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Deficiency of Toll-like receptors 2, 3 or 4 extends life expectancy in Huntington's disease mice

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Abstract

Huntington's disease (HD), an autosomal dominant neurodegenerative disorder characterized by progressive striatal and cortical atrophy, has been strongly linked with neuroinflammation. Toll-like receptors, a family of innate immune receptors, are a major pathway for neuroinflammation with pleiotropic effects on neuronal plasticity and neurodevelopment. We assessed whether deficiency for TLRs 2, 3 or 4 affects life expectancy in the N171-82Q mouse model of HD. Our data indicate that homozygous TLRs 2 and 3 as well as heterozygous TLR4 deficiency significantly extends the life expectancy of HD mice. Our data suggest that multiple TLR pathways may be involved in the neuroinflammatory and degenerative processes during HD.

Keywords: Neuroscience, Neurology

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1. Introduction

Neurodegenerative diseases, including Alzheimer's disease (AD), Parkinson's disease (PD), multiple sclerosis (MS) and Huntington's disease (HD), are associated with chronic neuroinflammation and increased levels of various cytokines. HD is an autosomal dominant neurodegenerative disorder characterized by progressive striatal and cortical atrophy, along with autonomic, cognitive, psychiatric and motor symptoms [1, 2]. Neuroinflammation is strongly implicated in the neurodegenerative process of HD [3, 4, 5, 6]. An altered immune profile in HD patients is known to correlate with disease progression. Although it is clear that neuroinflammation and microglia activation are not the primary cause of HD, mice that express mutant Huntingtin (mHtt) exclusively in microglia showed an enhanced neuronal death in the presence of sterile inflammation. Specifically, microglial mHtt expression triggers cells autonomous pro-inflammatory activation characterized by the release of pro-inflammatory cytokines, reactive oxygen species (ROS), and neurotoxic metabolites. In parallel, expression of mHtt in astrocytes induces the cell-autonomous repression of factors involved in supporting neuronal integrity, such as chemokine (C-C motif) ligand (CCL)-5 and transforming growth factor (TGF)-β. This suggests a potential contribution of mHtt-expressing brain immune cells activation and neuroinflammation to the development of HD pathogenesis. Microglial- or astrocyte-specific mHtt expression, however, is not the primary cause of HD, as expression of mHtt in neurons triggers cell-autonomous neuronal degeneration and apoptosis. In addition, studies with BAC HD mice showed marked amelioration of disease with reduction of mHtt in both cortical and striatal neurons, without affecting its expression in microglia [7].

Genome-wide analysis of mRNA expression study conducted on human prefrontal cortex tissues from 20HD patients, found that RNA levels of four of the five Nuclear Factor kappa-B (NFkB) family members, namely, NFkB1, NFkB2, RELA and RELB are differentially expressed compared with healthy controls. Additionally, 15 cytokine receptors, including interleukin (IL)-17RB, IL13RA1 and IL4R are altered in prefrontal HD tissue although the corresponding cytokines are not altered [5]. Microgliosis, often correlated with neuroinflammation, involves functional morphological changes and transcriptional regulation of gene expression is thought to play an important role in the neurodegenerative process in HD. Chang and colleagues have examined 13 microglia-derived inflammatory factors in the R6/2 mouse HD model as well as in HD patients and found that plasma levels of IL-6, Matrix metalloproteinase (MMP)-9, Vascular endothelial growth factor (VEGF) and Tumor growth factor (TGF)-β were significantly increased in HD patients compared with healthy individuals. Curiously, plasma levels of IL-18 are significantly reduced in HD patients, suggesting that the classical inflammasome pathway is not involved in the overall inflammatory process in HD. These changes

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occur relatively late as they are not observed in pre-HD carriers [8]. These changes occur at differential progressive stages of the disease; R6/2HD mice exhibit higher VEGF levels than WT littermates as early as 7 weeks of age, higher levels of IL-6 as early as 9 weeks of age (early symptomatic) and higher levels of MMP-9 and TGF-β at 11 weeks of age (middle symptomatic) compared with WT littermates. In agreement with evidence from human studies, 11 weeks old (middle symptomatic) R6/2 mice exhibit reduced plasma IL-18 levels compared with WT littermates. The existence of similar inflammatory markers in both humans and mice HD indicates that immune activation in HD is widespread and detectable in peripheral plasma across disease stages [9]. Some of the key inflammatory mediators in HD, namely, IL-6, IL-8 and tumor necrosis factor (TNF)-α, are elevated both centrally and peripherally [5]. IL-6 and IL-8 are not only strongly affected in the main area of HD pathology, the striatum, but also show significant changes in cortex and cerebellum [6]. Importantly, specific inhibition of soluble TNF-α, effectively improves functional outcomes in R6/2 mice [10].

