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Stressed (acute) mice display neuroimmunodysregulation and defective innate immune response against coliform infection

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ABSTRACT

We examined the impact of acute restraint stress (ARS) with(out) intraperitoneal *E. coli* infection on *TLR4* mRNA abundance in brain and spleen, clinical signs, cytokines and oxidative loads and peritoneal *E. coli* growth in balb/c mice. ARS exacerbated *E. coli* virulence and behavioral abnormality. At different post-stress hour the pattern and intensity of *TLR4* activity differed in brain and spleen. While *TLR4* stimulation in spleen of *E. coli*-infected mice was maximal, it superseded in brain of post-stressed *E. coli*-infected mice. ARS and *E. coli* infection elicited systemic pro-inflammatory and pro-oxidant status, with defective peritoneal *E. coli* clearance in post-ARS mice. Continuous *TLR4* activation in post-stressed mice partially disarms innate immune response, and contributes to inappropriate host–*E. coli* interactions and thus neuroimmune dysregulation/toxicity. The description of these observed novel effects induced by ARS will provide a basis for deeper investigations of the effects from increasingly stress-oriented rural/urban life upon neuroimmune system.

1. Introduction

Stress in animals and humans dysregulates innate immunity, in particular by affecting the hypothalamic–pituitary–adrenal (HPA) axis, resulting in adverse effects on innate immune cells and related molecules [1–3]. As key factors in immune surveillance, toll-like receptors (TLR) are pivotal innate molecules [4–6] that are expressed by dynamic immune cells in the brain (e.g., on microglia and astrocytes) and spleen (e.g., on dendritic cells, macrophage, lymphocytes, and neutrophils). TLR are also involved in many neurodegenerative diseases [3,6–8]. TLR have also been increasingly used as novel immunemolecules to extrapolate dysregulatory/toxicity of hazard materials [9–13]. Studies on the effects of acute restraint stress (ARS) on TLR are rare. Whether and how *TLR4* is differentially activated in the brain and other organs during/due to ARS are also unclear.

Little research has been conducted on the comparative impacts of ARS and ARS in combination with a bacterial challenge on *TLR4* in the brain and spleen; effects from TLR4 activation in stressed hosts are also not well defined. In general, it remains to be investigated why stress is associated with worse outcomes during infections and in oxidative stress-related neurodegenerative diseases. Overall, the impact of stress on particularly brain's immune surveillance has been under-investigated.

Stressed animals/humans do not properly respond to therapeutics/medicines [14]. This inadequacy might be due to a persistent pro-inflammation and/or pro-oxidant status [14] and thus immune disarmament in a stressed host. This implies that stressed animals/humans are accordingly more vulnerable to [microbial] infections. Examining immune/inflammatory/oxidative responses to an infectious pathogen in post-ARS hosts would allow key information on these processes to thus be obtained.

The present study aimed to broadly examine the effects of short-term stress on the dynamics of *TLR4* transcription in key (non)-immune organs. Clinical signs of ARS and ARS followed by an infection with *Escherichia coli* (*E. coli*) infection were also assessed to discern any link between stress, *TLR4* expression in the CNS, and systemic inflammation-oxidative [stress] status in a murine model.

2. Materials and methods

2.1. Mice and experimental procedure

Forty healthy Balb/c mice (male, ~5 weeks, ~25–30 g) were obtained from Razi Institute, Mashhad, for use in this study. All mice were housed at 4/cage in a pathogen-free facility maintained at 22 ± 2 °C with a 12 h light (07:00–19:00) and 12 h dark cycle. Throughout, all mice had *ad libitum* access to standard rodent chow and filtered water. All mice were acclimated for 1 week prior to use in any experiment. All experimental procedures were in accordance to the local human/animal welfare regulations and were approved by the Ethical Committee of Ferdowsi University of Mashhad.

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For each treatment group, the mice were divided into four treated and control (unstressed and/or intraperitoneally (IP) injected with phosphate-buffered saline [PBS, pH 7.4]) groups (Fig. 1, upper). For the induction of ARS, very gently select mice were individually placed into a plastic restrainer. For infections, *E. coli* (O157:H7) was grown overnight in selective nutrient broth at 37 °C; levels of bacteria were then calculated by colony forming unit and diluted as needed for the IP injections outlined below.

Twenty-four mice were placed under acute restraint stress (ARS) for 2 h (18:00–20:00), group/stage I; of 8 non-stressed mice four mice were IP challenged with *E. coli* (0.5 ml, 2×10^8 cfu/ml), group/stage II; of 8 post-stressed mice four mice were IP challenged with the *E. coli* (0.5 ml, 2×10^8 cfu/ml), group/stage III.

During ARS ± *E. coli* infection, behavioral signs and/or the clinical severity score were monitored (at least 5 times) and scored. Endpoints such as lethargy, biting, diarrhea, defecation, and urination were scored *en toto* from 1 to 6, with ranking from 1 = none, 2 = very mild, 3 = mild, 4 = moderate, 5 = severe, or to 6 = very severe symptoms. Peritoneal bacterial counts from mice in stages II and III were also determined according to protocols outlined in [15] by placing 100 μl of serially-diluted peritoneal exudate onto sheep blood agar plates (Thermo Fisher Scientific) and culturing at 37 °C in a 5% CO_2 incubator for 24 h.

