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Stimulatory Toll-like receptor 2 suppresses restraint stress-induced immune suppression

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ABSTRACT

Stress can enhance or suppress immune functions depending on a variety of factors. Our previous studies observed that Toll-like receptor 2 (TLR2) participates in chronic restraint stress-induced immune dysfunction. However, the mechanism by which TLR2 prevents immune suppression remains elusive. Our investigation found that stimulation of TLR2 by peptidoglycan (PGN) significantly attenuates splenocyte apoptosis and markedly blocks alterations of anti-apoptotic and apoptotic proteins. Activation of TLR2 inhibits chronic stress-reduced phosphorylation of c-Jun N-terminal kinase (JNK) and diminishes chronic stress-induced up-regulation of corticosterone production. Additionally, our data show that chronic stress causes a dramatic decrease of cytokine IL-2 level but an increase of IL-4 and IL-17 in $CD4^+$ T cells. Interestingly, PGN could block these alterations of cytokine levels. Collectively, our studies demonstrate that stimulation of TLR2 attenuates chronic stress-induced immune suppression by modulating apoptosis-related proteins and immunoregulatory agents.

1. Introduction

It has been shown that stress can either increase or decrease immune functions depending on a variety of factors including duration of stressful situation. Acute stress results in an enhancement of both cellular and humoral immunity, while chronic stress has significant suppressive effects on the immune system [1–4]. Reduction of lymphocyte numbers is demonstrated in a chronic restraint stress mouse model [1]. A recent study has shown that chronic stress induces a regulatory phenotype in macrophages with decreased tumor necrosis factor (TNF)-α and interleukin 6 (IL-6) but increased IL-10 production [2].

Toll-like receptor 2 (TLR2), a crucial member of TLR family, plays a critical role both in the innate and adaptive immune responses [5–7]. TLR2 is also involved in the regulation of cell survival and apoptosis [8]. As for apoptosis, a variety of molecules, especially caspases and members of the Bcl-2 family, play pivotal roles in this process. Among the caspases, caspase-3 is a key protein and a marker in apoptosis with the ability of cleaving and destroying hundreds of cellular protein substrates, and poly(ADP-ribose) (PARP) is one of the main cleavage targets of caspase-3 [9,10]. The Bcl-2 family, central regulators of cell survival and apoptosis, is a group of proteins that either promote (e.g., Bax and Bak) or prevent (e.g., Bcl-2 and Bcl-x_L) apoptosis [11]. We previously observed that TLR2 plays a role in stress-induced reduction in the number of splenocytes [12]. However, the mechanisms by which TLR2 protects against stress-induced loss of splenocytes are still unclear.

C-Jun N-terminal kinase (JNK) is an important mediator of intracellular signaling cascade during innate and adaptive immune responses [13]. As a downstream target of TLR2 signaling pathway, JNK exerts strong regulatory effects on T help 1 (Th1) and Th2 responses [13,14]. Glucocorticoids (GCs), a class of steroid hormones, are the end products of the hypothalamic–pituitary–adrenal (HPA) axis activity in response to inflammatory and stress-related stimuli [15,16]. In adaptive immune system, GCs induce selective suppression of Th1 response and lead to a shift towards Th2-mediated humoral immunity [17]. So far, it is unclear whether these two immunoregulatory agents could be modulated by TLR2 stimulation which has been shown to rescue the Th1/Th2 disequilibrium induced by chronic stress.

Abbreviations: TLR2, Toll like receptor 2; PGN, peptidoglycan; JNK, c-Jun N-terminal kinase; TUNEL, terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling.

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In the current study, we investigated whether activation of TLR2 could block chronic stress-induced cell apoptosis and immune suppression. Specifically we determined the involvement of anti-apoptotic Bcl-2 and pro-apoptotic Bax. We also examined the involvement of JNK, corticosterone and cytokines.

2. Materials and methods

2.1. Mice

TLR2 knockout (TLR2 KO) mice on a C57BL/6 background and wild type C57BL/6 mice were purchased from Jackson Laboratory and maintained in the Division of Laboratory Animal Resources at East Tennessee State University (ETSU), a facility accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC). All animal studies were approved by the ETSU Committee on Animal Care.

2.2. Experimental model of restraint stress

Six- to eight-week-old male mice were subjected to an established chronic physical restraint protocol used in our laboratory as well as others [1,18]. Briefly, mice were placed in a 50-ml conical centrifuge tube with multiple punctures to allow ventilation. Mice were held horizontally in the tubes for 12 h. Control littermates were kept in their original cage without food and water for 12 h. After physical restraint, mice were sacrificed by CO_2 asphyxiation, and the spleens were harvested.

2.3. Experimental protocols

To investigate the effects of PGN on splenocyte apoptosis and immune dysfunction in chronic stress, mice were treated with or without PGN (50 μg/25 g body weight, Sigma, St. Louis, MO) by intraperitoneal (i.p.) injection 1 h before the mice were subjected to restraint stress. To determine whether TLR2 is essential for the effects of PGN, TLR2 KO mice were also administered with or without PGN (50 μg/25 g body weight, i.p.) 1 h before the initiation of stress.