α-2-Macroglobulin (A2 M) is an acute-phase protein, whose release is stimulated by IL-6. Complement (C)7 and C9 are components of membrane attack complex (MAC), whose formation is modulated by clusterin. Clusterin expression is correlated with disease progression in plasma and CSF of HD patients. IL-6 is a pleiotropic cytokine that is also involved in the regulation of energy balance by decreasing food intake and increasing energy expenditure, possibly through its action on the hypothalamus, which causes the rapid weight loss observed in the different HD mouse models [11].

Toll like receptors (TLRs) are type-I trans-membrane receptors that are best known as sensors of microbial-associated molecular patterns (MAMPs) by cells of the innate immune system [12]. In addition, TLRs recognize danger-associated molecular patterns (DAMPs), also termed ‘endogenous ligands’, generated in response to traumatic tissue injury or as a by-product of inflammation [13]. TLR activation by either endogenous or exogenous ligands promotes signaling events which result in the production of inflammatory cytokines by central and peripheral immune response [14]. While the release of immunogenic molecules from dying neurons triggers an immune response, or sensitize the immune cells to the inflammatory environment, the intrinsic microglial mHtt expression might compromise their physiological function and exacerbate the release of pro-inflammatory cytokines and oxygen species [15].

Within the brain, TLR 2, 3 and 4 are predominantly expressed on microglial and glial cells [16]. TLR2 is expressed on the plasma membrane and can form heterodimers with either TLR1 or TLR6. TLR2 can dimerize with TLR1 or TLR6 to recognize triacylated or diacylated lipopeptides respectively from bacteria. TLR3 is localized in endosomal compartments and acts as a sensor for

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double-stranded RNA (dsRNA), primarily as a product of replicating viruses, but also from endogenous RNA sources such as mRNA [17] or products of cell damage such as stathmin [18]. TLR4 is activated during infections by gram-negative bacterial lipopolysaccharide (LPS). Central activation of TLR4 by LPS reduces hippocampal pyramidal neuron dendrite length and impairs hippocampal-dependent spatial reference memory in an inflammation-dependent manner, suggesting a neuroinflammatory role for TLR4 following activation with its cognate MAMP ligands [19, 20].

Many different endogenous ligands have been described for TLRs 2, 3 and 4 [21], including heat-shock proteins or nucleic acids that are present after the integrity of an adjacent cell has been compromised, as well as proteins normally present in tissues such as hyaluronan or fibrinogen [22, 23]. TLRs are present in the brain, where their expression is strongest on microglial and glial cells [24, 25]. Data suggest that as HD progresses, DAMPs capable of activating TLRs are generated, including protein aggregates and constituents released from dead neurons [7]. Thus, degenerating neurons and aggregating mHtt likely trigger a trans-cellular cross-amplifying pro-inflammatory process involving activation of microglial and glial cells, which in turn produce cytokines that further damage neurons.

To investigate the contribution of TLRs 2, 3 and 4 to the neuroinflammatory process in the progression of HD, we crossed mice deficient for either TLR2, 3 or 4 with the N171-82Q HD mouse strain and assessed the impact on survival and weight loss. Our data indicates that deficiency for TLR2, 3, and 4 extends survival in HD mice, and warrants further mechanistic investigation into the roles of TLRs in HD.

2. Materials and methods

2.1. Animals and breeding scheme

B6C3-Tg(HD82Gln)81Dbo/J (N171-82Q) HD model mice, congenic TLR2$^{-/-}$ mice (B6.129-*Tlr2*^{tm1Kir}/J, TLR3−/− mice (B6N.129s1-Tlr3tm1Flv/J) and TLR4$^{-/-}$ mice (B6.B10ScN-*Tlr4*$^{lps-del}$/JthJ) were purchased from Jackson Laboratories (Bar Harbor, ME, USA). HD mice heterozygous for TLRs were generated by crossing HD mice with homozygous TLR2, TLR3 or TLR4 null mice. This study was conducted under the National Institute on Aging's Institutional Animal Care and Use Committee (NIH animal welfare assurance number is: A4149-01). In accordance with these guidelines, mice were euthanized if they lost more than 20% of their initial body weight. When the animal exhibited clear neurological signs (unsteady gait, tremors/shakiness, lack of grooming) the animals were monitored on a daily basis by the caretakers and investigator in consultation with the attending veterinarian. to prevent dehydration in the animal due to a possible lack of food/water intake, Fruit jello or water soaked food, was

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placed on the floor of the cage, including daily injection of warm sterile saline (1.5–2 ml, intraperitoneally) if necessary. Mice were euthanized promptly when they were unable to ingest food or water. The aim of this study was to assess effects of genetic crosses on life expectancy, an experimental endpoint that was established at the beginning of the experiment.

