*Title:* Acute neuroinflammation elicited by TLR-3 systemic activation exacerbates early life stress induced working memory impairments in male adolescent mice

*Authors:* Thiago Wendt Viola[1], Luis Eduardo Wearick-Silva[1], Kerstin Creutzberg[1], Érika Kestering[1], Saulo Tractenberg[1], Marco Riva???? Rodrigo Grassi-Oliveira[1]

*Corresponding author:* Rodrigo Grassi-Oliveira
*Address:* PUCRS, Avenida Ipiranga 6690 – Prédio 63; Jardim Botânico – Porto Alegre, RS, Brasil; Email: rodrigo.grassi@pucrs.br

**Abstract**

Evidence has implicated Toll-like Receptors (TLRs) in the pathogenesis of cognitive impairment induced by systemic inflammation. Early life stress is associated with altered trajectories of neuroimmune signaling with implications for cognitive development. However, few experimental studies have examined the mechanisms responsible for the impact of TLR-3 activation on early life stress-related cognitive outcomes. This study investigated the effects of maternal deprivation (MD) during childhood and a viral immune challenge during adolescence on working memory performance of mice. Animals exposed to MD were separated from their dams daily for 180-min from postnatal day (PND) 2 to 15. At PND 45 animals were challenged with a single injection of either Poly (I:C) or sterile saline, and then subjected to a spatial working memory test in a Y-maze apparatus. Gene expression was determined by qPCR. We demonstrated that a single peripheral administration of a TLR-3 agonist can exacerbate MD-induced working memory impairments in adolescent mice. At a molecular level, exposure to MD was associated with lower mRNA levels of *Il6*, *Nfkb1* and *Tlr3* in the medial prefrontal cortex (mPFC). However, when MD animals were exposed to Poly (I:C), a more robust activation of *Il6*, *Nfkb1* and *Tlr3* gene transcription was observed in these mice compared with standard reared animals. Finally, higher mRNA levels of *Nfkb1* in the mPFC was correlated with lower working memory performance, suggesting a role for NF-κB signaling in the mechanisms underlying poor cognitive functioning induced by early life stress and systemic inflammation produced by viral mimetics.

Key words: Early life stress, Cognition, Working memory, Inflammation, Toll-like receptors, Neuroimmunomodulation.

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## 1. Introduction

Toll-like Receptors (TLRs) are the first line of defense against exogenous and endogenous pathogens and are responsible for triggering innate immune responses (1). These receptors are expressed in a variety of immune cells, while in the brain, they are identified predominantly on microglia, astrocytes, and with limited expression levels on neurons (2). Upon stimulation, TLRs can impact numerous aspects of central nervous system homeostasis by the production of inflammatory mediators, such as cytokines and chemokines, particularly through activation of the transcription factor nuclear factor kappa B (NF-κB) (3). Therefore, evidence has implicated TLRs in the pathogenesis of cognitive impairment induced by systemic inflammation or brain inflammatory illnesses (4), including Epilepsy, Bacterial Meningitis, Alzheimer's disease (5), but also stress-related neuropsychiatric disorders (6, 7).

