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Social disruption induces lung inflammation

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Abstract

Social disruption (SDR) is a well-characterized mouse stressor that causes changes in immune cell reactivity in response to inflammatory stimuli. In this study, we found that SDR in the absence of an immune challenge induced pulmonary inflammation and increased pulmonary myeloperoxidase activity. The percentage of neutrophils within the lungs increased 2-fold after social disruption. Monocyte accumulation in the lungs was also significantly increased. In addition, SDR increased the percentage of neutrophils that expressed CD11b, indicating that more neutrophils were in an activated state. In the lungs, we observed an increased level of the inflammatory cytokine, IL-1 β , as well as higher levels of KC/CXCL1, MIP2/CXCL2, and MCP-1/CCL2, which are chemokines responsible for neutrophil and monocyte recruitment. Furthermore, social disruption led to increased lung expression of the adhesion molecules P-selectin, E-selectin, and ICAM-1, which localize and recruit immune cells. These data support previous findings of an inflammatory environment induced by SDR. We demonstrate that this effect also occurs in the pulmonary milieu and in the absence of an inflammatory stimulus.

Keywords

Innate immunity; social stress; psychoneuroimmunology; lung; inflammation; Social disruption (SDR)

Introduction

A stressor is defined as a threat or perceived threat against the body's homeostasis. The body reacts to stressors by activating conserved behavioral and physiological stress responses in an

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attempt to re-establish homeostasis. Changes in the environment such as temperature extremes, physiological difficulties such as injury or nutrient deficiency, perceived threats such as public speaking, and psychosocial burdens such as social subordination or loneliness are some example stressors (Avitsur et al., 2006). Physiological and psychological stressors provide an important experimental approach to investigate the pathogenesis of the stress response and the multiple host factors that could alter the course of an infection or exacerbate underlying diseases.

An appropriate immune response is necessary to control and clear infectious agents from the host. Cells of the innate immune system, specifically phagocytes such as monocytes/macrophages and neutrophils, are the first line of defense against infectious agents (Dale et al., 2008). Stress-induced immunosuppression of these cells, generally through glucocorticoids, can result in increased susceptibility to infection, increased disease severity, and the establishment of persistent infections (Atkinson et al., 1973; Cohen et al., 1998; Fauci et al., 1976; Rinehart et al., 1974). In contrast, the development of stress-induced glucocorticoid resistance in innate immune cells may prevent the control of inflammation in response to microorganisms or worsen tissue damage through excessive host inflammatory responses (Barnes and Adcock 2009; Meduri and Yates 2004; Quan et al., 2001).

The social disruption (SDR) paradigm has been developed to investigate the effects of psychosocial challenges on the stress response in a murine model. During SDR, an aggressive, male intruder mouse is introduced into a cage of resident male mice with an established social hierarchy and subsequently disrupts the existing social environment by attacking and defeating the resident mice. This paradigm is repeated for six cycles to mimic a reoccurring stressor and induces a 2-fold increase in plasma corticosterone levels, indicating an activated stress response (Avitsur et al., 2001). As opposed to other models of stress where increased levels of circulating glucocorticoids lead to suppression of immune function, mice subjected to SDR display enhanced inflammatory responses (Sheridan et al., 1998). SDR is associated with increased cell mobility and myelopoiesis in the bone marrow, paralleled by an accumulation of neutrophils and monocytes in the circulation and the spleen (Engler et al., 2004). Splenocytes taken from mice subjected to SDR and cultured *in vitro* produce more IL-6 and TNF- α when stimulated with LPS, supporting a pro-inflammatory environment (Avitsur et al., 2003; Stark et al., 2002). Furthermore, splenocytes taken from SDR mice are resistant to glucocorticoid-induced apoptosis, lending to a phenotype of cells that can survive in the body during times of stress and high corticosteroid levels (Avitsur et al., 2001). Importantly, mice subjected to SDR also have a higher mortality and increased levels of IL-1 β and TNF- α in their lungs when endotoxic shock is induced by an intraperitoneal LPS injection (Quan et al., 2001).

As a result of previous studies demonstrating increased levels of inflammatory cytokines in the lungs of mice after SDR, we investigated the effects of SDR on the lung microenvironment. In this manuscript, we show that SDR induced pulmonary inflammation in mice by increasing levels of inflammatory cytokines and chemokines and by altering the expression of adhesion molecules.