2.2. Genotyping

Genotyping of the mice for the different TLRs as well as huntingtin was conducted by extracting DNA from the ears of the mice immediately following weaning at 4 weeks of age. A sterile single-use blade was used to excise tissue from each mouse. Briefly, DNA extraction was conducted on a 0.25 cm^2 mouse ear punch in a 0.5 ml lysis buffer (100 mM Tric-HCl, pH = 8.5, 5 mM EDTA, 0.2% SDS and 200 mM NaCl with 0.2 mg/ml Proteinase K added fresh (10 μl/ml of 20 mg/ml Proteinase K). The lysate was incubated overnight at 55 °C and subsequently incubated 65 °C, 20′ to inactivate Proteinase K. Lysates were then centrifuged at 18,000 RPM for 10 min and the supernatant was transferred to a fresh tube. An equal volume of isopropanol was added and the lysates were centrifuged for 8 min to pellet the DNA. The DNA was then re-suspended in 100 μl TE (10 mM Tris, 1 mM EDTA, pH = 8.0). 1 μl of DNA solution was used for subsequent PCR reactions. PCR reactions to determine the genotype status of the mice were conducted using the primer pairs indicated in Table 1.

The expected size of the amplification products for genotyping TLR2 are the following: mutant TLR2: 334 bp; wild type TLR2, 499 bp. The expected bands for genotyping TLR3 are the following: mutant TLR3: 208 bp; wild type TLR3: 341

Table 1. Primers used for genotyping of TLRs2–4 and Huntingtin. WT: wildtype.

Gene name	Primer sequences	Annealing temperature
Huntingtin	Forward: 5′ ATGGCGACCCTGGAAAAGCTG 3′	60 °C
	Reverse: 5′ GGCTGAGGAAGCTGAGGAG 3′	
TLR2	Wildtype forward: 5′ ACGAGCAAGATCAACAGGAGA 3′	58 °C
	Mutant forward: 5′ GGGCCAGCTCATTCCTCCCAC 3′	
	Common reverse: 5′ CTTCCTGAATTTGTCCAGTACA 3′	
TLR3	Mutant forward: 5′ GCCAGAGGCCACTTGTGTAG 3′	58 °C
	Wildtype forward: 5′ GCAACCCTTTCAAAAACCAG 3′	
	Common reverse: 5′ AATTCATCAGTGCCATGAGTTT 3′	
TLR4	Mutant forward: 5′ GCAAGTTTCTATATGCATTCTC 3′	56 °C
	Mutant reverse: 5′ CCTCCATTTCCAATA GGTAG 3′	
	WT forward: 5′ ATATGCATGATCAACACCACA G 3′	
	WT reverse: 5′ TTTCCATTGCTGCCCTATAG 3′	

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bp. The expected size of the amplification products for genotyping TLR4 are the following: mutant TLR4: 140 bp; wild type TLR4: 390 bp. The expected size of the amplification product for genotyping the transgenic Huntingtin is 331 bp. Denaturing was performed at 94 °C for 30 s, annealing for 10 s (temperature for each primer pair is indicated in Table 1), and elongation was performed at 72 °C for 10 s. No tissues other than ear tissues were extracted from the mice, and analysis was restricted to survival and weight measurements.

2.3. Weight and survival assessment

Animal weight was measured on a weekly basis since the animals were genotyped at the weaning age of 4 weeks, from weaning at 4 weeks of age until the time of death or euthanasia. N171-82Q mice are known to rapidly lose weight due to inability to reach food and water when they are close to the end of their life [26]. Therefore, humane endpoints were set for this survival study, namely, when mice exhibited reduced movements and weight, food and water were placed on the bedding of their home cage. When the mice exhibited immobility, they were euthanized using 95% CO2. In cases in which mice died between health monitoring, the cause of death was constipation. Since we euthanized the mice when the disease prevented them from reaching food and water, no analgesics or anesthetic were administered routinely to the mice.

2.4. Statistical analysis

Weight data was analyzed using two-way analysis of variance (ANOVA) and Bonferroni post hoc correction to determine pairwise comparisons amongst multiple data sets. Significance was set at $P < 0.05$. Statistical analysis was carried out using GraphPad Prism 5 software. Survival data was analyzed using SPSS, using the Breslow (generalized Wilcoxon) and the Log rank (Mantel-Cox) tests.