Mice were IP injected with mix of 4 mg ketamine and 0.8 mg xylazin/ml, and after aseptical cardiocentesis of ~300 μl blood (all stages for further analyses) and ~100 μl peritoneal flushes (stages II and III), the mice were eventually euthanized and spleen and brain of all experimental mice were then removed under sterile conditions. Splenic immune cells and brain (sagittal sections of frontal part of whole brain) were separated accordingly [10,16]. The harvested RBC-free splenic cell suspensions were then washed (400 × *g*, 10 min, 4 °C) twice with RPMI-1640, counted (in nigrosin solution) in a Neubauer chamber, and after cell viability assessment using nigrosin solution and the Neubauer chamber, in which necrotic cells appeared bright and dark-blue in the mixture [10–16], respectively, the cells and brain were eventually used for further cellular and molecular analyses.

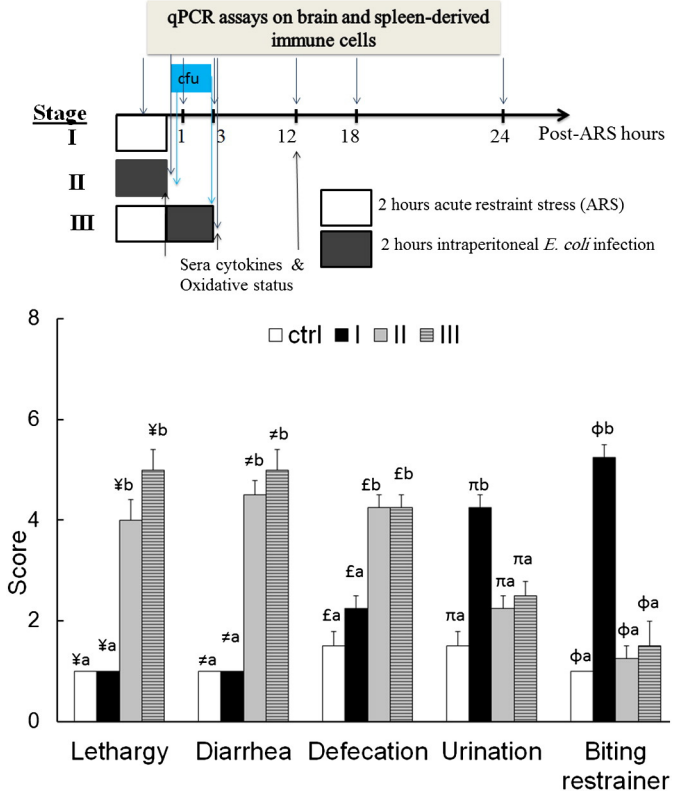


Fig. 1. Upper panel, experimental scheme of mice subjected to stress and infection. Mice were kept in restrainer for 2 h then study performed (see Materials and methods), with behavioral and clinical signs. Lower panel, behavioral and general clinical signs observed in mice during stages I, II and III. For the definition of behavioral signs and/or the clinical severity scores (0–6), see Materials and methods. Different letters with their symbols (i.e., ¥, #, £, π and ϕ for lethargy, diarrhea, defecation, urination and biting restrainer, respectively) indicate significance ($p < 0.05$) from their respective control, stages I, II and III treatments. After each time point and stage, brain and spleen-derived immune cells were used for qPCR.

2.2. Effect of ARS on TLR4 gene expression in brain and spleen

Myeloid-derived mononuclear splenic cells (MDMSC; 7×10^6) and sections of whole brain were each used for RNA extraction and cDNA synthesis [11,12]. *TLR4* expression in each set of samples was analyzed using qPCR assays. Exon junction or intron-spanning primers (Table 1) were designed for *TLR4* and *β-ACT* (housekeeping gene) using Allele ID7 (Premierbiosoft, Palo Alto, CA) and CLC main workbench software (clcbio Co. Aarhus, Denmark). "5× HOT FIREPol® EvaGreen®qPCR Mix Plus" (Solis BioDyne, Tartu, Estonia) was used to perform the qPCR, according to manufacturer protocols. Each qPCR reaction was done in a 20 μl final volume containing 10 pM of specific forward and reverse primers, 4 μl EvaGreen master mix, and 1 μl cDNA template. To optimize the qPCR assays, different concentrations (1, 0.1, 0.01, 0.001, 0.0001 and 0.00001 μg/μl) of cDNA were analyzed with the primers. qPCR conditions for *TLR4* and *β-ACT* genes were carried out (in duplicate) using a Rotter gen 6000 light-cycler system (QIAGEN, Venlo, the Netherlands) with a cycling program including holding for 15 min at 95 °C, followed by cycling 45 times at 94, 56, and 72 °C (20 s for each temperature) with melting curve analyses (included ramping from 50 to 99 °C, rising 0.5 °C/step and waiting 10 s for each step afterward; a single peak was obtained in each qPCR product reaction) accompanied by agarose gel electrophoresis to ascertain the absence of non-specific qPCR products.