2.4. Western blot analysis

Western blotting was performed as described previously [19]. Briefly, the cellular proteins were separated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis and transferred onto Hybond ECL membranes (Amersham Pharmacia, NJ). The ECL membranes were incubated overnight at 4 °C with the appropriate primary antibody [anti-TLR2 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-Bcl-2, anti-Bax, anti-phospho-JNK, anti-JNK, anti-cleaved-caspase-3, anti-caspase-3, anti-PARP, anti-GAPDH (Cell Signaling Technology, Beverly, MA)], followed by incubation with peroxidase-conjugated secondary antibodies (Cell Signaling Technology, Inc.). The blot was again washed three times with TBS before being exposed to the SuperSignal West Dura Extended Duration substrate (Pierce Biotechnology, Rockford, IL). The signals were quantified by scanning densitometry using a Bio-Image Analysis System (Bio-Rad).

2.5. TUNEL staining

TUNEL staining was performed as described previously [20]. Briefly, spleens from mice were fixed in 10% buffered formalin and embedded in paraffin. After deparaffinization and hydration of sections, TUNEL staining for apoptotic nuclei was performed using an In Situ Cell Death Detection kit (Roche Diagnostic, Indianpolis, IN). Briefly, sections were exposed for 10 min to a permeabilization solution (0.1% Triton X-100, 0.1% sodium citrate). After washing, 50 μl of TUNEL reaction mixture was placed on the sections and then incubated in a humidified atmosphere for 60 min at 37 °C. 50 μl of substrate solution was added following convert-AP incubation. Finally, sections were counterstained with haematoxylin. Sections were examined with the light microscope using a 40× objective. The percentage of apoptotic cells was calculated (TUNEL-positive cells/total cells) and averaged across at least five randomly chosen microscopic fields for each slide.

2.6. Determination of corticosterone

Blood was collected from all experimental and control mice immediately after stress. Samples were allowed to clot for 30 min at room temperature before centrifugation for 15 min at 1000*g*. Then serum was removed and stored at −20 °C for subsequent assay. The serum level of corticosterone was determined using a corticosterone mouse ELISA kit (IBL America, Minnuapolis, MN).

2.7. Culture and stimulation of $CD4^+$ T cells

Splenic $CD4^+$ T cells were negatively selected by using the MagCellect™ Mouse $CD4^+$ T Cell Isolation Kit (R&D Systems, Minneapolis, MN). Isolated $CD4^+$ T cells were plated in 96-well culture plates that were pre-coated with anti-CD3 (BD Biosciences Pharmingen, San Diego, CA) at 5 μg/ml in PBS for 24 h at 4 °C. Cells were seeded at 2.5×10^5 per well. The culture medium used was Gibco® RPMI Media 1640 (Invitrogen, Carlsbad, CA) supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mM L-glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin, and 1 μg/ml anti-CD28.

2.8. Enzyme linked immunosorbent assay (ELISA) for cytokines

The supernatants from anti-CD3/anti-CD28-stimulated $CD4^+$ T cells cultures were collected after 48 h. The amount of IL-2 and IL-4 in the supernatants was examined by using a Quantikine Mouse ELISA kit (R&D Systems, Minneapolis, MN) [1]. IL-17 was quantified using a mouse IL-17 platinum ELISA kit (eBioscience, Vienna, Austria).

2.9. Isolation of RNA and real-time quantitative RT-PCR

Total RNA was isolated from $CD4^+$ T cells using a RNeasy Plus Mini Kit (QIAGEN Sciences, Maryland, MD) according to the manufacturer's instructions. The real-time PCR was performed as described previously [1,21]. Briefly, one microgram of RNA from each sample was used for reverse transcription and synthesis of cDNA using a Reaction Ready™ first strand cDNA synthesis kit (SABiosciences, Frederick, MD). PCR was performed using RT^2 real-time™ SYBR Green Fluorescien PCR Master Mix (SABiosciences). GAPDH expression was used as internal control. The primer sequences used were as follows: IL-2 forward 5′-AGC AGC TGT TGA TGG ACC TA-3′, IL-2 reverse 5′-TAC TTG AAC CTG GAG ACG C-3′, IL-4 forward 5′-GGT CTC AAC CCC CAG CTA GT-3′, IL-4 reverse 5′-TAG TGA ACT CTC TCT AGT AGC CG-3′, IL-17 forward 5′-GCC CTC AGA CTA CCT CAA CC-3′, IL-17 reverse 5′-GAA TTC ATG TGG TGG TCC AG-3′, GAPDH forward 5′-TGA CCA CAG TCC ATG CCA TC-3′, GAPDH reverse 5′-GAT GGG GGT TAC ACA GGC AG-3′.