Repeated social defeat increases the bactericidal activity of splenic macrophages through a Toll-like receptor-dependent pathway

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Am J Physiol Regul Integr Comp Physiol 293: R1180–R1190, 2007. First published June 27, 2007; doi:10.1152/ajpregu.00307.2007 —Phagocytes of the innate immune system provide a first line of defense against invading microorganisms. Psychological stress is often thought to suppress the functioning of these cells, in part due to the immunosuppressive activity of stress-induced glucocorticoid hormones. However, exposure to the stressor social disruption (SDR) has been shown to increase cytokine production by monocytes/macrophages and to reduce their sensitivity to corticosterone. Thus, it was hypothesized that splenic monocytes/macrophages from socially stressed mice would be primed to be more physiologically active than cells from nonstressed controls. Flow cytometry was used to demonstrate that exposure to SDR significantly increased the expression of Toll-like receptors (TLR) 2 and 4 on the surface of splenic macrophages. In a follow-up experiment, exposure to SDR also increased the ability of these macrophages to kill Escherichia coli ex vivo and in vivo. However, SDR failed to increase the bactericidal activity of splenic macrophages from C3H/HeJ mice, which lack functional TLR4. In mice with functional TLR4, the stress-induced increase in bactericidal activity was associated with a significant increase in macrophage gene expression for inducible nitric oxide synthase and subunits of the NADPH oxidase complex, which are responsible for generating reactive nitrogen and oxygen intermediates, respectively. This stress-induced increase in gene expression was not evident in the TLR4-deficient mice. These data indicate that SDR increases TLR expression, which in turn enhances the bactericidal activity of splenic macrophages, in part by increasing pathways responsible for reactive oxygen and nitrogen intermediate production.

innate immunity; Escherichia coli; social stress; psychoneuroimmunology

PHAGOCYTES OF THE INNATE IMMUNE system provide a first line of defense against a variety of infectious microorganisms, particularly those that can be cleared from the host without the need for an ensuing adaptive immune response. Pattern recognition receptors, such as Toll-like receptors (TLRs), are well known for their role in the recognition of infectious microbes and play a prominent role in initiating the expression of genes encoding for proinflammatory cytokines. In addition to this cytokine production, ligation of TLRs facilitates multiple aspects of microbial killing by phagocytes. For example, TLRs have been linked with maturation of the phagosome (10) and are known to be essential for the production of antimicrobial reactive

nitrogen and oxygen intermediates (RNI and ROI, respectively) (29, 43, 51). The production of ROI is dependent on the activation of NADPH oxidase (also called phagocyte oxidase), which is a multisubunit enzyme complex composed of membrane-bound (p22phox and gp91phox) and cytosolic (p47phox, p40phox, p67phox, rac1, and rac2) subunits. The production of RNI, on the other hand, is triggered by the expression of a single gene, inducible nitric oxide (NO) synthase (iNOS).

It is widely accepted that psychological stress affects the immune response, and chronic or repeated exposure to a stressor has been shown to be immunosuppressive. In large measure, suppression of immunity is due to the well-known anti-inflammatory effects of adrenal glucocorticoid (GC) hormones. Ligation of GC receptors on mononuclear cells suppresses the expression of cytokines, chemokines, and adhesion molecules, in part, through a negative regulation of NF-κB activation and function (1, 59). This immunosuppression can have significant effects on immunity to microbial infection. For example, activation of the hypothalamic-pituitary-adrenal axis and the resultant increase in GC hormones significantly increased the susceptibility of splenic macrophages from BALB/c mice to Mycobacterium avium growth ex vivo, an effect that can be reversed by administration of the type II GC receptor antagonist RU-486 (12, 13). Increased susceptibility to M. avium was, in part, due to the GC-induced decrease in RNIs and TNF-α production (12).

Psychological stress, however, is not always immunosuppressive (23, 31), particularly if the stressor induces a state of functional GC resistance. Our laboratory has shown that the murine social stressor social disruption (SDR) causes splenic macrophages to become resistant to the suppressive effects of GC hormones (5–8, 21, 48, 49). After exposure to SDR, splenic monocytes/macrophages, which traffic from the bone marrow to the spleen during the stressor (20), are resistant to corticosterone-induced programmed cell death in culture (5–7, 21, 48, 49). This is, in part, due to the inability of splenic CD11b+ cells from mice exposed to SDR to translocate cytosolic GC receptors to the nucleus upon GC exposure and ligation of TLR2 or TLR4 (4, 41).

In addition to the development of GC resistance, splenic CD11b+ cells from mice exposed to SDR produce significantly higher levels of cytokines after LPS stimulation than cells from nonstressed control (HCC) mice (3, 5, 6, 16, 33, 41, 42, 48, 53). This is reflective of a primed phenotype and suggests that TLR expression or signaling is increased by exposure to SDR. It is known that physical stressors, such as injury, surgery, and
sepsis, can affect the expression of TLR2 and TLR4 on mononuclear cells (16, 33, 37, 40, 53), but it is not known whether psychological stressors that do not involve surgical tissue damage can also affect TLR expression. Thus, the first aim of the present study was to test the hypothesis that exposure to SDR would increase the surface expression of TLR2 and TLR4. Because of the impact that TLRs can have on the ability of phagocytes to kill bacteria, a second aim of the study was to test the hypothesis that SDR primes splenic macrophages for enhanced microbicidal activity through a TLR-dependent pathway.

