical neurons cultured from wild-type mice. Because initial experiments suggested that the quantity of primary microglia we obtained in our experiments might not be sufficient to secrete high enough concentrations of toxic substances (if any) in the media, instead of using the conditioned media approach, we performed direct primary microglia-neuron co-cultures. This method might allow the effects of primary microglia to be more sensitive

on neurons. However, there was no neurotoxicity in these direct co-cultures of primary microglia from YAC128 mice and wild-type cortical neurons (Fig. 5C).

Discussion

Here we describe the direct effects of mutant htt on the biology of microglia. We generated a new cellular model in which microglia express a mutant htt fragment. Using these microglial cell lines, we found that expression of htt 72Q led to increased levels of cytokines IL-1β and IL-6 and decreased production in NO upon LPS/IFN-γ stimulation. Production of insulin growth factor 1 (IGF-1) was also decreased at the basal level. Primary microglia isolated from HD mouse models also had similar changes in these specific cytokines and IGF-1 levels. We also found that conditioned media from microglia expressing a mutant htt fragment led to toxicity in neurons. Our results together suggest that mutant htt may impair microglial functions in a manner that might contribute to pathogenesis in HD.

Our microglial cell lines that express a mutant htt fragment may be useful for future mechanistic studies. The changes we saw in the microglial cell lines expressing htt 72Q were mostly reproduced in primary microglia from the R6/2 mice, a highly characterized transgenic HD mouse model. Primary microglia isolated from the YAC128 full length htt mouse model also suggested similar results, thereby suggesting that these microglial cell lines, though expressing a mutant htt fragment, might also be a useful tool to identify the changes found on microglia that express full length mutant htt. In addition, we also found that conditioned media from microglial cell lines expressing 72Q htt are toxic to primary neurons. This property might be useful for additional studies that evaluate the mechanisms of mutant htt expression in microglia pertaining to toxicity in neurons. In fact, microglia-specific genetic modifiers of mutant htt are currently being investigated in these microglial cell lines to evaluate effects in co-cultures with neurons (Mason *et al*., unpublished data).

Our results showing increased expression of pro-inflammatory cytokines IL-1β and IL-6 suggest that mutant htt can induce an inflammatory response. IL-1β is the key cytokine that leads to induction of the NF-κB signaling pathway that triggers IL-6 production[31,32]. This could explain the increased levels of IL-6 in our study, which is also observed in other HD studies[25,33]. Interestingly, mutant htt interacts directly with the IκB kinase complex[34], a key regulator of the NF-κB signaling pathway, which is activated in neurons in HD[34]. It would be interesting to see if the pathway is also activated by mutant htt expression in microglia, which could potentially explain the higher production levels of IL-6 that we observed. Pro-inflammatory cytokines IL-6 and IL-8 are also evident in the peripheral immune system of presymptomatic HD patients[25]. Monocytes from HD patients' blood and macrophages from YAC128 mice also lead to elevated IL-6 levels upon LPS/IFN-γ[25]. These results, together, suggest an overall dysfunction that might be caused by cell autonomous effects of mutant htt in myeloid cells in the brain and peripheral immune system.

To our surprise, despite some of these changes we observed in microglia expressing mutant htt upon activation, we were unable to identify changes in ROS levels and the expression of surface antigen marker MHC-II in these cells compared to the controls (Supplementary Fig. 1). This finding could suggest that the increased levels of cytokines might a specific mutant htt-mediated effect in microglia, such as the association of mutant htt with the NF-κB signaling pathway as postulated above.

Furthermore, we found decreased IGF-1 levels in microglia expressing mutant htt, which might also contribute to HD pathogenesis. IGF-1 is produced predominantly by microglia in the brain[30] and it has strong anti-apoptotic properties; its absence leads to

neuronal death[35]. It also induces autophagy-mediated clearing of mutant htt aggregates[36]. We recently found that mutant htt affects the transcription level of IGF-1 in microglia, and that over-expression of IGF-1 in mouse neural progenitor cells rescued behavioral deficits in HD mouse models (Silvestroni *et al*., manuscript in preparation). Thus, mutant htt in microglia may lead to decreased IGF-1 production in HD, which may have important implications for pathogenesis.

Our result showed that conditioned media from htt-expressing microglial cell lines could lead to neuronal death; however, the molecular mechanisms involved in neuronal death remains unclear. One possibility is that microglia might produce neurotoxic substances, such as 3-hydroxykynurenine (3-HK) and quinolinic acid in the kynurenine pathway, a pathway thought to contribute to HD pathogenesis. Consistent with this scenario, we found that microglial cell lines that express htt 72Q produced higher 3-HK levels; the presence of an inhibitor of kynurenine 3-monoxygenase (an enzyme in the kyurenine pathway that produces 3-HK) also rescued neurotoxicity from conditioned media of microglia that expressed htt 72Q (Appendix 1).

We also performed co-culture experiments with primary microglia from the YAC128 full-length model and primary WT neurons. Although our experiments did not indicate any neurotoxicity, it is possible that the neurons were dysfunctional but not yet dying. For example, the neurotoxic factors that are present may damage the fine neuronal processes and dendritic spines, which in turn might change the electrophysiological properties of the neurons, all of which would not be detected unless there is more thorough examination[37]. Furthermore, there is also the possibility that microglia that express full-length htt is not as toxic as the cells that express the exon 1 htt fragment, as full-length

mutant htt is thought to elicit some deleterious effects upon the enzymatic cleavage of this large protein and subsequently generate toxic fragments[38]. Overall, further experimentation is needed to determine the effects of primary microglia that express full-length htt on neurons.

Altogether, our results from this study suggest that mutant htt expression is sufficient to induce cell-autonomous effects in microglia. The newly generated microglial cell lines expressing a mutant htt fragment facilitated our studies and were confirmed in primary microglia from HD mice. In the next chapter, we describe a study that shows mutant htt also impairs the migration of microglia. These findings, originally obtained in microglial cell lines, led us to additional studies that found peripheral immune cells in HD patients are also defective in migration. Thus, taken together, these initial results that were obtained in microglia might lead to the identification of novel players that contribute to HD pathogenesis.

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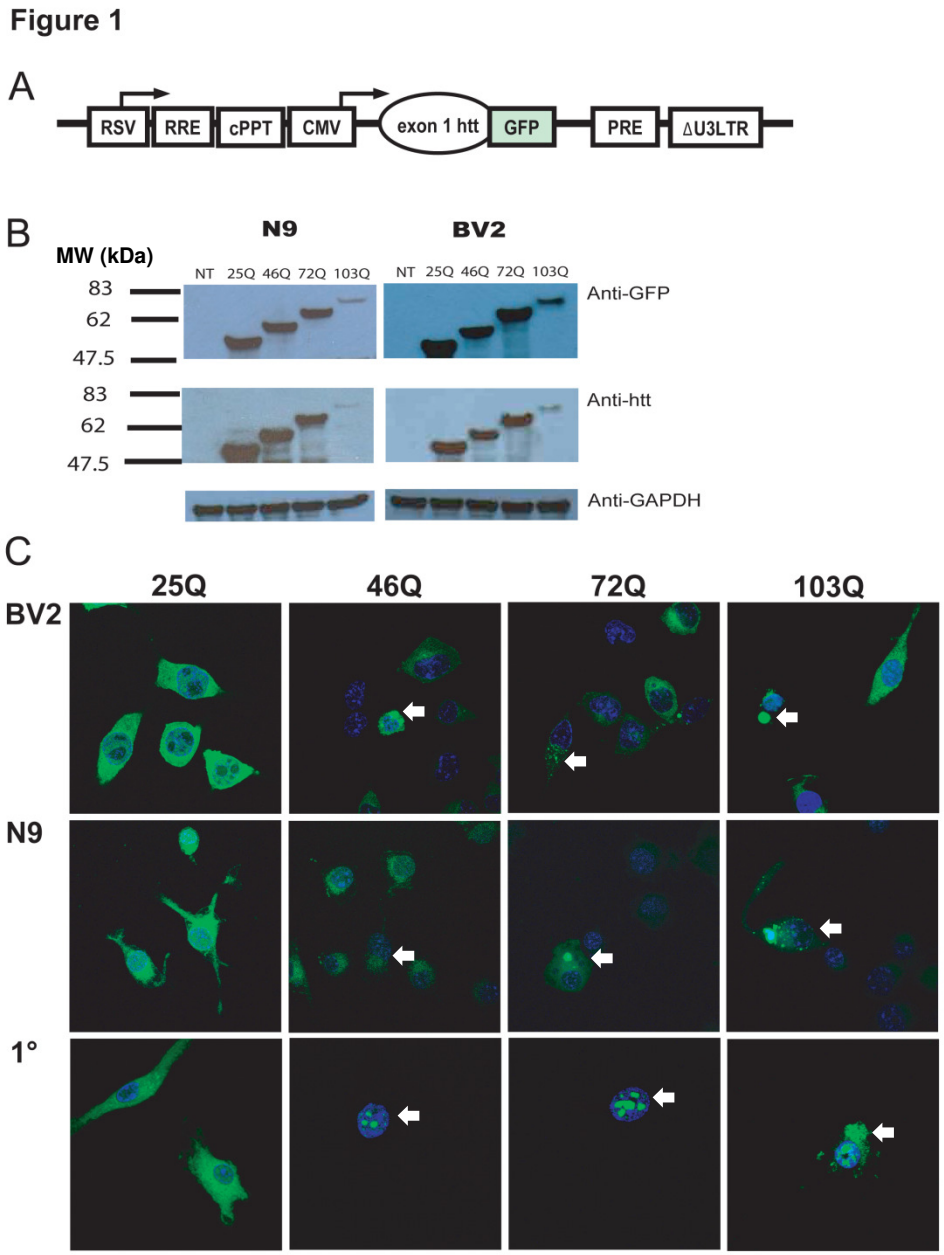


Figure 1. Generation of microglial cell lines that stably express a mutant htt fragment. ***A,*** Schematic of a lentiviral construct that was used to express a mutant htt fragment (exon 1) with 25, 46, 72, and 103 polyQ repeats fused to the green fluorescent protein (GFP). RSV, Rous sarcoma virus; HIV chimeric long terminal repeat; RRE, HIV Rev response element; cPPT, HIV-1 central polypurine tract; MSCV,murine stem cell virus promoter; PRE, human hepatitis virus posttranscriptional regulatory element;

ΔU3LTR, HIV-1 3' long-term repeat with deletion of U3 sequence. ***B,*** Mutant htt expression in transduced N9 or BV2 microglial cell lines. Cell lysates were obtained from non-transduced microglia (NT) and from microglia transduced with lentivirus that express htt exon 1 with 25, 46, 72 and 103Q. Western blots were probed with GFP, htt (EM48), and GAPDH antibodies as indicated. ***C,*** Fluorescent images of of N9 (top) and BV2 (middle) microglial cell lines, and primary mouse microglia (bottom) after transduction with lentivirus that expresses exon 1 htt with 25, 46, 72, or 103Q fused to GFP (green); inclusion bodies (white arrows) are formed in microglia expressing >25Q htt fragment. Nuclei were stained with DAPI (blue). Images were obtained at 60X using LSM 510 Zeiss confocal microscope.

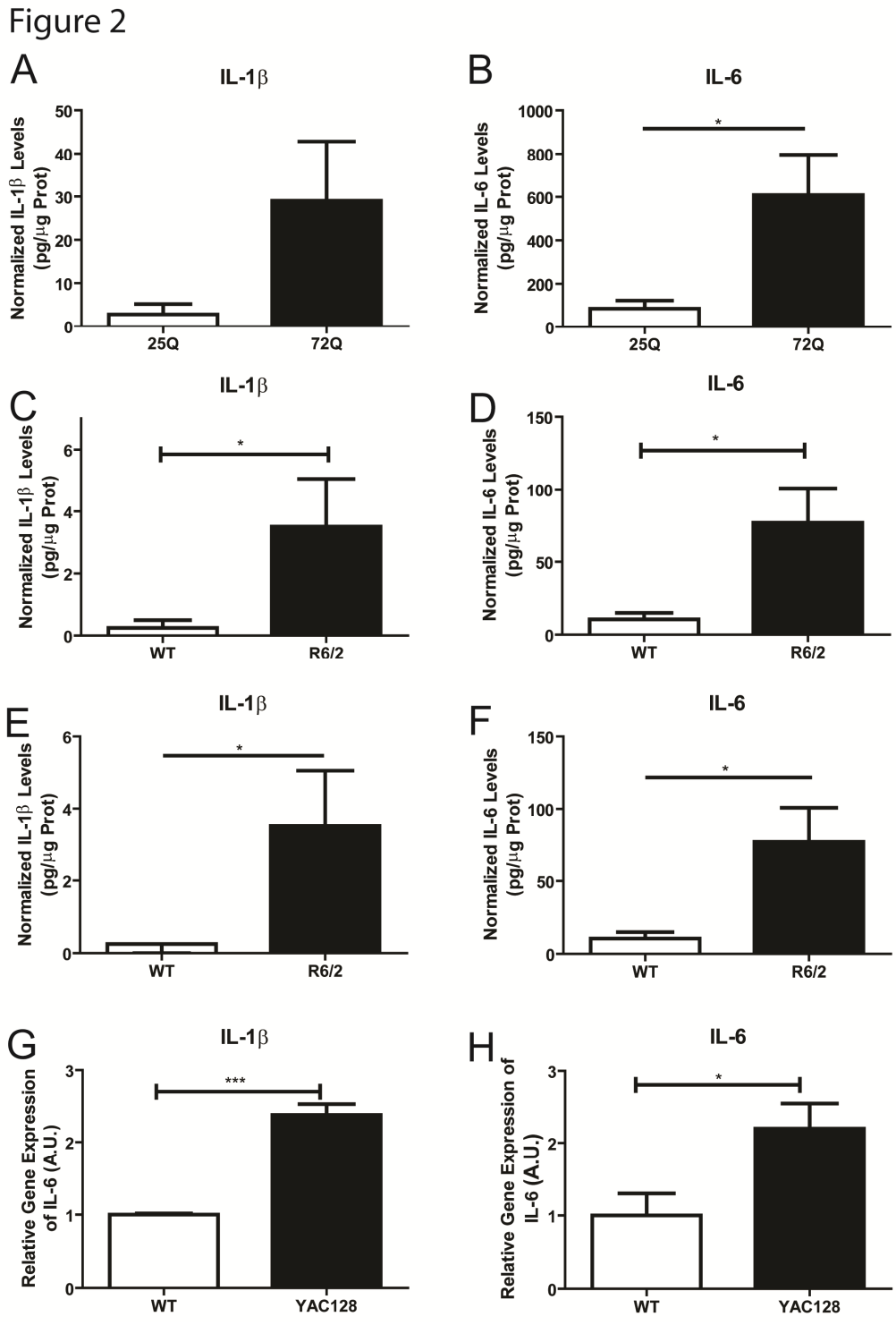


Figure 2: Mutant htt expression in microglia causes increased expression of IL-1β and IL-6 in response to LPS/IFNγ. ***A, B,*** IL-1β and IL-6 protein levels are increased in N9 microglial cell lines expressing htt 72Q compared to htt 25Q upon stimulation with

LPS (100 ng/ml) and IFN-γ (10 U/ml). IL-1β and IL-6 protein levels are increased in (***C, D***) primary microglia upon stimulation with LPS (100 ng/ml) and IFN-γ (10 U/ml) and (***E, F***) striatal brain tissues, from 14 week old R6/2 mice. ***G, H,*** Gene expression levels of IL-1β and IL-6 are increased in primary microglia from YAC128 mouse models upon LPS/IFNγ stimulation, compared to the controls. Values are mean ± SEM (**P*<0.05, ****P*<0.001; *t*-test; n=3-9).

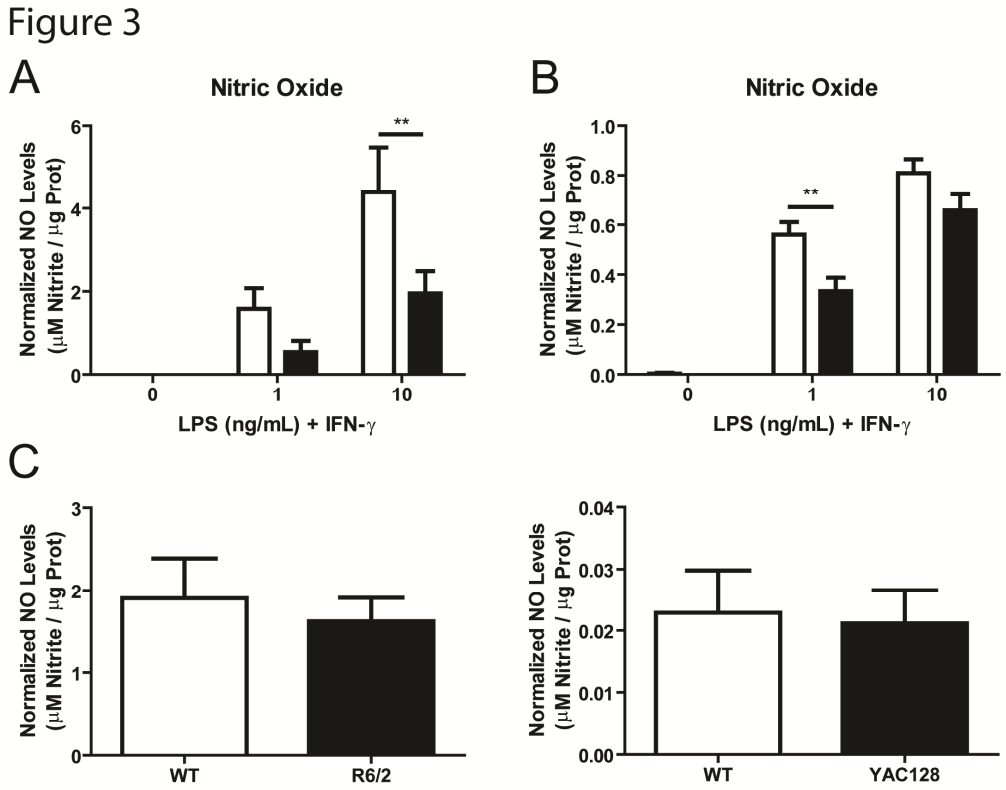


Figure 3: Microglial cell lines expressing a mutant htt expression have decreased levels of NO in response to LPS/IFN-γ stimulation. NO production is decreased in (A) N9 and (B) BV2 microglial cell lines expressing htt 72Q (black bars) upon LPS (0-10 ng/ml) and IFN-γ stimulation (10 U/ml), compared to those expressing htt 25Q (white bars). Values are mean ± SEM (*P*<0.01; two-way ANOVA, Bonferroni posttest; n=3).

NO production levels was not changed in primary microglia from (C) R6/2 and (D) YAC128 mouse models with LPS (100 ng/ml) and IFN-γ (10 U/ml) stimulation. Values are mean ± SEM (P<0.01; two-way ANOVA, Bonferroni posttest; n=4-5).