2.5. Ethical approval

This study was carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) revised 1996. Formal approval to conduct the experiments described has been obtained from the animal subjects review board of the National Institute on Aging, NIH, and could be provided upon request. All efforts were made to minimize the number of animals used and their suffering.

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3. Results

3.1. TLR deficiency extends life expectancy in HD mice

We have examined the impact of deficiency for either TLR2, TLR3 or TLR4 on life expectancy of the N171-82Q HD mouse model. To examine the contribution of TLR2 to HD progression, TLR2-deficient mice were crossed with HD mice. HD mice with normal TLR2 levels (TLR2+/+, n = 15), heterozygous TLR2 (TLR2 +/−, n = 17) and homozygous TLR2 loss (TLR2−/−, n = 22) were monitored for weight and survival on a weekly basis. While TLR2+/− had a trend to increase life expectancy of HD mice ($P > 0.05$, Wilcoxon), homozygous TLR2−/− HD mice exhibited significantly extended life expectancy compared to TLR2+/+ HD mice ($P < 0.05$, Wilcoxon, Fig. 1A). HD progression in the N171-82Q HD mouse

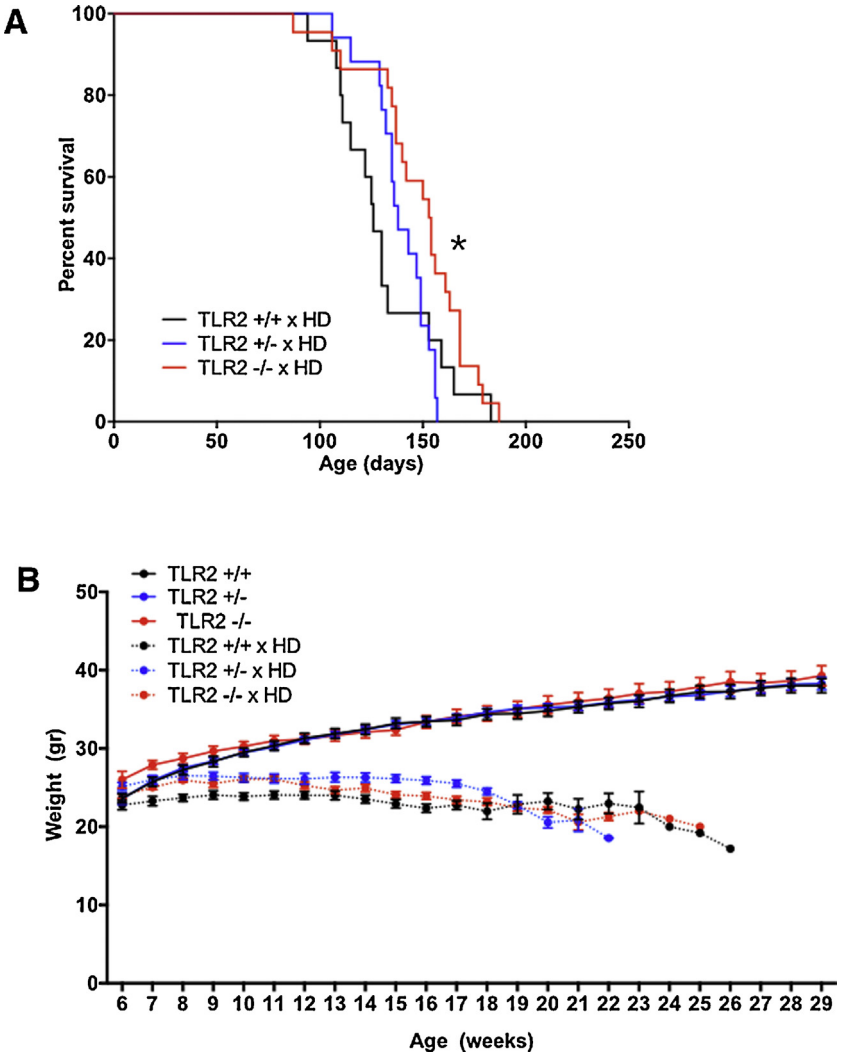

Fig. 1. TLR2 deficiency extends life expectancy in HD mice. (A) TLR2−/− mice were crossed with HD mice to generate TLR2+/+ × HD, TLR2+/− × HD and TLR2−/− × HD mice, and survival rates and (B) weights were recorded. * $P < 0.05$.

