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Enhanced Resistance of Restraint-Stressed Mice to Sepsis¹

Yu Wang, Ying Lu, Duo Yu, Yongqiang Wang, Fuyong Chen, Hanchun Yang,²
and Shijun J. Zheng²

Sepsis remains a major health concern across the world. The effects of stress on host resistance to sepsis are still not very clear. To explore the effects of chronic stress on sepsis, we examined the impact of restraint stress on the resistance of mice to sepsis. Interestingly, it was found that restraint stress enhanced the antiseptic resistance of mice and the concentrations of the proinflammatory cytokines IL-1, IL-6, IL-12, and TNF- α in the blood of stressed mice were dramatically reduced post *Escherichia coli* infection or LPS treatment as compared with that of controls ($p < 0.05$). In addition, the mRNA expressions of glucocorticoid-induced leucine zipper (GILZ) were up-regulated in the spleen and peritoneal macrophages of mice receiving restraint stress or dexamethasone treatment. These results demonstrate that restraint stress enhances the resistance of mice to sepsis, supporting corticotherapy for sepsis and proposing restraint-stressed mouse as an animal model to elucidate mechanisms of stress-associated, antiseptic resistance. *The Journal of Immunology*, 2008, 181: 3441–3448.

The physical restraint or immobilization of animals is used as a “stressor” for the induction of stress response syndromes in animals (1). The physical restraint stress has been widely used as a stress model to dissect the stress-associated alteration of physiological, immunological, and neurobiological status in mammals (2, 3). Although stress has been recognized for a long time, its impacts on immunity and underlying mechanisms are still not completely known. It has been shown that the enhancement of immune responses by acute stress is dependent on the hypothalamic-pituitary-adrenal axis (4), while the mechanisms by which chronic stress exerts its immunosuppressive effects are via the reduction of immune cells by Fas-mediated apoptosis (5, 6), which may involve, at least in some cases, the up-regulated expression of Fas by endogenous opioids (3).

Stress response is associated with a rise in glucocorticoid concentration in blood, which is regulated by the hypothalamic-pituitary axis with the aim of maintaining homeostasis (7). Glucocorticoids can interact with receptors in both the hypothalamus and pituitary gland to reduce the release and synthesis of the corticotrophin-releasing hormone and the adrenocorticotrophic hormone, respectively, which is the principal feedback regulation (8). Glucocorticoids may also modulate immune responses by direct binding to their high-affinity receptors on immune cells. Consequently, the glucocorticoid-receptor complex enters the nucleus and interacts directly with specific DNA sites (glucocorticoid-responsive elements), exerting both inhibitory and activating actions on transcription (7, 9, 10). As such, stress may have significant impacts on immune responses via glucocorticoids that may alter the susceptibility of hosts to various diseases.

Sepsis is clinically caused by Gram-negative (G^-)³ or Gram-positive (G^+) bacterial infections associated with the uncontrollable release of high levels of proinflammatory cytokines such as TNF- α , IL-1, IL-6, IL-12, and other inflammatory mediators, leading to multiple critical organ failure in host (11). Although both G^- and G^+ bacterial infection can cause sepsis, the essential components of G^- and G^+ bacteria triggering sepsis may vary. The prime mediator of G^- bacterial sepsis is LPS, an essential component of the outer membrane of G^- bacteria, whereas peptidoglycan, a major component of the G^+ bacterial cell wall, together with lipoteichoic acid, triggers sepsis (12, 13). In addition, bacterial DNA may also cause sepsis (14). Currently the precise mechanisms underlying G^+ and G^- bacterial sepsis are still not very clear.

Because sepsis is associated with an intense inflammatory response, glucocorticoids are clinically used to treat sepsis due to its anti-inflammatory effect. Although many effects of glucocorticoids on the immune and inflammatory responses have been described in vitro, their clinical relevance remains controversial (7). The use of glucocorticoids (corticotherapy) in severe sepsis is one of the main controversial issues in critical care medicine. The high-dose glucocorticoids in the treatment of severe sepsis and septic shock have not yielded a positive clinical outcome (15, 16), while the low-dose glucocorticoids had anti-inflammatory effects as demonstrated by a decrease in inflammatory response and an increase in anti-inflammatory cytokines (17). In addition, the use of low-dose glucocorticoids in treatment of patients with septic shock associated with adrenal insufficiency or adrenal failure have shown decreased mortality (7, 18). It seems that the outcome from the use of glucocorticoids in the treatment of severe sepsis depends on the dosage of glucocorticoids. Although it is generally accepted that chronic stress often results in immunosuppression while acute stress has been shown to enhance immune responses (3, 4), the effects of restraint stress on host resistance to sepsis are still not clear. In this study we show that restraint stress enhances the resistance of mice to sepsis via inhibiting the

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³ Abbreviations used in this paper: G^- , Gram negative; DEX, dexamethasone; EIA, enzyme immunoassay; G^+ , Gram positive; GILZ, glucocorticoid-induced leucine zipper.

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production of proinflammatory cytokines by macrophage, providing novel evidence to dissect the underlying mechanisms of the antiseptic effects of restraint stress. These findings support the clinical use of glucocorticoids in the treatment of sepsis and may allow restraint stress to be used as a model to elucidate mechanisms of corticotherapy for sepsis.

Materials and Methods

Mice

Six- to 8-wk-old inbred BALB/c male mice were purchased from Vital River. All mice were maintained in our animal facility with food and water ad libitum for a minimum of 7 days before experimentation. All procedures were approved by the Animal Care and Use Committee of China Agricultural University (Beijing, China).