MATERIALS AND METHODS

Animals. Male CD-1 and C3H/HeN mice were purchased from Charles River Laboratories (Wilmington, MA). Male C3H/HeJ mice were purchased from Jackson Laboratories (Bar Harbor, ME). All mice were 6–8 wk of age and were allowed to acclimate to the animal vivarium for 1 wk before experimentation. The mice were housed in groups of three to five per cage and kept on a 12:12-h light-dark schedule with lights on at 0600. Food and water were available ad libitum. All experimental procedures were approved by The Ohio State University’s Animal Care and Use Committee.

SDR. The SDR stressor occurred over a 2-h period between 1630 and 1830, i.e., the transition from the end of the light cycle to the beginning of the dark (i.e., active) cycle. SDR was initiated by placement of an aggressive male mouse into the home cage of the resident mice, as previously reported (5, 7, 8, 20, 21, 48, 49). The aggressor was the same species as the residents and was originally isolated from the rest of the colony because of aggressiveness toward cagemates. During SDR, agonistic interactions between the aggressor and the residents were observed for the first 20 min to ensure that the aggressor attacked and defeated all the residents. If fighting did not begin within the first 5 min of the interactions, a different aggressor was placed in the cage. After fighting was initiated, the aggressors were left in the cages for 2 h. At the end of the 2-h period, the aggressor was removed and the residents were left undisturbed until the following day, when SDR was repeated. The residents were exposed to a total of six 2-h cycles of SDR. The subjects of the experiments were the residents that were repeatedly defeated by the aggressor. When the mice were killed, wounds that were incurred during SDR were counted and scored. Scoring was based on a three-point scale, with 0 indicating no wounds, 1 indicating one to three superficial skin wounds, 2 indicating four to five superficial skin wounds, and 3 indicating more than five superficial skin wounds. In no case did wounding exceed superficial skin wounds from biting and scratching.

Bacteria. Escherichia coli strain K12 (ATCC 10798, American Type Culture Collection) was grown in trypticase soy broth overnight at 37°C. After incubation, stock vials of E. coli were prepared by centrifugation of the bacteria for 10 min at 1,000 g. The resultant pellet was resuspended in PBS + 10% glycerol and divided into cryovials for storage at −70°C until use in an experiment. Before each experiment, E. coli stock vials were thawed and placed into 30 ml of trypticase soy broth and incubated for ~20 h (without shaking) at 37°C. Thus the E. coli used in all the experiments was in a stationary phase of growth.

In vitro killing assay. Immediately after the mice were euthanized by CO2 asphyxiation, cardiac blood was obtained. Spleens from individual mice were removed, placed into 5 ml of ice-cold Hanks’ buffered saline solution, and macerated (Stomacher 80 Biomixer, Seward, UK). The resultant cell suspension was washed by centrifugation at 600 g for 10 min at 4°C, and red blood cells were lysed with red blood cell lysis buffer (0.16 M NH4Cl, 10 mM KHCO3, and 0.13 mM EDTA). After they were washed a second time, the cells were filtered through a 70-μm nylon mesh filter and then resuspended in RPMI 1640 (0.075% sodium bicarbonate, 10 mM HEPES buffer, 100 U/ml penicillin G, 100 μg/ml streptomycin sulfate, 1.5 mM l-glutamine, and 0.00035% 2-mercaptoethanol) + 10% heat-inactivated FBS. The number of cells in the preparation was enumerated using a particle counter (model Z2, Beckman Coulter, Fullerton, CA) and adjusted to 5 × 10⁷ cells/ml for bacterial killing assays involving total splenocytes. In follow-up bacterial killing assays, cell concentrations were adjusted to 5 × 10⁸ monocytes/macrophages per milliliter.

CD11b+ cell enrichment. Twelve microliters of anti-CD11b magnetic microbeads (Miltenyi Biotec, Auburn, CA) were added per 10⁷ total splenocytes. The mixture was incubated at 4°C for 25 min and then washed twice in PBS supplemented with 0.5% bovine serum albumin and 2 mM EDTA. After they were washed, the cells were filtered through a 70-μm filter and then loaded onto a magnetic cell-sorting separation column (Miltenyi Biotec). The columns were washed three times with the wash buffer and then removed from the magnet, so that CD11b+ cells could be flushed from the column with a syringe plunger. After they were washed again, the cells were counted and resuspended at 5 × 10⁶ cells/ml in CTLL RPMI with 10% heat-inactivated FBS.