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model is characterized by failure to gain weight after 2 months of age and weight loss during disease progression, particularly at the "end stage" in the last 4 weeks before death [26]. Accordingly, TLR2-deficient HD mice did not consistently gain weight over their life span. However, in contrast to its effect on disease onset and progression, weight loss in both heterozygous (TLR2+/−) TLR2 deficiency and homozygous (TLR2−/−) TLR2 deficiency was similar to that of HD mice. (Fig. 1B).

To test the contribution of TLR3 to HD progression, TLR3-deficient mice were crossed with HD mice. HD mice with normal TLR3 levels (TLR3+/+, n = 17), heterozygous TLR3 (TLR3+/−, n = 19) and homozygous TLR3 loss (TLR3−/−, n = 14) were measured for weight and life expectancy on a weekly basis. While heterozygous deficiency for TLR3 (TLR3+/−) did not significantly alter life expectancy ($P > 0.05$, Wilcoxon, Fig. 2A), homozygous TLR3 deficiency (TLR3−/−) increased life expectancy compared with TLR3+/+ HD mice ($P < 0.05$, Wilcoxon, Fig. 2A). Consistent with the N171-82Q model, TLR3-deficient HD mice failed to gain weight over their life span compared to WT mice. No significant difference in weight was observed between TLR3−/− HD or TLR3+/− HD compared with TLR3+/+ HD mice (Fig. 2B).

To test the contribution of TLR4 to HD progression, TLR4-deficient mice were crossed with HD mice. HD mice with normal TLR4 levels (TLR4+/+, n = 15) and heterozygous TLR4 (TLR4+/−, n = 9) were measured for weight and life expectancy on a weekly basis. Heterozygous deficiency for TLR4 (TLR4+/−) significantly increased survival of TLR4+/+ HD mice ($P < 0.05$, Wilcoxon, Fig. 3A). Similar to TLR2- and TLR3-deficient HD mice, TLR4+/− HD mice failed to gain weight and, in fact, lost more weight than HD mice ($P < 0.05$, two-way ANOVA, Fig. 3B).

4. Discussion

Accumulating evidence indicates that neuroinflammation plays a significant role in the pathological course of neurodegenerative disorders in general, and HD in particular. Extensive neuronal cell death is evident in both human HD brains as well as mouse HD models [27], with both cell autonomous and non-cell autonomous mechanisms acting as contributing factors to brain tissue damage [7]. Further, evidence suggests that a significant portion of the brain damage in HD patients results from the release of Danger Associated Molecular Patterns (DAMPs) [28]. DAMPs are known to activate innate immune receptors including TLRs, resulting in translocation of pro-inflammatory transcription factors such as NFkB to the nucleus. This in turn triggers subsequent neuroinflammatory processes orchestrated by pro-inflammatory cytokines such as IL-6, IL-8 and

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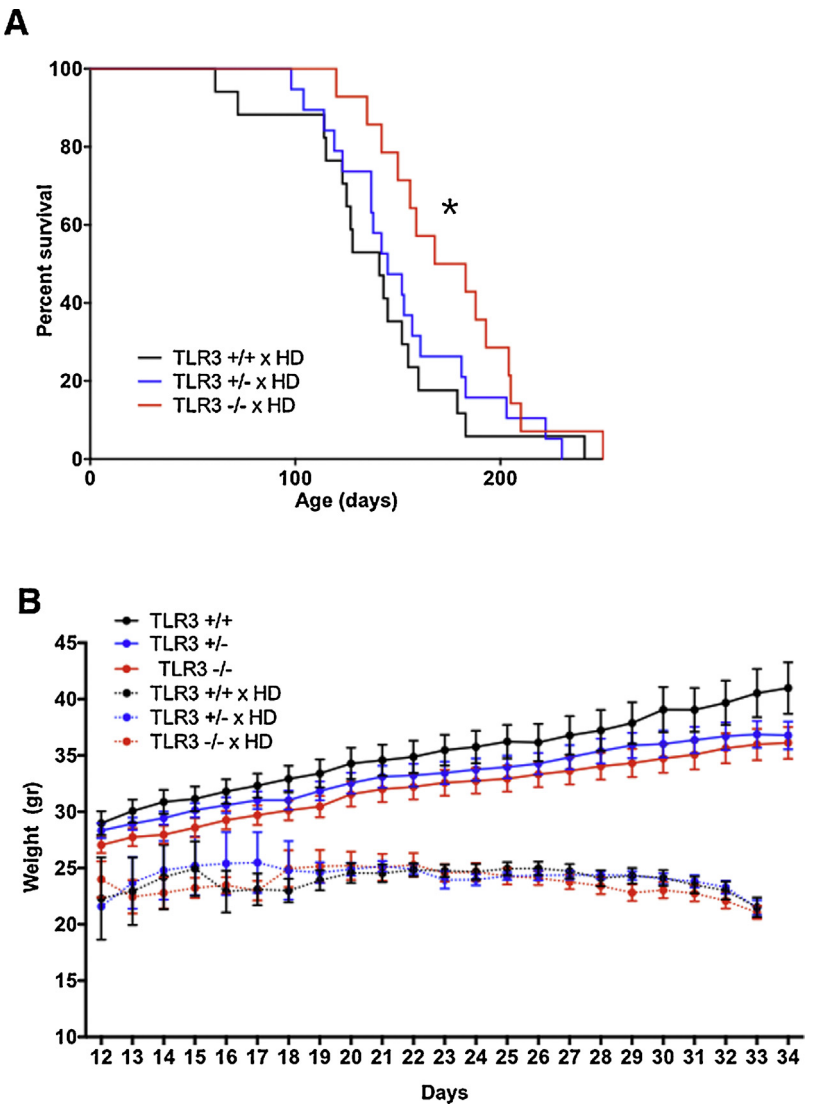