Methods

Mice

C57BL/6 male mice at 6–8 weeks of age were purchased from Jackson Laboratory (Bar Harbor, ME) and allowed to acclimate to their surroundings for 7–10 days before initiation of any experimental procedures. Mice were housed in ventilated polypropylene cage racks with *ad libitum* access to water and rodent chow. The animal room was maintained on a 12-hour light-dark cycle (lights on at 6AM). Sentinel screening was routinely performed in the rooms where experimental mice and older aggressor mice were housed showed and showed no indications

of illness, as the mice were negative for all tested pathogens during the time period that the experiments were conducted. All experiments were approved by The Ohio State University Institutional Laboratory Animal Care and Use Committee and were in accordance with the NIH Guidelines for the Care and Use of Laboratory Animals.

Social disruption paradigm

To induce social disruption, an aggressive, intruder male mouse was introduced to a cage of resident mice from 17:00 and 19:00 for two, four, or six cycles. Aggressors were C57BL/6 or CD-1 males, selected based on having a history of agonistic behavior. Different aggressors were used during each cycle to prevent habituation. During the SDR period, the aggressor mice were observed for dominant behavior, such as back biting and tail rattling, and the resident mice were observed for submissive behavior, including upright submissive posture, fleeing, and crouching/huddling. If the intruder mouse did not attack within 5–10 minutes from the beginning of the session or was attacked and defeated by any of the resident mice, then a new intruder was introduced, replacing the initial intruder. After the two hour session, the intruder mouse was removed and food was returned to the cages. As we have done in previous studies (Engler et al., 2008), the resident mice were carefully inspected for bite injuries after each confrontation cycle and mice with severe wounds were removed from the study. Residents were monitored for signs of infection of existing wounds, and no cutaneous infections were observed throughout the course of the experiments (Powell et al., 2009). Control mice were left undisturbed in their home cages and housed in a different room from the SDR cages (Avitsur et al., 2006). For all experiments, resident mice were sacrificed the morning after the last cycle of SDR, unless otherwise stated.

Tissue processing

Lungs were removed and insufflated under 20cm pressure. For later assessment of myeloperoxidase levels, cytokine analysis, or RNA expression, lungs were snap frozen in liquid nitrogen then RNA or protein was extracted by standard freeze fracture protocols. In separate experiments, single-cell suspensions of lung homogenate were obtained by first removing the lungs and placing them into 10mls of a solution containing 0.525mg/ml Collagenase Type1 (Worthington Biochemical Corporation, Cat# LS004196) and 0.02mg/ml Deoxyribonuclease I (Sigma, Cat# D5025) in RPMI (Cellgro, Cat# 10-040-CM) with 10% FBS (Atlanta Biologicals, Cat# S11550). After 30 minute incubation at 37°C, lungs were mechanically homogenized, passed through a 100µm filter (Fischer Scientific, Cat# 08-771-19) and spun at 1400 rpm for 10 minutes. Red blood cells were lysed by adding 1ml RBC lysis buffer (0.034M Aluminum Chloride, 0.01M Potassium Bicarbonate, and 0.1mM EDTA in dH₂O) for 30 seconds, immediately followed by the addition of 10mls RPMI containing FBS. Pelleted cells were re-suspended in PBS and then cytopun or subjected to flow cytometry analysis.

Myeloperoxidase (MPO) activity

The right upper lung lobe was homogenized in 0.5% Hexadecyltrimethylammonium bromide (HTAB) in 50mM potassium phosphate buffer (pH=6.0), followed by 3 cycles of sonication, snap freezing in liquid nitrogen, and thawing in 37°C water. Samples were then centrifuged at 16,100 × g for 15 minutes at 4°C. The supernatant (10µL) or MPO standard (Sigma, Cat#M6908) was transferred to a 96-well plate with 290µL of substrate (0.167mg/mL *o*-dianisidine dihydrochloride and 0.0005% H₂O₂ in 50mM potassium phosphate buffer, pH=6.0). MPO activity was measured over a 5 minute period at 450nm on a spectrophotometer (Molecular Devices, Menlo Park, CA). 1U of MPO activity is the equal to the amount of MPO required to degrade 1µmol of peroxidase/min at room temperature (Wilgus et al., 2002).