Flow cytometry. A total of 2.5 × 10⁵ CD11b+-enriched splenocytes were incubated at 4°C for 45 min with FITC-conjugated anti-mouse Gr-1/Ly-6G (clone RB6-8C5) and allophycocyanin-conjugated anti-mouse CD11b/Mac-1 (clone M1/70) alone or with one of the following: phycoerythrin (PE)-conjugated anti-mouse TLR2 (clone 6C2), PE-conjugated anti-mouse TLR4 (clone MTS510), PE-conjugated anti-mouse CD86 (clone GL1), PE-conjugated anti-mouse CD69 (clone H1.2F3), or PE-conjugated anti-mouse I-A/E (clone 2G9). Monoclonal antibodies were purchased from BD Pharmingen (San Diego, CA), except TLR2 and TLR4, which were purchased from eBioscience (San Diego, CA). Antibody labeling was performed by a standard lyse-wash procedure using FACS lysing solution (BD Immunocytometry Systems, San Jose, CA) and calcium- and magnesium-free Dulbecco’s PBS with 2% FBS + 0.1% NaN₃. A total of 10⁵ cells from each sample were analyzed on a dual-laser flow cytometer (FACSCalibur, BD Immunocytometry Systems) using CellQuest Pro (version 4.0.2) and Attractors (version 3.1.0) software. Monocytes/macrophages and granulocytes were identified by forward- and side-scatter characteristics and differences in CD11b and Gr-1 expression (28). Matched isotype controls were used for all antibodies to set negative staining criteria.

Bacterial killing assay. A total of 5 × 10⁶ splenocytes were enriched for adherent mononuclear cells by culture in a 24-well tissue culture plate for 3 h at 37°C in an atmosphere of air + 5% CO2. This was done in duplicate for each animal, so that phagocytosis/bacterial killing by the adherent splenocytes could be determined after two time points. After incubation, nonadherent cells were washed three times with 1 ml of RPMI and removed from the plates. After the last wash, 1 ml of a mixture containing 5 × 10⁷ CFU/ml E. coli in RPMI was added to each well. The E. coli were opsonized by addition of 5% fresh serum (pooled from nonstressed HCC mice) to the bacterial suspension. After addition of the bacteria, the plates were incubated for 20 min at 37°C in air + 5% CO2 to allow the adherent splenocytes to phagocytize the bacteria. After 20 min of incubation, 1 ml of RPMI was pipetted into the well for a total of three washes, and extracellular bacteria were washed away. After the final wash, 1 ml of H2O was added to one of the wells to lyse the splenocytes, and the lysate was collected into a sterile tube for four-plate enumeration of phagocytized bacteria. RPMI + 10% FBS was added to the duplicate wells, and the plates were incubated for an additional 70 min. At the end of the incubation, the above-described procedures were repeated to enumerate the bacteria that remained alive within the adherent splenocytes.

Adherent mononuclear phagocyte standardization and bacterial killing. Because SDR increases the percentage of phagocytic cells in the spleen (6, 20), it was important to determine whether changes in
bacterial killing by splenocyte cultures were due to altered activity of these cells or an increased prevalence of phagocytes. Therefore, after single-cell suspensions were created and the cells from the spleens of CD-1 mice were counted, the suspensions were stained with FITC-labeled anti-Gr-1 and allophycocyanin-labeled anti-CD11b antibodies to determine the number of monocytes/macrophages in the cultures. Cell concentrations were then adjusted, so that the same number (i.e., 5 × 10^5) of monocytes/macrophages would be added to cultures from control, as well as stressed, mice. After 3 h of adherence, 5 × 10^6 opsonized E. coli were added to the wells, and the bacterial killing assay was conducted as described above.

In vivo bacterial killing. CD-1 male mice were left undisturbed (HCC, n = 30) or exposed to six daily cycles of SDR (n = 30) during the week before infection. At ~15 h after the last cycle of SDR, the mice were intravenously infected with 1 × 10^8 CFU of E. coli. Sterile capillary tubes were used to sample blood from the retroorbital plexus immediately, as well as 15, 30, 60, and 120 min, after infection. The mice were then euthanized via CO2 asphyxiation, and the spleen was excised under sterile conditions. Spleens were macerated with sterile frosted glass slides in sterile PBS, and bacteria were enumerated in the spleen suspension, as well as in the blood, via standard pour-plating methodology using trypticase soy agar. Bacterial levels were determined via backcalculation of dilutions and are expressed as log10 CFUs per gram (spleen) or per milliliter (blood).

To determine whether SDR-induced effects on microbial killing were dependent on TLR4 signaling, C3H/HeN mice, which have functional TLR4, were compared with C3H/HeJ mice, which have a mutation in TLR4 that renders the receptor nonfunctional. The mice were left undisturbed (n = 30) or exposed to SDR (n = 30) during the week before E. coli infection. At ~15 h after the last cycle of SDR, all the mice were intravenously infected with 5 × 10^6 CFU of E. coli. Bacterial levels in the spleen were assessed before, as well as 15, 45, 75, and 135 min after infection via standard pour-plate methodology on trypticase soy agar. In addition, a piece of the spleen was snap frozen in liquid nitrogen for later analysis of gene expression using real-time PCR.