Reagents

Both LPS (*Escherichia coli* O111: B4) and dexamethasone (DEX) were purchased from Sigma-Aldrich, a corticosterone enzyme immunoassay (EIA) kit was from Cayman Chemical, ELISA sets for IL-1 β , IL-6, IL-12, and TNF- α were from BD Pharmingen, mouse IFN- γ was from Peprotech, TRIZol reagent was from Invitrogen, oligo d(T) and RNase inhibitor were from TaKaRa, Moloney murine leukemia virus reverse transcriptase was from Promega, dNTPs were from Tiangen, and the LightShift chemiluminescent EMSA kit was from Thermo Fisher.

Mouse model of restraint stress

Mice were subjected to restraint stress as previously described (6). Seven- to 9 wk old male BALB/c mice were placed in a 50-ml conical centrifuge tube filled with multiple punctures and held horizontally in the tubes for a continuous 14 h followed by a 10-h rest. The control littermates were kept in their original cage. Food and water were provided ad libitum for both stressed and control mice during the rest period of the experimental groups. Mice were physically restrained for two cycles before *E. coli* infection or LPS treatment via i.p. injection.

Thymocyte and splenocyte count

Mice were sacrificed by cervical dislocation. The thymus and spleen were surgically removed and weighed before a single cell suspension was prepared using a 200 μ m wire mesh. Thymocytes and splenocytes were counted using a hemocytometer under the microscope after lysing RBC with a lysis solution containing 1.78 M NH₄Cl and 0.03 M NH₄HCO₃.

Determination of level of corticosterone in plasma

Blood samples were collected with tubes containing an anticoagulant solution of 4% sodium citrate. The blood samples were centrifuged at 1,000 \times g for 10 min before the plasma was separated and stored at -70°C until use. The concentration of corticosterone was determined using a corticosterone EIA kit from Cayman Chemical per the manufacturer's instructions.

E. coli challenge

E. coli (O149), a clinical isolate from diseased pigs and identified by the Chinese Ministry of Agriculture Institute of Veterinary Drug Control (Beijing, China), were grown in Luria-Bertani medium. The log-phase growing cultures were washed twice with PBS and stored at -70°C until use. The number of viable bacteria was determined by CFU assay before the infection of mice. Each mouse was i.p. injected with 5×10^7 , 7.5×10^7 , or 1×10^8 CFU of *E. coli* in a total volume of 200 μ l for the survival test. The sublethal dose of *E. coli* (2.5×10^7 CFU/mouse) was i.p. injected into the mice for the examination of bacterial growth in the organs.

Staphylococcus aureus challenge

S. aureus (CVCC-C56010), which was purchased from the Ministry of Agriculture Institute of Veterinary Drug Control in China, were grown in tryptic soy broth. The log-phase growing cultures were washed twice with PBS and stored at -70°C until use. The number of viable bacteria was determined by CFU assay before infection of mice. Each mouse was i.p. injected with 4×10^7 or 2×10^8 CFU of *S. aureus* in a total volume of 200 μ l for the survival test.

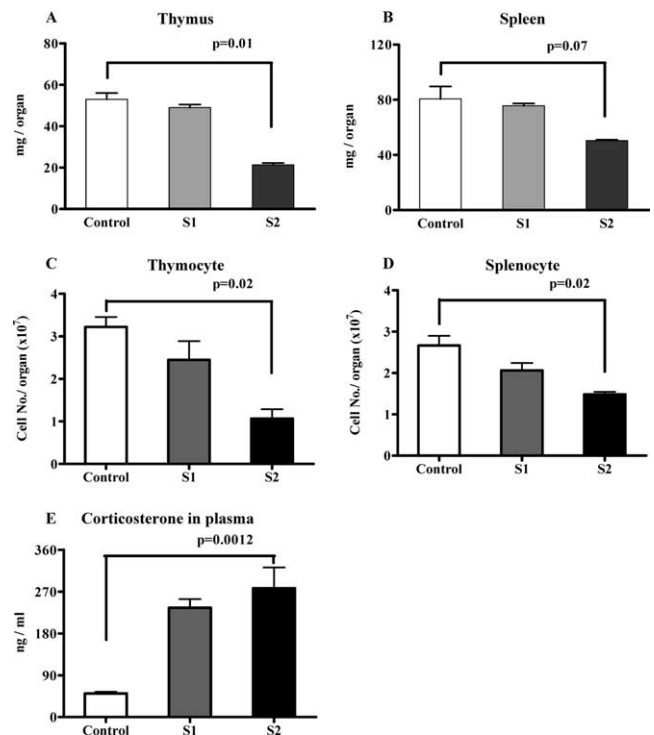


FIGURE 1. Organ weight and cell numbers were reduced in restraint-stressed mice. *A–D*, The thymus and spleen organs were surgically removed from the stressed mice ($n = 5$) and controls ($n = 5$) and weighed (*A* and *B*). The total numbers of thymocytes (*C*) and splenocytes (*D*) were determined. *E*, The concentrations of corticosterone in plasma were measured by using a corticosterone EIA kit. S1 stands for one cycle of restraint stress and S2 represents two stress cycles. The differences between the S2 and control groups are statistically significant ($p < 0.05$ as determined by ANOVA). Data are representative of three independent experiments.

LPS treatment

Control and stressed mice were i.p. injected with different doses of LPS in a total volume of 200 μ l and observed every 8 h until the last survivor recovered.

Recovery of bacteria from organs

Mice were i.p. injected with a sublethal dose of *E. coli* and sacrificed at 24, 36, and 72 h postinfection. The livers and spleens were surgically removed and homogenized in PBS. Serial 10-fold dilutions of organ homogenates in PBS were plated on Luria-Bertani agar culture medium and cultured for 24 h before the number of bacteria was determined by CFU on the plate.

Flow cytometry

Cells from peritoneal cavity, spleen, and peripheral blood were labeled at 4°C with the following Ab reagents: allophycocyanin-conjugated rat anti-mouse CD11b (clone M1/70; BD Pharmingen) and Tri-color-conjugated rat anti-mouse F4/80 (clone BM8; Invitrogen). After washing, cells were fixed in 1% paraformaldehyde in PBS. Cells were analyzed on a FACS-Calibur flow cytometer (BD Biosciences) using the CellQuest program (BD Biosciences).