Fig. 2. TLR3 deficiency extends life expectancy in HD mice. (A) TLR3−/− mice were crossed with HD mice to generate TLR3+/+ × HD, TLR3+/− × HD and TLR3−/− × HD mice, and survival rates and (B) weights were recorded. * $P < 0.05$.

TNF-α. Due to its generalized nature, this vicious cycle is common to all neurodegenerative disorders including HD.

Loss of TLRs by itself does not seem to be significantly detrimental to the organism, but carries implications on metabolism, central nervous system and autonomic nervous system functions. Mice deficient for TLR5 are susceptible to spontaneous gut inflammation, metabolic syndrome, and exhibit microbiotal-dysbiosis [29, 30, 31, 32]. Mice lacking TLR2 or TLR4 exhibit reduced basal heart rate, which results from an increase of parasympathetic tone. In addition, thermoregulatory responses to stress are altered in TLR2−/− and TLR4−/− mice, and brown fat-dependent thermoregulation is altered in TLR4−/− mice. Moreover,

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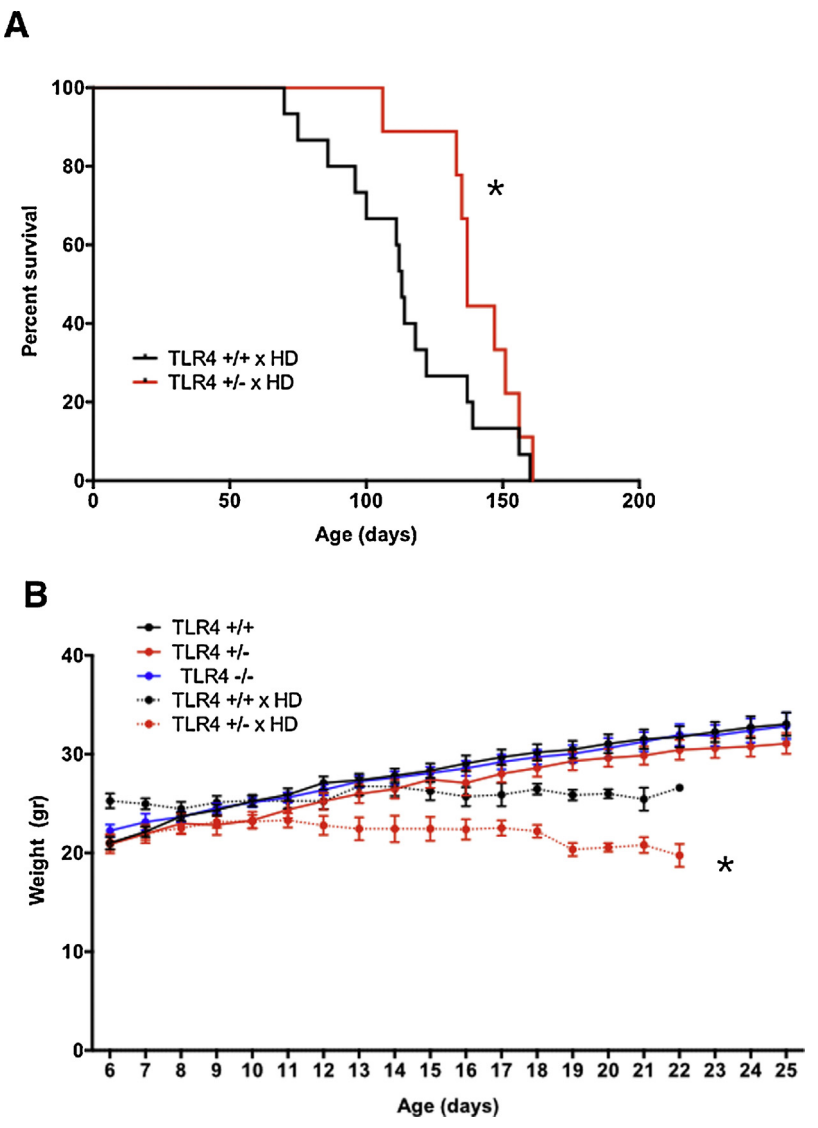