RNA isolation and cDNA synthesis. The spleens were placed into 1 ml of TRIzol reagent (GIBCO, Rockville, MD) and homogenized using a Tissue Tearor (Biospec Products, Bartlesville, OK). RNA was isolated according to the TRIzol protocol provided by the manufacturer (GIBCO). Total RNA was reverse transcribed using a commercially available kit (Promega, Madison, WI) according to the manufacturer’s instructions. Briefly, 2 μg of total RNA were combined with 5 mM MgCl2, dNTPs at 1 mM each, 1 U/μl RNasin, and 15 U/μg avian myeloblastosis virus RT and primed with 0.5 μg/μl random hexamers and diethyl pyrocarbonate-H2O. The reaction was incubated for 2 min at 50°C followed by 10 min at 95°C. The change in fluorescence was measured using Applied Biosystems 7000 Prism Sequence Detector (PE Applied Biosystems) and analyzed using Sequence Detector version 1.0. The relative amount of transcript was determined using the comparative cycle threshold (Ct) method, as described by the manufacturer. In these experiments, gene expression in the spleens of noninfected, nonstressed HCC mice was used as the calibrator. Gene expression, therefore, is expressed as the fold increase in expression of these noninfected, nonstressed control mice.

Statistical analyses. Changes in bacterial levels after the in vitro bacterial killing assay were analyzed using a mixed-factor ANOVA with the group (HCC vs. SDR) as the between-subjects variable and the end-point time after infection as the within-subjects variable. Changes in bacterial levels, as well as gene expression in vivo, after the in vivo bacterial killing assay were analyzed using a two-factor ANOVA, since each time point used different groups of mice. The group (HCC vs. SDR) and the end-point time after infection were used as the between-subjects variables. Tukey’s honestly significant difference tests were used as post hoc tests. Changes in the percentage of bacteria killed in vitro, splenic leukocyte subsets, and spleen mass were assessed using a t-test. Real-time PCR data were assessed with a two-factor ANOVA, with the groups (HCC vs. SDR) and the end-point time after infection as the between-subjects variables. The dependent variable was ΔCt, which is calculated by determining the amplification cycle number at which the gene of interest begins exponential amplification minus the amplification cycle number at which the internal housekeeping gene (i.e., 18S) begins exponential amplification. This ΔCt calculation controls for differences in reverse transcription and sample loading. The ΔCt is derived from normal biological activity and, thus, can be assumed to follow a normal distribution. Therefore, parametric statistics were used to determine the difference in ΔCt between groups and time after infection. ΔCt, however, is not intuitively informative from a biological relevance standpoint. Therefore, for descriptive purposes, ΔCt was standardized and transformed to depict the n-fold change in gene expression compared with gene expression in nonstressed (i.e., HCC), noninfected mice, which was set to equal 1. During the transformation process, variability within samples becomes positively skewed, resulting in visually misleading error bars. For this reason, such error bars are not shown, and Figs. 5 and 6 simply depict the mean n-fold difference in gene expression. The level of significance was set at P < 0.05 for all tests. All statistics were calculated using SPSS for Windows version 11.5 (SPSS, Chicago, IL).

RESULTS

Repeated social defeat during SDR resulted in a significant increase in spleen mass [t(11) = 6.1, P < 0.01; Table 1], as well as the number of splenic monocytes/macrophages [t(11) = 3.9, P < 0.01] and granulocytes [t(11) = 4.2, P < 0.01; Table 1]. Moreover, exposure to this stressor also significantly affected the expression of a variety of cell surface markers. Flow cytometry revealed that SDR significantly increased the expression of CD86 on splenic monocytes/macrophages (Table 1). This difference was limited to splenic monocytes/macrophages, since the expression of CD86 on granulocytes from the spleens of mice exposed to SDR was not significantly different from that on granulocytes from HCC mice (data not shown). Other markers of macrophage activation, however, were not significantly affected by SDR. For example, staining for the activation marker CD69 was too low to be detectable on splenic CD11b+ cells (i.e., monocytes/macrophages and granulocytes), whereas major histocompatibility complex II (MHCII) expression was not significantly
different between splenic monocytes/macrophages from HCC and SDR mice [t(11) = 1.05, not significant; Table 1]. As expected, MHCII was absent on granulocytes.

In addition to changing the expression of these cell activation markers, exposure to SDR also increased the expression of TLR2 and TLR4. The mean fluorescence intensity of anti-TLR2 antibody staining on splenic CD11b+ monocytes/macrophages was significantly higher in cells from mice exposed to SDR than in cells from HCC mice (t(11) = 4.25, P < 0.01; Fig. 1A). In addition to the SDR-induced increase in anti-TLR2 staining, the mean fluorescence intensity of anti-TLR4 antibody staining on splenic CD11b+ monocytes/macrophages was also significantly higher for mice exposed to SDR [t(11) = 5.47, P < 0.01; Fig. 1B]. Again, this stress-induced increase in TLR staining was not evident on splenic granulocytes (data not shown).

In vitro bacterial killing. Splenocytes from mice exposed to SDR, as well as from HCC mice, were cultured with E. coli for 20 or 90 min to determine the capacity of the splenocytes to kill the E. coli. Bacterial levels in wells cultured in the presence of splenocytes from SDR mice were significantly different across the two time points from bacterial levels in wells cultured with splenocytes from HCC mice [significant group × time interaction: F(1, 8) = 13.08, P < 0.01]. Post hoc testing indicated that this effect was due to significantly lower bacterial levels in the SDR group at 90 min (P < 0.01); bacterial levels at 20 min did not differ between SDR and HCC cultures (Fig. 2A). Similarly, the percentage of bacteria that was killed was significantly higher in cultures containing splenocytes from SDR mice than HCC mice [t(1, 8) = 4.19, P < 0.01; Fig. 2A]. However, because SDR significantly increased the number of CD11b+ phagocytes in the spleen (Table 1), there would have been a higher proportion of these cells in the cultures, making interpretation of the data difficult. Therefore, a follow-up experiment in which the same number of monocytes/macrophages was placed into each well was conducted to determine whether the increase in bactericidal activity was due to increased cell activity or increased number of phagocytes.