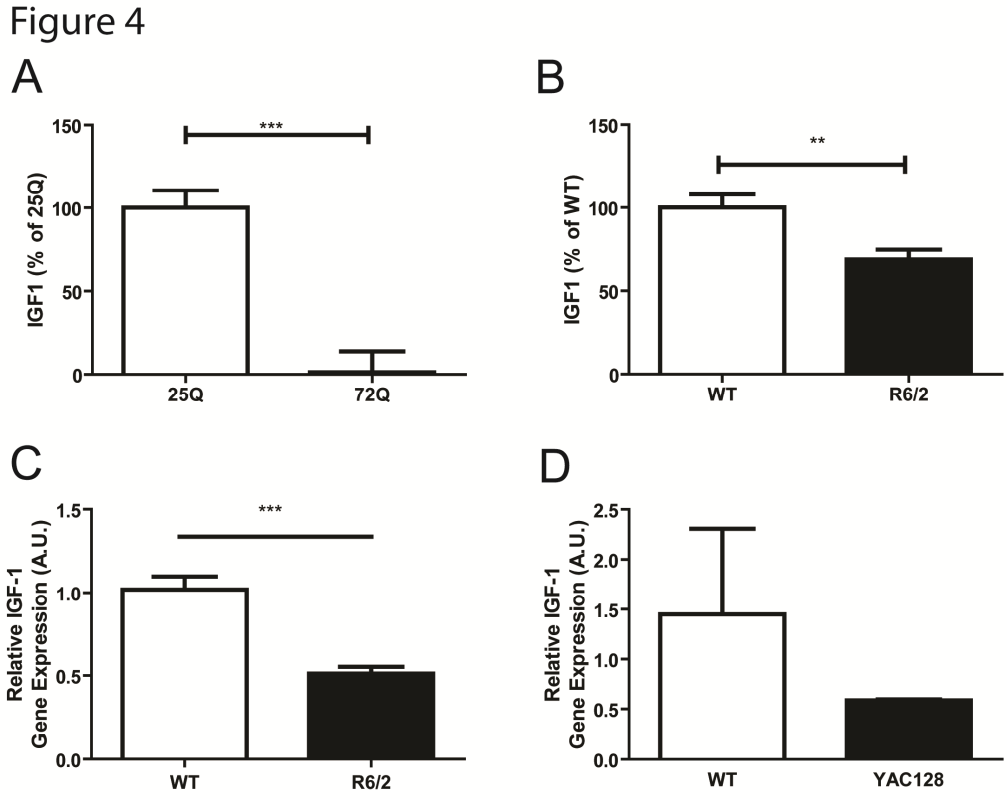


Figure 4: IGF-1 gene expression and/or protein levels are decreased in microglia that express mutant htt and striatal tissues from HD mouse brains. IGF-1 protein levels are lower than controls in (***A***) BV2 microglial cell lines expressing htt 72Q and (***B***) primary microglia from R6/2 mice. ***C, D,*** The gene expression levels of IGF-1 in striatal tissues from (***C***) R6/2 and (***D***) YAC128 mouse brains are decreased compared to their respective wild-type littermates. Values are mean ± SEM (**P<0.01, ***P<0.001; t-test; n=3-8).

Figure 5

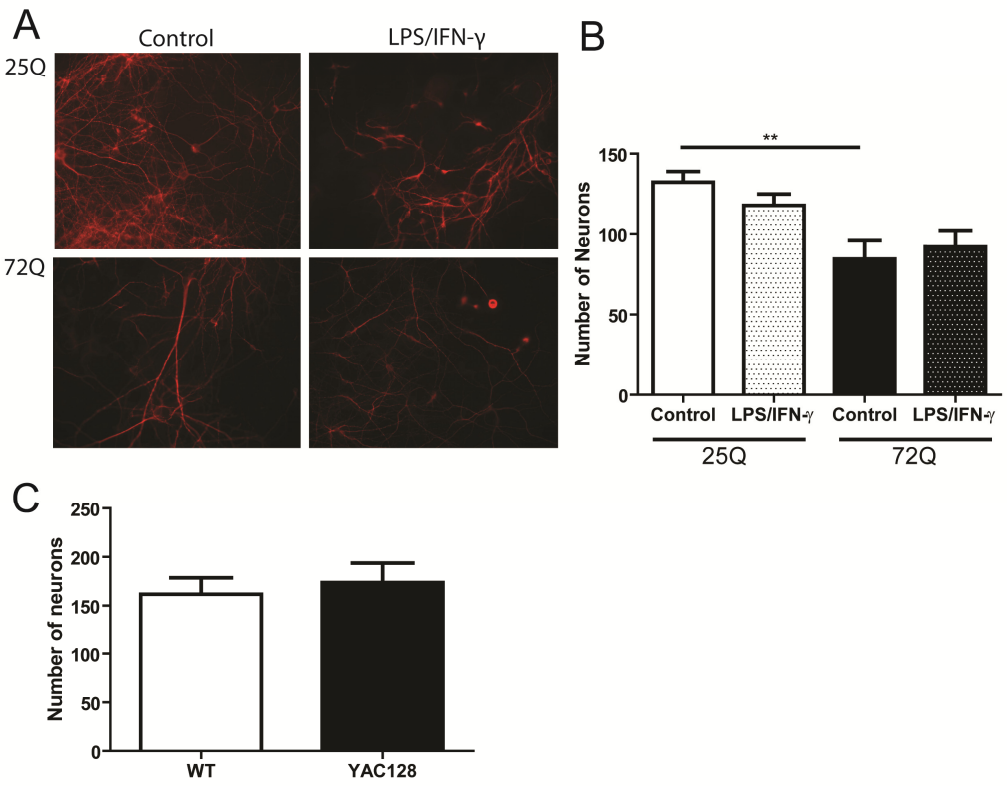
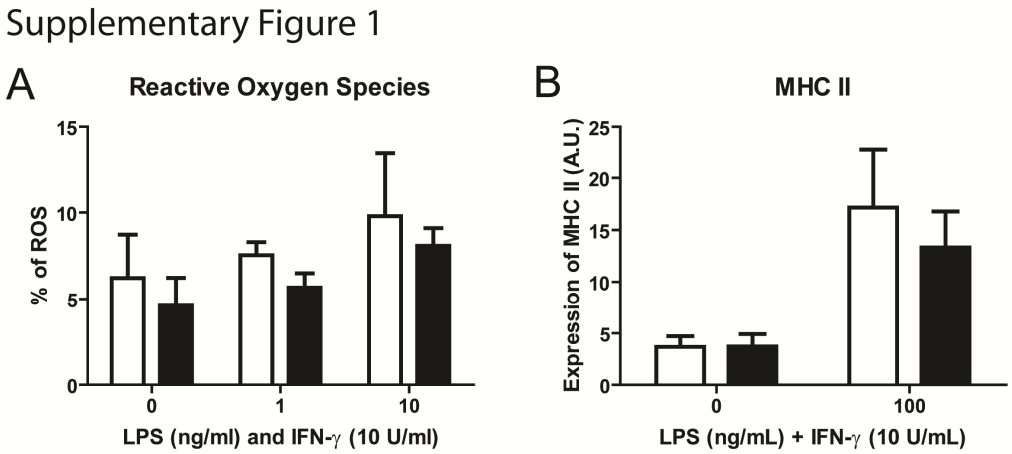


Figure 5: Conditioned media from microglia expressing a mutant htt fragment is toxic to neurons. ***A*****,** Representative images of wild-type primary neurons treated with conditioned media from N9 microglial cell lines expressing htt 25Q and 72Q under control and stimulated conditions (100 ng/ml LPS, 10 U/ml IFN-γ). ***B,*** Conditioned media from microglia expressing 25Q and 72Q mutant htt (+/- pre-treatment of 100 ng/ml LPS and 10 units/ml IFN-γ) were cultured with DIV 7 primary mouse neurons for 24 h, using a coverslip coculture approach. Values are mean ± SEM (**P<0.01; one-way ANOVA, Bonferroni post-test; n=4). ***C,*** Direct microglia-neuron coculture of YAC128 microglia and wild-type neurons did not cause neuronal cell death. Values are mean ± SEM (n=3). Neurons were stained with anti-MAP2 antibody (1:200) and anti-mouse Texas Red antibody (1:200). Neuronal viability was quantified by counting the number of

neurons in images collected by fluorescent microscopy from 20 random fields using a 40x objective. 4 independent experiments were performed.



Supplementary Figure 1. Primary microglia from YAC128 mice did not have increased levels of ROS and expression of MHC-II surface antigen. Primary microglia from YAC128 mice (black bars) were stimulated with LPS/IFN-γ and evaluated for (***A***) ROS production levels by dihydrorhomadine-1,2,3 and (***B***) expression of MHC-II surface antigen marker with anti-MHC-II conjugated with APC. White bars are results from WT littermates. Values are mean ± SEM (n=3).

Chapter 3

Mutant Huntingtin Impairs Migration of Immune Cells in Huntington's Disease

Abstract

The activation of peripheral immune cells and microglia, and increased levels of pro-inflammatory cytokines have been described in Huntington's disease (HD) patients before the onset of motor symptoms. Whether these changes are due to cell autonomous effects of mutant huntingtin (htt) in immune cells, and/or compensatory response to htt-induced degeneration in neurons, remains unclear. Here we report that mutant htt expression in microglial cell lines and primary microglia isolated from brains of HD mouse models have significant defects in migration towards chemotactic stimuli. We also observed a delayed functional response of microglia in a mouse model of HD upon laser-induced injury as determined by *in vivo* two-photon imaging. Mouse models of HD displayed profound deficits in thioglycollate-induced peritonitis, prior to motor deficits, that persisted over time. Migration deficits were also observed in peripheral immune cells isolated from HD patients, and the magnitude of these deficits were not influenced by disease stage. We further provide evidence that the molecular mechanism of mutant htt-induced migration defects in immune cells involves decreased membrane ruffling and cofilin levels. These findings suggest mutant htt expression in immune cells is sufficient to induce cell autonomous changes in their function that may contribute to immune dysfunction and neurodegeneration in HD.

Introduction

Huntington's disease (HD) is a devastating, incurable neurodegenerative disorder caused by an extended polyglutamine length in huntingtin (htt), a protein that is ubiquitously expressed. Mutant htt can lead to dysfunction in neurons; however, it may also cause dysfunction in non-neuronal cells. In fact, pathological phenotypes in peripheral tissues, such as weight loss and altered glucose homeostasis, are robust in HD patients and are thought to be in part caused by mutant htt in non-neuronal cells[1-4]. As well, other peripheral cell types such as fibroblasts, lymphocytes, and erythrocytes from HD patients and/or HD mouse models also appear to be abnormal, most likely due to expression of mutant htt[1,5]. These changes in HD peripheral tissues emphasize that the brain may not be the only contributing system to HD pathogenesis.

Mutant htt may elicit dysfunction in the immune system. Levels of serum soluble immune markers such as tumor necrosis factor (TNF) receptor, interleukin (IL)-2-receptor, and immunoglobulins are elevated in HD patients[6]. Plasma samples from HD patients also have increased levels of pro-inflammatory cytokines (i.e., IL-6, IL-8, and TNF-α) that correlate with disease progression[7]. These increases were significant 16 years before the onset of other HD symptoms, like chorea. Monocytes and macrophages isolated from HD patients and/or mouse models were hyperactive in the production of these pro-inflammatory cytokines. Additionally, IL-6 can lead to activation of the complement cascade. In fact, clusterin, a key modulator of the complement cascade, is upregulated in peripheral blood in HD[8]. Peripheral chemokines (eotaxin-3, macrophage inflammatory protein-1β, eotaxin, monocyte chemoattractant protein-1,-4), which are central to processes related to infection, migration of leukocytes into the CNS, and

modulation of the function of blood brain barrier, are increased in HD (Wild *et al.*, in press). The transcription of genes in HD blood is dysregulated and associated with disease progression[1,9,10]. For example, immediate early response 3 (IER3), a gene that functions to protect cells from TNF-α-induced apoptosis, is upregulated in HD blood[11]. The mitochondrial dysfunction and apoptotic marker Bax is also increased in lymphocytes and monocytes from HD patients[12]. Together, these studies provide strong evidence that the peripheral immune system is abnormal in HD.

Microglia are the resident immune cells in the brain. They have the same lineage as peripheral myeloid cells such as monocytes and macrophages; therefore, microglia have many similar capabilities and functions as their peripheral counterparts. In the inactivated state, microglia extend and retract their processes to actively survey their microenvironment[13]. They also provide trophic support for neurons and physically interact with synapses[14]. When the brain is exposed to pathological insults, microglia become activated, transform from a ramified morphology to one that is amoeboid/phagocytic, release cytotoxic substances (i.e., oxygen radicals, nitric oxide) or cell-signaling molecules (i.e., pro-inflammatory cytokines, TNF-α), and migrate or extend their processes toward the site of injury. Microglia may also increase expression of cell surface antigens as part of their normal function as antigen-presenting cells (APCs) to elicit further immune responses[15]. These changes in activated microglia may be protective or toxic to the normal or diseased brain.

Microglia, just as the peripheral immune cells, may play a role in HD pathogenesis. Reactive microglia and gliosis occur in vulnerable regions of HD brains[16,17]. Ferritin accumulation is often indicative of cellular dysfunction and is thought to contribute to

pathology in various neurodegenerative diseases[18]. Microglia in the R6/2 HD mouse model show more ferritin immunostaining than their wild-type (WT) littermates early in the disease state[19]. These changes also increased with disease severity[19]. Brains from mid-stage HD patients also had increased ferritin immunostaining in microglia, many of which are dystrophic[19]. Positron emission tomography from HD patients also showed an increase in binding of ^{11}C-(R)-PK11195, a surrogate marker for microglial activation *in vivo*, in the striatum and cortex that correlates to HD severity[20,21]. Furthermore, microglial activation in brain regions required for cognitive function can predict disease onset[22].

One hallmark that enables the rapid response of immune cells is their ability to undergo morphological changes and migrate in response to pathological insults in tissues. The chemoattraction of immune cells to sites of infection or injury is an early and essential step in normal immune responses. After being activated, immune cells localize together in affected tissues and communicate through short-range cytokines and/or direct cell-cell contact[23]. Immune cells, including but not limited to monocytes, macrophages, and microglia express chemoattractant receptors and adhesion molecules to enable their migratory response to inflammatory cues[24]. These cells rely on the remodeling of actin cytoskeleton, which is a key mediator of cell polarization and chemotaxis[25,26]. Peripherally, transmigration of immune cells from the bloodstream into perivascular tissue allows their accumulation at the site of injury or infection[23]. Similarly, microglia quickly rearrange their actin networks and form membrane ruffles and leading edges for chemotaxis[27,28]. In the live brain, microglia respond rapidly by extending their processes

toward laser ablation injury. Overall, migration of immune cells is a critical step to initiate a response to injury or inflammation.

In this chapter we describe data which show that the expression of mutant htt in microglia and peripheral immune cells causes a severe impairment in their migration towards chemoattractants, tissue injury and inflammation *in vitro* and *in vivo*. We further show that peripheral blood monocytes (PBMCs) isolated from blood of HD patients are profoundly impaired in their migration at the earliest stages of disease. We provide evidence that functional impairment in the migration of mutant htt expressing immune cells may be due to perturbations in actin remodeling that are required for migration. Our observations indicate that mutant htt in microglia and immune cells confers cell-autonomous deficits that may have important implications for pathogenesis in HD.

Methods

Animals and breeding strategy. The University of California, San Francisco, IACUC Committee approved all experiments and procedures involving mice. Mice were maintained and bred in accordance with National Institutes of Health guidelines. YAC128 founder mice (FVB/NJ background) were kindly provided by Dr. Blair Leavitt (University of British Columbia). BACHD founder mice (C5BL/6, FVB/NJ background) were provided by Dr. William Yang (University of California, San Diego). These mice were crossed with breeder mice to generate litters as required. $CX3CR1^{GFP/GFP}$ mice were obtained from Dr. Israel Charo (Gladstone Institute of Cardiovascular Diseases) and crossed with BACHD mice to generate BACHD:$CX3CR1^{GFP/+}$ for *in vivo* microscopy.

Cell culture. Primary microglia were isolated from P1-3 mice as described[29]. Briefly, brains were dissected, trypsinized and triturated into single cells and cultured in Dulbecco's modified Eagle's medium with high glucose (DME-H21) with 10% heat-inactivated FBS and penicillin plus streptomycin. After 10-20 d, cultures were gently shaken by hand, and microglia collected as floaters, resulting in >99% purity. Granulocyte macrophage-colony stimulating factor (GM-CSF, 2 ng/ml) was used to help stimulate microglial yield. Isolated cells were incubated with macrophage serum-free medium (MSFM, Invitrogen) for 24 h, unless otherwise specified, and used as required. Microglial cell lines BV2 and N9 were stably transduced with lentiviral constructs expressing htt 25Q or 72Q (Kwan *et al.,* manuscript in preparation). Cells were cultured in DME-H21 with 5% heat-inactivated FBS.

Chemotaxis assays

Boyden chamber assays. Microglial cell lines and isolated primary microglia were collected and prepared as described[30]. Briefly, harvested cells were stained with 700 nM DRAQ5 for 20 min, and washed with MEM, and then washed with MEM with 10% Cellgro (Mediatech). Migration was measured in a chemotaxis chamber (Neuroprob) using 10 µm filters. Lower wells were filled with chemoattractants (0-300 µM ATP, Sigma MO; 0-100 nM Complement protein C5a, Calbiochem). Primary microglia were allowed to migrate from upper wells through the filter for 3 h at 37°C and 5% CO_2. Non-migrated cells were wiped off, and the filter was scanned and analyzed by an Odyssey Infrared Imaging System (LI-COR).

Dunn chamber assay. Cells were spotted onto fibronectin-coated coverslips (Becton Dickinson) and incubated with serum-free DME-H21 for 4 h as described[31]. The Dunn chamber was used to form an ATP gradient of 0 to 100 µM. Distance and direction of movement by the cell's leading edge was monitored over a 30 min period by time-lapse microscopy. Images were processed and analyzed using Image J software (manual tracking and chemotaxis plug-ins).

In vivo imaging of microglia. GFP-expressing microglia were imaged by two-photon time-lapse microscopy as described[32]. A z-stack (~30 µm depth, 1 µm z-steps) was acquired every 3 min for 60 min. A maximum projection of the ~30 z-steps centered on the laser ablation was constructed for each time point. Results were analyzed by determining the change in GFP fluorescence as the processes enter the space surrounding the center of the laser ablation. The fluorescence was normalized to an outer region that is

3x the area of the space around the ablation. Baseline images were similarly obtained by imaging the area every 30 sec for 10 min.

HD patient monocyte migration. These experiments were conducted in accordance with the declaration of Helsinki and were approved by local ethics review boards; all subjects gave informed written consent. Blood samples were obtained from control subjects and genetically-diagnosed HD patients and processed as described[8]. Clinical assessment was carried out by a neurologist experienced in assessment of HD patients. Blood samples were obtained from HD patients and age-matched controls. Sorted blood monocytes were seeded on 0.5 μm transwells and stimulated with chemoattractant 50 ng/ml monocyte chemotactic protein 1 (MCP-1). After 2 h, the migrated monocytes in the bottom chamber were counted; FACS analysis with anti-CD11b (BD Pharmingen) confirmed that the cells were monocytes/leukocytes.

Membrane ruffling. Assays were performed as described[31] by culturing the cells on poly-L-lysine (10 μg/ml) and incubating the cells with MSFM containing ATP (100 μM) or macrophage colony stimulating factor (M-CSF, 100 ng/ml) for 10 min at 37°C. Cells were fixed with 4% PFA and stained with rhodamine-conjugated phalloidin (Molecular Probes). Cells with membrane ruffling, in which the cell edges have dense staining, were counted as a percentage of the cells in the field of view.