Cell differential analysis

A portion of the single-cell suspension isolated from the lungs was cytopun for 3 minutes at 1500 rpm. Slides were stained using Protocol™ Hema 3™ Manual Staining System per the manufacturer's instructions (Fischer Scientific). Cells were classified as neutrophils, monocytes, or lymphocytes based on morphology, and 200 total immune cells were counted per slide in a blinded manner.

Flow cytometry

Lung homogenate from each mouse was placed into separate eppendorf tubes, while a portion of homogenate from each mouse was combined for the isotype control. Cells were washed 2X with buffer (PBS/ 0.1% Sodium Azide/1% BSA) and then Fc receptors were blocked for 10 minutes with mouse IgG (Jackson ImmunoResearch). Immune cell surface antigens were detected by a 30 minute incubation with the following antibodies Ly6G-PE (BD Pharmingen, Cat# 551461) and CD11b (BD Pharmingen, Cat# 553312). Adhesion molecule expression was assessed by dual staining for CD31-APC ((BD Pharmingen, Cat# 553373) and either CD62P-FITC (BD Pharmingen, Cat# 553744) and CD62E (BD Pharmingen, Cat# 553751) or CD54-FITC (BD Pharmingen, Cat# 553252). Cells were then washed 3X and fixed with 1% paraformaldehyde for analysis using a BD FACS Calibur. 10,000 events were collected per sample. Leukocytes were gated based on forward versus side scatter and data was analyzed using Denovo FCS Expression V3.

Evans blue dye (vascular permeability) assay

Mice were subjected to six cycles of SDR and then retro-orbitally injected with 200µl volume of 2% (wt/vol) solution of Evan's blue (Sigma, Cat# E2129) in PBS (Beghdadi et al., 2008). After 30 minutes, mice were anesthetized with isofluorene and the lungs were perfused through the right ventricle with PBS at a speed of 1ml/min for five minutes. The lungs were then homogenized in 1ml PBS and then incubated overnight at 37°C in an additional 2mls formamide. After centrifugation, supernatants were assessed for absorbance at 620nm and 740nm. As a positive control, a group of mice that were not subjected to SDR were interperitoneally injected with either PBS or 400µg LPS and then sacrificed 24 hours later and subjected to the Evans blue protocol described above (Standiford et al., 1995). The amount of Evan's blue dye in the lungs was determined using the following equation, Absorbance 620 – Absorbance 740 (removes hemoglobin contamination), and the concentration was extrapolated from a standard curve.

Cytokine level assessment

Mice were sacrificed immediately after the second cycle of SDR. First blood was collected by heart stick, and serum was separated by centrifugation. The large left lobe was removed and frozen for protein analysis and the remaining four lobes were frozen for RNA analysis. The left lobe was homogenized and incubated for two hours at 4°C in 200µl 2X PBS containing protease inhibitors. After centrifugation, supernatant was collected and assayed for cytokine levels along with serum samples using the Bio-Rad Bioplex System according to manufacturer's protocol.

mRNA analysis

Total RNA was extracted from lung tissue by freeze fracture and Trizol extractions. cDNA was generated using Superscript II First-Strand Synthesis with Oligo-dT primers (Invitrogen). Real-time PCR was performed using SYBR Green (Applied Biosystems) and the following sets of primer pairs: for IL-1β 5'-CCTGAACTCAACTGTGAAATGC-3' (forward) and 5'-GTGCTGCTGTGAGATTTGAAG-3' (reverse); for IL-6 5'-CCACGGCCTTCCCTACTTC-3' (forward) and 5'-

TTGGGAGTGGTATCCTCTGTGA-3' (reverse); for KC 5'-GCAGACCATGGCTGGGATT-3' (forward) and 5'-CCTGAG GGCAACACCTTCAA-3' (reverse); for MIP2 5'-CTCAAGGGCGGTCAAAAAGTT-3' (forward) and 5'-TGTTTCAGTATCTTTTGGATGATTTTCTG-3' (reverse); for MCP-1 5'-AGGTCCTGTCTATGCTTCTG-3' (forward) and 5'-TCTGGACCCATTCTTCTTG-3' (reverse); for P-selectin 5'-AATGCTCCGAATTGCACATG-3' (forward) and 5'-TTCCCCAGGGATTGGAACA-3' (reverse); for E-selectin 5'-CTCCTGCGAAGAAGGATTTGA-3' (forward) and 5'-CCCCCTCTTGGACCACACTGA-3' (reverse); for ICAM-1 5'-GGGACCACGGAGCCAATT-3' (forward) and 5'-CTTGC GGCCCTGAGATCCA-3' (reverse); and for GAPDH 5'-CATGGCCTTCCGTGTTCTTA-3' (forward) and 5'-GCGGCACGTCAGATCCA-3' (reverse). Using Ct values, the ΔC_t for each sample was calculated: $\Delta C_t = C_{t_{\text{target gene}}} - C_{t_{\text{control gene}}}$. Then the values for each mouse (mouse) were compared with the average value of the control mice (average HCC): $\Delta \Delta C_t = \Delta C_{t_{\text{mouse}}} - \Delta C_{t_{\text{average HCC}}}$. Finally the fold change for each mouse was calculated: $\text{fold change} = 2^{-\Delta \Delta C_t}$.