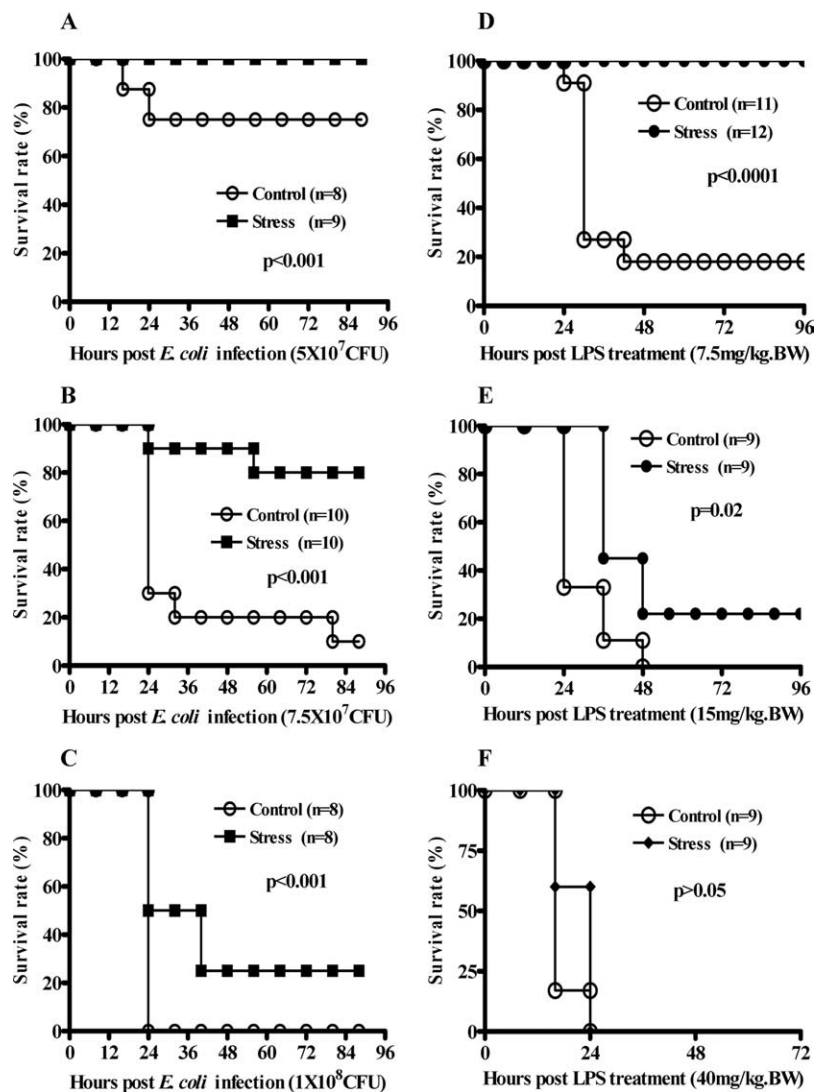
Collection of serum samples

Blood samples were collected by orbital puncture from stressed mice and controls at the different time points following *E. coli* infection or LPS treatment. Serum was separated by centrifugation after the blood clotted and saved at -70°C until use.

Cytokine assay

The concentrations of IL-1 β , IL-6, IL-12, and TNF- α in the serum and the culture supernatant were measured by the ELISA sets (BD Pharmingen) per the manufacturer's instruction.

FIGURE 2. Enhanced survival of stressed mice from sepsis with *E. coli* infection and LPS treatment. *A–C*, Stressed mice ($n = 8–10$) and controls ($n = 8–10$) were infected with *E. coli* at 5.0×10^7 CFU (*A*), 7.5×10^7 CFU (*B*), and 1.0×10^8 CFU (*C*) per mouse via i.p. injection. Data shown are survival rates of stressed and control mice postinfection with *E. coli*. The difference between the two groups is statistically significant as determined by Mann-Whitney *U* test ($p < 0.001$). Results are representative of three independent experiments. *D–F*, Stressed mice ($n = 9–12$) and controls ($n = 9–11$) were i.p. injected with LPS at the doses of 7.5 mg (*D*), 15 mg (*E*), and 40 mg (*F*) per kilogram of body weight (BW). Data shown are survival rates of stressed mice and controls post-LPS treatment. The difference between the two groups is statistically significant for the doses of 7.5 and 15.0 mg per kilogram of body weight (*D* and *E*) ($p < 0.05$), but not for the dose of 40.0 mg per kilogram of body weight (*F*) as determined by Mann-Whitney *U* test. Results are representative of two independent experiments.



Cell culture

Stressed mice and controls were i.p. infected with *E. coli* at 2.5×10^7 CFU/mouse. Peritoneal macrophages were harvested 24 h after *E. coli* infection and cultured for 48 h at 1×10^6 cells/ml in complete DMEM containing 10% FBS with or without 200 pg/ml LPS and 10 U/ml IFN- γ . The concentrations of cytokines in the culture supernatant were measured as described above.

DEX treatment and sample collection

Mice were i.p. injected with DEX at different doses (0.02, 0.2, and 2 mg per kg of body weight). Three hours after DEX treatment, mice were sacrificed before tissues and peritoneal macrophages were harvested and stored at -70°C until use.