Among the family of TLRs, the neuroinflammatory signaling mediated by TLR-2 and TLR-4 have been the most widely investigated, which are receptors that recognize infections of bacterial origin (4). For instance, administration of a TLR-4 agonist that induces peripheral and central inflammation, also results in increased hippocampal amyloid-beta, tumor necrosis factor (TNF)-α, and interleukin (IL)-6 levels with concomitant cognitive deficits in contextual fear-related memory (8, 9), suggesting that TLRs activation can be detrimental to brain functioning during acute inflammatory response. On the other hand, some TLRs are implicated in the recognition of viral infections, such as the TLR-3, TLR-7, TLR-8 and TLR-9. As a sensor for double-stranded RNAs of viral origin, TLR-3 is predominantly expressed intracellularly (10) and recent evidence demonstrated its functions on the regulation of hippocampal glial excitability (11). Although TLRs that respond to bacteria or viral ligands have important differences regarding their immune functions, the majority of evidence suggests that upon activation these receptors have a similar effect on brain functioning, in particular related to hippocampal-dependent spatial memory deficits (4, 12). However, intracerebral infusion of a TLR-3 agonist - Polyinosinic: polycytidylic acid [(Poly (I:C)] into the lateral ventricle was recently shown to induce working memory impairments in mice (13). As a key function of the medial prefrontal cortex (mPFC), working memory is a short-term memory process engaged during active maintenance and manipulation of information in order to guide behavior (14, 15). Despite that, few experimental studies have examined the mechanisms responsible for the impact of TLR-3 activation on mPFC-related cognitive outcomes (11).

Moreover, compelling evidence suggests that the nature and severity of cognitive deficits later in life depend upon many factors involved with brain development processes, including the experiences that occur during the sensitive period of early life (16). Stress

exposure in the earliest years of childhood has been related to cognitive impairment including poor working memory performance (17). Beyond behavioral outcomes, early life stress is associated with a chronic inflammatory state (18) and glia cells abnormalities (19), which points out to altered neuroimmune signaling as a candidate pathway for mediating the consequences of postnatal stress on brain and cognitive functioning. For example, persistent higher levels of inflammatory markers in the brain, including TLR-4 and NF-κB, were detected in adult animals exposed to infant maternal deprivation (MD), which is a well-known experimental model of early life stress (20). In addition, NF-κB gene expression was upregulated in the mPFC of adolescent mice exposed to early life adversity, and this effect was associated with higher levels of cocaine-contextual memory (21).

Given the potential relationship between early life stress, altered cognitive functioning and TLR-3 mediated neuroinflammation, the current study investigated the effects of maternal care deprivation during childhood and a viral immune challenge during adolescence on the working memory performance of mice. The cumulative effect of both factors was also assessed at the molecular level in the mPFC, since mRNA levels of NF-κB, IL-6, TNF-α and TLR-3 were measured following TLR-3 systemic activation or following saline administration. Considering that early life stress *per se* is associated with cognitive impairments, we hypothesized that Poly (I:C) administration would enhance stress-mediated working memory deficits as a immunological "second hit", and that differential expression of the immune-related genes would be correlated with cognitive outcomes upon TLR-3 stimulation.

## 2. Methods

### *2.1. Animals*

This study was performed with male BALB/c mice. All animals were housed under a 12 h/12 h light–dark cycle in ventilated cages with temperature maintained at 21 ± 1 °C. Food and water were available ad libitum. The experiments were conducted in accordance with the NIH laboratory animal care guidelines and approved by the Ethical Committee on the Use of Animals of the Pontifical Catholic University of Rio Grande do Sul, Brazil.

### *2.2. Infant maternal care deprivation model*

The early life stress model consists of exposing infant animals to daily episodes of maternal care deprivation during the first days of life. Thus, pregnant females were visually checked daily for the presence of pups. On the day of birth, the litters were randomly assigned to one of two groups: MD or animal facility rearing (AFR) control animals. The AFR litters

were left undisturbed until weaning, except for cage cleaning at postnatal day (PND) 10. The MD litters were subjected to a procedure that was used in previous studies with BALB/c mice (21, 22). In this procedure, pups were separated from their dams daily for 180 min (15:00–18:00), from PND 2 to PND 15. To do this, first, the dam was transferred to another cage. Then, the whole litter was transferred to another clean cage with bedding material and placed in another room, to prevent vocal communication between the dam and pups. The temperature of pups' cage (33 ± 2 °C) was controlled using a digital heating pad placed under the cage to compensate for the dams' body heat. After the maternal care deprivation period, the pups were returned to their home cage, followed by the dam. All pups were weaned at PND 21 and remained together with their same-sex littermates (two or three animals per cage) under standard housing conditions.