Because our data indicated that ~99% of adherent cells were mononuclear, flow cytometry was used to count and characterize the cells in the splenocyte suspension, so that an equal number of monocytes/macrophages could be placed into each well. Even after standardization of the number of these cells, monocytes/macrophages from mice exposed to SDR killed more E. coli than did monocytes/macrophages from HCC mice. This was evidenced by a significant decrease in the number of E. coli remaining in the cultures after 90 min of incubation [F(1, 16) = 10.33, P < 0.01; Fig. 2B], as well as a corresponding significant increase in the percentage of bacteria killed [(t(16) = 3.38, P < 0.01; Fig. 2B).

In vivo bacterial clearance. To determine whether this enhanced killing also occurs in vivo, CD-1 mice were exposed to SDR or left undisturbed before infection with 1 × 10^8 CFU of E. coli. At various times after infection, E. coli were enumerated from the blood and the spleen. Bacterial levels in the blood decreased rapidly during the first 2 h of infection in the stressed and nonstressed animals (an ~3-log-unit decrease). This decrease in bacterial levels was greater in the blood of mice exposed to SDR before infection than in the nonstressed HCC mice. This difference, however, did not quite reach statistical significance [F(4, 44) = 2.27, P = 0.08; Fig. 3A]. Bacterial levels in the spleen, however, were significantly different between the HCC and SDR mice [F(4, 44) = 2.87, P < 0.05; Fig. 3B]. Bacterial levels were only modestly decreased in the nonstressed HCC mice during the first 135 min after infection (Fig. 3B) but were decreased by

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**Table 1. Spleen mass and splenocyte cell characteristics in CD-1 male mice**

<table>
<thead>
<tr>
<th>Group</th>
<th>Spleen Mass, g</th>
<th>Cells per Spleen</th>
<th>Fluorescence Intensity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Granulocytes</td>
<td>Monocytes/Macrophages</td>
</tr>
<tr>
<td>HCC</td>
<td>115.5±4.2</td>
<td>5.78×10^6±1.4×10^6</td>
<td>1.74×10^6±1.7×10^5</td>
</tr>
<tr>
<td>SDR</td>
<td>189.8±14.3*</td>
<td>2.35×10^7±5.3×10^6*</td>
<td>4.75×10^6±9.7×10^5*</td>
</tr>
</tbody>
</table>

Values are means ± SE [n = 5 (SDR) and 8 (HCC)]. HCC, control nonstressed mice; SDR, mice exposed to stressor social disruption. *P < 0.01 vs. HCC.

**Fig. 1.** Significant increase in expression of Toll-like receptor (TLRs) 2 and 4 (TLR2 and TLR4) on splenic CD11b+ monocytes/macrophages as assessed via flow cytometry using fluorescent anti-TLR antibody staining. SDR, mice exposed to social disruption; HCC, nonstressed control mice. Values are means ± SE [n = 5 (SDR) and 8 (HCC)]. *P < 0.01 vs. HCC.


~1 log unit at 75 min after infection ($P < 0.05$) and 1.5 log units by 135 min after infection in the mice exposed to the stressor ($P < 0.05$; Fig. 3B). These data argue for increased killing, rather than a reduction in the number of bacteria initially trapped in the spleen.

In vivo bacterial clearance in C3H/HeN and C3H/HeJ mice. In a follow-up experiment, C3H/HeJ mice with a functional mutation in TLR4 were compared with their C3H/HeN control strain, which have functional TLR4, to determine whether the enhanced in vivo bacterial clearance in the spleen was dependent on TLR4. As in the outbred CD-1 mice, bacterial levels in spleens of C3H/HeN mice exposed to SDR were different from those in the nonstressed HCC mice during the first 135 min after infection [$F(3, 40) = 3.10, P < 0.05$; Table 2]. Post hoc testing indicated that this was due to significant differences in bacterial levels at 45 and 135 min after infection ($P < 0.01$).

Interestingly, the difference in bacterial levels was not statistically significant between the two groups of animals 15 min after infection, indicating that the number of bacteria phagocytized and/or initially trapped in the spleen was not affected by exposure to SDR (Table 2). Moreover, when the percent change from bacterial numbers at 15 min was calculated, there was a statistically significant group × time interaction [$F(3, 40) = 3.54, P < 0.05$]. Post hoc testing indicated that SDR in C3H/HeN mice resulted in a significant percent change in bacterial levels in the spleen at 135 min after infection compared with nonstressed HCC mice ($P < 0.05$; Fig. 4A).