Western blotting analysis. Cell lysates were obtained using standard RIPA buffer (150 mM NaCl, 1% NP-40, 0.1% SDS, 0.5% sodium deoxycholate, 50 mM Tris, pH 7.4) with complete protease inhibitors (Roche). Proteins were separated by SDS-PAGE and transferred onto nitrocellulose membrane (Schleicher and Schuell), and blocked for 1 h with 0.1% Tween and 5% nonfat dry milk. The membrane was probed overnight with primary antibodies anti-phospho cofilin (1:1000, Cell Signaling), anti-cofilin (1:1000, Abcam), or anti-GAPDH (1:500, Chemicon), washed, and incubated for 1 h with horseradish peroxidase-conjugated anti-rabbit or anti-mouse (1:10,000). After washing, the blots were revealed using ECL kit as per manufacturer's instructions (Amersham).

Thioglycollate-induced peritonitis. Peritonitis was induced with thioglycollate (Sigma) as described[33]. Mice were given intraperitoneal injection of 3% thioglycollate or saline control. Peritoneal leukocytes were collected 24 h later by flushing the peritoneal cavity with 10 ml of PBS. Peritoneal cells were washed with staining buffer (PBS with 5% FBS), blocked with anti-CD16/32 (1:200, R&D Systems) and stained with anti-F4/80-APC (1:100, Caltag, CA). Cells were analyzed by LSR-II (BD Biosciences) and FlowJo software (Tree Star). To determine the infiltrating macrophages, cells were gated for F4/80 dim population amongst unstained and F4/80 dim cells.

Results

Mutant htt in microglia impairs migration to chemotactic stimuli

We have previously described that mutant htt expression leads to cell autonomous effects in microglial cell lines that were also observed in primary microglia from HD mouse models. We found that mutant htt leads to increased levels of pro-inflammatory cytokines IL-1β and IL-6 upon stimulation (Chapter 2), similar to a reported study[7]. We wanted to further evaluate other cell autonomous effects that mutant htt might have on these cells, and in the current chapter we describe the effects of mutant htt on microglial cell migration.

Microglial cell lines that stably express mutant htt were tested in a Boyden chamber assay to determine their migration towards different chemoattractants. Microglia were stained with the fluorescent dye, Draq 5, and the migrated cells were quantified based on fluorescence signal on the filter in which the cells were trapped. We found that, in the absence of a chemoattractant, BV2 microglial cell lines that expressed htt with 25Q and 72Q (described in Chapter 2) were not different than control cells in their ability to migrate. In contrast, upon stimulation with ATP (100 μM), microglia that expressed htt with 72Q, but not 25Q, displayed a significant migration deficit in comparison to control cells (Fig. 1A). Similar findings were obtained using primary microglia isolated from the YAC128 and BACHD mouse models (Fig. 1B, C). The YAC128 and BACHD mouse models are two well-characterized full-length htt mouse models that recapitulate behavioral and pathological features of HD[34,35]. In the presence of 100 μM ATP, microglia from the two HD models had a strong defect in their migration response compared to WT microglia. Similar migration deficits were observed in response to

complement protein C5a, where primary microglia from YAC128 and BACHD mice had minimal responses (Fig. 1D, E). Using quantitative PCR, we found that microglia expressing mutant htt and control cells had similar mRNA levels of P2Y12, the purinergic receptor in microglia that mediates their migration response to ATP[31,36] (data not shown). We are in the process of determining the gene expression for the C5a receptor. Because C5a is a chemoattractant known to stimulate microglial migration via a different signaling pathway than ATP[30], our results suggest that mutant htt induced migration deficits in microglia may not be specific to chemoattractants and their cognate receptors, but rather may reflect an impairment in the ability to engage appropriate downstream signaling mechanisms and machinery required for proper migration.

Mutant htt impairs process extension in microglia

We next examined the ability of microglia to extend processes and migrate in real time using a Dunn chemotaxis chamber in a gradient of ATP (0-100 μM) with time-lapse microscopy. Microglia from WT mice showed a clear and robust polarization of their leading edges toward the ATP source within 30 min, whereas YAC128 microglia showed a significant reduction in the extension and movement of their processes (Fig. 2A, B). By tracking the path and leading edge displacement of each cell, we determined the average distance and *x*- and *y*-axis displacements of cells. We determined the average length of overall process displacement, the average velocity of cellular movement, and the overall average accumulated distance of the processes (Fig. C-E). YAC128 microglia had significant deficits relative to WT control cells for all of these parameters, suggesting that

mutant htt-induced migration deficits in microglia could be due to defective process polarization and extension.

Mutant Htt Impairs Microglial Function *In Vivo*

As cultured microglia in isolation may not faithfully represent their natural physiological environment in the brain, we next evaluated microglial process extension and retraction in the intact cortex of 12 month old BACHD mice by time-lapse *in vivo* two-photon imaging. Microglia were fluorescently labeled by crossing BACHD mice with $CX3CR1^{GFP/GFP}$.

We first recorded microglial process extension and retraction of $BACHD^{Tg/+}$;$CX3CR1^{GFP/+}$ mice and controls under basal conditions (Fig. 3A). $BACHD^{Tg/+}$;$CX3CR1^{GFP/+}$ mice have a lower frequency of process extensions in comparison to control mice (WT;$CX3CR1^{GFP/+}$) and a higher frequency of process retractions (P <0.01). Microglial cell size and density were not significantly different between WT and BACHD mice (data not shown).

We next induced brain injury by focal laser ablation and examined the response of microglia in these mice by two-photon time-lapse microscopy (Fig. 3B-E). As described[31,32], microglia from WT control mice showed a robust response to laser induced injury that was characterized by the rapid and complete extension of processes toward the injury site. In contrast, microglial processes from $BACHD^{Tg/+}$;$CX3CR1^{GFP/+}$ mice showed a significant (P<0.001) delay in their response, and were unable to fully encompass the site of injury (Fig. 3B,C). At 12 min after the injury, the response of microglia from $BACHD^{Tg/+}$;$CX3CR1^{GFP/+}$ mice was less than 50% of that observed in

control mice (Fig. 3D). Indeed, the average velocity of microglial processes in BACHD$^{Tg/+}$;CX3CR1$^{GFP/+}$ mice over the first 30 min (the typical time it takes for microglia in WT control mice to reach the site of the laser ablation) was 30% less than that observed in control mice (Fig. 3E). Taken together, these results indicate microglia in the brains of aged BACHD mice are dysfunctional under basal conditions and in response to injury.

Migration of macrophages to an inflammatory stimulus is severely impaired in mouse models of HD

As microglia are thought to be mononuclear phagocytes that are derived from the same common myeloid progenitor cell as macrophages and monocytes[37-39], we next asked if the migration of monocytes and macrophages might also be impaired. We first evaluated the *in vivo* recruitment of macrophages in response to a peritoneal injection of 3% thioglycollate (a non-specific inflammatory stimulus). Cells were collected from the peritoneum and labeled for the macrophage marker F4/80; the F4/80 dim population which characterizes infiltrating macrophages was quantified by FACS. BACHD mice that were 8 weeks of age had a marked reduction (~43%) in the recruitment of macrophages 24 h after thioglycollate injection (Fig. 4A). This deficit was even more pronounced in BACHD mice aged 8 months. We next quantified macrophage infiltration in the R6/2 transgenic mouse model of HD that expresses a short fragment of mutant htt. This model is characterized by rapid and robust disease progression. At 8 weeks, an age where the mice are symptomatic, R6/2 mice also had a significant reduction in macrophage recruitment in response to thioglycollate (Fig. 4C). Thus, in two independent

mouse models of HD periotoneal macrophage infiltration to an inflammatory stimulus is profoundly impaired.

Peripheral blood mononuclear cells isolated from HD patients also display migration defects

To determine if migration deficits of myeloid cells are also observed in HD patients, we isolated PBMCs from blood samples at various disease stages and tested their migration towards the chemokine MCP-1 using a transwell approach. Monocytes from HD patients were severely impaired in this migration assay, and a similar degree of impairment was observed across all disease stages (Fig. 4D). Surprisingly, even monocytes isolated from pre-manifest HD patients were impaired, suggesting that this defect may be due to a cell autonomous effect of mutant htt in these cells.

Microglia from HD mouse models have decreased membrane ruffling and cofilin levels

We next performed a series of experiments to gain insight into possible mechanisms that may underlie mutant htt-induced migration deficits in microglia. When microglia and other myeloid cells migrate towards chemotactic stimuli, they rapidly reorganize the actin cytoskeleton and plasma membrane (also defined as membrane ruffling), and this typically precedes the formation of a lamellipodium. Cells were stained with rhodamine-phalloidin and the fraction of cells with membrane ruffling was assessed and quantified. Upon stimulation with ATP (100 μM) for 10 min, YAC128 microglia showed a significant (P<0.01) deficit in membrane ruffling in comparison to microglia from

littermate controls (Fig. 5A). We also tested if YAC128 microglia were sensitive to stimulation with M-CSF (100 ng/ml), which induces membrane ruffling through Rac-mediated activation of actin polymerization as opposed to the G protein-coupled receptor pathways[36,40,41]. While membrane ruffling increased >3-fold in WT microglia treated with M-CSF, microglia from YAC128 mice were unresponsive to this treatment (Fig. 5A). Similar observations were observed with BACHD microglia upon stimulation with ATP (Fig. 5B).

An actin binding protein called cofilin plays an essential function in regulating actin depolymerziation that is required for proper cell migration[42]. Phosphorylation of cofilin at Ser-3 (p-cofilin) inhibits its actin-severing activity. The transient phosphorylation of cofilin after stimulation with ATP results in actin polymerization required for migration. Both under basal conditions and in response to ATP stimulation, we found that p-cofilin levels were significantly reduced in primary microglia from BACHD mice in comparison to littermate controls (Fig. 5C). Interestingly, levels of total cofilin were also decreased in primary microglia from BACHD mice (Fig. 5D), in N9 microglial cell lines that stably express mutant htt 72Q, and in brain homogenates from BACHD mice (Fig. 5E, F). These results indicate mutant htt expression in microglia is sufficient to impair actin remodeling by decreasing levels of cofilin in a manner that might contribute to migration deficits, and support a recent study which showed that mutant htt causes defective actin remodeling during stress[43].

Discussion

In this study, we provide *in vitro* and *in vivo* evidence that expression of mutant htt in microglia and peripheral myeloid cells causes pronounced defects in cellular migration in response to chemotactic stimuli. Migration deficits were observed with a variety of stimuli (ATP, C5a, laser injury, MCP-1, M-CSF) that signal through different cognate receptors, suggesting that mutant htt may impair intracellular machinery involved in migration. Consistent with this hypothesis, mutant htt caused a significant reduction in membrane ruffling, cofilin and phospho-cofilin levels in microglia. Importantly, migration deficits were observed in PBMCs isolated from the blood of HD patients, even prior to the onset of neurological and motor symptoms. These observations indicate that mutant htt in microglia and peripheral myeloid cells confers cell-autonomous deficits that may have important implications for pathogenesis.

Recent *in vivo* imaging studies have demonstrated that under basal conditions microglia are important sentinels in the CNS whose highly motile processes are constantly surveying the brain parenchyma and transiently contacting synapses in an activity-dependent manner[13,14]. In this study we found that microglia from aged BACHD mice have deficits in process extension and retraction that were most pronounced in response to a laser induced injury. These results are consistent with previous immunohistochemical studies in which microglia in R6/2 mice appeared dystrophic and decreased in number with age[19,44]. Increased binding of the positron emission tomography (PET) ligand ^{11}C-PK11195, which binds to the the benzodiazepine receptor expressed by reactive microglia, has also been reported in HD patients, and its levels correlate inversely to levels of ^{11}C-raclopride, which binds dopamine D2/D3 receptors,

that are found in the striatum[20,21]. Given that increased ^{11}C-PK11195 binding was observed even in pre-manifest HD patients, and that microglia cultured from early postnatal BACHD and YAC128 mice have migration deficits, these results collectively suggest that mutant htt microglia impairs the normal function of these cells in a manner that is at least partially independent of mutant htt induced neuronal dysfunction. Our *in vivo* imaging results also suggest that mutant htt-expressing microglia are not able to survey the brain parenchyma appropriately, and we hypothesize that their ability to survey synapses is also likely impaired.

Our results further showed that peripheral macrophages, which are derived from the same myeloid lineage as microglia, are also dramatically impaired in their migration to chemotactic stimuli in two independent mouse models of HD *in vivo*. Importantly, profound migration defects were also found in PBMCs isolated from HD patients. Pro-inflammatory cytokines and chemokines are elevated in pre-manifest HD patients, and remain elevated throughout the course of disease[7] (Wild *et al.*, in press). We hypothesize that migration deficits in peripheral immune cells could potentially explain chronically elevated levels of pro-inflammatory cytokines and chemokines, like interleukin-6 (IL-6), IL-8 and tumor necrosis factor-α (TNF-α), which in turn may contribute to other peripheral symptoms in HD. Taken together, these results indicate that the innate immune system in HD patients is abnormal, and our results would suggest that HD patients are likely to be at least partially immune compromised. Further studies are also warranted to examine if mutant htt induces abnormalities in the adaptive immune system.

The molecular mechanisms that are responsible for mutant htt-induced migration defects in microglia and peripheral monocytes/macrophages are likely to be complex and

will require further investigation. Our initial studies indicate that mutant htt perturbs the proper regulation of actin remodeling due to decreased levels of cofilin and phospho-cofilin. Consistent with our data in microglia, a recent study demonstrated that mutant htt colocalizes with nuclear actin-cofilin rods in neurons under stress conditions[43]. This study also detected cross-linked complexes of actin and cofilin in HD patient lymphoblasts that correlated with disease progression. Ongoing studies are testing if similar complexes can be found in myeloid cells from HD patients.

In summary, this study provides evidence for pronounced migration deficits in microglia and peripheral monocytes/macrophages in mouse models of HD, and in PBMCs isolated from HD patients, which may partially explain the early and chronic elevation of pro-inflammatory cytokines and chemokines in this disease. While the focus of this study was solely to determine if mutant htt impairs the function of myeloid cells in a cell autonomous manner, we hypothesize that these abnormalities are very likely to contribute to at least some aspects of pathogenesis in HD. These results further suggest that drugs that are currently used for lowering pro-inflammatory cytokines, including antibodies to TNF-α and IL-6, might provide some benefits to HD patients. The migration deficits that are observed in PBMCs isolated from the blood of HD patients, if confirmed in larger cohorts of patients, could also potentially serve as a useful biomarker in clinical trials.

Acknowledgements

Dr. Ralph Andre in Dr. Sarah Tabrizi's laboratory performed the human monocyte migration assay (Fig. 4A). Austin Chou contributed the thioglycollate-induced peritonitis

experiments (Fig. 4B,C). Dr. Aaron Miller in Dr. Nephi Stella's performed the initial Boyden migration assays in microglial cell lines (data not shown). In collaboration with Drs. Dimitrios Davalos and Katerina Akassalgou, we performed the *in vivo* two-photon microscopy to evaluate microglial response to tissue injury (Fig. 3). All other data were obtained by myself under that supervision of Dr. Paul J. Muchowski.

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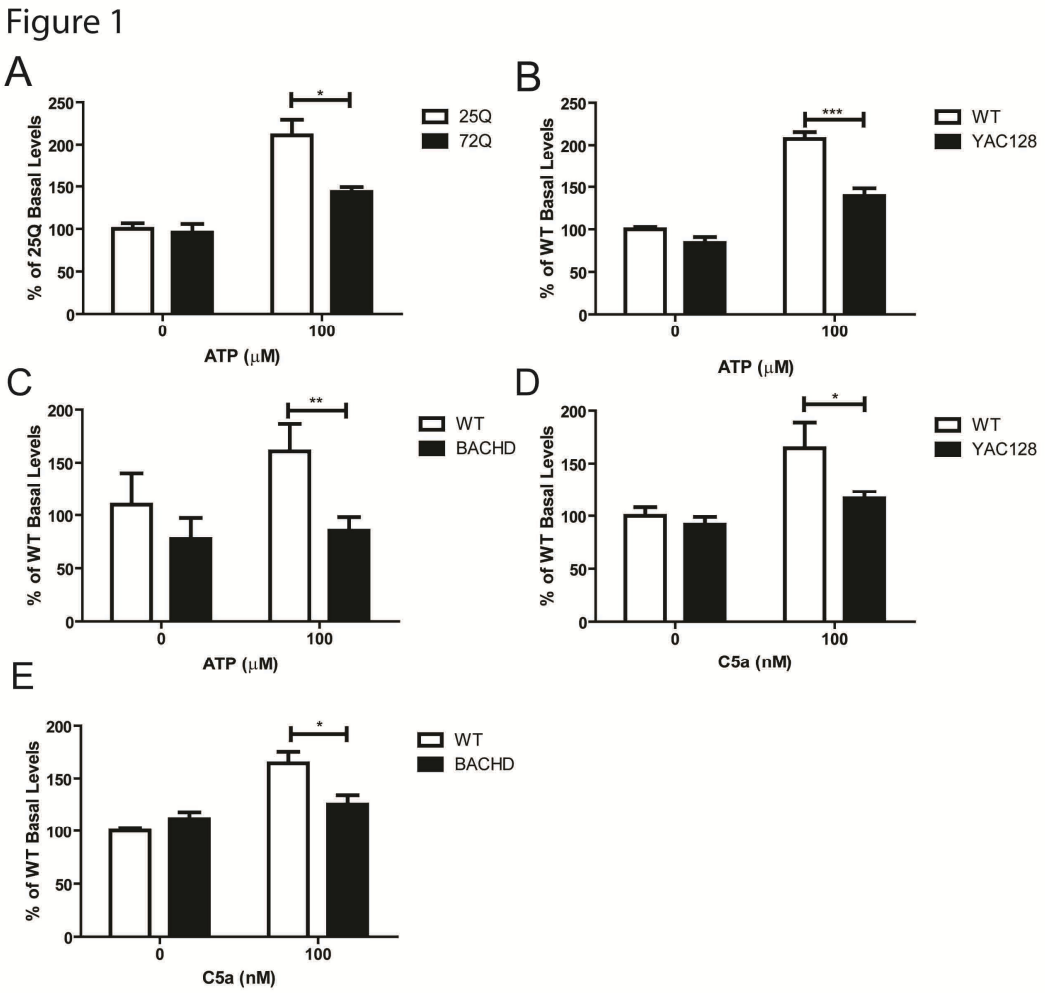

Figure 1. Mutant htt expression in microglia impairs their chemotactic response to ATP and C5a. A, BV2 microglial cell line expressing htt 72Q showed significant migration defects in response to stimulation with the chemoattractant ATP (100 μM) compared to 25Q htt-expressing cells. Primary microglia from ***B,D,*** YAC128 and ***C,E,*** BACHD mice showed a similar migration defect in response to stimulation with ATP (100 μM) and C5a (100 nM). Cells were cultured in a Boyden chamber assay and allowed to migrate through the filter for 3 h. Values are mean ±SEM (**P*<0.05, ***P*<0.01, ****P*<0.001; *t*-test).