*2.3. Working memory test*

At the PND 45 animals were subjected to a spatial working memory test in a black Plexiglas Y-maze apparatus (23). The Y-maze apparatus consists of three symmetrical arms (30 cm x 5 cm x 10 cm, each arm) and it uses the natural tendency of mice to explore novel over familiar arms. This working memory test consisted of two phases, called the sample and test phases. The allocation of arms (start, familiar and novel arm) to a specific spatial location was counterbalanced across the experimental groups.

In the sample phase, animals were allowed to explore two arms (referred to as 'start arm' and 'familiar arm'), while the access to the remaining arm ('novel arm') was obstructed by a removable barrier wall. To begin a trial, the animal was introduced at the end of the start arm and was allowed to freely explore both the start and the familiar arms. After 5 minutes of exploration, the animal was removed from the apparatus and kept in a holding cage for 1 minute, while the barrier wall was removed.

In the test phase, the animal was reintroduced to the maze and could freely explore all arms of the maze for 2 min. After the test, the animal was removed from the apparatus and placed back in their home cage. The wood shavings that covered the floor of the apparatus was changed in preparation for the next animal. On each trial, the time spent in each of the three arms was recorded. The relative time spent in the novel arm during the choice phase was calculated by the formula [time spent in the novel arm/[time spent in all arms]) × 100 and used as the index for working memory performance. In addition, total distance moved on the entire maze was recorded and analyzed in order to assess general locomotor activity.

*2.4. TLR-3 agonist administration*

Poly (I:C) was used to mimic the acute phase of a viral infection, and it was prepared for injection by re-suspension in sterile saline and administered at a dose of 7.5 mg/kg (i.p.). This mild to moderate dose was chosen based on previous studies showing that this concentration of the TLR-3 agonist could induce behavioral and cognitive alterations in mice (24-26). Therefore, experimental groups were challenged with a single injection of either Poly I:C or sterile saline at PND 45.

*2.5. Gene expression analysis*

Six hours after the Poly (I:C) or saline injection animals were euthanized. We selected this time point since previous evidence indicated enhanced gene expression of brain pro-inflammatory genes after 6hrs of Poly (I:C) administration (26). The tissue from the mPFC was rapidly hand-dissected with a scalpel and stored at -80°c until molecular analysis. Total RNA was isolated from 6 samples per group using QIAzol (Qiagen; Hilden, Germany) and chloroform standard protocols. RNA concentration was measured using the NanoDrop spectrophotometer. Total of 500 ng of RNA from each sample was reverse transcribed using the miScript II RT Kit (Qiagen). The following primers (IDT) were designed, tested and used: *Il6* Forward (CCCCAATTTCCAATGCTCTCC), *Il6* Reverse (GACCACAGTGAGGAATGTCCA), *Tnf* Forward (CCTGTAGCCCACGTCGTAG), *Tnf* Reverse (GGGAGTAGACAAGGTACAACCC), *Tlr3* Forward (GTGAGATACAACGTAGCTGACTG), *Tlr3* Reverse (TCCTGCATCCAAGATAGCAAGT), *Pgk* Forward (TGCACGCTTCAAAAGCGCACG), *Pgk* Reverse (AAGTCCACCCTCATCACGACCC). The Quantitect primer (QT00154091) for *Nfkb1* was purchased from Qiagen. Each SYBR Green PCR reaction was run in duplicate for each sample using a Rotor Gene Real-Time PCR machine (Qiagen). The fold change relative expression was calculated using the ΔΔCt method with the AFR-vehicle group as a reference. PGK ct values were used as endogenous control for mRNA analysis. To verify primer specificities, melting curve analyses and agarose gels were performed.

*2.6. Statistical Analysis*

Group differences were assessed by two-way ANOVAs (group and treatment effects). The ANOVAs were followed by Tukey post-hoc tests. Pearson's correlation analysis was used to evaluate the association between behavioral and gene expression data. Statistical significance was defined as $p < 0.05$ and results are expressed as the mean ± SEM.