A different pattern emerged when bacterial levels in the spleens of the TLR-4 deficient C3H/HeJ mice were assessed. In this case, exposure to SDR decreased *E. coli* levels across all time points [main effect for stress: $F(1, 39) = 35.85, P < 0.01$; Table 2]. Moreover, in contrast to differences in the C3H/HeN mice, bacterial levels in the spleens of C3H/HeJ mice exposed to SDR were already different from the nonstressed HCC mice at 15 min after infection, suggesting that the level of phagocytosis and/or trapped bacteria in the spleen was lower in the stressed mice (Table 2). Despite the lower level of bacteria, the rate of bacterial clearance was not significantly affected by exposure to SDR. In contrast to findings in the C3H/HeN mice, when the percent change from number of bacteria at 15 min...
After infection were calculated in TLR4-deficient mice, there was not a statistically significant treatment effect \(F(1, 39) = 0.31, P > 0.5\), nor was there a significant group \(\times\) time interaction \(F(3, 39) = 0.45, P > 0.5\), indicating that SDR did not affect the percent change in number of bacteria during the first 135 min of infection (Fig. 4B).

**Gene expression for iNOS and NADPH oxidase subunits.** The expression of genes encoding for iNOS, as well as the NADPH oxidase subunits, were significantly affected in the C3H/HeN mice exposed to SDR. iNOS gene expression was significantly higher in the spleens of C3H/HeN mice exposed to SDR than in the nonstressed HCC mice [main effect for stress: \(F(1, 49) = 0.2021, P < 0.05\); Fig. 5A]. This effect is most likely due to the higher mean level of gene expression 75 and 135 min after infection; however, the stress \(\times\) time interaction did not reach statistical significance \(F(4, 49) = 2.26, P = 0.07\), precluding post hoc analysis of these specific time points. In contrast to results from the C3H/HeN mice, SDR did not significantly affect iNOS gene expression in the spleens of C3H/HeJ mice exposed to SDR at any time point \(F(1, 49) = 0.06, P > 0.5\); Fig. 5B).

Similar results were evident when gene expression for the \(p40^{phox}\) subunit of NADPH oxidase was assessed. SDR caused a significant increase in \(p40^{phox}\) gene expression in the spleens of C3H/HeN mice compared with the nonstressed HCC mice \(F(1, 49) = 16.84, P < 0.001\); Fig. 6A]. Again, this was a generalized effect of the stressor, since the stress \(\times\) time interaction was not statistically significant \(F(4, 49) = 0.71, P > 0.5\); Fig. 6B]. In addition to the effects on \(p40^{phox}\), SDR also increased \(p22^{phox}\) gene expression in the spleens of C3H/HeN mice \(F(1, 49) = 3.99, P = 0.05\); Fig. 6C]. As with \(p40^{phox}\), there was not a statistically significant stress \(\times\) time interaction \(F(4, 49) = 0.30, P > 0.5\), demonstrating that this stress effect is a generalized effect across the different time points. SDR in the TLR4-deficient C3H/HeJ mice, on the other hand, did not significantly affect \(p22^{phox}\) gene expression \(F(1, 49) = 0.12, P > 0.5\); Fig. 6D]. As with the other genes, the mean level of \(p47^{phox}\) gene expression in C3H/HeN mice increased after SDR (Fig. 6E], a difference that approached, but did not quite reach, statistical significance \(F(1, 49) = 3.13, P = 0.08\). In contrast, exposure to SDR before infection had no effect on the mean level of \(p47^{phox}\) gene expression in the TLR4-deficient C3H/HeJ mice exposed to SDR \(F(1, 49) = 0.13, P > 0.5\); Fig. 6F]. In contrast to the other NADPH oxidase subunit genes, \(p67^{phox}\) expression was not significantly affected by SDR, regardless of the mouse strain [main effect for stress in C3H/HeN mice: \(F(1, 49) = 0.81, P > 0.5\); Fig. 6G].

**DISCUSSION**

The results of this study indicate that repeated social defeat during the SDR stressor enhances innate immunity to *E. coli* infection and extend previous studies demonstrating that SDR significantly impacts splenic monocytes/macrophages (6, 20, 49). In addition to significantly increasing the number of these cells in the spleen, repeated social defeat also changed the expression of various surface markers on these monocytes/macrophages (Table 1), including markers that have been associated with macrophage activation. For example, CD11b and CD86 (32) were significantly elevated in splenic macrophages from mice exposed to SDR compared with macrophages from nonstressed HCC mice (Table 1). On the other hand, expression of MHCI, which is also known to be in-

![Fig. 3. SDR before intravenous infection with \(1 \times 10^6\) CFU of *E. coli* resulted in significantly lower bacterial levels in blood and spleen as determined via standard pour-plate analysis. Values are means \(\pm\) SE (\(n = 6\) per group per time point). *P < 0.05 vs. HCC at 75 and 135 min.](http://ajpregu.physiology.org/)}
increased on classical and type II-activated macrophages (36), from SDR mice was not significantly different from that on splenic monocytes/macrophages from nonstressed HCC mice. Additionally, the activation marker CD69 was absent on all CD11b/H11001 cells, indicating that these cells were not fully activated by the stressor. This pattern of cell surface marker expression, along with previous findings demonstrating enhanced cytokine production (3, 4, 6, 42, 48), suggested that the macrophages were primed but were not yet fully activated during repeated social defeat (11, 36, 44).