Figure 2

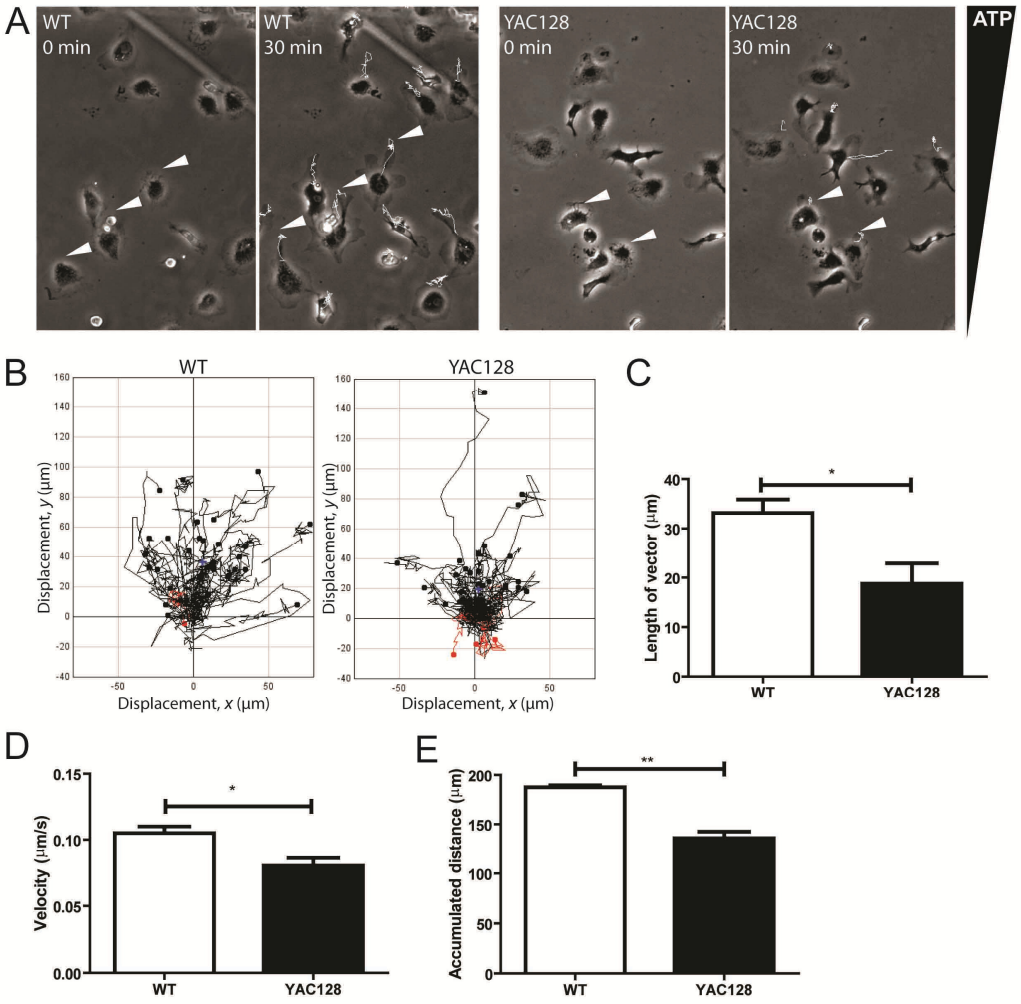

Figure 2. Mutant htt expression in microglia diminishes the extension of microglial processes towards ATP. ***A,*** Microglia from WT and YAC128 mice were placed in a gradient of ATP (0-100 µM) using a Dunn chemotaxis chamber and imaged by phase contrast time-lapse microscopy. Microglia from WT mice showed robust extensions of their processes (arrowheads) towards the ATP gradient (white lines indicate the movement of the leading edge of cell over 30 min), whereas YAC128 microglia showed a significantly decreased response. ***B,*** Chemotaxis plots indicating tracks and distance (µm) traveled by the leading edge of WT (left) or YAC128 (right) microglia towards

ATP after 30 min. Values are plotted in the *x* and *y* directions relative to starting location. Black lines indicate the processes that had a net extension; red lines indicate the processes that had a net retraction. ***C-E,*** Summary of the average length of the vector (**C**), average velocity (**D**), and average accumulated distance (**E**) of WT (n=2) and YAC128 (n=3). Values are mean SEM (*P<0.05, **P<0.01; *t*-test).

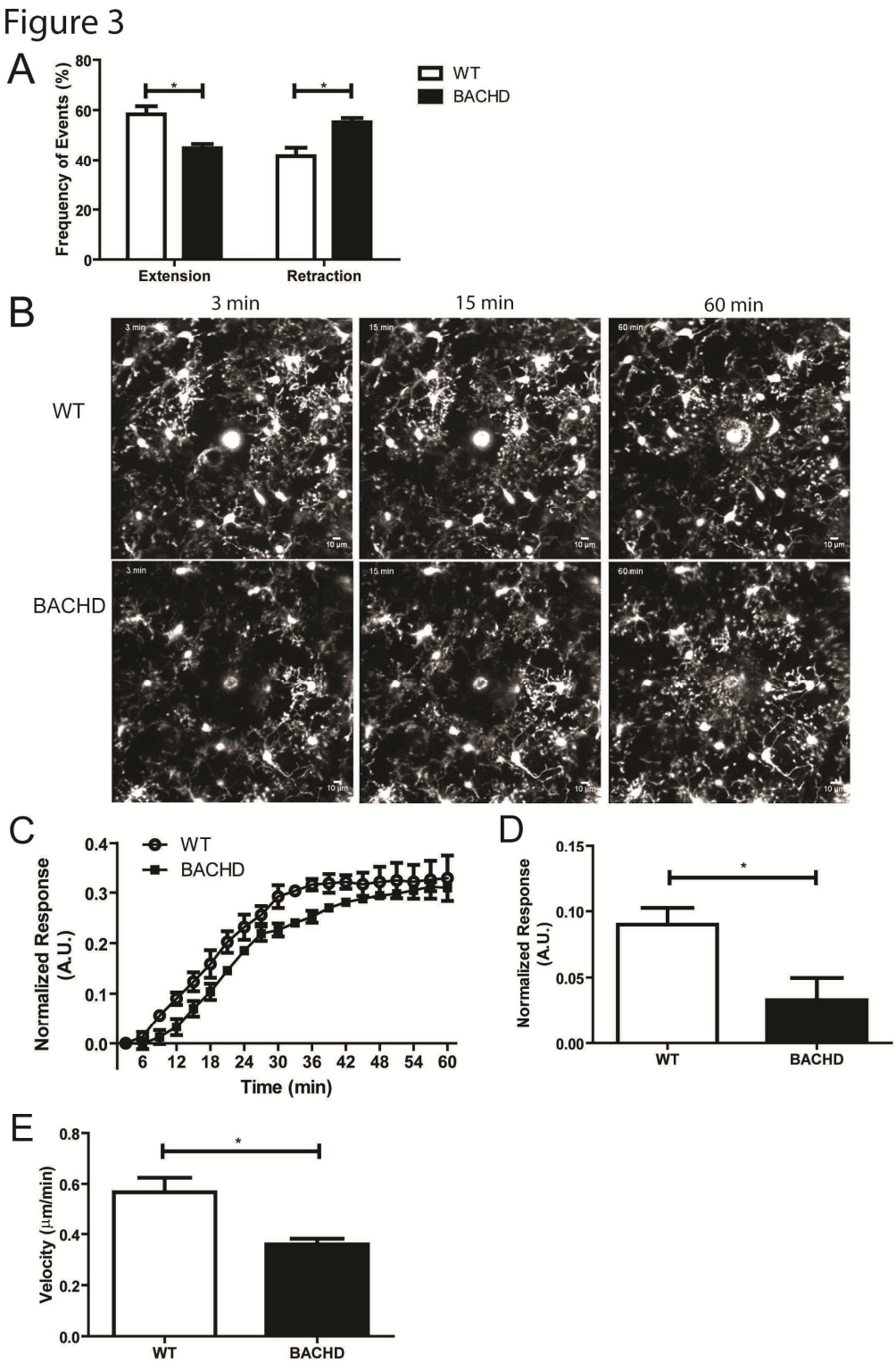

Figure 3. Microglia have increased baseline retraction of processes and a delayed response to focal laser ablation in BACHD mice. Microglia from WT;CX3CR1$^{GFP/+}$

(WT) or BACHD$^{Tg/+}$;CX3CR1$^{GFP/+}$ (BACHD) mice were imaged using *in vivo* two-photon time-lapse microscopy. ***A,*** Baseline time-lapse imaging of microglia demonstrates less extension and more retraction of microglial processes over 10 min in BACHD$^{Tg/+}$;CX3CR1$^{GFP/+}$ than WT;CX3CR1$^{GFP/+}$ mice (*P<0.05; t-test). ***B,*** Tissue ablation with a laser (white zone in center) results in the rapid extension of microglial processes toward the site of injury in WT;CX3CR1$^{GFP/+}$ mice. In contrast, microglia from BACHD$^{Tg/+}$;CX3CR1$^{GFP/+}$ mice showed delayed responses over a 60 min observation period. ***C,*** Quantification of process extension toward the site of laser ablation (P<0.001; two-way ANOVA). ***D,*** Microglial response is lower in BACHD$^{Tg/+}$;CX3CR1$^{GFP/+}$ than WT;CX3CR1$^{GFP/+}$ mice when measured at the 12 min time point after laser ablation (*P<0.05; t-test). ***E,*** The average velocity was determined over the 30 min period for WT;CX3CR1$^{GFP/+}$ and BACHD$^{Tg/+}$;CX3CR1$^{GFP/+}$ mice (*P<0.05; t-test). Values are mean ± SEM; n = 2 for WT and n = 3 for BACHD.

Figure 4

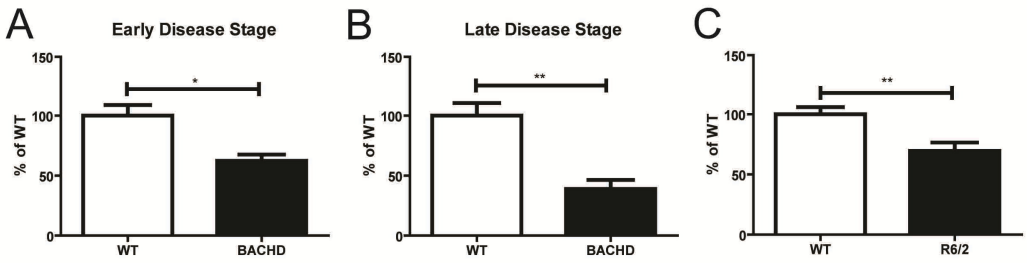

Figure 4. Migration of macrophages into the peritoneum, induced by thioglycollate, is defective in mouse models of HD. ***A-C,*** 3% thioglycollate was injected in the peritoneum of BACHD mice at (***A,B***) 8 weeks and 8 months, respectively, and (***C***) R6/2 mice at 8 weeks and their respective WT littermates to evaluate the recruitment of peripheral macrophages. Cells were stained with anti-F4/80-APC and analyzed by FACS.

Recruited populations were expressed as the percentage of F4/80 dim cells to total cells. Results are normalized to WT controls. Values are mean ± SEM (P<0.05, **P<0.01; *t*-test; 1 experiment for BACHD, 4 independent experiments for R6/2).

Figure 5

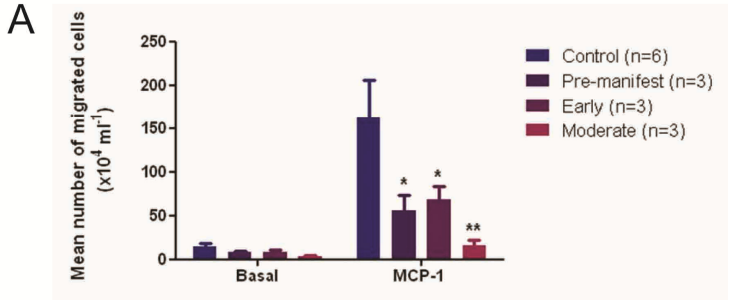

Figure 5. Severe impairment in migration in PBMCs isolated from HD patients. PBMCs were isolated from blood samples of age-matched controls, pre-manifest, early and moderate symptomatic HD patients. Cells were stimulated with MCP-1 (50 ng/ml) to stimulate migration in an 8 µm transwell. Values are mean ± SEM (*P<0.05, **P<0.01; two-way ANOVA, Bonferroni posttest; n = 3-6).

Figure 6

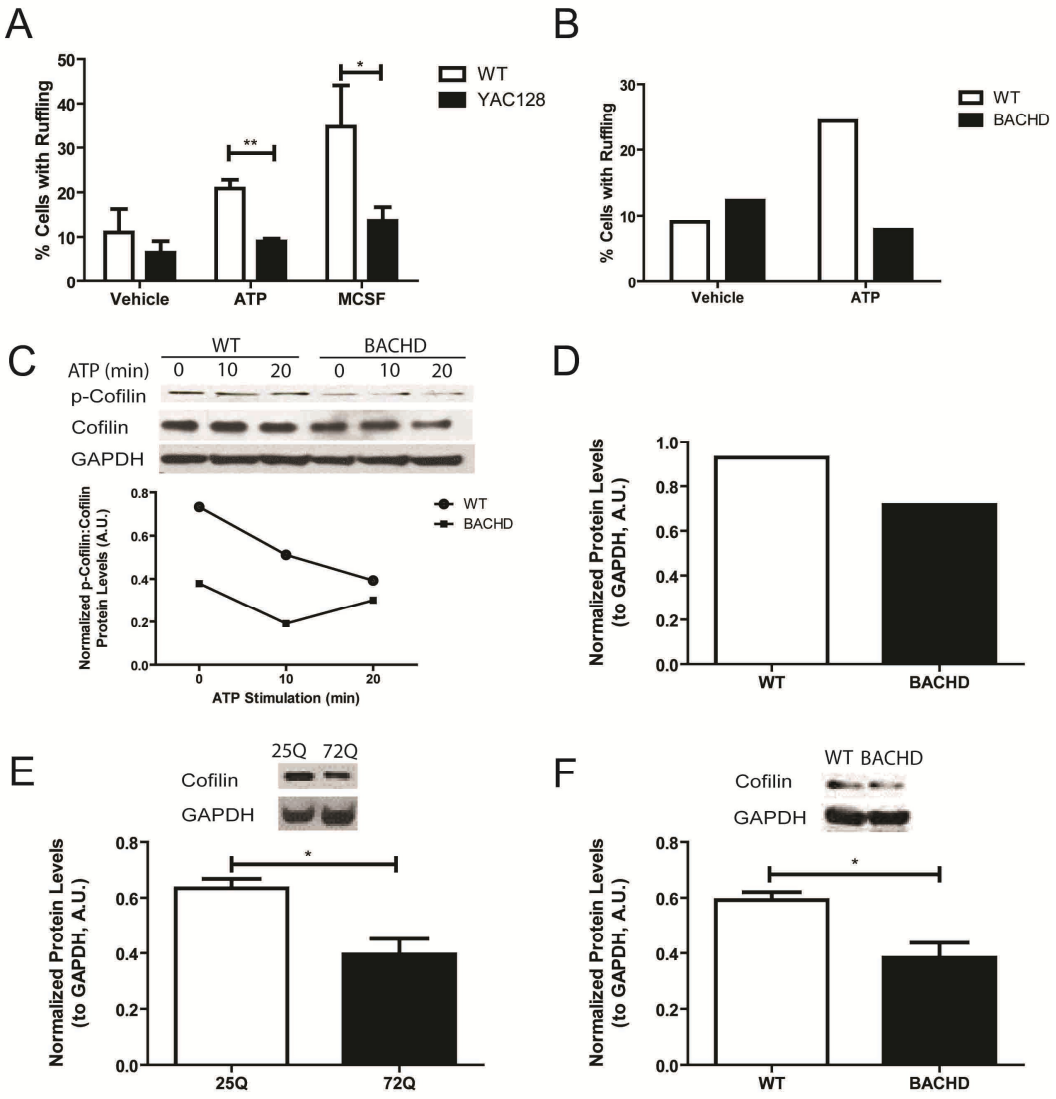

Figure 6. Mutant htt expression in microglia decreases membrane ruffling and cofilin levels. A, Primary microglia from YAC128 mice have decreased membrane ruffling upon stimulation with ATP (100 µM) and M-CSF (100 ng/ml), in comparison to WT microglia. Membrane ruffling in microglia from YAC128 mice treated with M-CSF is not statistically different from untreated cells. Cells were stained with rhodamine-phalloidin and the fraction of cells with membrane ruffling was assessed and quantified within 10 min. Values are mean ± SEM (**P*<0.05, ***P*<0.01; 3 independent experiments;

t-test). ***B,*** Primary microglia from BACHD mice also have decreased membrane ruffling upon ATP stimulation (preliminary result). ***C,*** Western blot analysis using phospho-cofilin [(p)-cofilin], cofilin and anti-GAPDH antibodies in homogenates from primary microglia isolated from BACHD mice at selected time points after stimulation with ATP (100 μM). Data were quantified as the ratio of (p)-cofilin:cofilin, both normalized to GAPDH levels. ***D,*** Quantification of cofilin levels in primary BACHD microglia, normalized to GAPDH levels. ***E,F,*** Western blot analysis using monoclonal anti-cofilin shows that there is a significant decrease in cofilin protein levels in (***E)*** N9 microglial cell lines expressing htt 72Q and (***F)*** BACHD brain lysate, compared to controls ($P<0.05$). Protein levels were normalized to GAPDH levels. Values are mean ± SEM; *t*-test; n = 2-3.

Chapter 4

Bone Marrow Transplantation Confers Modest Benefits in Mouse Models of Huntington's Disease

Abstract

Huntington's disease (HD) is caused by an expanded polyglutamine tract in the protein hungtintin (htt). Although HD has historically been viewed as a brain-specific disease, htt is expressed ubiquitously and recent studies indicate it causes changes to the immune system that might contribute to pathogenesis. Monocytes from HD patients and mouse models are hyperactive in response to stimulation, and increased levels of pro-inflammatory cytokines and chemokines are found in pre-manifest patients and their levels correlate with pathogenesis. In this study, wild-type bone marrow cells were transplanted into two lethally irradiated transgenic mouse models of HD that ubiquitously express full-length htt. Bone marrow transplantation partially attenuated hypokinetic and motor deficits in HD mice. Increased levels of synapses were found in HD mice that received bone marrow transplants. Importantly, serum levels of interleukin (IL)-6, 10, CXC chemokine ligand 1 (CXCL1), and interferon-gamma (IFN-γ) were significantly higher in HD than wild-type mice, but were normalized in mice that received a bone marrow transplant. These results suggest that immune cell dysfunction might be an important modifier of pathogenesis in HD, and that therapies aimed at normalizing levels of pro-inflammatory cytokines and chemokines might confer benefits to HD patients.

Introduction

Immune dysfunction has been implicated in multiple neurodegenerative diseases. Peripheral inflammatory markers including pro-inflammatory cytokines, such as interleukin (IL)-1, IL-6 and tumor necrosis factor (TNF)-α are increased in Alzheimer's disease (AD) patients[1-3]. Monocyte chemoattractant protein (MCP)-1, IL-6, and TNF-α are also increased in spinal cord tissues, cerebrospinal fluid and/or sera of amyotrophic lateral sclerosis (ALS) patients[4-6]. The mechanisms for these changes in the immune system remain unclear; however, it is speculated that the immune cells respond to the pathological changes in the brain, and their modulation can contribute to disease pathogenesis.