## 3. Results

### *3.1. Effects of early life stress and TLR-3 activation on working memory performance*

The experimental design of the study is illustrated in figure 1A. Significant group [$F(3,39) = 4.58$, $p < 0.05$] and treatment [$F(3,39) = 4.69$, $p < 0.05$] effects were detected on the performance of mice in the Y-maze, showing that both MD and Poly (I:C) exposure reduced the percentage of time exploring the novel arm during testing (Figure 1B). Post-hoc analysis revealed that only animals exposed to both MD and to Poly (I:C) presented significant less working memory performance compared with AFR-vehicle group ($p < 0.05$). These results demonstrated that MD-induced working memory impairments are exacerbated by Poly (I:C) systemic administration.

A significant treatment [$F(3,39) = 5.49$, $p < 0.05$] effect was observed on the distance traveled by mice during the working memory test, showing that Poly (I:C) administration reduced locomotor activity. However, exposure to MD did not affect locomotor activity given that no group [$F(3,39) = 0.14$, $p > 0.05$] differences were observed (Figure 1C).

### *3.2. Effects of early life stress and TLR-3 activation on gene expression levels*

Significant group [$F(3,20) = 12.89$, $p < 0.01$] and treatment [$F(3,20) = 36.06$, $p < 0.01$] effects were detected on mRNA levels of *Tlr3*, showing that while MD exposure reduced, Poly (I:C) exposure increased *Tlr3* expression in the mPFC. Post-hoc analysis revealed the MD-vehicle group had significant less *Tlr3* expression compared with the AFR-vehicle group ($p < 0.05$). In addition, the AFR-Poly (I:C) group ($p < 0.01$) and the MD-Poly (I:C) group ($p < 0.01$) had significant higher *Tlr3* expression compared with the MD-vehicle condition (Figure 2A).

A significant treatment [$F(3,20) = 26.75$, $p < 0.01$] effect was observed on mRNA levels of *Il6,* showing that Poly (I:C) exposure increased *Il6* expression in the mPFC, while no group differences were detected [$F(3,20) = 0.50$, $p > 0.05$]. However, post-hoc analysis revealed that only animals exposed to both MD and to Poly (I:C) presented significant higher *Il6* expression compared with the MD-vehicle group ($p < 0.05$), while no significant differences were detected regarding AFR groups (Figure 2B). No significant treatment [$F(3,20) = 0.28$, $p > 0.05$] or group [$F(3,20) = 2.84$, $p > 0.05$] effects were detected regarding mRNA levels of *Tnf-α* (Figure 2C).

A significant treatment [$F(3,20) = 11,48$, $p < 0.01$] effect was observed on mRNA levels of *Nfkb1,* showing that Poly (I:C) exposure increased *Nfkb1* expression in the mPFC, while no group differences were detected [$F(3,20) = 2.71$, $p > 0.05$]. However, post-hoc analysis revealed that only animals exposed to both MD and to Poly (I:C) presented significant higher

*Nfkb1* expression compared with the MD-vehicle group ($p < 0.01$), while no significant differences were detected regarding AFR groups (Figure 2D).

These results demonstrated that MD and Poly (I:C) had opposite effects on gene expression levels of *Tl3, Il6 and Nfkb1.* In basal conditions, MD exposure was associated with lower mRNA levels of these genes in the mPFC. However, when stressed animals were exposed to Poly (I:C), a more robust activation of gene transcription was observed in animals exposed to MD compared with standard reared mice. Moreover, correlation analysis revealed a significant negative association between *Nfkb1* gene expression and the percentage of time exploring the novel arm during the Y-maze test ($R^2 = 0.20$; $p < 0.05$), showing that higher mRNA levels of *Nfkb1* in the mPFC was associated a lower working memory performance (Figure 2E).