In addition to affecting CD11b and CD86 expression, exposure to SDR significantly increased TLR2 and TLR4 expression on monocytes/macrophages, but not on neutrophils. This increase in TLR expression was not simply due to the minor bite wounds that occurred during the SDR paradigm, since there was not a significant correlation between wound severity and TLR2 expression (r = 0.02, not significant; data not shown). Although wounding was weakly associated with TLR4 expression in mice exposed to SDR (r = 0.75, P = 0.15), two SDR-exposed mice had no visible signs of wounding. In these unwounded mice, the mean fluorescence intensity of anti-TLR4 staining was still higher than that found on any of the control mice (mean fluorescence intensity for the unwounded mice exposed to SDR was 48.96 and 65.36; highest mean fluorescence intensity for the HCC mice was 41.55). This suggests that wounding alone was not the reason for the increase in TLR4 expression after exposure to SDR, which is consistent with our previous findings indicating that SDR-induced differences in leukocyte subsets in the spleen are the greatest in mice that are wounded during the SDR paradigm but still occur at lower levels in unwounded mice exposed to SDR (20).

These SDR-induced increases in TLR expression are consistent with the notion that SDR primes splenic monocytes/macrophages, since others have suggested that increased TLR expression is associated with a primed monocyte/macrophage phenotype (11, 44). However, a central hallmark of any primed cell is heightened reactivity upon stimulation (44). It was known that splenic monocytes/macrophages from SDR mice produced higher levels of proinflammatory cytokines upon ex vivo LPS stimulation (3, 4, 6, 42, 48), but it was not known whether the cells would also have increased microbicidal activity against gram-negative bacteria. In the present experiment, monocytes/macrophages from mice exposed to SDR killed a significantly higher percentage of E. coli in cocultures than did cells from nonstressed HCC mice. This was not simply due to an increase in the number of monocytes/macrophages in the cultures, since E. coli killing was also higher in cultures from SDR mice when the number of splenic monocytes/macrophages added to each culture was standardized. Moreover, the effects of SDR on bacterial killing were not dependent on superficial skin wounds incurred during SDR; wounding scores did not correlate with the percentage of bacteria killed in the assay (r = −0.09, not significant; data not shown). In addition to the enhanced killing in culture, bacterial levels were significantly lower in the spleens of mice exposed

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**Fig. 4.** A: exposure to SDR significantly decreased E. coli bacterial levels in spleens of C3H/HeN mice, expressed as the percent change in the log10 CFU E. coli per gram of spleen mass (CFU/g) at 45, 75, and 135 min after infection of that at 15 min. B: percent change in E. coli levels in spleens of C3H/HeJ mice exposed to SDR was not significantly different from that in nonstressed control mice (HCC) at any time after infection. Values are means ± SE (n = 6 per group per time point from duplicate experiments). *P < 0.05 vs. HCC at 135 min.

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**Fig. 5.** Inducible nitric oxide synthase (iNOS) gene expression was significantly increased by SDR during the first 135 min after intravenous E. coli infection in TLR4-sufficient C3H/HeN mice (A), but not in TLR4-deficient C3H/HeJ mice (B). Values are means ± SE (n = 6 per group per time point from duplicate experiments). *P < 0.05 (main effect of stress).
to SDR before intravenous *E. coli* infection than in nonstressed HCC mice. It cannot be concluded, however, that this is exclusively due to the enhanced microbicidal activity of the monocytes/macrophages, since other factors, such as complement activity and acute-phase reactants, may also be affected by stress (19, 35). However, when considered with the ex vivo data, it is likely that SDR-induced increases in monocyte/macrophage number and microbicidal activity contributed to the lower bacterial levels in the spleens.

 Shortly after macrophages phagocytize bacteria, there is a rapid increase in oxygen consumption due to the activity of NADPH oxidase, a phenomenon generally referred to as the oxidative burst. NADPH oxidase becomes activated when the catalytic core of the complex (consisting of p22^phox^ and
gp91<sub>phox</sub>) associates with the cytosolic subunits consisting of p47<sub>phox</sub>, p67<sub>phox</sub>, Rac1 or Rac2, and p40<sub>phox</sub> (22, 39, 56). The oxidative burst results in the production of superoxide anion, which alone can have bactericidal effects, can interact with other molecules to produce additional antimicrobial compounds (collectively referred to as ROI), or can react with NO to produce highly toxic molecules such as nitrogen dioxide, hypochlorous acid, and peroxynitrite (22). NO itself maintains some bactericidal activity, but additional nitrogen-containing compounds (collectively referred to as RNI) tend to be more potent (22).

The generation of ROI and RNI are crucial for controlling bacterial infection (22). Recent research has provided a strong link between TLR activation and the generation of ROI and RNI. Laroux et al. (29) showed that culturing elicited peritoneal macrophages with <i>E. coli</i> significantly increases assembly of the NADPH oxidase system and generation of ROI. Interestingly, elicited peritoneal macrophages from mice lacking the TLR adaptor protein myeloid differentiation factor 88 (MyD88), which results in ineffective TLR signaling, were unable to assemble the NADPH oxidase system and produced low levels of ROI on stimulation with <i>E. coli</i>, showing the importance of TLR signaling for this response (1a, 29). Similarly, LPS stimulation of macrophages results in the production of NO, which is now known to be dependent on MAP kinase phosphorylation (27, 30). In vivo models have also demonstrated that disruption of TLR signaling significantly reduces NO production. For example, NO production in gnotobiotic mice monoassociated with <i>E. coli</i> was significantly reduced in mice lacking TLR4 (25).