The immune system is also implicated in Huntington's disease (HD), which is a neurodegenerative disease caused by a polyglutamine expansion in the protein huntingtin (htt). Levels of serum soluble immune markers such as TNF receptor, IL-2-receptor, and immunoglobulins are elevated in HD patients[7]. Plasma samples from HD patients also have increased levels of pro-inflammatory cytokines (i.e., IL-6, IL-8, and TNF-α) that correlate with disease progression[8]. These increases are significant 16 years before the onset of other HD symptoms, like chorea. Monocytes and macrophages isolated from HD patients and/or mouse models were hyperactive in the production of these pro-inflammatory cytokines. Peripheral chemokines (eotaxin-3, macrophage inflammatory protein-1β, eotaxin, MCP-1,-4), which are central to processes related to infection, migration of leukocytes into the CNS[9], and modulation of the function of blood brain barrier, are also increased in HD (Wild *et al.*, in press). Immediate early response 3 (IER3), a gene that functions to protect cells from TNF-α-induced apoptosis, is

upregulated in HD blood[10]. Mitochondrial dysfunction and apoptotic marker, Bax, is also increased in lymphocytes and monocytes from HD patients[11]. Together, these studies provide strong evidence that the peripheral immune system is abnormal in HD.

In addition to changes in the peripheral immune system in HD, microglia, the immune cells in the brain, are also affected in the disease. Reactive and dystrophic microglia occur in the brains of HD mouse models and patients in regions that are vulnerable to the disease[12-14]. Positron emission tomography from HD patients also showed an increase in binding of ^{11}C-(R)-PK11195, a surrogate marker for microglial activation *in vivo*, in the striatum and cortex that correlates to HD severity[14-16]. Furthermore, microglial activation in brain regions required for cognitive function can predict disease onset[16]. Similar to the peripheral immune changes, microglia are activated in the pre-manifest stages of the disease. Our studies, along with others[8], showed that the changes in microglia may be due to cell autonomous changes induced by mutant htt expression. We found microglia that express mutant htt, when stimulated, have increased levels of pro-inflammatory cytokines and impairment in their migration response (Chapter 2 and 3).

To address the role the peripheral immune system and the role of BMDCs in neurodegenerative diseases, studies have used bone marrow transplantation in several disease mouse models in order to replace the peripheral immune system and experimentally allow bone marrow (BM)-derived cells to enter the brain. In an inherited ALS mouse model, bone marrow transplantation delays the onset and progression of disease[17,18]. In a mouse model of prion disease, bone marrow transplantation of wild-type (WT) cells prevents peripheral prion infection[19]; BM-derived brain macrophages are also

abundant in regions of prion deposition[19]. In AD mouse models, BMDCs enter the brain parenchyma and reside by amyloid plaques[20]. However, it is important to emphasize that the recruitment of BMDCs in the brain in these experiments is likely a result of the irradiation procedure. Despite this potential caveat, the recruitment of BMDCs in the brain clearly has beneficial effects in multiple independent mouse models of neurodegeneration.

Here we explored the use of bone marrow transplantation as a method to investigate if replacement of mutant htt-expressing BMDCs could ameliorate disease phenotype. We examined the HD phenotype based on three components: 1) replacement of the immune system, 2) recruitment of BM–derived microglia/macrophages in the brain, and 3) amelioration of disease phenotypes in the presence of normal myeloid cells peripherally and in the CNS. We generated bone-marrow chimeras in two well-characterized full-length htt mouse models of HD, YAC128 and BACHD. Our goal was to determine if bone marrow transplantation of WT cells would improve 1) behavior, 2) neuropathology, and 3) the increased levels of peripheral cytokines in these models. We find that bone marrow transplantation conferred modest behavioral benefits, improved brain pathology, and normalized peripheral cytokine levels. These results suggest mutant htt in immune cells may contribute to pathogenesis in HD.

Materials and Methods

Animals and breeding strategy. All experiments and procedures involving mice were approved by the IACUC Committee of the University of California, San Francisco. Mice were maintained and bred in accordance with National Institutes of Health guidelines. YAC128 founder mice (FVB/NJ background) were kindly provided by Dr. Blair Leavitt (University of British Columbia). These mice were crossed once with C57BL/6 mice (Jackson Laboratory) to generate F1 mixed background mice. Transgenic Iba1-GFP mice, kindly provided by Dr. Shinichi Kohsaka (National Institute of Neuroscience, Japan), were crossed once with FVB/NJ mice also to generate F1 mixed background mice for donors to YAC128 mice. BACHD founder mice (FVB/NJ background) were provided by Dr. William Yang (University of California, San Diego). These mice were bred with FVB/NJ mice (Jackson Laboratory). To generate donor mice for bone marrow transplants in BACHD mice, WT or BACHD mice were bred with β-actin GFP (Jackson Laboratory).

Bone marrow transplantation. Bone marrow cells were harvested as described[21]. Briefly, cells were harvested from femurs and tibias from donor animals, resuspended, and injected retro-orbitally into lethally irradiated mice (600 rads x 2, 3 h apart). In the YAC128 study, Iba1-GFP mice served as donors. Recipient mice were irradiated and transplanted at 3–4 weeks of age. Four groups were included in the study (n = 16–25): WT mice transplanted with WT BM (WT BM→WT), YAC128 mice transplanted with WT BM (WT BM→YAC128), non-transplanted WT, and non-transplanted YAC128 (Fig 1A). In the BACHD study, β-actin GFP served as donors. Recipient mice were irradiated

and transplanted at 8 weeks of age. Six groups were included in the study (n = 18–26): WT mice transplanted with WT BM (WT BM→WT), BACHD mice transplanted with WT BM (WT BM→BACHD), WT mice transplanted with BACHD BM (BAC BM→WT), BACHD mice transplanted with BACHD BM (BAC BM→BACHD), non-transplanted WT, and non-transplanted BACHD. Blood samples were collected and the efficiency of bone marrow transplantation was quantified by fluorescent-assisted cell sorting (FACS) using anti-CD11b-PE (1:4000, eBioscience) and GFP expression as a readout (FACS-Calibur, BD Biosciences).

Behavioral tests. Open field. Spontaneous locomotor activity in an open field arena was measured in an automated Flex-Field/Open Field Photobeam Activity System (San Diego Instruments). Before testing, mice were transferred to the testing room and acclimated for at least 1 h. Mice were tested in a clear plastic chamber (41 x 41 x 30 cm) for 10 to 30 min, with two 16 x 16 photobeam arrays detecting horizontal and vertical movements. The apparatus was cleaned with 70% alcohol after testing of each mouse. Total movements in the open field were recorded for additional data analysis.

Rotarod and balance beam motor experiments. Motor coordination was assessed by accelerating rotarod performance and balance beam crossing. Mice were trained on the rotarod (San Diego Instruments, San Diego, CA) at 16 rpm for 5 min. Mice were then tested for three consecutive accelerating trials of 5 min each with a speed at 4–40 rpm and an inter-trial interval of 60 min. The sequence was repeated for 3 days. Values were averaged across all trials. Balance beam crossing was evaluated as described[22]. Briefly,

mice were trained for three trails on the balance beam and evaluated for time to cross for three trials. The inter-trial interval was 60 min.

Neuropathology. YAC128 mice were sacrificed at 18 months; BACHD mice were sacrificed at 12–14 months. Mice were deeply anesthetized with Avertin (tribromoethanol, 250 mg/kg) and transcardially perfused with 0.9% saline. One hemibrain was drop-fixed in 4% paraformaldehyde for 48 h and stored at 2% paraformaldehyde until use. The other hemibrain was snapped-frozen at -70°C. Vibratome sagittal sections (50 µm) were prepared using Vibratome-3000. Sections were labeled with anti-synaptophysin (1 µg/ml; Boehringer Mannheim, Indianapolis, IN) and FITC-conjugated horse anti-mouse IgG (1:75; Vector Laboratories, Burlingame, CA) and imaged by confocal microscopy (Nikon C1si Spectral Confocal). To quantify microglial immunoreactivity, we immunostained sections with a rabbit monoclonal antibody against Iba-1 (1:1000, DakoCytomation), biotinylated secondary antibody, avidin-coupled to horseradish peroxidase and reacted with DAB. To evaluate BM–derived brain macrophages/microglia, we co-labeled sections with anti-Iba-1 (secondary with anti-rabbit Alexa 549, Jackson Immunoreasearch) and anti-GFP (secondary with anti-mouse FITC, Vector Labs). For all immunohistochemistry analyses, at least three fields of views were imaged randomly and blinded and percent of immunoreactivity was evaluated by MetaMorph software (Molecular Devices).

Serum cytokine analysis. Blood samples were obtained from animals at 6, 9, and 12 months after behavioral testing. Serum cytokine levels were quantified using Meso Scale

Discovery (MSD) assays as per the manufacturer's protocol and analyzed on a SECTOR 2400 instrument (MSD). The operator was unaware of the disease state of each sample during processing, and statistical analysis was performed independently. The serum cytokines were normalized to the non-transplanted WT or chimeric WT control samples as irradiation, and bone marrow transplantation may elevate peripheral cytokines in the early stages.

Statistics. All data are expressed as the mean ± SEM. For each outcome measure, one-way ANOVA analysis with Bonferroni post-tests was performed to determine levels of significance, unless otherwise indicated.

Results

Bone marrow transplantation is well tolerated and leads to efficient reconstitution of host-derived cells in HD mouse models

Bone marrow transplants were performed in 4 and 8 week old YAC128 and BACHD mice, respectively (Fig. 1). The reconstitution of BMDCs was determined in the blood of chimeric WT and BACHD mice using FACS 12–14 weeks after transplantation by gating for GFP+ and CD11b+ cells. The percentage of $GFP^+/CD11b^+$ cells in the blood of chimeric mice was similar to that found in β-actin GFP donor mice, indicating that the bone marrow transplant procedure led to an efficient reconstitution of donor derived cells in WT and BACHD mice (Fig. 2A-C). Similar results were obtained in studies with YAC128 mice (data not shown).

We next used immunohistochemistry to determine if BMDCs were found in the brains of chimeric mice. At 12–13 months of age, brain sections from bone marrow chimeras were labeled with antibodies against the microglia/macrophage marker Iba1 and GFP to evaluate their colocalization. As expected, the cortex of transgenic Iba-1 GFP control mice, the majority of cells that expressed GFP were positive for Iba-1 (Fig. 2D, top row). $GFP^+/Iba\text{-}1^+$ cells were also detected in the cortex of chimeric WT (data not shown) and YAC128 mice brains (Fig. 2D, middle panel), but not in brain sections from mice that did not receive a transplant (Fig. 2D, bottom panel). Similar results were obtained in BACHD mice (data not shown). As determined by FACS, these GFP-expressing cells were composed of both CD45-low and CD45-hi populations (data not shown), characteristic of parenchymal microglia and infiltrating macrophages, respectively. We observed no significant effect of genotype on the levels of $GFP^+/Iba\text{-}1^+$

cells in the cortex of chimeric mice (Fig. 2E). However, a significant increase in GFP+/Iba-1^{+} cells in the striatum of BACHD mice that received BACHD bone marrow was observed relative to other genotypes (Fig. 2F). Overall, our results indicate that bone marrow transplantation after lethal irradiation is well tolerated and leads to a significant recruitment of BMDCs into the blood and brains of two independent mouse models of HD.

Bone marrow transplantation confers modest protection against behavioral deficits in mouse models of HD

We first evaluated the effects of bone marrow transplantation in the YAC128 transgenic mouse model of HD that expresses full-length htt and displays behavioral and neuropathological deficits that may resemble those found in HD patients[23-25]. Bone marrow from Iba1-GFP transgenic mice was transplanted into lethally irradiated 3-week-old WT and YAC128 mice. As reported[24], non-transplanted YAC128 mice were significantly (P<0.001) heavier than WT littermate controls. Although mice that received transplants weighed significantly (P<0.001) less than non-transplanted littermate controls, there was no effect of genotype on body weight in chimeric WT and YAC128 mice (Fig. 3A and B).

We next determined the effects of bone marrow transplantation on hypokinetic deficits in YAC128 mice using an open field apparatus. As described[24], YAC128 mice that did not receive a transplant showed a significant (P<0.01) decline in total open field activity at 12 months of age. In contrast, WT and YAC128 mice that received WT bone marrow transplantation were not significantly different (Fig. 3C and D).

We also quantified the effects of bone marrow transplantation on motor performance in YAC128 mice, as measured in rotarod and balance beam traversal assays. While YAC128 mice, as described[24], showed a significant ($P< 0.0001$) rotarod deficit at 12 months of age, this deficit was not rescued in mice that received a bone marrow transplant (data not shown). In contrast, while YAC128 mice performed worse than WT littermates in a balance beam traversal assay, chimeric YAC128 mice were not significantly different than WT littermates that received a transplant (Fig. 3E).

We further examined the effects of bone marrow transplantation in BACHD mice, which express full-length htt but may have more robust neuropathological and behavioral deficits than YAC128 mice[25]. Bone marrow from β-actin-GFP transgenic mice was transplanted into lethally irradiated 8-week-old WT and BACHD mice. Similar to YAC128 mice (Fig. 3A), BACHD mice were significantly ($P<0.001$) heavier than WT littermates, and all mice that received a bone marrow transplant weighed significantly less than those that did not (Fig. 4A and B). Moreover, in transplanted mice there was no significant effect of genotype on body weight.

We next evaluated the effects of bone marrow transplantation on hypokinetic deficits in BACHD mice using an open field apparatus. As described[26], BACHD mice that did not receive a transplant showed significant deficits in total open field activity at 12, but not 6 months of age (Fig. 4C and D). In contrast, WT and BACHD mice that received WT bone marrow were not significantly different at 12 months of age (Fig. 4D). Notably, BACHD mice that received BACHD bone marrow were also not significantly different to BACHD mice that received WT bone marrow. Furthermore, WT mice that received

BACHD bone marrow were not significantly different than those that received WT bone marrow (Fig. 4D).

We next evaluated the effects of bone marrow transplantation on motor performance in BACHD mice, as measured by rotarod analysis. While BACHD mice showed significant rotarod deficits at 6 and 12 months of age, as described[26], bone marrow transplantation did not rescue these deficits (data not shown).

We also evaluated the effects of bone marrow transplantation using a balance beam traversal assay. While BACHD mice were slower than non-transplanted WT littermates at 6 months of age, BACHD mice that received WT bone marrow were not significantly different than WT mice that received WT bone marrow at this time point (Fig. 4E). However, at 12 months of age, a significant effect of genotype on balance beam performance was observed in BACHD mice that received WT bone marrow (Fig. 4F). At this time point, BACHD mice that received WT bone marrow and BACHD mice that received BACHD bone marrow were not significantly different (Fig. 4F). Overall, our results suggest that bone marrow transplantation confers modest, but significant benefits on some, but not all, behavioral deficits that were examined in YAC128 and BACHD mice.

Bone marrow transplantation in BACHD mice increases brain levels of synaptophysin

The loss of striatal neurons in BACHD mice is observed at late stages of disease, after behavioral manifestations are clearly evident[26]. As previous studies described the loss of synapses in R6/2 mice[27,28], which may correlate better with behavioral deficits in HD

mouse models, we quantified the effects of bone marrow transplantation on synaptophysin levels in BACHD mice after completion of behavioral assays. Similar to R6/2 mice, synaptophysin levels in the cortex of BACHD mice were significantly lower than in WT littermate controls (Fig. 5A). While BACHD mice that received a WT bone marrow transplant had a significant increase in synaptophysin levels, a similar increase was also observed in WT mice that received WT bone marrow. Moreover, WT or BACHD mice that received a BACHD bone marrow transplant also had increased synaptophysin levels. Similar results were obtained in the striatum of BACHD mice that received bone marrow transplants (data not shown). Interestingly, the increase in synaptophysin levels relative to non-transplanted controls was significantly higher in BACHD mice than in WT mice (Fig. 5B). As synaptophysin levels were not decreased in YAC128 mice relative to littermate controls at 12 months of age (data not shown), we did not evaluate the effects of bone marrow transplantation on this outcome measure.

Bone marrow transplantation prevents cytokine and chemokine changes in YAC128 mice

Inflammatory cytokines are increased in blood samples from HD patients and in YAC128 mice[8]. Using a mesoplex assay that measures multiple cytokines and chemokines simultaneously, we found significant increases in IL-6, CXCL1 (a functional homolog of IL-8 in mice), IFN-γ, and IL-10 in serum samples of YAC128 mice relative to WT littermate controls mice (Fig. 6). In contrast, there was little or no significant difference among genotypes for these cytokines in mice that received bone marrow transplants. As BACHD mice did not have significant increases in cytokines and chemokines relative to

WT littermate controls at 12 months of age (data not shown), we did not evaluate the effects of bone marrow transplantation on this parameter.

Discussion

HD is widely recognized as a movement disorder that is associated with the massive loss of medium spiny neurons in the striatum. However, many studies have indicated that pathology is also observed in other brain regions and in non-neuronal tissues[9,29-33]. A number of studies have described the early and chronic elevation of pro-inflammatory cytokines[8,34] and chemokines (Wild *et al*., in press) that are hypothesized to be derived from cell autonomous effects of mutant htt in monocytes and macrophages. In this study we formally tested if reconstituting the peripheral immune system by performing allogeneic bone marrow transplants with WT cells in two HD mouse models influenced pathogenesis. We found that this procedure, when performed in pre-symptomatic mice is well tolerated and modestly, but significantly, prevented a number of behavioral and pathological deficits, and normalized levels of cytokines and chemokines. While the magnitudes of protective effects in this study are modest, it is worth emphasizing that the observed benefits are occurring while mutant htt is still exerting its toxic effects in neurons. As such, our results suggest that immune cells may play an important disease-modifying role in HD that may be amenable to therapeutic intervention.

Bone marrow transplantation led to a highly significant increase in levels of synaptophysin in the brains of BACHD mice. To our surprise, synaptophysin levels were increased in all mice that received a bone marrow transplant, even when the bone marrow was derived from BACHD mice. Increased synaptophysin levels have also been described in mouse models of Alzheimer's disease and stroke after bone marrow transplantation[35-37]. The mechanisms that underlie increased synaptogenesis after bone marrow transplantation are not well understood, but several studies have shown that total

body irradiation leads to a breakdown in the blood-brain-barrier concomitant with increased levels of cytokines and chemokines, which leads to the infiltration of BMDCs into the brain parenchyma[38,39]. We hypothesize that BMDCs that enter the brain are attracted to vulnerable brain regions by local production of chemokines and cytokines, and may support synaptogenesis by secreting neurotrophic factors[8,36,39]. Infiltrating BMDCs might also directly survey and protect synapses in an effort to restrict further damage, as described for parenchymal microglia[40]. Interestingly, while all mice that received transplants had increased levels of synaptophysin, the relative increase of synaptophysin levels was highest for BACHD chimeras that received bone marrow transplants, suggesting that mutant htt specific changes in brain microenvironments might contribute to the danger signals that occur after total body irradiation that serve to attract BMDCs into the CNS. As synapse loss may precede frank neuronal loss in HD patients[27,41], we believe that our data showing increased synaptophysin levels after bone marrow transplantation might have important clinical implications.