**4. Discussion**

Exposure to stressful experiences during childhood, such as abuse and neglect, is associated with altered trajectories of brain and cognitive development (27). Structural and functional cortical modifications in line with deficits in cognitive processes such as executive functions (17), behavioral inhibition (28), and working memory (29), have been robustly documented in individuals with histories of early life adversity. Pre-clinical studies have explored the molecular underpinnings of the cognitive phenotype produced by early life stress, and most of the studies have focused on the role of altered neurotransmission and neuroplasticity (30, 31), as well as the consequences of overactivation of the neuroendocrine system (32). Taking into account the relevant data that childhood stress could also affect the neuroimmune response (18), combined with the idea that systemic inflammation leads to activation of brain neuroimmune response (33), the current study provided evidence that a single peripheral administration of a TLR-3 agonist can modulate and exacerbate early life stress induced working memory impairments in adolescent mice. At a molecular level, we have identified that early life stress and Poly (I:C) had opposite effects on gene expression of neuroinflammatory markers IL-6 and NF-κB, as well as concerning TLR-3 expression. In particular, exposure to maternal care deprivation was associated with lower mRNA levels of these genes in the mPFC. However, when maternally deprived animals were exposed to Poly (I:C) during adolescence, a more robust activation of *Il6*, *Nfkb1* and *Tlr3* gene transcription was observed in these mice compared with standard reared animals. Finally, higher mRNA levels of *Nfkb1* in the mPFC was correlated with lower working memory performance,

suggesting a role for NF-κB signaling in the mechanisms underlying poor cognitive functioning induced by early life stress and systemic inflammation produced by viral mimetics.

Our behavioral results are consistent with previous findings showing that either early life stress (29) or acute inflammation (13) could produce cognitive deficits that include working memory performance. Specifically, we demonstrated that only following a viral immune challenge the MD effect on cognition reached statistical significance in comparison to normally reared animals without acute inflammation. Therefore, these data support the hypothesis that an immunological "second hit" could not only aggravate the effects of postnatal stress on working memory, but it could also be a pathological trigger for cognitive decline in vulnerable individuals due to postnatal stress exposure (34). This combinatory effect has already been documented regarding viral diseases, such as HIV infection, given that HIV induced neurocognitive impairments are more pronounced in individuals with histories of early life stress (35), particularly on cognitive flexibility and working memory processes (36).

Although there is little information on brain and behavioral responses to TLR-3 *in vivo*, previous evidence demonstrated that direct activation of TLR-3 by intracerebroventricular infusion of Poly (I:C) impaired working memory (13), and that TLR-3 signaling can suppress neuronal plasticity and induce neuroglial immune responses by driving gene expression of pro-inflammatory cytokines (11). In our experiments we observed increased mRNA levels of *Il6*, *Nfkb1* and *Tlr3* in the mPFC after 6 hours of a single injection of Poly (I:C) in a mild to moderate dose. Although we did not provide direct evidence of cellular specificity, it is possible that our gene expression data is closely involved with the response of glia cells, taking into account previous findings showing that astrocytes and microglia cells express TLR-3, and that these cells can synthesize pro-inflammatory cytokines in response to Poly (I:C) stimulation (37). Furthermore, when cortical glia cells are activated upon TLRs stimulation, the neuroinflammatory toxicity can be extended to primary cortical neurons, an effect that has been shown to be involved with cognitive impairment (11). For instance, activated microglia cells produce IL-6, IL-1β, and TNF-α, and when these cytokines are released extracellularly they directly affect synaptic plasticity and working memory performance (33).