Analyses of qPCR data were calculated using GenEX version 5 software (MultiD, Goteborg, Sweden) and relative expression software tool (REST; Qiagen, Hilden, Germany). In each qPCR reaction, the cycle number at which the fluorescence rose appropriately above background was determined as the crossing point (CP). Cycle threshold values were means of duplicates in each qPCR run and four biological repeats after inter-plate calibration and optimization to ensure that the efficiency of the target and the reference genes was appropriate. The Pfaffl equation [17] was first used to calculate the relative gene expression ratio, i.e., the change in *TLR4* expression divided by that of *β-ACT*. A slope was determined from the exponential phase. Expression of *TLR4* was calibrated to that of *β-ACT* using: relative fold change (rcf) = $[((E_{TLR4})^{(\text{Control CP}_{TLR4} - \text{treated CP}_{TLR4})})/((E_{\beta\text{-}ACT})^{(\text{Control CP}_{\beta\text{-}ACT} - \text{treated CP}_{\beta\text{-}ACT})})]$.

2.3. Effect of ARS on the innate immune response against E. coli challenge

To assess the effect of ARS on the functional activity of splenic phagocytes, MDMSC of post-stress hour (psh) 12 were isolated by plastic adherence, after incubation of splenic immune cells for 2 h in a 95% humidity with 5% CO_2 at 37 °C, as previously described in porcine [10,16] and human [13]. In brief, 2×10^5 MDMSC were treated with 2×10^6 FITC-loaded polystyrene microparticles (1.0 μm, Sigma-Aldrich; St. Louis, MO) at 10 beads/MDMSC and incubated for 3 h (37 °C, 5%

Table 1
Designed primers for use in qPCR assays.

Gene (accession number)	Sequence (5′ → 3′)	qPCR product (bp)	Annealing (°C)
β-ACT (NM_007393)	F:CTTCTTTGCAGCTCCTTC R: CCATTCCCACCATCACAC	191	56
TLR4 (NM_021297)	F: TCCCTGATGACATTCCTT R: AGTTTGAGAGGTGGTGTA	177	56

CO_2 and 95% humidity). Thereafter, the cells were harvested on ice, washed with ice-cold PBS (phosphate-buffered saline, pH 7.4), and the internalization of beads then assessed by flow cytometry using a FACSCalibur system (Beckton Dickinson, Franklin Lake, NJ). A minimum event count of 10,000 was applied for each sample. Phagocytic activity was presented in terms of mean fluorescence intensity (MFI) for the entire population analyzed. The actual number of $FITC^+$ MDMSC was also used to reflect on the functional ability of the MDMSC.

Other sets of peritoneal cells were sonicated and the supernatants used for assessments of MPO activity. This assay was based on oxidation of *o*-dianisidine (0.8 mM, final concentration) by the extract when stimulated with 0.1 mM H_2O_2. Five minutes after addition of the *o*-dianisidine and H_2O_2, the OD 450 nm was measured using a microtiter plate spectrophotometer (Multiskan Plus Type 314, Labsystems, Helsinki, Finland). Results were expressed as % of the non-stressed mice.

2.4. Effect of ARS on inflammatory/oxidative status of mice

Murine IL-1β, IL-6, TNF-α, INFγ, and IL-10 concentrations in sera were determined with commercially available ELISA kits (eBioscience, Frankfurt, Germany), according to manufacturer instructions. Optical densities (OD) were measured in an automated ELISA plate reader (Bio-Tek, ELx50™, Vermont, USA). Cytokine concentrations (all in terms of pg/ml) were calculated using curve-fitting algorithms applied for standard curve calculations. The sensitivities of the IL-1β, IL-6, TNF-α, INFγ and IL-10 kits were <1 pg/ml.

To assess host oxidative status, heparinized blood (whole) and serum were evaluated using chemiluminescence (CL) assays [9,18]. Specifically, the CL of whole blood (10 μl, diluted 1:10 with PBS) from each mouse was performed in 96-well white flat-bottom microtiter plates (Nunc, Wiesbaden, Germany) with 200 μl/well. Phorbol-12-myristate-13-acetate (PMA, Sigma) at a final concentration of 200 ng/ml (with final volume of 20 μl) was used as an activator and luminol (at a final concentration and volume of 0.3 mM and 60 μl, respectively, Sigma) as a CL enhancer. Immediately before CL measurement PMA and luminol were separately added to the wells with automatic injector. The CL assay was performed using a multi-label reader Mithras LB 940 luminometer (MicroWin 2000, Berthold, Bad Wildbad, Germany). The area under the curve (AUC) was calculated (counts/min) over the entire measurement period of 30 min. Each sample/mouse was assayed in duplicate.