Bone marrow transplantation in HD mice also resulted in the normalization of cytokine and chemokine levels. Levels of IL-6, IL-8, IFN-γ, and IL-10 are increased in the blood of HD patients and mouse models[32,34]. Confirming previous studies, these cytokines were increased in YAC128 mice, and these changes were largely attenuated in mice that received bone marrow from WT mice. It was previously hypothesized that increased levels of cytokines and chemokines that occur in HD patients are due to a cell autonomous effect of mutant htt expression in these cells, and our data strongly support this hypothesis. High chronic levels of cytokines and chemokines that are uncontrolled are likely to have deleterious consequences that could ostensibly contribute to

pathogenesis in peripheral tissues as well as ongoing neurodegeneration in the CNS. For example, IFN-γ and IL-6 are key signaling molecules in the immune system that activate macrophages and the production of other cytokines, that mediate fever and the acute phase response and might also contribute to muscle wasting and cachexia that is observed in HD patients. IL-8 is a chemokine produced by macrophages and epithelial cells that binds CXCR1/CXCR2 and is a chemoattractant that induces chemotaxis of neutrophils. Increased levels of this cytokine would suggest that diseased tissues in HD patients are sending danger signals to induce an innate immune response, and high chronic levels of this cytokine might reflect an impairment in immune cells (such as neutrophils) in their ability to mount an effective innate immune response. Consistent with this scenario, we identified a profound impairment in the ability of peripheral blood monocytes isolated from HD patients to migrate towards the chemokine MCP-1, dramatic deficits in the migration of macrophages from HD mouse models to respond to a peritoneal thioglycollate challenge, and decreased process extension and retraction of microglial processes in BACHD mice as determined by two-photon in vivo imaging (Chapter 3). It is also important to emphasize that changes in peripheral levels of cytokines can also indirectly influence brain function and dysfunction by several different communication pathways from the periphery to the brain that include the humoral, neural and cellular pathways[42]. For example, activation of endothelial cells in the brain parenchyma by cytokines such as IL-6 is responsible for the subsequent release of second messengers that act on brain targets[43].

Two recent studies in mouse models provide further support for the hypothesis that dysfunction of the peripheral immune system might play an important disease-modifying

role in HD. Genetic deletion of the cannabinoid receptor 2 (CB2), a protein expressed predominantly in peripheral immune cells that regulates production of pro-inflammatory cytokines via NFκb[44-46], exacerbates pathogenesis in a mouse model of HD[47]. More recently, peripheral inhibition of the mitochondrial enzyme kynurenine 3-monooxygenase (KMO) in blood cells increased survival and prevented synaptic loss and CNS inflammation in the R6/2 mouse model of HD (Zwilling and Huang *et al.*, Cell in press). The neuroprotective effects of KMO inhibition are likely due not only to changes in neurotransmitter release that are controlled by secretion of tryptophan metabolites from peripheral immune cells that cross the blood-brain-barrier, but also by dampening of innate immune responses.

Chronic elevation of cytokines and chemokines are almost certainly bound to have important clinical ramifications for HD patients. A recent study showed that acute and chronic systemic inflammation, associated with increases in serum TNF-α, is associated with an increase in cognitive decline in Alzheimer disease, and based on our results we would predict similar findings in HD patients. However, in contrast to AD patients, elevated levels of cytokines in HD patients are found at the *earliest* disease stages and persist chronically throughout the entire disease course[8]. Thus, studies to examine the consequences of peripheral depletion of cytokines for which therapeutic monoclonal antibodies are widely used in humans, such as TNF-α and IL-6, in mouse models of HD are clearly warranted.

Finally, it is worth emphasizing that bone marrow transplantation is a widely used clinical procedure for numerous diseases including sickle cell anemia, aplastic anemia, congenital neutropenia, leukemia, lymphoma, multiple myeloma, etc. Although this

procedure involves well-characterized risks, it is generally well tolerated and in two independent HD mouse models our studies showed no significant differences in their ability to tolerate total body irradiation and bone marrow transplantation. As an alternative to bone marrow transplantation, mesenchymal stem cells, which can be derived from bone marrow, may also hold promise for HD. Transplantation of mesenchymal cells engineered to over-express brain-derived neurotrophic factor (BDNF) or nerve growth factor (NGF) in the striata of YAC128 mice improved behavioral deficits[48]. In a mouse model of cerebellar degeneration, transplantation with bone marrow derived mesenchymal cells resulted in increased transcripts of synaptic proteins and amelioration of electrophysiological deficits[49]. Overall, these studies and the data presented in the current study support the continued investigation of potential disease-modifying effects of immune cells in HD.

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Figure 1

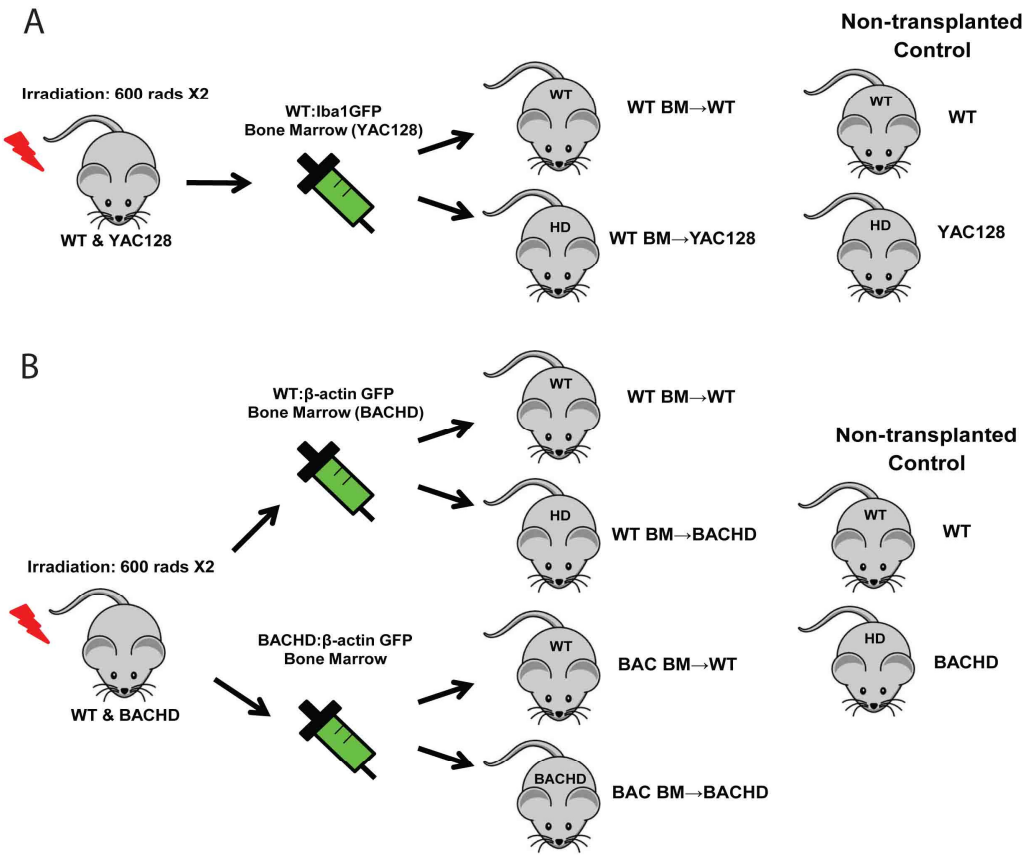


Figure 1. Generation of bone marrow chimeras in YAC128 and BACHD full-length transgenic htt mouse models. ***A,*** YAC128 mice and WT littermates were lethally irradiated at 3–4 weeks of age and transplanted with bone marrow from transgenic Iba1-GFP mice. ***B,*** BACHD mice and WT littermates were lethally irradiated at 2 months of age and transplanted with bone marrow from β-actin GFP mice or BACHD; β-actin GFP mice. Non-transplanted WT and YAC128/BACHD mice were also tested in these studies.

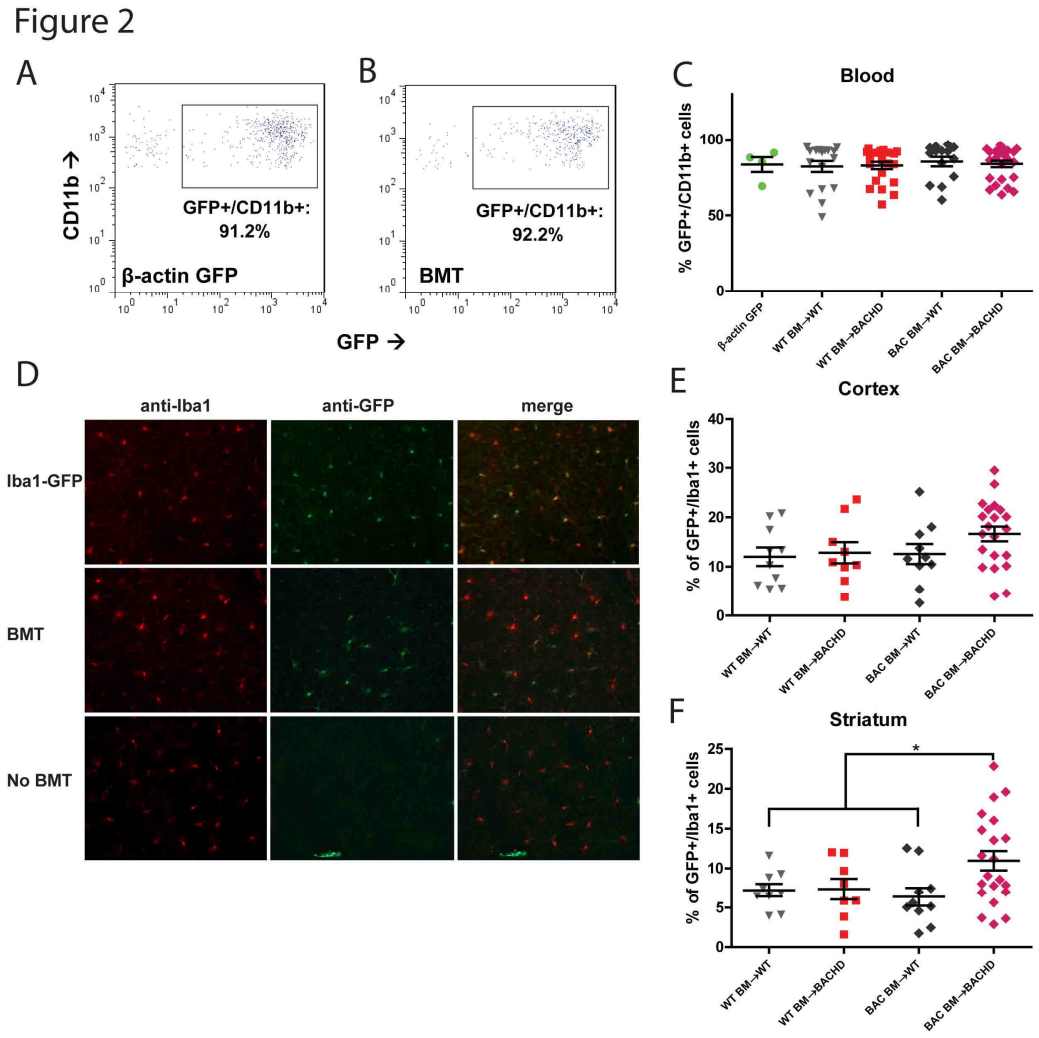


Figure 2. GFP-positive monocytes and bone marrow derived microglia/macrophages were detected in blood and brains of bone marrow chimeras. ***A, B,*** Representative plots of fluorescence-assisted cell sorting (FACS) demonstrate that bone marrow chimeras of BACHD have similar GFP+ monocyte (CD11b+) populations (%) in blood as β -actin GFP. ***C,*** Summary of GFP+ monocyte (GFP+ cells within CD11b+ population) in β-actin GFP mice, WT mice receiving normal bone marrow (WT BM → WT), BACHD mice receiving normal bone marrow (WT BM → BACHD), WT mice receiving BACHD bone marrow (BAC BM → WT), and BACHD mice receiving BACHD bone marrow (BAC BM → BACHD). ***D,***

Representative images from the cortex of perfused brains from IBA1-GFP mice (top panel), bone marrow chimeras (middle panel), and non-transplanted mice (bottom panel) at 12 months, using anti-Iba1 (1:1000) and anti-GFP (1:1000). ***E–F,*** Percentage of cells labeled with anti-GFP and anti-Iba1 in various groups of bone marrow chimeras of BACHD. The percentage of GFP+ and Iba1+ microglia/brain macrophages is not significantly differently in cortex ***(E)*** but is higher in BAC BM → BACHD group in striatum **(*F*).** Values are mean ± SEM (P<0.01; one-way ANOVA).

Figure 3

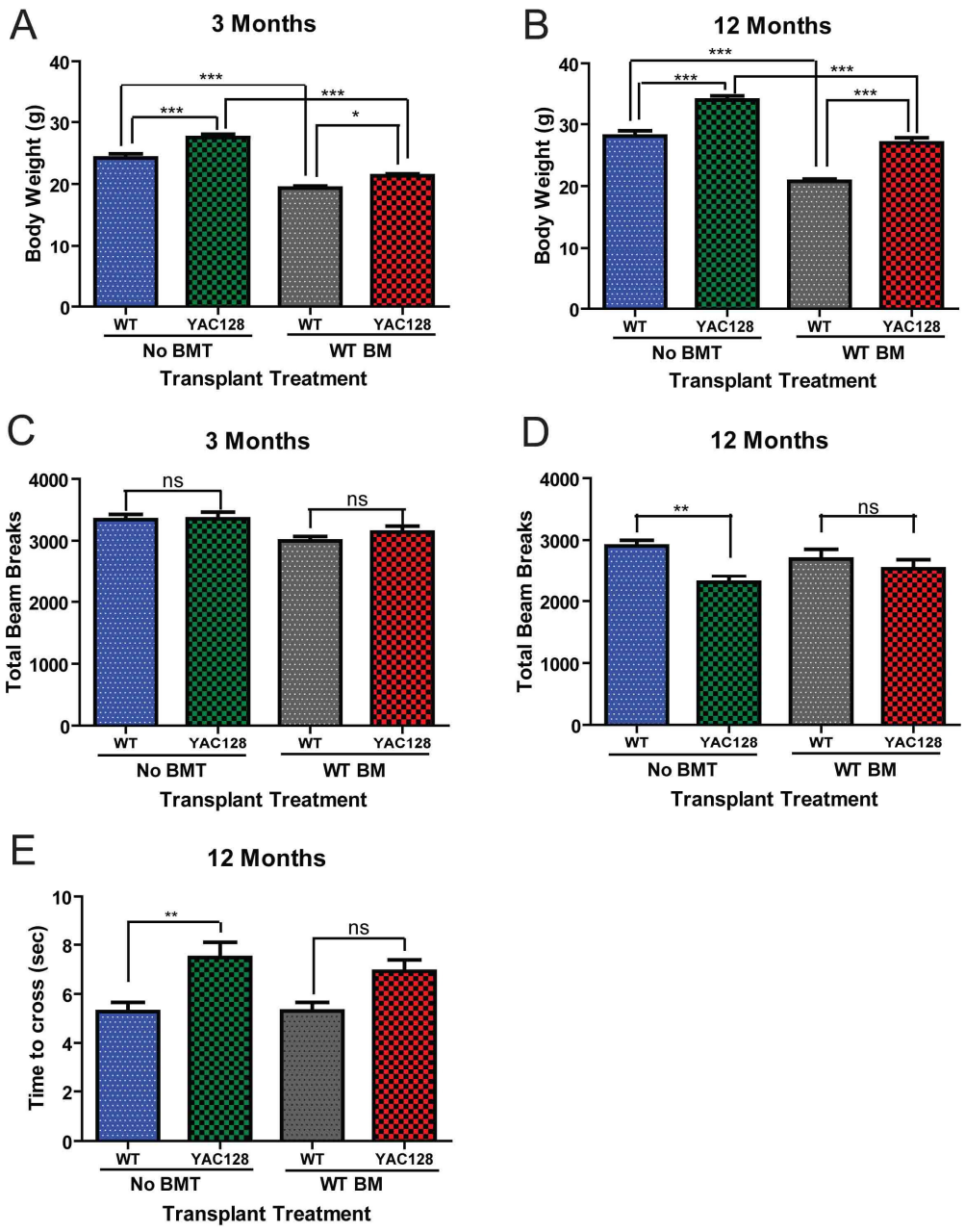


Figure 3. Bone marrow transplantation in YAC128 mice confers modest behavioral changes. ***A, B,*** Non-transplanted YAC128 mice were significantly (P<0.001) heavier than WT littermate controls at 3 and 12 months. Similarly, transplanted YAC128 mice were significantly (P<0.001) heavier than WT transplanted mice. Transplanted WT and

YAC128 mice weighed less than the non-transplanted controls ($P<0.001$). ***C, D,*** At 3 months, there was no genotype effect on the general activity using open field apparatus. However, at 12 months, non-transplanted YAC128 mice displayed hypokinetic activity ($P<0.01$), which was absent in WT and YAC128 mice that received WT bone marrow transplant. ***E,*** Balance beam performance was worse in nontransplanted YAC128 mice compared to the WT littermates ($P<0.01$) at 12 months; however chimeric YAC128 mice were not significantly different than chimeric WT littermates. Values are mean ± SEM (*$P<0.05$, **$P<0.01$, ***$P<0.001$; one-way ANOVA, Bonferonni posttest).

Figure 4

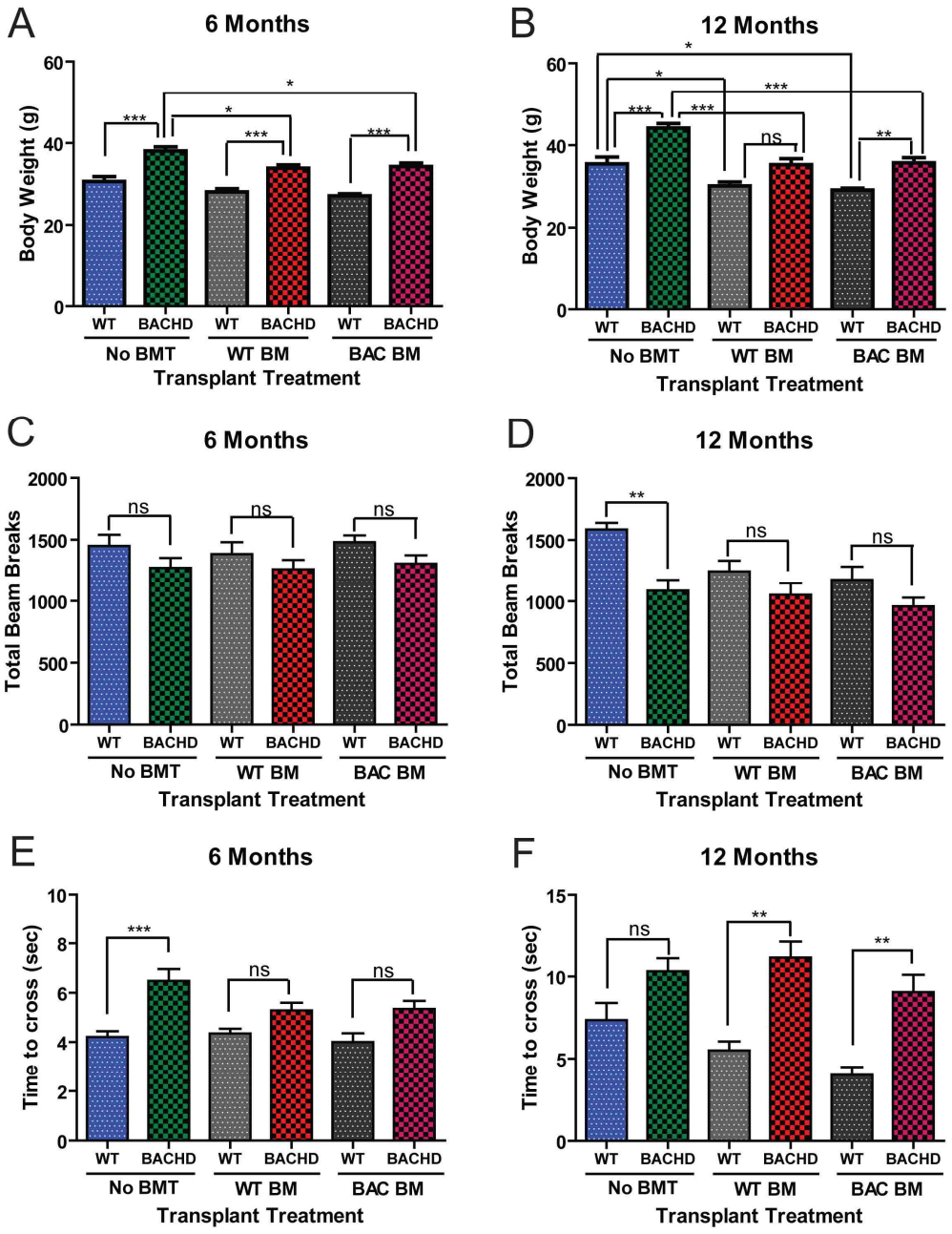


Figure 4. Bone marrow transplantation in BACHD mice confers modest behavioral changes. ***A, B,*** At 6 and 12 months, non-transplanted BACHD mice weighed heavier than non-transplanted WT mice (P<0.001). Transplanted BACHD mice weighed heavier than transplanted WT mice (P<0.001). At 12 months, transplanted mice weighed less

than the non-transplanted mice ($P<0.05$, $P<0.001$). ***C, D,*** At 6 months, there was no genotype effect on the general activity using open field apparatus. However, at 12 months, non-transplanted BACHD mice displayed hypokinetic activity ($P<0.01$), which was absent in WT and BACHD mice that received WT bone marrow transplant. BACHD mice that received BACHD bone marrow transplant were also not significantly different to BACHD mice that received WT bone marrow. ***E, F,*** At 6 months, BACHD mice were slower than non-transplanted WT littermates on the balance beam traversal assay. BACHD mice that received WT bone marrow were not significantly different than WT mice that received WT bone marrow at this time point. BACHD mice that received WT bone marrow and BACHD that received BACHD bone marrow were not significantly different. The changes induced by bone marrow transplantation did not persist at 12 months of age. Values are mean ± SEM (*$P<0.05$, **$P<0.01$, ***$P<0.001$; one-way ANOVA, Bonferroni post-test).