Statistics

Mann-Whitney U test for spleen weight comparison, flow cytometric analysis, and to compare MPO and Evans blue dye levels. A Student's T-test was used to compare cytokine levels, and an ANOVA with Tukey posthoc testing was used to compare cell differential data and adhesion molecule expression. We recognize that animal responses to SDR can be highly variable and therefore we did perform Grubb's outlier tests on all data (Avitsur et al., 2001). However we chose to represent all data points in this manuscript. For all studies, $p < 0.05$ was considered statistically significant.

Results

Social disruption causes lung inflammation

To illustrate the effectiveness of SDR, we evaluated spleen size at the time of sacrifice. As previously reported, splenomegaly was observed in mice subjected to SDR ($p = 0.008$) (Figure 1A) (Avitsur et al., 2001). After six cycles of SDR, lungs were removed, fixed, embedded, and sectioned for pathological evaluation. A marked increase in inflammatory cells was observed in mice subjected to SDR (Figure 1B). To quantitatively evaluate inflammation in the lungs, we measured myeloperoxidase (MPO) activity in lung samples. Produced during the inflammatory response, MPO is a major neutrophil protein and is also present in monocytes and macrophages (Winterbourn et al., 2000). Social disruption caused a significant increase in the amount of MPO present in the lungs of mice ($p = 0.013$) (Figure 1C).

Neutrophils accumulate in the lungs following SDR

Next, we investigated the specific cell populations that accumulated in the lungs after SDR. First, we counted cell differentials from whole lung homogenates and observed a significant increase in the percentage of immune cells that were neutrophils in the mice subjected to SDR ($p = 0.041$ after four SDR cycles and 0.006 after six SDR cycles) (Figure 2A and B). To confirm the observed increase in neutrophils after SDR, we isolated a single-cell lung suspension and performed flow cytometry for Ly6G, a cell surface marker specific to neutrophils (Daley et al., 2008). After six cycles of SDR, the percentage of Ly6G⁺ cells in the lung increased 2-fold ($p = 0.001$) (Figure 2C and D).

To further delineate cell populations present after SDR, cells were dually stained with Ly6G and CD11b. CD11b (Mac-1) is expressed on both neutrophils and monocytes, and its expression is enhanced when cells are activated to aid binding to adhesion molecules on the vasculature (Ross 2002). In addition to increasing the percent of Ly6G⁺ cells, SDR

significantly increased the percentage of Ly6G+ cells that also expressed CD11b ($p=0.003$) (Figure 3A and B). A slight shift in CD11b fluorescence intensity on Ly6G+ cells was observed with SDR; however, the shift was not significant. These data indicate that SDR increased the percentage of neutrophils that were activated (Ly6G+ cells also expressing CD11b) but that the levels of CD11b expression was not affected on a per cell basis. Further, monocytes (Ly6G-CD11b+ cells) were significantly increased from 10.5 to 13.5% of total cells in the lung ($p=0.01$) (Figure 3C).

SDR does not affect vasculature leakage

To determine if vascular leakage was contributing to the increased levels of inflammatory cells within the lungs of mice after SDR, we intravenously injected mice with Evans blue dye, which binds to albumin in the bloodstream. After 30 minutes, the lungs were flushed through the pulmonary artery to remove dye in the bloodstream, leaving only dye that leaked into the interstitium of the lung. We observed no change in the amount of Evans blue dye in the lungs of mice subjected to SDR, indicating that vasculature leakage is not the cause of the increased inflammation observed with SDR ($p=0.203$) (**Data not shown**). Further, LPS induced vascular leakage in home cage control mice as previously reported ($p=0.058$) (**Data not shown**) (Standiford et al., 1995).