To quantify serum oxidative status, a cell-free CL assay was used by applying 10 μl of serum. Cell-free CL was measured during 30 min in the luminometer after addition of 0.1 mM luminol and 0.1 mM H_2O_2 to the serum sample. The CL response (in terms of AUC) was measured for registered impulse rates (CPM) over the entire 30 min period. The cell-free CL index was then calculated using the AUC of each sample from treated mice divided by the mean corresponding value from non-treated control mice. Further, to gain a view on systemic antioxidant/scavenging/quenching capacity of the experimental mice's sera on ROS, CL of the sera was measured after injecting sodium hypochlorite (NaOCl; 100 μl of a stock prepared by diluting NaOCl 1:1000 in PBS) into the wells of flat-bottomed white microtiter plates, containing PBS, 0.04 mM of luminol and 10 μl of sera from treated and control mice, in a luminometer. The flashing-like reaction was recorded simultaneously and immediately during injection of luminol and the cumulative RLU was measured for 5 s. All assays were carried out in duplicate at 37 °C.

2.5. Statistical analyses

Statistical differences between treatments and controls were analyzed using SPSS version 21 (SPSS Inc., Chicago, IL). For the analyses, a one-way analysis of variance (ANOVA) was employed; data were compared further using a Bonferroni test for multiple comparisons. A p-value ≤ 0.05 was considered significant.

3. Results

3.1. Behavioral and clinical observations

With 100% survival rate, behavioral and general clinical signs of the examined mice revealed that excitement urination, defecation and biting the restrainer in ARS mice were more intense and frequent (see the median scores for each behavior in Fig. 1, lower panel); diarrhea and lethargy in ARS mice which underwent *E. coli* challenge were more pronounced.

3.2. ARS disrupts TLR4 activity in brain and spleen

In the brain, *TLR4* expression at psh 1 and 3, 12, 18, and 24, respectively, decreased and increased significantly (Fig. 2). In the spleen, *TLR4* expression at psh 3 and 24 was unchanged (versus non-stressed mice), but at psh 1 and 12, and 18, respectively, increased and decreased significantly (Fig. 2) (this suggests random outcomes if no clear trend in/over time). Immediately at ARS hour 1 *TLR4* mRNA significantly increased only in the brain (Fig. 2A). Though the intensity of *TLR4* alteration differed, *TLR4* mRNA levels in the brain and spleen increased (compared to levels in non-stressed, non-infected mice) in mice infected with *E. coli* alone and those who were both stressed and infected with *E. coli* (Fig. 2B and C). Further, mice with preceding ARS showed stronger *TLR4* activation after *E. coli* infection in brain than spleen (Fig. 2C).

3.3. ARS potentiates pro-inflammatory/pro-oxidant status in bloodstream of stressed mice

As shown in Fig. 3A, there was a significant increase in IL-1β, IL-6, TNF-α and INFγ levels in the bloodstream of post-ARS, *E. coli*-infected and stressed *E. coli*-infected mice; this was most pronounced in the stressed *E. coli*-infected mice. In contrast, the IL-10 levels in blood decreased remarkably in these hosts. Though cellular CL of whole blood revealed a weaker leukocyte functions in mice at psh 12, the cell-free CL of sera at psh 12, *E. coli*-infected and stressed *E. coli*-infected mice was significantly higher compared to their control counterparts (Fig. 3B). As such, bloodstream of post-ARS and *E. coli*-infected mice was remarkably at the pro-oxidant status.

3.4. ARS weakens the innate immune response against E. coli

A fluorescence-based phagocytosis assay revealed that there was a significant decrease in the percent of phagocytic MDMSC in stressed mice (Fig. 3C, upper) and that ARS led to decreased MDMSC phagocytic activity. This was consistent with the results of the killing capacity of the peritoneal innate immune cells against the *E. coli* as observed on diapedesed peritoneal leukocytes' MPO activity (Fig. 3C, middle). A weaker *in situ* peritoneal *E. coli* clearance in ARS mice was also observed (Fig. 3C, lower).

4. Discussion

The main questions addressed in this *in vivo* study were: (1) whether and how differently short-term stress could affect the dynamics of *TLR4* transcription in the brain and spleen; (2) how *TLR4* mRNA abundance might be affected in the brain and spleen of stressed *E. coli*-infected mice; (3) how these changes might be related to immunopathologic events in the body (especially in CNS); and, (4) to evaluate the eventual implications of this murine model in regard to the idea/concept of "acute stress-mediated neuroimmunotoxicity". The present studies showed the relationship between acute stress and *TLR4* expression in brain and MDMSC, and *TLR4* activation by ARS occurred differently in brain and spleen. Indeed, high blood glucocorticoids in post-ARS sheep [19], affect CNS/PNS, thereby disrupting inflammatory responses via (in)activation of NF-κB pathways [2,3,5]. To eliminate the influence