Fig. 3. TLR4 deficiency extends life expectancy in HD mice. (A) TLR4−/− mice were crossed with HD mice to generate TLR4+/+ × HD and TLR4+/− × HD mice, and survival rates and (B) weights were recorded. * $P < 0.05$.

TLR2−/− and TLR4−/− mice consume less food and exhibit a greater mass compared to wild type mice [33]. With respect to impacts on cognitive performance, TLR3 is a suppressor of hippocampal cellular plasticity and memory retention [34], whereas developmental TLR4 deficiency enhances spatial reference memory acquisition and memory retention, impairs contextual fear-learning and enhances motor functions [35].

TLRs mediate signaling via two main signaling mechanisms, the MyD88-dependent and TRIF-dependent pathways (Fig. 4). TLR2 mediates signaling via the MyD88 pathway, whereas TLR3 mediates signaling via the TRIF-dependent

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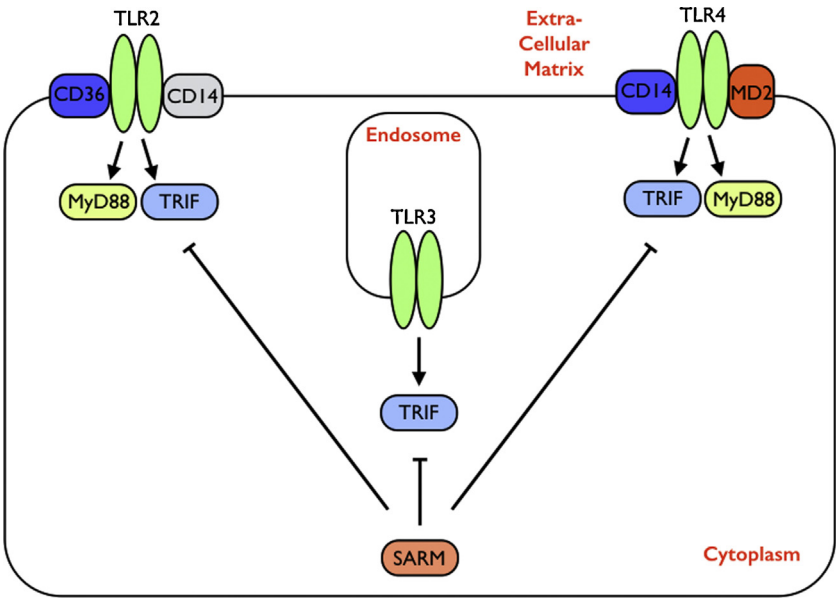

Fig. 4. Intracellular signaling by TLR2, TLR3 and TLR4. TLRs 2 and 4 are located on the cell membrane and mediate signaling via MyD88 and TRIF-dependent pathways, while TLR3, located on the endosomal compartment, mediates signaling via TRIF. Sarm1, an inhibitory TIR-adaptor protein can inhibit TRIF-mediated signaling which can originate by both TLR2, 3 and 4.

pathway and TLR4 mediates signaling via both pathways (Fig. 4). Assessing the impact of these TLRs can therefore provide insights as to the relative contribution of these various TLR-related signaling pathways to the onset and progression of HD. In order to assess whether these members of the TLR family are involved in the pathophysiology of HD, we assessed the impact of developmental deficiencies for TLR2, TLR3, or TLR4 on the life expectancy of HD mice. Our results indicate that homozygous developmental deficiency for either TLR2, TLR3 or TLR4 significantly increases the life expectancy of HD mice. The fact that homozygous TLR2, TLR3 and heterozygous TLR4 deficiencies all significantly extended life expectancy in HD mice is striking. This suggests that during the neurodegenerative process in HD, a milieu of DAMPs is released into the inflamed brain tissue, affecting multiple converging TLR pathways to induce neuroinflammation and alter disease progression. Since the three TLR-deficient transgenic strains we used independently extended life expectancy to a similar extent, it is plausible that a certain inflammatory threshold is reduced when a specific TLR is absent. Under this scenario, DAMPs that typically activate TLR2, 3 or 4, no longer exert their effect, blunting the cumulative inflammatory load. Other TLRs, however, will continue to be activated by different DAMPs to produce neuroinflammation. Indeed, while we have no indication whether baseline inflammatory markers are lower when TLR2, 3 or 4 is deficient in the context of HD pathology, studies on TLR3 deficiency in epilepsy, for example, indicate that TLR3 deficiency blunts the expression of pro-inflammatory cytokines such as TNF-α and interferon-β [36].