On the other hand, we observed that regardless of Poly (I:C) administration, exposure to maternal care deprivation was associated with lower mRNA levels of *Il6*, *Nfkb1* and *Tlr3* in the mPFC of adolescent animals, suggesting that early life stress has persistent effects on the transcription of neuroimmune genes. However, following Poly (I:C) administration, animals exposed to MD presented a significant enhancement in the expression of *Il6*, *Nfkb1* and *Tlr3* compared with the MD-vehicle group, an effect that was not significant when analyzing gene

expression changes in standard reared animals. In this sense, these findings are in accordance with the concept of "glial priming". Previous studies demonstrated that "primed" glia cells are characterized by an activated morphology with enlarged cell bodies, accompanied by persistent lower expression levels of pro-inflammatory cytokines (33). However, the inflammatory response produced by primed glia to a subsequent immune challenge is significantly exaggerated when compared to typical glia cells that receive a similar challenge, and this aberrant response is associated with detrimental brain functioning and cognitive impairment (38). Previous evidence supports this idea since infant maternal care deprivation was associated with increased number, density, and surface area of glia cells in the brain (39).

Interestingly, we also found that higher mRNA levels of *Nfkb1* in the mPFC was correlated with lower working memory performance, suggesting a close link between NF-κB signaling and cognition. This is consistent with previous findings showing that enhanced cortical activation of NF-κB signaling pathway is accompanied by impairment of working memory functioning, particularly due to the deleterious effect of neuroinflammation on cognitive abilities (40). NF-κB is a critical transcription factor implicated in the regulation of inflammatory responses, as it enhances the transcription of pro-inflammatory cytokines, including TNF-α, IL-1β and IL-6, as well as the oxidative stress related enzyme cyclooxygenase 2 (41). In this sense, our data fits well with the hypothesis that by controlling NF-κB signaling in the brain, it would be possible to prevent working memory impairment in cognitively-impaired individuals (40), including those affected by early life stress. However, it still remains to be revealed whether the blockage of cortical NF-κB gene expression would rescue the cognitive deficits induced by the exposure to TLR-3 activation and infant maternal care deprivation.

In conclusion, the present study provides further evidence to support the idea that early life stress is a major risk factor to cognitive impairment, and that one possible pathway involved with this phenotype is through altered neuroinflammation (34). In particular, we demonstrated that the systemic activation of TLR-3 during adolescence is capable of exacerbate working memory impairments attributed to infant maternal care deprivation, and that this effect is accompanied by modifications in cortical gene expression of *Il6*, *Nfkb1* and *Tlr3*. In this sense, our study adds further weight to the notion that interventions targeting anti-inflammatory pathways may be highly promising for the treatment of neurocognitive disorders (42), especially those that early life stress is accounted as a major risk factor.

Figures

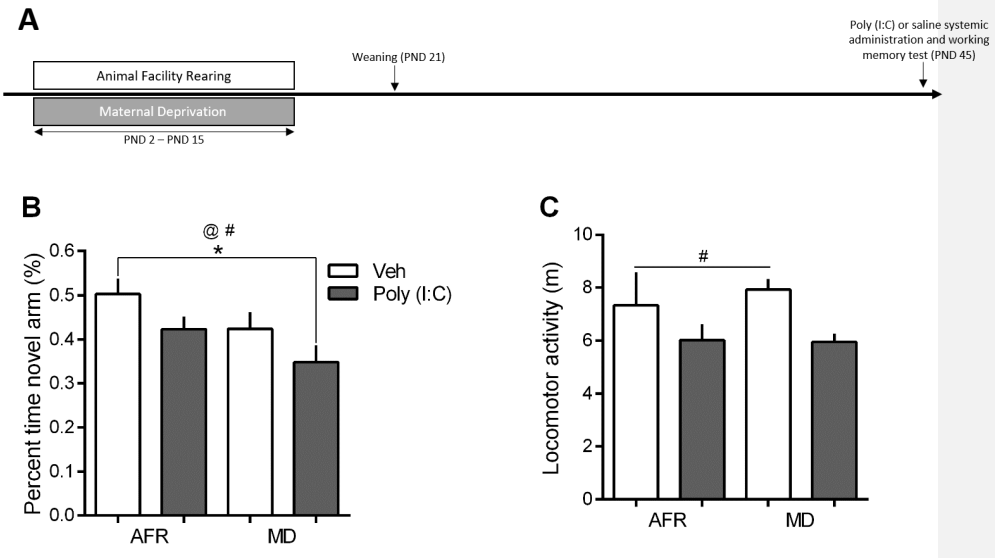


Figure 1.