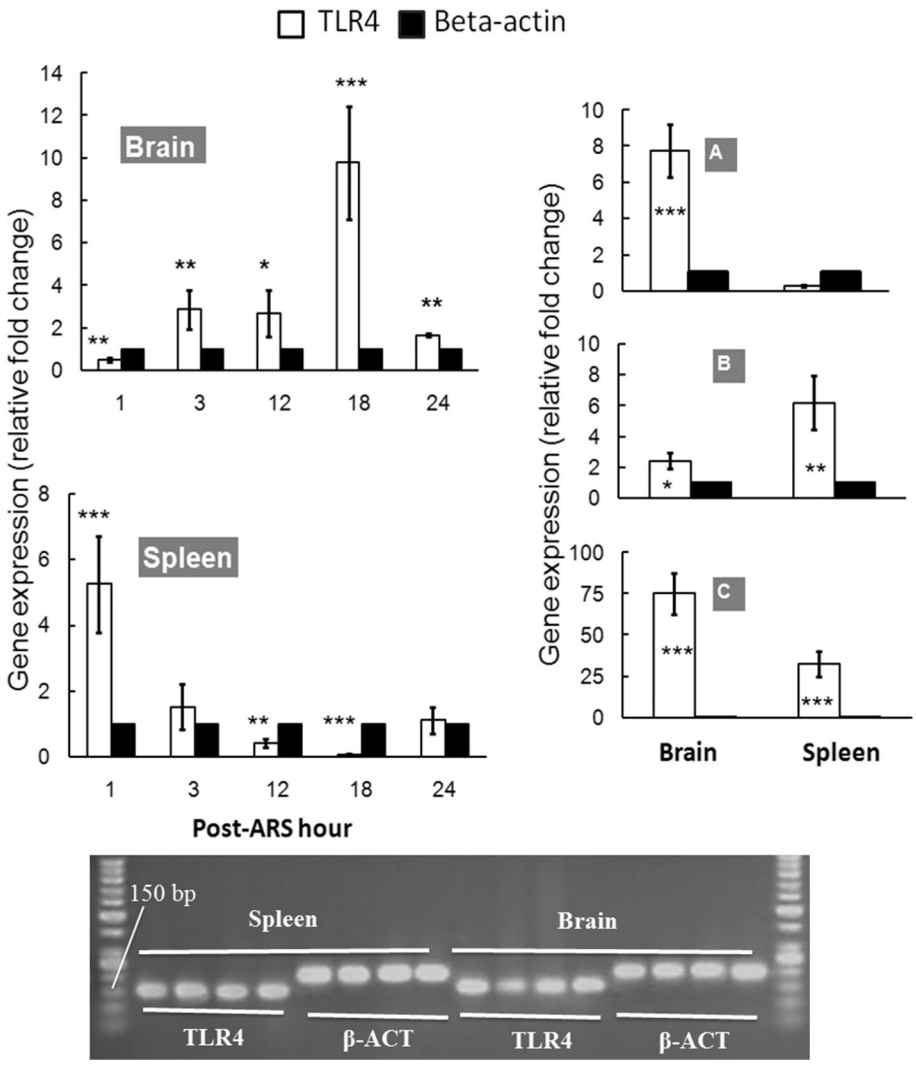


Fig. 2. Dynamic changes in TLR4 transcription in murine brain and spleen at different times after acute restraint stress (ARS), and during (1 h) ARS (A), 2 h after only *E. coli* infection (B) and post-ARS 2 followed by 2 h after *E. coli* infection (C). The relative expression level of each gene in control for both brain and spleen was 1, see Materials and methods. Data are mean ± sem ($n = 4$). *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$ versus control mice. Insert, agarose gel electrophoretic image of qPCR product of *TLR4* and *β-actin* of brain and spleen-derived immune cells.

of other physiological factors such as hormonal changes, pregnancy, lactation, etc., only male mice were used here, and 2 h was set for ARS in accordance with some of our previous studies on TLR and immune co-stimulatory molecules [10–12,16].

Astrocytes and microglia in CNS highly express TLR [3,6,20]; (and first investigator's unpublished toxicological *in vitro* experiments on primary murine astrocytes and glials); over-expression of, e.g., *TLR4* results in the production of large amounts of IL-1, TNF-α and IL-6, exacerbating neuron injuries [3,6,21]. The observed higher level of pro-inflammatory cytokines and pro-oxidant status in our study supports the notion that the ARS disturbs appropriate immune responses. Also the weaker peritoneal *E. coli* clearance in ARS mice emphasizes a weaker innate immune responses against invading pathogens; this could be due mainly to less phagocytosis and killing capacities of innate immune cells in the infection site as observed here.

The dynamics of the *TLR4* expression in brain revealed consistent up-regulation; this might be due to the changes in glucocorticoids level, of which high and low levels, respectively, inhibit and stimulate *TLR4* expression [3]. Lymphocytes, macrophages and dendritic cells in spleen are crucial, and chronic restraint stress via TLR4 up-regulation, stimulates immune system [22]. Though spleen is inaccessible to endogenous levels of steroids [23], the observed dual effects of ARS on *TLR4* transcript in spleen are intriguing. Sudden increase of glucocorticoids stimulates *TLR4* expression, and thus inflammation. Conversely, gradual increase of glucocorticoids suppresses immune and inflammatory responses [2], restoring homeostasis. So, increase glucocorticoids might have been involved in *TLR4* down/up-regulation, and measuring glucocorticoids is valuable.