In the present experiment, mice lacking functional TLR4 (i.e., C3H/HeJ mice) did not show the same SDR-induced increase in clearance of bacteria from the spleen as did stressed mice containing functional TLR4 (i.e., CD-1 and C3H/HeN mice exposed to SDR). Concomitant with this stress-induced increase in bacterial clearance in the mice with functional TLR4, there was a stress-induced increase in gene expression for iNOS and three of the four NADPH oxidase subunits (i.e., p40<sub>phox</sub>, p22<sub>phox</sub>, and p47<sub>phox</sub>; marginally significant; Fig. 6). Of these, the increase in p40<sub>phox</sub> is perhaps the most striking, since this subunit is thought to play a regulatory role in the assembly and activation of the NADPH enzyme complex (1a, 18, 22, 50, 54). As with the clearance of bacteria from the spleen, this SDR-induced increase in gene expression was dependent on functional TLR4; iNOS or NADPH oxidase gene expression was not significantly elevated in C3H/HeJ mice exposed to SDR (Figs. 5 and 6). Although we did not measure the levels of specific ROI and RNI, the increased iNOS and NADPH oxidase gene expression, together with the increased bacterial killing, strongly suggests that SDR increased the microbicidal activity of splenic macrophages, in part, by increasing the production of ROI and RNI through a TLR4-dependent mechanism.

The increased gene expression of p40<sub>phox</sub>, p47<sub>phox</sub>, and p22<sub>phox</sub> after SDR was evident even before the mice were infected with <i>E. coli</i>, an effect that was dependent on functional TLR4 (Fig. 6). Although the reasons for this are not clear, it is known that factors such as granulocyte-macrophage colony-stimulating factor (GM-CSF), which are increased in the spleen after SDR (20), can prime phagocytes for enhanced NADPH oxidase activity. For example, GM-CSF has been shown to prime neutrophils by partially phosphorylating p47<sub>phox</sub> (14), and it induces the expression of gp91<sub>phox</sub> (which was not measured in the present study) in other cell lines (46). Therefore, it is possible that stress-induced GM-CSF or other as yet undefined factors primed phagocytes in the spleen by enhancing the expression of genes encoding the NADPH oxidase subunits. Further studies are needed to test this hypothesis.

SDR did not affect the rate of bacterial clearance from the spleens of the TLR4-deficient C3H/HeJ mice. However, bacterial levels across all the time points were lower in these stressed TLR4-deficient animals than in their nonstressed controls. The reason for this reduction is not immediately clear, although it could be related to a reduced ability of spleen cells from stressed C3H/HeJ mice to recognize and phagocytize bacteria. Our data are in agreement with previous reports (24) that nonstressed C3H/HeN and C3H/HeJ mice are not significantly different in their ability to phagocytize or kill bacteria. Others, however, have shown that during challenge, such as with polymicrobial sepsis, bacterial levels are lower in the blood of C3H/HeJ mice than in controls (1a). Sepsis and endotoxic shock are considered potent physiological stressors because of the cytokine-induced activation of the hypothalamic-pituitary-adrenal axis and the sympathetic nervous system (2, 15, 17, 44, 58). Thus it is possible that the neuroendocrine environment affects phagocytosis, but not bacterial killing, in the spleens of the TLR4-deficient C3H/HeJ mice.

Psychological stress is often considered immunosuppressive, in large part, because of the anti-inflammatory effects of stress-induced GC hormones. GC actions are mediated through intracellular GC receptors, which are found in most leukocyte subpopulations (9, 34) and which, when activated, translocate into the nucleus. In the nucleus, the translocated GC receptor acts as a ligand-dependent transcription factor to modulate the expression of GC-responsive genes or to suppress the activity of other transcription factors, such as NF-kB (9). Data from this laboratory indicate that exposure to SDR significantly decreases the ability of the GC receptor to translocate to the nucleus (41). Therefore, microbicidal mechanisms that have been found to be suppressed by GCs, such as cytokine and iNOS production (12, 13), are intact, even in the presence of stress-induced GC. Although the mechanisms through which SDR induces GC resistance are not clear, it is known that MAP kinases, primarily p38, are involved in IL-1-induced GC resistance (38, 57). Moreover, p38 has been shown to be a crucial link between TLR4 activation and the production of iNOS and NADPH oxidase (26, 47, 52). Therefore, it is possible that the stress-induced enhancement in microbial killing by macrophages from mice exposed to SDR is linked to the development of GC insensitivity.

The inflammatory response must be tightly regulated to maintain good overall health in the face of a microbial infection. When the inflammatory response is weak, bacteria and viruses may overcome the host; on the other hand, excessive inflammation may also result in adverse effects, such as lung consolidation during an influenza viral infection (3, 5, 6, 45) or systemic inflammatory disease during endotoxic shock (42). Thus, discovering and, ultimately, manipulating the mechanisms through which the nervous system influences the inflammatory response to an infectious challenge have tremendous therapeutic potential.
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