A, Experimental design. B, Percent time in novel arm in the Y-maze working memory test. C, Locomotor activity in the Y-maze working memory test. #, treatment (Poly I:C x Vehicle) effect in the ANOVA. @, group (AFR x MD) effect in the ANOVA. *, $p < 0.05$. AFR, animal facility rearing. MD, maternal deprivation. $n = 10/12$ per group.

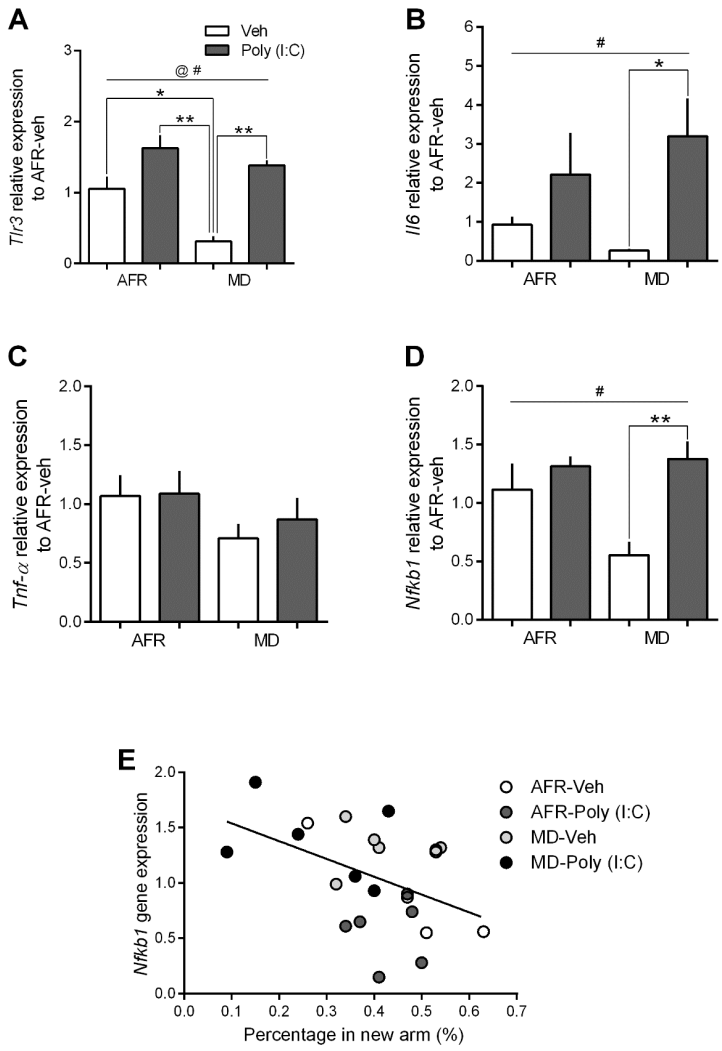


Figure 2.
A, *Tlr3* gene expression levels in the mPFC. B, *Il6* gene expression levels in the mPFC. C, *Tnf-α* gene expression levels in the mPFC. D, *Nfkb1* gene expression levels in the mPFC. E. correlation analysis between *Nfkb1* gene expression and working memory performance score. #, treatment (Poly I:C x Vehicle) effect in the ANOVA. @, group (AFR x MD) effect in the ANOVA. *, $p < 0.05$. **, $p < 0.01$. AFR, animal facility rearing. MD, maternal deprivation. $n = 6$ per group.

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