Because *TLR4* expression increased at psh >3, we then decided to evaluate key cytokine pattern in serum at psh 12. The observed higher level of pro-inflammatory cytokines here supports the notion that the ARS disturbs appropriate immune response, disarming the immune cells. This was confirmed by noting a far weaker peritoneal *E. coli* clearance in post-ARS mice experimentally challenged with *E. coli*. The pattern of redox status of bloodstream was parallel to those observed on pro-inflammatory cytokines. This is constant enough to support conclusion that ARS disturbs redox and immune systems. As such, ARS is central contributor to lead a weaker innate immunity in mice.

Immune and nervous systems are strongly interrelated to maintain homeostasis. For example, *TLR4* activation by *E. coli*/endotoxin [13] causes release of pro-inflammatory cytokines and thus stimulating HPA axis [19]. Increased expression of TLR in murine astrocytes exposed to *E. coli* [4] is not surprising. Consistently [24], our study reveals that 2 h after *E. coli* challenge the abundance of TLR4 mRNA in brain, but less pronounced in spleen, was not surprising. This might be due to the proximity of spleen to the IP infection site.

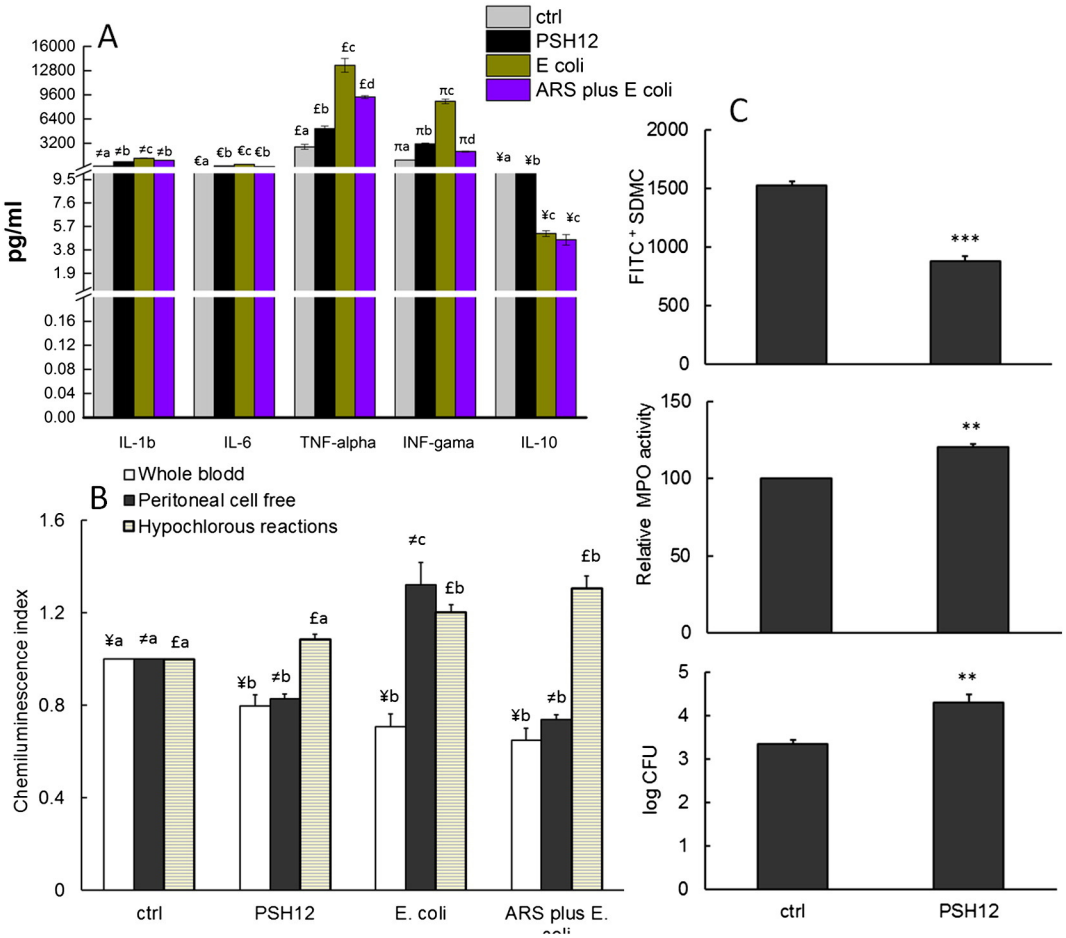


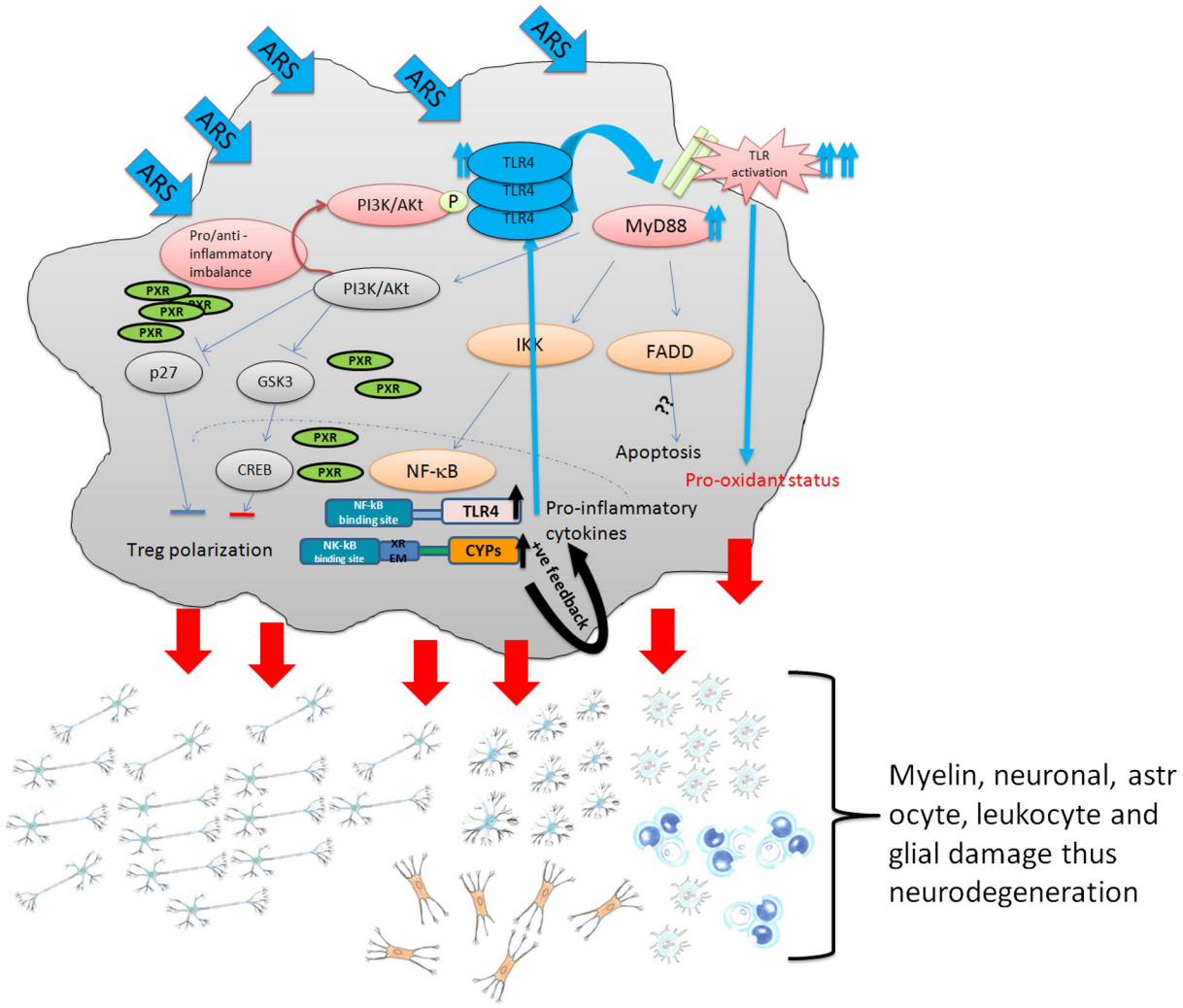