RNA extraction, cDNA synthesis, and RT-PCR

Total RNA was extracted from peritoneal macrophages and spleen tissue using TRIzol reagent (Invitrogen) per the manufacturer's instructions. One microgram of total RNA was incubated with 0.5 μg of oligo d(T)₁₅ (TaKaRa) for 5 min at 70°C to melt the secondary structure within the sample before cooling immediately on ice and was subsequently combined with 40 U of RNase inhibitor (TaKaRa), 1.25 mM dNTP (Tiagen), and 200 U of Moloney murine leukemia virus reverse transcriptase (Promega) in reverse transcriptase buffer in a total volume of 25 μl before incubation at 40°C for 90 min. To enable appropriate amplification in the exponential phase for the target gene, PCR amplification of β -actin, TLR4, and glucocorticoid-induced leucine zipper (GILZ) genes were conducted in separate reactions using specific primers for β -actin (sense, 5'-TGGGAATCCT GTGGCATCCATGAAAC-3'; antisense, 5'-TAAAACGCAGCTCAGTAAC

AGTCCG-3') (19), TLR4 (sense, 5'-CTGGGTGAGAAATGAGCTGG-3'; antisense, 5'-GATACAATTCCACCTGCTGCC-3') (19), and GILZ (sense, 5'-CAGCAGCCACTCAAACCAGC-3'; antisense, 5'-ACCACATCCCCTC CAAGCAG-3') (20). PCR was performed in a thermal cycler (PTC-200; Bio-Rad) with a program containing an initial step at 94°C for 5 min followed by 25 cycles for the amplification of β -actin, 30 cycles for that of TLR4, and 28 cycles for that of GILZ genes, each cycle consisting of 94°C for 20 s, 60°C for 20 s, and 72°C for 30 s. The resulting products were quantified by electrophoresis on 2% agarose gel containing SYBR Green and analyzed by AlphaEase software (Alpha Innotech). The mRNA level of GILZ in each sample was normalized to the level of β -actin and calculated as a ratio to the level of the control.

Nuclear protein extract and EMSA

Crude nuclear proteins were extracted from peritoneal macrophages using a nuclear-cytosol extraction kit (Appligen) with a protease inhibitor mixture (Roche). 5'-Biotin-labeled NF- κB consensus double stranded oligonucleotides (5'-AGTTGAGGGGACTTTCCAGG-3') (21) were synthesized by AuGCT Biotechnology. Detection of the NF- κB -oligonucleotide complex was performed using a LightShift chemiluminescent EMSA kit (Thermo Fisher) per the manufacturer's instruction. Briefly, nuclear protein (3–5 μg) was incubated with 20 fmol of biotin-labeled oligonucleotides for 30 min at room temperature in a 20- μl reaction volume containing 10 mM HEPES-KOH (pH 7.9), 50 mM KCl, 2.5 mM MgCl_2 , 1 mM DTT, 10% glycerol, 1 μg of DNase-free BSA, and 2.5 μg of polydeoxyinosinic-deoxycytidylic acid. The resulting products were resolved by electrophoresis on a 6% polyacrylamide gel using $0.5 \times$ Tris-borate EDTA (TBE) buffer. NF- κB -oligonucleotide complex was electroblotted to a nylon membrane

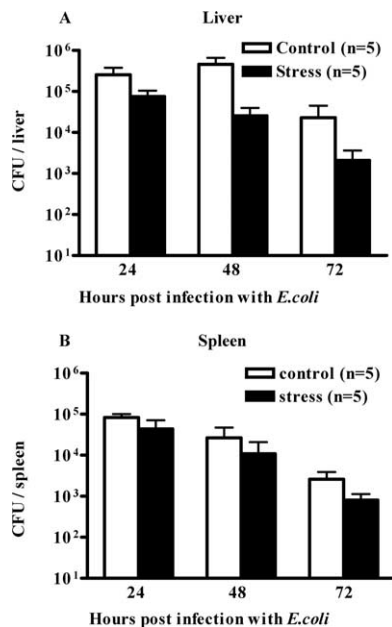


FIGURE 3. *E. coli* counts in the liver and spleen of mice postinfection. Mice pretreated with restraint stress ($n = 15$) and controls ($n = 15$) were infected with 2.5×10^7 *E. coli* via i.p. injection. Five mice from each group were sacrificed 24, 48, and 72 h postinfection for the determination of bacterial loads in the liver (A) and spleen (B). There were no statistically significant differences between the two groups at each time point as determined by ANOVA ($p > 0.05$). Data are representative of three independent experiments and presented as mean \pm SEM.

(Millipore). After incubation in blocking buffer for 15 min at room temperature, the membrane was incubated with streptavidin-HRP conjugate for 15 min at room temperature. Color was developed using LightShift chemiluminescence detection reagents (Thermo Fisher).

Statistical analysis

The significance of the differences between stressed mice and controls in organ weight, cell numbers, cytokine concentrations and mouse survival data was determined by Mann-Whitney U test and ANOVA accordingly.

Results

Restraint-stressed mice are partially resistant to sepsis

Sepsis remains a major health concern worldwide. To explore the effects of chronic stress on septic shock, we stressed mice using the physical restraint stress model as described in *Materials and Methods* before the mice were infected with pathogenic bacteria. As shown in Fig. 1, after two rounds of stress mice had reduced mass of thymus and spleen (Fig. 1, A and B), reduced numbers of thymocytes and splenocytes (Fig. 1, C and D), and elevated concentrations of corticosteroid in the blood (Fig. 1E), which demonstrates that the mice were severely stressed after two rounds of physical restraint (6). Interestingly, the stressed mice showed enhanced resistance to sepsis postinfection with a lethal dose of pathogenic *E. coli* as compared with the controls (Fig. 2, A–C). However the stressed mice also succumbed to death if the dose continued to increase, which indicates that the stressed mice are partially resistant to *E. coli*-induced sepsis. Because LPS is the major endotoxin of Gram-negative bacteria and also a prime mediator of sepsis, we further tested the resistance of stressed mice to LPS-mediated sepsis. We injected mice with the different doses of LPS via the i.p. route and examined the survival of stressed mice vs controls post-LPS treatment. As expected, the stressed mice survived better than the controls post-LPS treatment (Fig. 2, D and E) ($p < 0.05$) but died of high-dose LPS treatment (Fig. 2F),