Figure 5

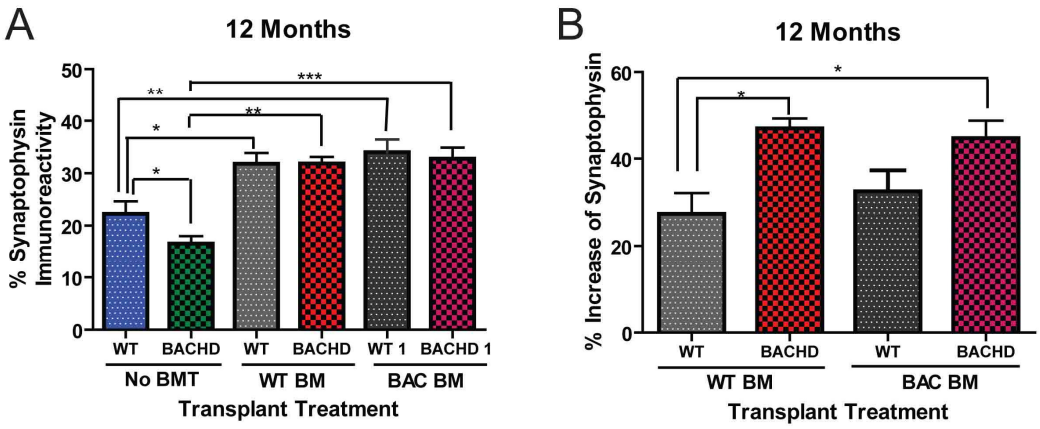
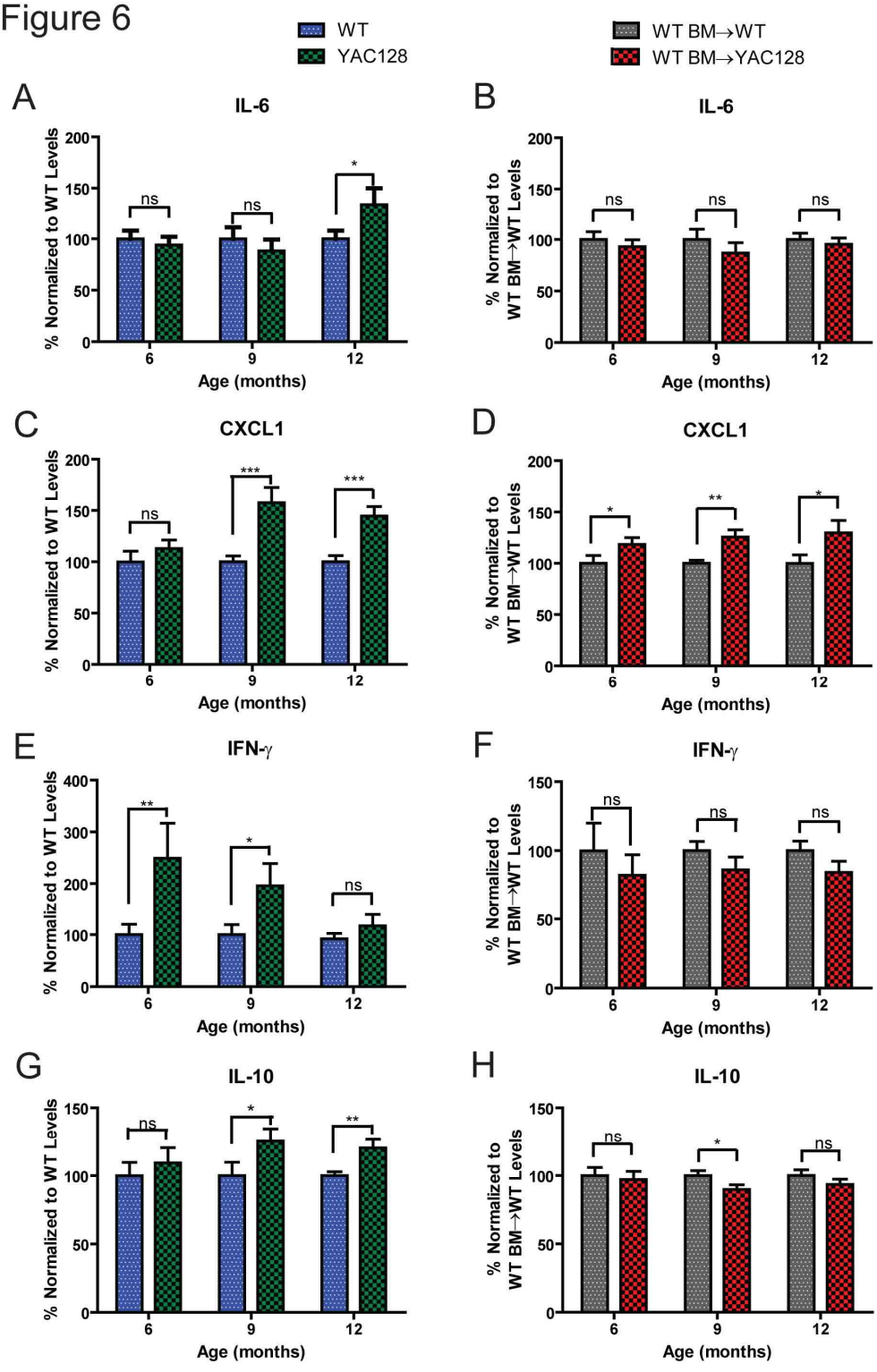


Figure 5. Mice receiving bone marrow transplants have increases in synaptophysin immunoreactivities in the cortex. ***A,*** Synaptophysin levels were decreased in the cortex of BACHD mice compared to WT controls (**P*<0.05*; t*-test). ***A,*** Synaptophysin levels in transplanted WT and BACHD mice were higher than the non-transplant animals in the cortex. Values are mean ± SEM (**P*<0.05, ***P*<0.01, ****P*<0.001; one-way ANOVA, Bonferroni posttest). ***B,*** The percent increase of synaptophysin levels, relative to the non-transplanted controls, was higher in transplanted BACHD mice than that of the transplanted WT mice. Values are mean ± SEM (**P*<0.05; one-way ANOVA, Bonferroni posttest).

Figure 6


WT
YAC128
WT BM→WT
WT BM→YAC128
A
IL-6
% Normalized to WT Levels
Age (months)
B
IL-6
% Normalized to WT BM→WT Levels
Age (months)
C
CXCL1
% Normalized to WT Levels
Age (months)
D
CXCL1
% Normalized to WT BM→WT Levels
Age (months)
E
IFN-γ
% Normalized to WT Levels
Age (months)
F
IFN-γ
% Normalized to WT BM→WT Levels
Age (months)
G
IL-10
% Normalized to WT Levels
Age (months)
H
IL-10
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6
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Figure 6. Bone marrow transplant prevents inflammatory cytokine and chemokine changes in YAC128 mice. At 6, 9, and 12 months, serum cytokines were determined in non-transplanted and transplanted WT and YAC128 mice. ***A, C, E, G,*** Interleukin (IL)-6, CXCL1 (similar functions as IL-8), interferon-gamma (IFN-γ), IL-10 were elevated in non-transplanted YAC128 mice relative to WT mice. Values are mean ± SEM (**P*<0.05, ***P*<0.01; *t*-test). ***B, D, F, H,*** These changes were not observed or observed at reduced levels in chimeric YAC128 mice, relative to chimeric WT mice. Values are mean ± SEM (**P*<0.05, ***P*<0.01; *t*-test).

Chapter 5

Conclusions and Future Directions

This thesis addresses the role of microglia and the immune system in Huntington's disease (HD) using *in vitro* and *in vivo* approaches. Because huntingtin (htt) is ubiquitously expressed, we hypothesized that the expression of mutant htt contributes directly to the dysfunction of microglia and the peripheral immune cells. In addition, we hypothesized that these changes may contribute to the pathogenesis of HD. In **Chapter 2**, we established new microglial cell lines that express htt of various polyglutamine (polyQ) lengths. We found that mutant htt expression leads to cell-autonomous effects in microglia. We found that the expression of mutant htt in microglial cell lines and primary microglia from HD mouse models, upon stimulation, have increased levels of pro-inflammatory cytokines IL-1β and IL-6. In addition, microglial cell lines or primary microglia that expressed mutant htt have decreased expression of insulin growth factor (IGF)-1. In **Chapter 3**, we provide *in vitro* and *in vivo* evidence that expression of mutant htt in microglia and peripheral immune cells (i.e., monocytes and macrophages) causes defects in cellular migration in response to chemotactic stimuli. Importantly, migration deficits were observed in monocytes derived from the blood of HD patients even prior to the onset of neurological and motor symptoms. In **Chapter 4**, we found that bone marrow transplantation is well-tolerated and confers modest benefits in two mouse models of HD. We found that bone marrow transplantation can normalize the hypokinetic and some motor deficits that are otherwise seen in non-transplanted animals. As well, we found that HD mice that received bone marrow transplants had increased levels of synapses. Serum levels of IL-6, chemokine ligand 1 (CXCL1, similar to IL-8), interferon (IFN)-γ, and IL-10 were significantly increased in the HD mice compared to wild-type animals; however, the levels of these cytokines in wild-type and HD mice that received

bone marrow transplants were not different. Collectively, this thesis provides evidence that microglia and the peripheral immune cells may play a disease-modifying role in HD pathogenesis.

The generation of microglial cell lines that express htt exon 1 with various polyQ lengths allowed us to identify the cell-autonomous effects of mutant htt in microglia. We found that cells with the pathogenic polyQ lengths have increased pro-inflammatory cytokines and decreased IGF-1 levels. These cell lines recapitulate features of primary microglia cultured from the fragment mutant htt mouse model, R6/2. The cell lines resulted in the studies that showed the loss of endogenous brain IGF-1 in HD and the finding that mouse neural progenitor cells over-expressing this trophic factor could restore behavioral deficits in the R6/2 model (Silvestroni *et al.*, in preparation). More importantly, the neurotoxic effects of these cell lines will be useful for future mechanistic studies relating to microglia in HD. Indeed, these cell lines are already being used to verify the suppressors of mutant htt toxicity in microglia that were identified in a yeast genetic screen (Mason *et al*., in preparation). As well, we also used these cell lines to study the role of the kynurenine pathway (a tryptophan degradation pathway that produces neurotoxic metabolites) in microglia (**Appendix 1**).

The most consistent changes evaluated in our study are the increased production of pro-inflammatory cytokines and the migration defect. These changes are also observed in microglia and the peripheral immune cells[1]. While mutant htt expression might induce these changes via independent mechanisms, there might be a connection between the increased levels of cytokines and the migration impairment. As in the CNS, circulating levels of pro-inflammatory cytokines and chemokines are elevated in pre-manifest HD

patients, and remain elevated throughout the course of disease[1] (Wild *et al.*, in press). It is possible that the migration deficits in peripheral immune cells lead to chronically elevated levels of pro-inflammatory cytokines, like IL-6, as a compensatory response which may ultimately contribute to central and peripheral symptoms in HD. Taken together, these results indicate that the innate immune system in HD patients is abnormal, and would suggest that patients may be at least partially immune compromised.

Bone marrow transplantation in HD mice resulted in the normalization of cytokine and chemokine levels including those of IL-6, CXCR1 (similar to IL-8), IFN-γ, and IL-10. It was previously hypothesized that increased levels of cytokines and chemokines that occur in HD patients was due to a cell autonomous effect of mutant htt expression in these cells, and our data strongly support this hypothesis. High chronic levels of cytokines and chemokines that are uncontrolled are likely to have deleterious consequences that could ostensibly contribute to pathogenesis in peripheral tissues as well as ongoing neurodegeneration in the CNS. For example, IFN-γ and IL-6 are key signaling molecules in the immune system that activate macrophages and the production of other cytokines, that mediate fever and the acute phase response and might contribute to muscle wasting and cachexia that is observed in HD patients. IL-8 is a chemokine produced by macrophages and epithelial cells that binds CXCR1/CXCR2 and is a chemoattractant that induces chemotaxis of neutrophils. Increased levels of this cytokine would suggest that diseased tissues in HD patients are sending danger signals to induce an innate immune response, and high chronic levels of this cytokine might reflect an impairment in immune cells (such as neutrophils) in their ability to mount an effective immune response. Consistent with this scenario, and as alluded to earlier, the profound

impairment in the ability of peripheral blood monocytes isolated from HD patients to migrate towards the chemokine MCP-1, dramatic deficits in the migration of macrophages from HD mouse models to respond to a peritoneal thioglycollate challenge, and decreased process extension and retraction of microglial processes in BACHD mice as determined by two-photon in vivo imaging (Chapter 3). It is also important to emphasize that changes in peripheral levels of cytokines can also indirectly influence brain function and dysfunction by several different communication pathways from the periphery to the brain that include the humoral, neural and cellular pathways[2]. For example, activation of endothelial cells in the brain parenchyma by cytokines such as IL-6 is responsible for the subsequent release of second messengers that act on brain targets[3].

One important note, however, is that mutant htt expression in immune cells alone does not seem to cause HD phenotypes as determined by transplantation of HD bone marrow into wild-type mice. Therefore the peripheral response is likely a modifier, rather than a cause in the HD pathogenesis.

Our results from the bone marrow transplant study demonstrated some modest, but significant benefits in two HD mouse models. The increase in synaptophysin levels suggests that bone marrow-derived microglia/macrophages might provide some neurotrophic support to the surrounding tissues, perhaps via increases of neurotrophic factors, in order to stimulate synapses formation. We are still in the process of determining if levels of trophic factors like BDNF or IGF-1 levels are increased in a manner that correlates with synaptophysin levels.

Recent *in vivo* imaging studies have demonstrated that under basal conditions microglia are important sentinels in the CNS whose highly motile processes are constantly surveying the brain parenchyma and transiently contacting synapses in an activity-dependent manner[4,5]. We found that microglia have deficits in process extension at the baseline level and more pronounced in response to a laser induced injury. These results might partially explain the decreased level of synapses that are seen in the brains of HD mice[6] (Zwilling & Huang *et al*., Cell in press, Chapter 3), as the dynamics of process extensions are abnormal in HD mice. In addition, because process extension in microglia is important for maintenance of synapses, the wild-type BMDCs that have entered the brain parenchyma of chimeric HD mice would have normal process extension and might provide the trophic support that the synapses were missing, which could also be a contributing factor for the increased synapses levels we observed upon bone marrow transplantation.

Bone marrow transplantations have become new treatment avenues for many neurodegenerative diseases. Once we have clearly identified various mechanisms that are responsible for the mutant htt-mediated changes in immune cells, we can further utilize genetic modification of bone marrow stem cells to target specific disease-modifying pathways that are induced by immune cells. For example, if the production of neurotrophic factors by bone marrow-derived microglia/macrophages is indeed responsible for the increase in synapses that we observed, then bone marrow stem cells could be engineered to over-express these trophic factors and be delivered to neuronal tissues by bone marrow-derived microglia/macrophages. Another example could be the modification of the cannabinoid receptor 2 (CB2), a protein that is expressed

predominantly in peripheral immune cells that regulates production of pro-inflammatory cytokines via NFκb[7-9]. Deletion of CB2 exacerbates pathogenesis in a mouse model of HD[10]; therefore activating CB2 or its downstream effectors may also be beneficial. Altogether, our bone marrow transplant study provides an important platform for future HD research relating to BMDCs.

The bone marrow transplant study has unveiled some aspects of the role of inflammation in HD; however, there are still limitations regarding the understanding of how inflammation plays a role in the brain parenchyma and disease phenotypes. Firstly, by replacing the peripheral immune system, we show that PBMCs may play a role in the inflammation in the mouse models of HD since there was normalization of the inflammatory cytokines and chemokines. On the other hand, we do not have direct evidence that the normalization of these cytokines leads to the behavioral or neuropathological changes. One method to address this question could be the use of antibodies against the cytokines and evaluating the behavioral or neuropathological changes in the mouse models of HD. Furthermore, upon bone marrow transplantation in our study, BMDCs are recruited to the brains of the bone marrow chimeras, even though the resident microglia that express mutant htt still remain. While the presence of these BMDCs may contribute to the beneficial effects of the transplantation, our results may be more robust if the resident microglia that express mutant htt were absent, which could be possible if the HD mouse models were in the PU.1 knock-out background[11]. Alternatively, the BACHD mouse model could be crossed to the CD11b-cre mice to delete mutant htt from myeloid cells, which could be a way to further understand how the absence of mutant htt in the myeloid cells of the periphery and the brain would contribute

to disease pathogenesis. Because we did not observe any significant morophological or immunoreactivity changes in microglia in the YAC128 and BACHD mouse models, it was difficult to determine if bone marrow transplantation ameliorated the inflammation in the brains of these mice. However, of interest is that in the BACHD mice that were receiving BACHD bone marrow, there was increased Iba1 immunoreactivity than those that received WT bone marrow (data not shown). This result suggests that in the BACHD brain, there may be microenvironmental changes such as inflammatory factors in the brain that could render the parenchymal microglia to be more reactive. This effect may not be observed under normal conditions; however it might become pronounced upon a procedure such as the irradiation from the bone marrow transplantation which could stimulate some inflammatory changes in brain[12,13]. More importantly, Iba1 immunoreactivity was normal in the BACHD mice receiving WT bone marrow, which could be an indication of a reduction in inflammation by bone marrow transplantation of WT cells. Overall, the results of our bone marrow transplantation study open up a line of studies that could further examine how inflammation in the periphery and the brain can contribute to HD pathogenesis.