Fig. 3. Acute restraint stress (ARS) induced a higher pro-inflammatory cytokine secretion (A); this increase was more pronounced in post-stressed *E. coli*. Different letters with their symbols (i.e., #, €, £, π and ¥ for IL-1β, IL-6, TNF-α, IFN-γ and IL-10, respectively) indicate significance ($p < 0.05$). Bloodstream of post-ARS and *E. coli*-infected mice is remarkably at the pro-oxidant status (B). Three parameters [(i.e., whole blood luminol-dependent PMA-stimulated CL (¥), peritoneal cell-free CL (≠) and luminol-dependent CL from added NaClO/hypochlorite (£)] in relation to different treatments (i.e., control, post-stress hour (psh) 12, *E. coli* infection and ARS followed by *E. coli* infection) in mice; the values are CL index (see Materials and methods). Different letters represent significantly different from their respective treatments ($p < 0.05$). Innate immune cells of post-ARS mice are disturbed and weak (C). ARS interferes with phagocytotic activity and killing capacity of murine myeloid-derived mononuclear splenic cells (MDMSC). Phagocytosis of 1 μm fluorescent microparticles (upper), relative peritoneal MPO activity (middle) and log CFU (lower) of MDMSC in relation to psh 12. Data are represented as mean ± sem ($n = 4$). **, $p < 0.01$, ***, $p < 0.001$ versus control mice.