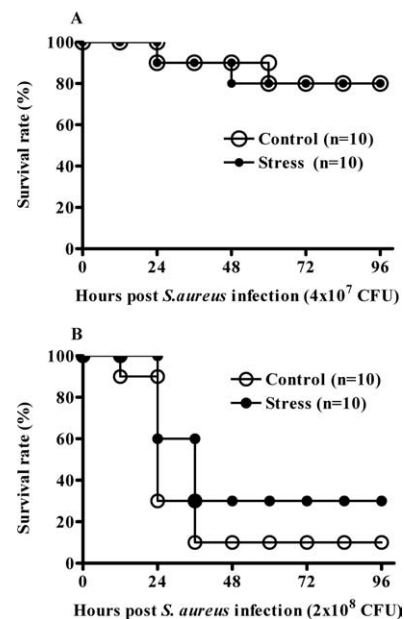


FIGURE 4. Restraint stress did not alter the susceptibility of the mice to *S. aureus* infection. Stressed mice ($n = 10$) and controls ($n = 8$ – 10) were infected with lethal doses of *S. aureus* at 4×10^7 CFU (A) or 2×10^8 CFU (B) per mouse via i.p. injection. Data shown are survival rates of stressed mice and controls postinfection with *S. aureus*. Mice that survived 120 h postinfection were recovered fully. There were no statistically significant differences between the two groups as determined by Mann-Whitney U test ($p > 0.05$). Results are representative of three independent experiments with the similar results.

which indicates that the stressed mice are partially resistant to G^- bacterial septic shock. However, when infected with a sublethal dose of the bacteria, the stressed mice did not show any enhanced capacity of inhibiting the bacterial growth or clearing the pathogen (Fig. 3, A and B) as compared with the controls ($p > 0.05$). These results suggest that restraint stress primarily affects the acute and intense inflammation of a host in response to bacterial infection rather than the regular process of immune response.

To determine whether the resistance of stressed mice to LPS-mediated sepsis could be generalized to G^+ bacterial sepsis, we examined the survival of stressed mice infected with *S. aureus* via i.p. injection. As shown in Fig. 4, when infected with the lethal dose of pathogenic *S. aureus*, the stressed mice did not show any advantage over the controls in terms of survival rate. These results suggest that the enhanced resistance of stressed mice to sepsis might be specific to LPS-producing G^- but not G^+ pathogenic bacteria.

The production of proinflammatory cytokines IL-1, IL-6, IL-12, and TNF- α was inhibited in stressed mice after *E. coli* infection or LPS treatment

Because sepsis is associated with the alteration of cytokine expression and cytokine response is modulated by glucocorticoids (7), it would be intriguing to examine the levels of cytokines in the blood of stressed mice in response to *E. coli* infection and post-LPS treatment. Although the concentrations of the proinflammatory cytokines IL-1, IL-6, IL-12, and TNF- α in both stressed mice and the control groups were barely detectable before infection, they were dramatically elevated in the blood of controls following *E. coli* infection. However, they were significantly reduced in stressed mice as compared with that of controls post-*E. coli* infections (Fig. 5, A–D) ($p < 0.05$). Similar results were also observed from