In addition, though not addressed in our studies, it is of interest that, despite the migration defect in HD immune cells, these cells do not appear to have problems in their reconstitution and migration out of the bone marrow (i.e., in mice transplanted with HD bone marrow). One possibility is that immune cells of earlier lineage retain signaling mechanisms from their micro-environment to prevent these impairments. Another possibility is that perhaps mutant htt expression is down-regulated in these earlier stem cells thereby reducing the migration impairment we saw in monocytes and macrophages.

In this vein, studies of the deletion of chemokine receptor 2 (Ccr2) in mice might be informative for what is happening in HD mice. Ccr2 is a receptor that is responsible for monocyte migration, including their migration from the bone marrow to the bloodstream[14]. However, the deletion of Ccr2 in mice surprisingly does not affect BMDCs reconstitution. Although there is clear evidence that the number of monocytes is decreased in the blood, these cells are still able to transmigrate from the bone marrow. As well, the mice are not immune compromised. This observation suggests that there are likely some intrinsic or extrinsic signals that are intricate to the hematopoietic stem cell system and its microenvironment. These signals may, in turn, help tolerate the migration defects of monocytes from the bone marrow to the bloodstream. If we can identify the key signals that are relevant to the microenvironment in the bone marrow, we may be able to identify new targets in the dysfunctional immune cells.

Finally, the work in this thesis has important clinical implications for HD patients and suggests that current drugs in use for chronic inflammatory conditions that target pro-inflammatory cytokines, such as IL-6 and TNF-alpha (which are elevated in HD patients), might provide therapeutic benefits in HD patients. While mutant htt clearly wreaks havoc on neurons, our studies suggest its effects in microglia and immune cells might also contribute to the pathogenic process in HD.

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Appendix I

Mutant Huntingtin in Microglia Modulates the Kynurenine Pathway

Abstract

Huntington's disease (HD), for which there is no effective therapy, is a fatal neurodegenerative disorder caused by an expanded polyglutamine (polyQ) tract in the protein huntingtin (htt). Two neuroactive metabolites in the kynurenine pathway, 3-hydroxykyurenine (3-HK) and quinolinic acid (QUIN), are found at abnormally high levels in the cortex and striatum but not in unaffected brain regions in early-grade HD and in three mouse models of HD. Our lab reported a genome-wide loss-of-function suppressor screen in yeast and identified kynurenine-3-monoxygenase (KMO), an enzyme in kynurenine pathway that leads to the formation of 3-HK, as a potent suppressor of mutant htt toxicity (measured by yeast viability). Yeast cells expressing mutant htt (htt) have increased 3-HK and QUIN levels. Upon deletion or pharmacological inhibition of KMO, 3-HK and QUIN levels returned to control levels. In addition, a small pilot study showed that administration of a selective KMO inhibitor, Ro 61-8048, significantly improved behavior and survival in a HD mouse model (unpublished data). Interestingly, the majority of kynurenine synthesis in the brain occurs in glia and not neurons. KMO is expressed predominantly in microglia in human brains. Furthermore, in HD patients and mouse models, microglia are activated and become dystrophic in early stages of the disease. The goal of studies described in this appendix was to investigate if microglia expressing mutant htt cause an increase in production of 3-HK in a manner that contributes to neurotoxicity.

Background and Significance

Excitotoxicity and the Kynurenine Pathway in HD

One hypothesis for neurodegeneration in HD is excitotoxicity, a mechanism of neuronal death characterized by excessive stimulation of excitatory amino acid receptors (i.e., NMDA receptors)[1,2]. This hypothesis is based on the disruption of neuronal circuitry in the striatum, which receives excitatory input from the cortex. Many distinct neuropathological features and the chemical imbalance in HD can be duplicated in experimental animals by intrastriatal injection of excitotoxins, such as kainic acid and QUIN, a selective endogenous NMDA receptor agonist that occurs in the brain[3-5]. QUIN-induced striatal lesions, in particular, closely resemble those in HD brains, including axon-sparing lesions and survival of medium aspiny and large neurons[5,6]. These findings implicated QUIN in HD.

QUIN is a key metabolite in the kynurenine pathway, the major route of tryptophan degradation in mammals (Fig. 1). In addition to QUIN, two additional neuroactive metabolites are found in the kynurenine pathway – 3-HK, the bioprecursor for QUIN, and kynurenic acid (KYNA), an NMDA antagonist. 3-HK, similar to QUIN, is neurotoxic; it generates free radicals[7,8] and potentiates the toxic effects of QUIN[8]. Postmortem brains from early grade HD patients have significant increases in 3-HK and QUIN levels in the cortex and striatum, but not in areas unaffected by neurotoxicity[9]. Increased 3-HK and/or QUIN levels also occur in several HD mouse models.

We recently reported a genome-wide loss-of-function suppressor screen in yeast and identified 28 gene deletions that suppressed toxicity of mutant htt fragment[10]. *Bna4* (encodes kynurenine 3-monooxygenase) was identified to be one of the most potent

suppressors. Kynurenine 3-monooxygenase (KMO) is a mitochondrial enzyme that converts kynurenine into 3-HK, which then ultimately gets metabolized to QUIN. Deletion of *bna4* eliminated production of 3-HK, QUIN and consequently reactive oxygen species (ROS) levels in mutant htt yeast strains[10]. Moreover, pharmacological inhibition of KMO activity by Ro 61-8048[11], a potent bioavailable compound, reduced 3-HK and ROS to wild-type levels in mutant htt yeast cells and dose-dependently improved mutant htt-mediated toxicity.

In the brain, the enzymes of the kynurenine pathway are mainly localized in glial cells[12]. The rate-limiting enzyme of this pathway is indoleamine 2,3-dioxygenase (IDO). Even though IDO is expressed in astrocytes, neurons and microglia, only microglia produce detectable amounts of QUIN[13] because KMO is expressed predominantly in microglia. Thus, microglia in the CNS, through KMO activity, are the major source of 3-HK and QUIN.

Results

KMO Is Expressed Predominantly in Microglia

In human brains, most kynurenine pathway enzymes are expressed in microglia and astrocytes[12]. To confirm these studies in mice, a former rotation student in the Muchowski lab (Christine Cheah) performed quantitative RT-PCR (qRT-PCR) on RNA samples to measure levels of *KMO* mRNA in primary microglia, astrocytes, and cortical neurons from wild-type (WT) mice. *KMO* mRNA was not detected in primary neurons, but microglia expressed 50-fold more *KMO* mRNA than astrocytes (data not shown).

3-HK Levels Are Increased in R6/2 Primary Microglia

Levels of 3-HK, but not QUIN, are elevated in the striatum, cortex, and cerebellum of R6/2 mice[14]. Because KMO catalyzes the formation of 3-HK predominantly in microglia, in collaboration with Dr. Thomas Moeller (University of Washington), we determined if 3-HK or QUIN levels were abnormal in primary microglia isolated from R6/2 mice on postnatal day 3 (P3) by HPLC (high performance liquid chromatography) and gas chromatography mass spectrometry (GC-MS). 3-HK levels were 40% higher in R6/2 than WT microglia ($P<0.05$); however, no change was observed in QUIN (Fig. 1). These results are consistent with observations in dissected brain regions of R6/2 mice. In R6/2 microglia treated with 10 μM Ro-61-8048, 3HK levels were 16% lower than vehicle-treated cells ($P<0.03$). Thus, the kynurenine pathway is upregulated in microglia at early stage in a mouse model.

3-HK Levels Are Increased in Immortalized Microglial Cell Lines that Express a Mutant Htt Fragment with Expanded PolyQ Repeats

In collaboration with Dr. Paolo Guidetti (University of Maryland), 3-HK and QUIN levels were analyzed by HPLC and GC/MS in N9 and BV2 transduced with 25, 46, and 72Q htt (Fig. A-D). These cell lines are described in detail in Chapter 2. Expression of expanded polyQ mutant htt, but not the non-pathogenic polyQ repeat, was sufficient to activate the kynurenine pathway in N9 cells and produce more 3-HK ($P<0.001$). This effect was partially counteracted by treatment of 10 μM Ro 61-6048 ($P<0.05$). Although BV2 cells did not show a significant increase, there is a strong trend towards a 3-HK increase in 72Q htt-expressing cells, which was counteracted by the inhibitor. Interestingly, QUIN levels were unchanged. Overall, these results were consistent with data from dissected brain tissues and primary microglia from R6/2 mice.

A KMO inhibitor can partially rescue mutant htt microglia-mediated neurotoxicity

We next assayed the effects of conditioned medium from these cells on the viability of primary cortical neuron cultures using a coverslip co-culture approach. In the absence of stimuli, conditioned medium from 72Q cells caused a significant decrease in the percentage of viable neurons than 25Q cells, as determined by MAP2 immunostaining. This toxic effect was not seen when 72Q cells that were pretreated with the KMO inhibitor Ro 61-8048 (Fig. 5d). These results suggest that microglia that express a mutant htt fragment likely secrete concentrations of 3-HK that are toxic to neurons in co-culture and that inhibition of KMO in these cells attenuates this toxicity.

Summary and Conclusion

Our results demonstrate that mutant htt expression in microglia leads to aberrations in the kynurenine pathway thereby causing an increase in 3-HK and neurotoxicity. We demonstrated that mutant htt expression in microglial cell line and primary microglia from R6/2 all produce increased levels of 3-HK. Interestingly, in the presence of Ro 61-8048, microglia-induced neurotoxicity was partially rescued. This study provides additional support to the hypothesis that the kynurenine pathway plays an important role in HD pathogenesis. The conditioned media coculture approach has already been used by Mason *et al*. to validate modifiers of htt toxicity identified in microglia. Furthermore, our lab has demonstrated that modulation of KMO in the periphery additionally leads to elevation of the neuroprotective metabolite kynurenic acid (KYNA) in a mouse model of HD. As a result, this leads to increased levels of KYNA in the brain (Zwilling & Huang *et al*, Cell, in press). Overall, our studies of how mutant htt in microglia modulates the kynurenine pathway may help guide ongoing in vivo studies of this pathway in mouse models of HD.

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Figure 1

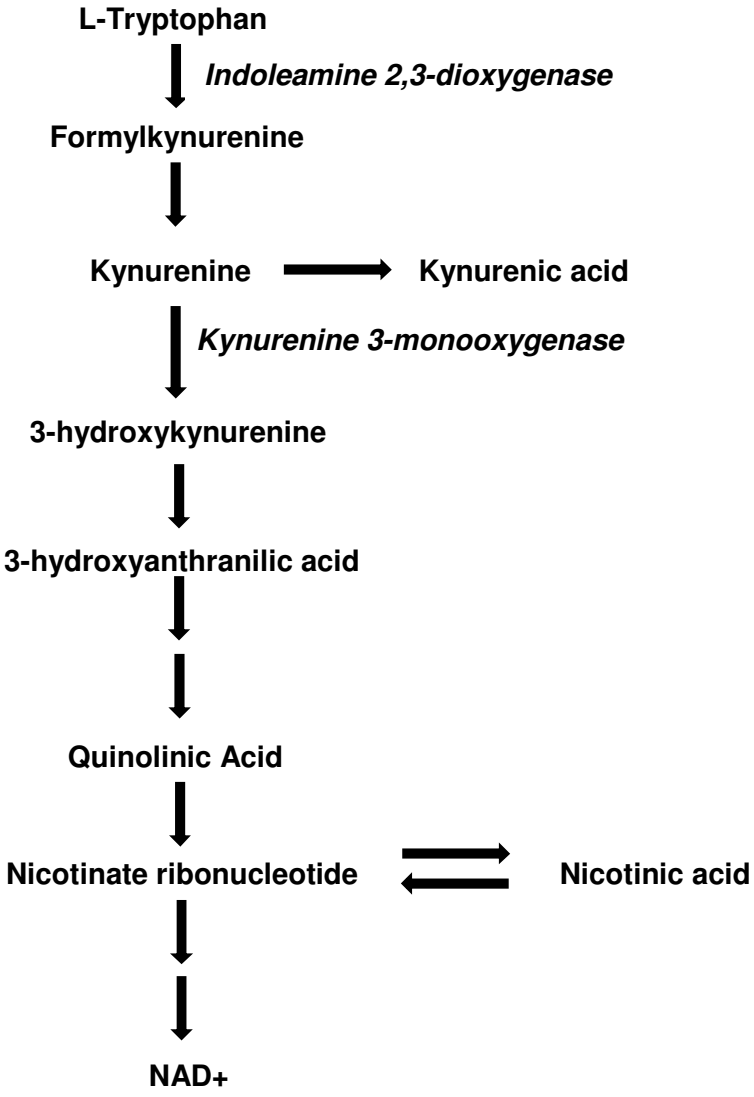

Figure 1. The kynurenine pathway. Arrows represent enzymatic steps in the pathway, with the selected enzymes listed to the right.

Figure 2

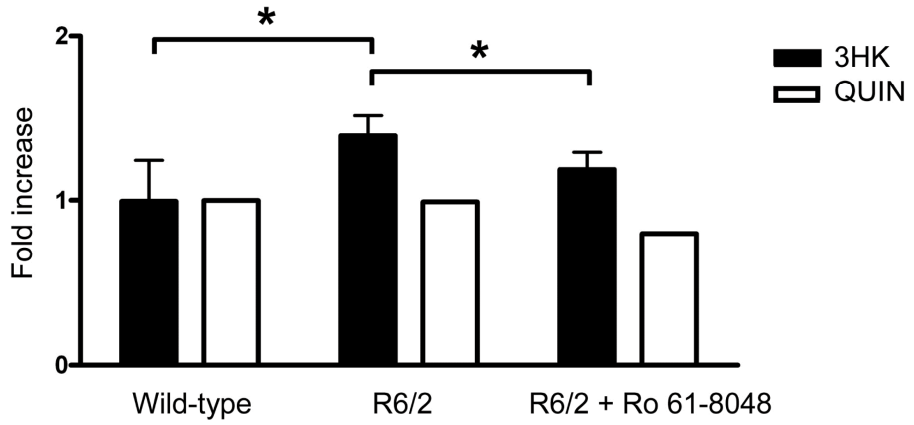

Figure 2. Elevated 3-HK levels in primary microglia from R6/2 mice. 3-HK and QUIN levels were analyzed in primary microglia after 6 h in culture with and without 10 μM Ro 61-4048. 3HK levels were ~40% higher (*P = 0.004) in R6/2 than WT microglia, and 16% lower (*P < 0.05) in Ro 61-8048 treated than mock-treated R6/2 microglia (ANOVA and Bonferroni posttest). No significant changes were observed in QUIN levels.

Figure 3

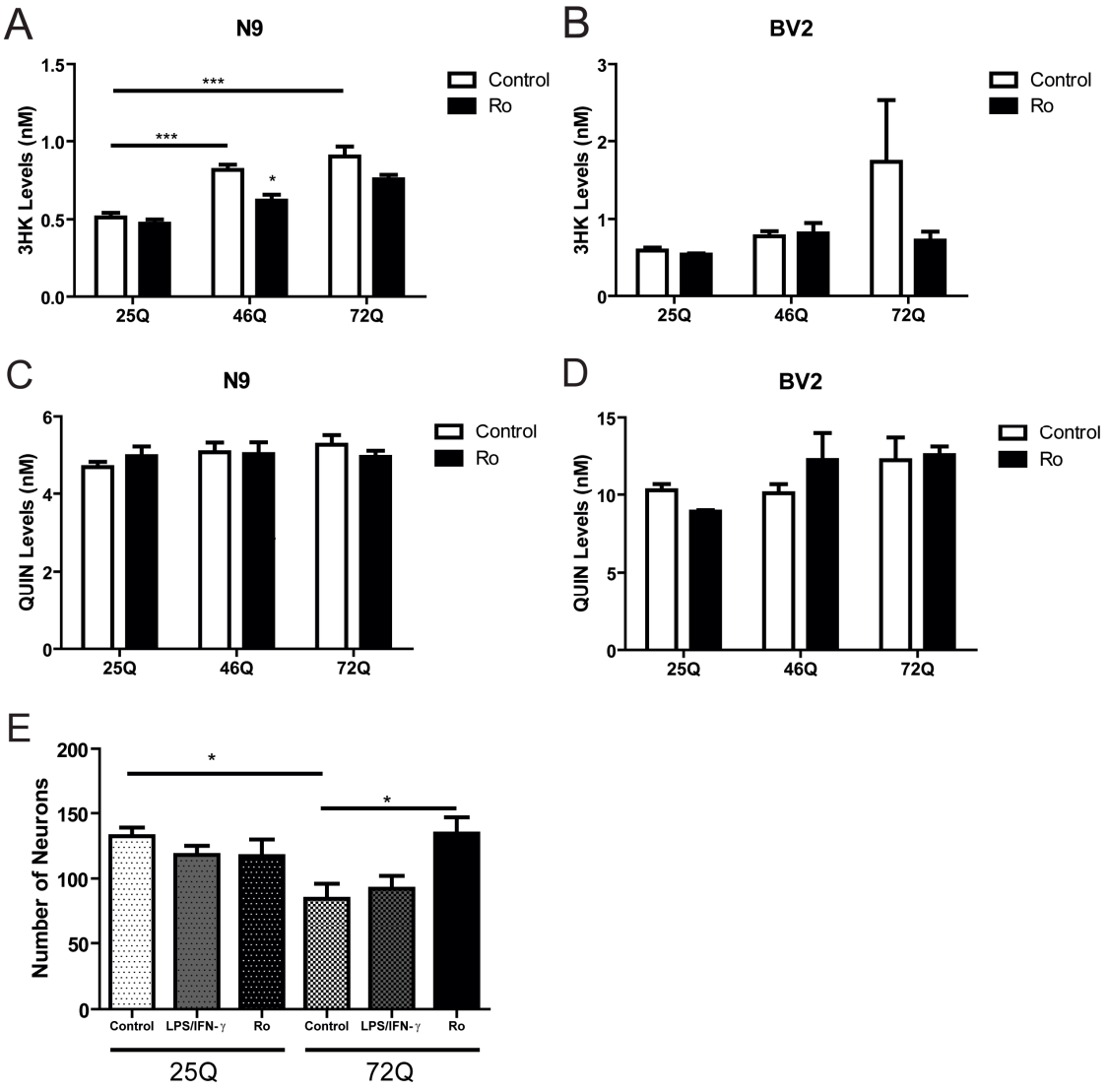

Figure 3. Microglia expressing polyQ htt longer than 25Q have higher levels of 3-HK and causes KMO-dependent neurotoxicity. ***A-D,*** Cell lysates were obtained from N9 and BV2 to determine 3-HK and QUIN levels. The elevated 3-HK levels were partially reduced by 10 μM Ro 61-8048 in N9 46Q cells ($P<0.05$), while there was a trend for the drug to decrease 3-HK levels in N9 72Q cells and BV2 72Q cells. ***E,*** Conditioned-medium from microglia expressing 25Q and 72Q mutant htt (control

untreated cells or cells stimulated with 100 ng/ml LPS and 10 units/ml IFN-γ) were cultured with DIV 7 primary mouse neurons for 72h, using a coverslip approach. Neurons were stained with anti-MAP2 antibody (1:200) and anti-Texas Red antibody (1:200). The effects of microglia conditioned medium on neuronal viability were quantified by counting the number of neurons in images collected by fluorescent microscopy from 20 random fields using a 40x objective. Values are mean ± SEM (*P<0.01, ***P<0.001; one-way ANOVA)

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