Post-stressed mice challenged with *E. coli*, showed increased IL-1β, TNF-α and IL-6 loads in the blood; this was consistent with other findings [25] who used LPS instead of *E. coli*. In contrast, in post-LPS challenged mice, acute stress decreased those cytokines [26]. The underlying reason for this discrepancy could mainly be due to down-regulatory effects of glucocorticoids, since corticosterone-exposed macrophages treated with LPS fail to produce TNF-α and IL-6 [27]. The contribution of glucocorticoids to regulation of immune genes is important [2]. Cytokine secretion patterns dictate the polarization of naive T-cells into effector T-cells which, in turn, drive the ensuing immune responses [28,29]. That is why in the present studies, the effect of ARS and *E. coli* infection on cytokine secretion profiles was determined. One key anti-inflammatory cytokine (IL-10) was also assessed to further confirm the immunodysregulation/toxicity effect of ARS; based on the findings here, the changes noted in IL-10 in the blood provided further support for the concept underlying a pro-inflammatory behavior for ARS. In addition to cytokines and TLR4, it is worth examining/performing bone marrow and circulating cell population analyses to determine which type of myeloid cells, particularly the pivotally myeloid-derived suppressor cells is recruited during immune challenge in a stressed/infections state.

The findings here will provide a basis for deeper investigations of the effects from increasingly stress-oriented macro/microenvironment upon neuroimmune system. The observed increase of *TLR4* expression in brain and spleen of post-stressed *E. coli* challenged mice, though more pronounced in brain, could mainly be resulted from the timely effect of low level of corticosterone, because induction of 2 h ARS does not lead to maximal corticosterone load [2].

At different time points (1, 3, 12, 18 and 24 h) after ARS, the fluctuation on the dynamics of *TLR4* transcription in brain and spleen emphasizes the point that innate immune molecules in different tissues behave differently at least dynamically. Since *TLR4* up-regulation is double-edged sword, it can be both helpful as an immune surveillance for appropriate and protective immune response. It can also cause damage to the tissue as a result of e.g., pro-inflammatory cytokine storm and partial blockage of immunetolerance (e.g., decreased IL-10). Also, continuous activation of TLR4 in immune cells may disarm them, exacerbating vulnerability of host to invading pathogens. As such, ARS disturbs protective immune response of brain and causes neuron damage [20,21] via proposed mechanisms (Scheme 1).

In short, indisputably, ARS more intensively stimulates *TLR4* activity in brain than in spleen, mechanistically explaining a more pronounced stress-induced immunodysregulation in CNS/PNS than in lymphoid organs *in vivo*. Continuous *TLR4* activation in post-stressed mice partially disarms innate immune response, and contributes to inappropriate host–*E. coli* interactions and thus neuroimmune dysregulation/toxicity. The increased *TLR4* activity in murine brain by very short-term stress would add one more molecular reason towards lessening stress at every level in animals and humans to ward off, at least, neuron damage. The confirmatory novel effects induced by acute stress will provide a



Scheme 1. Predicted impact of acute restraint stress (ARS) on induction of TLR4 activity in brain-derived immune cells and its consequences. Immunobiological behavior of ARS is dynamically complex; the scheme is our current-and-future scientific concepts of molecular neuroimmunopathology and ARS. Constant ARS-mediated stimulation of *TLR4* potentially leads to numerous cellular signaling (i.e., numerous nuclear pro-inflammatory and pro-oxidant molecules like *PXR*, *CYP*, *XREM* etc.), resulting in further pro-oxidant status, thereby accelerating formation of NF-κB activity and eventually pro/anti-inflammatory imbalance. The NF-κB possesses specific activation sites on TLR4 promoters in leukocytes/microglia/astrocytes. Activation of these promoters potentiates positive feed-back of pro-oxidant molecules, further synergizing the transcription and translation of TLR4. Here *TLR4* expression in brain was far more pronounced in ARS followed by *E. coli* infection and the differences between *E. coli* alone and ARS plus *E. coli* might give a clue for stress-mediated neuroimmunopathology. The activated *TLR4* with oxidative stress in microglia/astrocytes caused by ARS could lead to (de)activity of many other downstream signaling pathways and via (de)phosphorylation of e.g., PI3k/AKt, resulting in further immune dysfunction, e.g., diminished regulatory T-lymphocyte proliferation and polarization; these are a sign of further invisible brain damage caused by ARS. The scientific concepts of this model shed light on stress-associated immune-dysregulations and neuroimmunopathology in animals/humans. *IKK, inhibitor kB kinase; FADD, Fas-associated protein with death domain; PI3K, phosphoinositide 3-kinase, AKt, a kind of protein kinase B involved in cellular survival pathways; GSK3, Glycogen synthase kinase 3; CREB, cAMP response element-binding protein; CYP, cytochrome P450; P27, an inhibitor of cyclin dependent kinase involved in the regulation of the cell proliferation; PXR, pregnane X receptor; XREM, xenobiotic responsive enhancer module.*

basis for deeper investigations (i.e., evaluation of other TLR, cytokines and pivotally myeloid-derived suppressor cells in bone marrow, other lymphoid organs and CNS/PNS) of the effects from increasingly stress-oriented rural/urban life upon neuroimmune system.

Acknowledgments

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