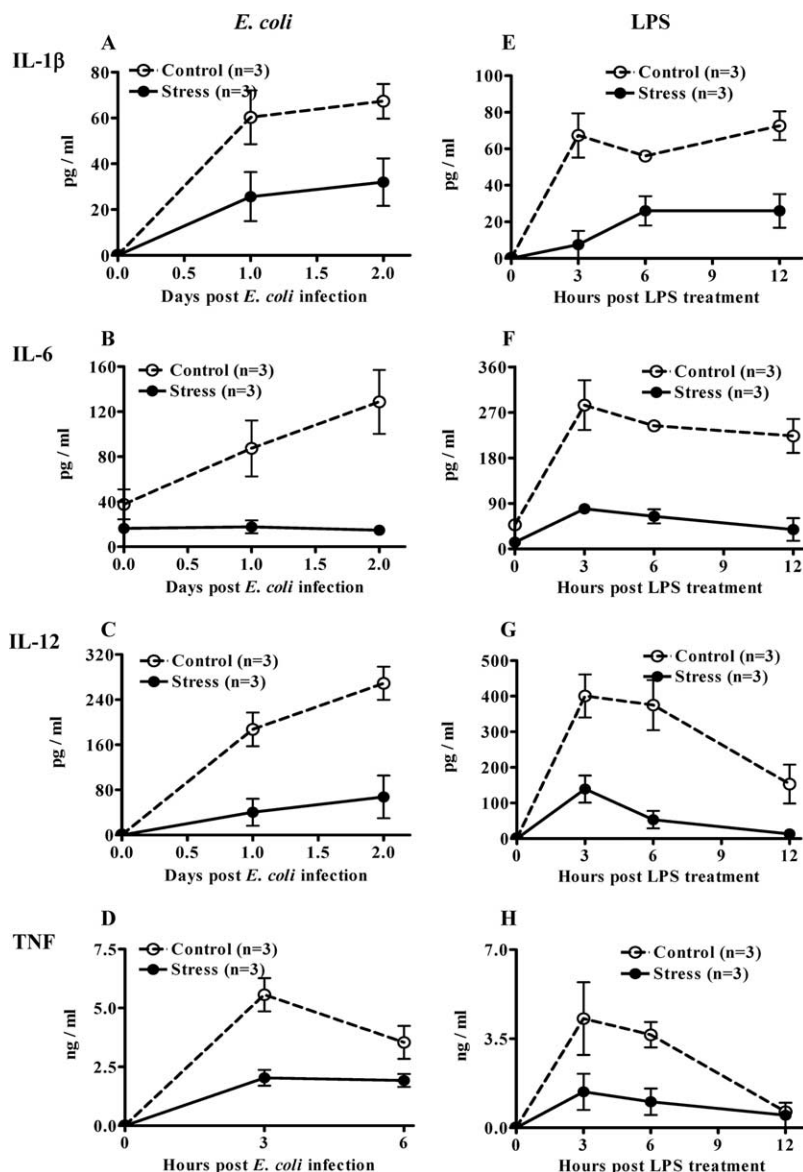


FIGURE 5. Reduced levels of proinflammatory cytokines IL-1 β , IL-6, IL-12, and TNF in the serum of stressed mice post-*E. coli* infection and post-LPS treatment. Stressed mice and controls were infected with 2.5×10^7 CFU of *E. coli* (A–D), and 15.0 mg of LPS per kilogram of body weight (BW) (E–H). Mice were sacrificed at different time points post-*E. coli* infection (A–D) or LPS treatment (E–H) for the measurement of cytokine concentrations in the serum as determined by ELISA kits. The differences between the two groups are statistically significant as determined by ANOVA ($p < 0.05$). Results are representative of three independent experiments and presented as mean \pm SEM.

the LPS treatment (Fig. 5, E–H). Because macrophages were the major sources of proinflammatory cytokines (22, 23), we examined expressions of proinflammatory cytokines by peritoneal macrophages of stressed mice. When cultured *in vitro*, less IL-1 β , IL-6, IL-12, and TNF- α were produced by the peritoneal macrophages of *E. coli*-infected stressed mice than those by controls ($p < 0.05$) (Fig. 6), which suggests that the decreased level of proinflammatory cytokines in stressed mice with *E. coli* or LPS sepsis might result from the inhibitory effects of stress on cytokine production by macrophages. These data demonstrate that the inflammation was dramatically suppressed in stressed mice in response to *E. coli* infection or LPS treatment, which may, at least in part, account for the reduced mortality in stressed mice.

To rule out the possibility that the decreased level of cytokines in stressed mice in response to *E. coli*-infection might be due to the decreased number of macrophages as a consequence of stress, we examined and compared the numbers of inflammatory monocytes/macrophages, CD11b $^+$ /F4/80 $^+$ double positive cells, in the peripheral blood, spleens, and peritoneal cavity of stressed mice vs controls using flow cytometry. As shown in Fig. 7, there were no differences in the numbers of CD11b $^+$ /F4/80 $^+$ double positive cells in blood, spleens and peritoneal cavity between stressed mice

and controls post-*E. coli* infection ($p > 0.05$), which clearly indicates that the reduced production of cytokines in stressed mice with *E. coli* or LPS sepsis might be attributed to the inhibitory effects of stress on cytokine expressions rather than the curtailed numbers of inflammatory macrophages.

mRNA expression of GILZ and activation of NF- κ B increased in stressed mice

A recent study demonstrated a novel link between the innate immune system and the adrenal stress response mediated by TLRs (24) that prompted us to explore the mechanisms underlying the reduced production of proinflammatory cytokines by the macrophages of stressed mice post-*E. coli* infection or post-LPS treatment. Because glucocorticoids stimulate the production of GILZ in macrophages and GILZ inhibits the expression of TLRs (20) and NF- κ B (NF- κ B) (25), we hypothesized that restraint stress may exert immune-suppressive effects via up-regulation of GILZ expression. As expected, the mRNA expression of GILZ was dramatically increased in a time-dependent manner in the peritoneal macrophages and splenocytes of mice post-restraint stress (Fig. 8, A and B), which indicates that GILZ is one of the molecules involved in chronic stress-associated immunosuppression.

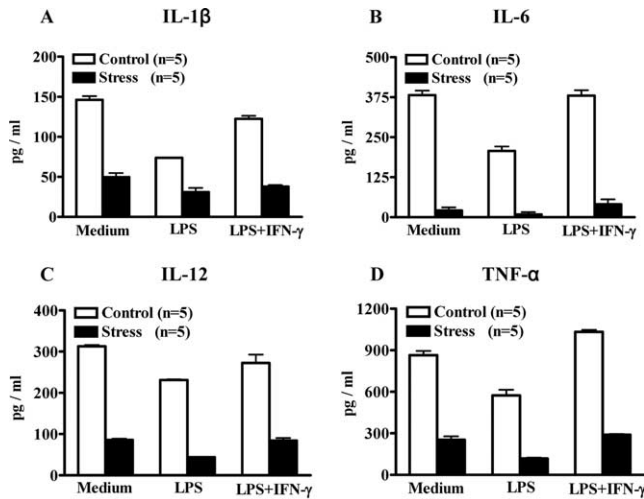


FIGURE 6. Reduced production of proinflammatory cytokines by the macrophages of stressed mice following *E. coli* infection. Mice were treated as in Fig. 5, A–D. Peritoneal macrophages were collected from stressed mice ($n = 5$) and controls ($n = 5$) 24 h following *E. coli* infection, and cultured for 48 h with or without 200 pg/ml LPS and 10 U/ml IFN- γ . IL-1 β (A), IL-6 (B), IL-12 (C), and TNF- α (D) concentrations in the supernatants were determined by ELISA. The differences between the stressed and control groups are statistically significant ($p < 0.001$ as determined by ANOVA). Data are representative of three independent experiments.