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That a deficiency for either TLR2, 3 or 4 independently extended life expectancy raises several question. First, it would be interesting to assess the impact of a triple-transgenic mouse deficient for TLRs 2, 3 and 4, on life expectancy when crossed with an HD mouse strain. Since deficiency for each receptor by itself provides an independent benefit to life expectancy, it would be expected to observe a significant beneficial effect on life expectancy when carrying the HD genotype. TLR2 and TLR4 mediate signaling via MyD88- and TRIF-dependent pathways (Fig. 4), while TLR3 mediates signaling solely via TRIF (Fig. 4). Thus, a similar approach can be also attempted with a double transgenic MyD88−/−/TRIF−/− mouse strain with a predictably similar outcome.

Interestingly, TLR4+/− HD mice exhibited longer life expectancy despite losing weight at a faster rate than TLR4+/+ HD mice. These data are in agreement with Lee and colleagues who recently reported that TLR4 and its endogenous ligand, high-mobility group box 1 (HMGB1), are increased during disease progression in hSOD1(G93A) mice, with TLR4 and HMGB1 are expressed by activated microglia and astrocytes. Similar to our study, ALS mice deficient for TLR4 exhibited significantly extended survival [37].

Several mouse models were developed to study HD, many of which are inherently flawed in various aspects. Both the N171-82Q model which expresses the a truncated protein containing the N-terminal 171 amino acids [26] and the R6/2 model [8] which expresses truncated mHtt with ~125CAG repeats in exon 1, suffer from loss of the natural genomic and protein context of the polyglutamine expansion. This deviation from the genomic context in the human disease can lead to altered regulation and a loss of potential disease-modifying post-translational modifications and protein interactions. In addition, it was shown that mHtt fragments generated by intracellular cleavage have a different subcellular localization than truncated mHtt [38]. Therefore, although truncated fragments in these model cause neuronal toxicity, this might not necessarily recapitulate the disease. Indeed, the N171-82Q HD mouse model utilized in this study expresses exons 1 and 2 of the huntingtin gene (with expanded polyQ82) under the control of a mouse prion promoter, resulting in neuronal-only transgene expression and at a level distinct from that of the endogenous Htt gene. Pathologies such as seizure activity and hyperkinesis that appear in the human conditions and that appear in other HD mouse models such as the R6/2 model are not present in N171-82Q mice. Interestingly, when mHtt is expressed at 160Q but not 98Q repeats in astrocytes, mice develop neurological phenotype [39, 40]. Moreover, when mice expressing 98Q repeats in astrocytes are crossed with N171-82Q mice, the resultant phenotype is more severe [40]. This implies that astrocytes play a role in disease progression in a manner that depends on the number of CAG-repeats, and that astrocyte-related pathology complements neuronal pathology in the disease. In this respect, the use of the N171-82Q model only partially reflects pathology of the disease in humans.

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Resultantly, whereas neuroinflammation, a characteristic of HD in humans is also prevalent in the N171-82Q model, our findings should also be tested in HD mouse models which express mHtt in astrocytes.

Identifying multiple TLR pathways as relevant for life expectancy in HD bears important therapeutic implications. If multiple TLR pathways converge on MyD88 or TRIF, it is possible that targeting these signaling hubs exerts beneficial effect in HD. The observations presented herein serve as a foundation for future studies examining the TLR-related mechanisms mediating neuroinflammation and degeneration in HD, highlighting TLRs as candidate pathway for intervention in HD. It is now well-established that neurodegenerative diseases in general and expanded repeat diseases in particular have an inherent auto-neuroinflammatory component mechanism, and the current results adds to the body of such evidence.

Declarations

Author contribution statement

Kathleen Griffioen, Eitan Okun: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Mark P. Mattson: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Competing interest statement

The authors declare no conflict of interest.

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Additional information

No additional information is available for this paper.

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