Because glucocorticoids inhibit the expression of TLRs (20) and NF- κ B (25), it would be tempting to determine the impact of restraint stress on the expression of TLR4, a pattern recognition receptor for LPS and a key mediator in the cross-talks between the innate immune system and stress response (26), and its impact on the activation of NF- κ B, a master regulator of all TLR-induced responses (27). Interestingly, we found that NF- κ B was dramatically activated in the peritoneal macrophages of mice ~3 h post-restraint stress as demonstrated by EMSA (Fig. 8C). In addition, the mRNA expression of TLR4 increased >2-fold in the splenic and peritoneal macrophages of mice post-restraint stress as examined by semiquantitative RT-PCR (our unpublished observation). These results suggest that the stress-associated GILZ expression (a target of GC signal) in macrophages may connect to the TLR and NF- κ B pathways.

mRNA expression of GILZ increased in the spleen and peritoneal macrophages of mice post-DEX treatment

To explore whether the up-regulation of GILZ expression in the peritoneal macrophages and splenocytes of stressed mice was due to the elevated level of GCs, mice were injected with DEX, a glucocorticoid commonly used in anti-inflammatory therapy (28), via the i.p. route before the peritoneal macrophages and splenocytes of mice were collected for the measurement of the mRNA expression of GILZ by semiquantitative RT-PCR. As shown in Fig. 9, the mRNA level of GILZ dramatically increased in a dose-dependent manner in the spleen and peritoneal macrophages of mice treated with DEX. These results indicate that DEX directly triggers the expression of GILZ in vivo and that restraint stress renders the mice resistant to sepsis via the GILZ-mediated immunosuppressive effects of GCs.

Discussion

Physical restraint stress is widely used as a stress model to investigate the stress-associated alteration of physiological, immunological, and neurobiological status in mammals (2). Although it is

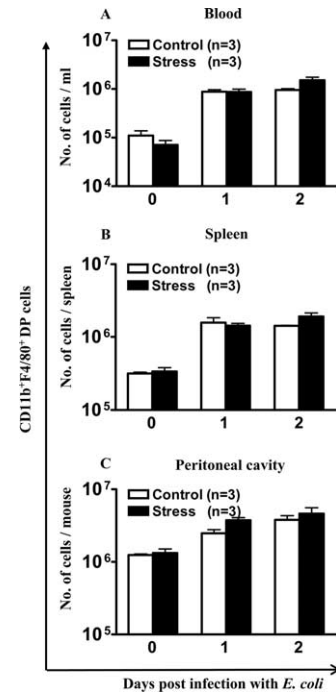


FIGURE 7. Comparison of monocytes/macrophages in the peripheral blood, peritoneal cavity, and spleen in stressed mice vs controls post-*E. coli* infection. Mice were treated as in Fig. 5, A–D. Peritoneal macrophages, splenocytes, and PMBCs were collected from normal ($n = 9$) and stressed mice ($n = 9$) at different time points post *E. coli* infection. Numbers of monocytes/macrophages were determined by flow cytometry with anti-CD11b and anti-F4/80 Abs and analyzed by CellQuest program. Data shown are mean and SD of monocytes/macrophages in the peripheral blood (A), spleens (B), and peritoneal cavity (C). There were no differences between *E. coli*-infected stressed mice and controls ($p > 0.05$ as determined by ANOVA).

known that chronic stress significantly affects immune responses and alters susceptibility to various diseases (3), the effects of restraint stress on host defense are still not clear. It has been reported that restraint stress exerted immune suppressive effects during influenza A/PR8 infection in mice via diminishing NK cell activity (29–31) and resulted in decreased inflammation within the CNS during Theiler's virus infection (32). Similar effects of stress on host defense were also observed in chronically stressed mice infected with *Listeria monocytogenes* (33, 34) or *E. coli* (35). In our experiment, we found that pretreatment of mice with physical restraint stress dramatically enhanced the resistance of mice to sepsis as demonstrated by an improved survival rate of stressed mice post-*E. coli* infection or post-LPS treatment as compared with that of the controls (Fig. 2). Furthermore, intense inflammatory responses in stressed mice to *E. coli* infection or LPS treatment were markedly reduced (Fig. 5). These observations provide strong evidence that restraint stress compromises acute inflammatory responses of host against infectious agents.

Although we found that restraint stress enhances the resistance of mice to sepsis due to *E. coli* infection or LPS treatment, there was no significant difference between stressed mice and controls in their resistance to *S. aureus* infection (Fig. 4). This is in agreement with a previous report showing that glucocorticoid treatment was effective at protection against *E. coli* but not so good in the case of *S. aureus* infection (36). These observations suggest that the enhanced resistance induced by stress or the use of glucocorticoids (corticotherapy) to sepsis may be specific to some species of pathogens. Because LPS is an essential component of the outer

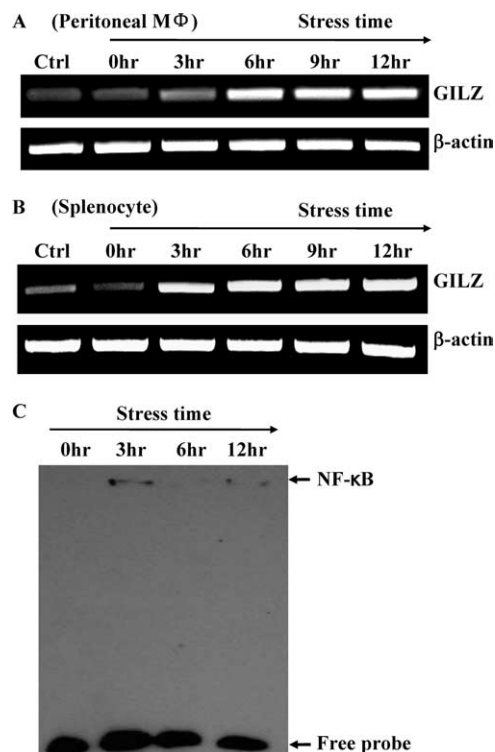


FIGURE 8. The mRNA expressions of GILZ and activation of NF- κ B were increased in stressed mice. *A* and *B*, Peritoneal macrophages (MΦ) and splenocytes were collected at different time points following restraint stress and subjected to semiquantitative RT-PCR using specific primers as described in *Materials and Methods* for determining the mRNA expressions of GILZ in peritoneal macrophages (*A*) and splenocytes (*B*) of stressed mice. β -actin indicates endogenous control (Ctrl). *C*, Crude nuclear proteins were extracted from the peritoneal macrophages of stressed mice sacrificed at different time points and subjected to EMSA for determining NF- κ B activation by restraint stress. The detection of the NF- κ B-oligonucleotide complex was performed using a LightShift chemiluminescent EMSA kit.

membrane of G^- bacteria while 90% of the cell wall of G^+ bacteria is composed of peptidoglycan (37, 38), the different components of G^- and G^+ bacteria may directly contribute to the differences in host responses to *E. coli* and *S. aureus* infections. More efforts will be required to generalize this observation using other species of bacteria.

Sepsis remains a major health concern across the world. However, no detailed information has yet been available as to the exact effects of restraint stress on host resistance to sepsis. Stress response is associated with a rise in glucocorticoid concentration in blood, while glucocorticoids are involved in modulating the immune response by inhibiting the production of the proinflammatory cytokines IL-1, IL-6, IL-12, and TNF by macrophages and monocytes (7). Thus, it is logical to hypothesize that chronic stress may suppress the inflammatory response by inhibiting macrophage functions. As such, the stressed mice might be resistant to sepsis. Consistent with this hypothesis, we found that the mice with an elevated level of corticosteroid in the blood due to physical restraint stress (Fig. 1E) had reduced inflammatory responses to bacterial infection or LPS treatment as demonstrated by decreased levels of IL-1, IL-6, IL-12, and TNF in the blood of stressed mice as compared with that of controls (Fig. 5). Proinflammatory cytokines such as IL-1 and TNF are produced by macrophages during infections and sepsis and contribute to mortality if produced in excessive quantities for extended periods of time (39, 40). Inhibi-

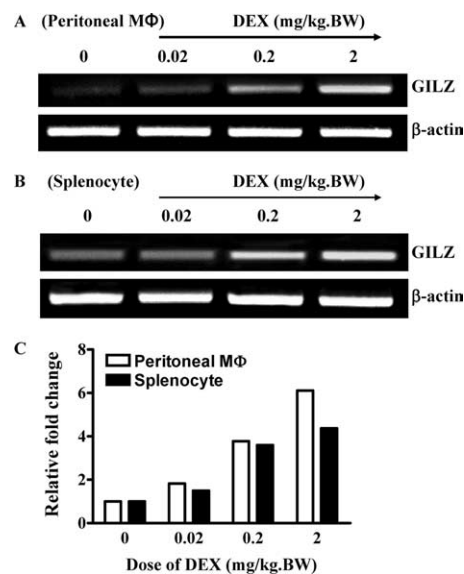


FIGURE 9. In vivo induction of GILZ by DEX. Mice were sacrificed 3 h after i.p. injection with DEX at different doses of 0.02, 0.2, and 2 mg per kilogram of body weight (BW). Semiquantitative RT-PCR was performed as described in Fig. 8 for the analysis of the mRNA expression of GILZ in peritoneal macrophages (MΦ) (*A*) and splenocytes (*B*) of mice post-DEX treatment. The density of bands in *A* and *B* was quantitated by densitometry. The relative level of GILZ mRNA expression in peritoneal macrophages and splenocytes (*C*) is calculated as follows: GILZ band density of stressed sample/GILZ band density of the control. β -actin indicates endogenous control.

tion of cytokine response by restraint stress (Fig. 5) or by heat stress (41) may directly contribute to the enhanced resistance of the host to sepsis.

Of note, our experiment was focused on the effects of chronic stress on antiseptic resistance. The results we show here were obtained from the mice receiving two cycles of restraint stress before infection with *E. coli* or LPS treatment. However, if mice continuously received chronic combined acoustic and restraint stress for up to nine cycles, they failed to combat bacterial infection (35). Although these results demonstrated the immune-compromised situation of mice receiving chronic stress, the mechanisms may not be the same. Our experiments were more focused on the effect of restraint stress on sepsis, the acute and intense inflammation of a host to pathogens, that is characterized by excessive proinflammatory cytokine production by monocyte/macrophages, supporting the relationship of adrenal response to innate immune response (24). In addition, our results clearly demonstrate the enhanced resistance to sepsis of stressed mice, which may directly benefit from the immune-suppressive effects of stress via the anti-inflammatory action of glucocorticoids.

Because stress is associated with an elevated level of glucocorticoids in blood, and the immunosuppressive effect of glucocorticoids is mediated by GILZ, we hypothesized that restraint stress may exert immune-suppressive effects via up-regulation of GILZ expression. As expected, the mRNA expression of GILZ dramatically increased in a dose-dependent manner in the spleen and peritoneal macrophages of mice post-restraint stress (Fig. 8) or postinjection with DEX (Fig. 9), which provides strong evidence that physical restraint stress reduces inflammatory response via GILZ-mediated immunosuppressive effects of GCs. These results support the anti-inflammatory and immunosuppressive effects of glucocorticoids via GILZ-mediated down-regulation of proinflammatory cytokine expression in macrophages (20). In addition to

GILZ, the transcription factor NF- κ B is also activated by restraint stress (Fig. 8C), which may account for the increased mRNA expression of TLR4 in macrophages of stressed mice (our unpublished data). As such, stress-associated GILZ expression may connect to TLR and NF- κ B pathways. Furthermore, other transcription factors such as AP-1 and NF-AT may also be involved in the anti-inflammatory process of chronic stress (9). Because pathogen-associated molecular patterns of bacterial components activate macrophages by binding to TLRs, thus triggering the NF- κ B pathway and stimulating the production of inflammatory proteins including cytokines (42–44), more efforts will be required to elucidate the involvement of NF- κ B, AP-1, or other transcription factors in the immune suppressive effects of restraint stress on sepsis.

In conclusion, our results demonstrate that physical restraint stress enhances the resistance of stressed mice to sepsis by inhibiting the production of proinflammatory cytokines and by the up-regulation of GILZ expression in macrophages, which provides strong evidence to support the role of corticotherapy in treatment of sepsis.

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Disclosures

The authors have no financial conflict of interest.

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