Pro-inflammatory cytokine and chemokine expression is increased during SDR

To further investigate the mechanism of leukocyte accumulation in the lungs of mice after SDR, we evaluated tissue and sera levels of pro-inflammatory cytokines and chemokines after two, four, and six cycles of SDR and found that levels of cytokines peaked at two cycles. Therefore, we measured cytokine levels immediately after the cessation of two cycles of SDR. Previous reports have demonstrated that the inflammatory cytokines IL-1 β and IL-6 are increased during stressful situations (Brydon et al., 2005; Kiecolt-Glaser et al., 2003; Quan et al., 2001; Stark et al., 2002). We observed significant increases in IL-1 β RNA and protein levels in the lungs of SDR mice but no change in sera levels (Figure 4A). Furthermore, we observed significant increases in IL-6 sera levels but no change in lung expression (Figure 4B). These data indicate that SDR caused compartmentalized effects on cytokines levels, inducing IL-1 β expression in the lungs, while stimulating IL-6 production systemically.

C-X-C chemokines include CXC1L (KC) and CXCL2 (MIP-2), which exert their pro-inflammatory activity and induce neutrophil recruitment through CXCR2, the mouse homolog of the IL-8 receptor (Yoshimura 2007). Monocyte chemoattractant protein-1 (MCP-1/CCL2) is a C-C chemokine that preferentially recruits mononuclear cells (Leonard and Yoshimura 1990). Both KC and MIP-2 were markedly increased at the lung protein and sera levels (Figure 4C and D), while MCP1 RNA and protein levels were significantly elevated in the lungs of mice subjected to SDR (Figure 4E). Other inflammatory cytokines, such as TNF- α , or those known to attract neutrophils, such as IL-17, or monocytes, such as M-CSF, were unchanged by SDR (**Data not shown**). These data indicate that the recruitment of neutrophils and monocytes after SDR is being regulated by specific cytokines.

SDR alters adhesion molecule expression

In order for neutrophils and monocytes to leave the circulation and enter the lungs, they must extravasate through the endothelium. To aid this process, endothelial cells express the adhesion molecules, E-selectin and P-selectin, which bind to leukocytes and initiate rolling to slow down the immune cells. This lag in movement facilitates the firm adhesion of leukocytes, which is largely regulated by the binding of β_2 integrins to Inter-Cellular Adhesion Molecule-1 (ICAM-1) and subsequent transendothelial migration (Fabbri et al., 1999). We hypothesized that SDR enhanced the expression of the adhesion molecules in the lungs, allowing the immune cells to enter the organ. Analysis at the mRNA level, revealed no change in P-selectin (Figure

5A). However, a significant increase in E-selectin mRNA was apparent after two cycles of SDR, followed by a significant increase in ICAM-1 mRNA after four cycles of SDR ($p=0.008$ and $p=0.001$, respectively) (Figure 5B and C). We further investigated protein expression of the adhesion molecules by performing flow cytometry on lung homogenate. Cell populations were gated on CD31+ endothelial cells, and then dual expression of P-selectin, E-selectin or ICAM-1 was determined. We observed an upwards trend in the percentage of cells expressing P- and E-selectin after two cycles of SDR, followed by a return to control levels of expression after four cycles of SDR. After six cycles of SDR the percentage of CD31+ cells expressing P-selectin was significantly increased ($60.99\% \pm 3.42$ for control mice vs. $73.14\% \pm 3.64$ for SDR mice, $p=0.041$) and the fluorescence intensity measured by geometric mean of P-selectin was increased (7.08 ± 0.50 for control mice vs. 9.81 ± 0.97 for SDR mice, $p=0.037$) (Figure 5D). Similarly, the percentage of CD31+ cells expressing E-selectin increased significantly after six cycles of SDR ($62.23\% \pm 3.76$ for control mice vs. $73.80\% \pm 2.68$ for SDR mice, $p=0.036$) and the increase in the geometric mean of E-selectin after six cycles of SDR was approaching significance (12.34 ± 1.11 for control mice vs. 16.09 ± 1.29 for SDR mice, $p=0.059$) (Figure 5E). ICAM-1 expression on CD31+ cells was influenced differently by SDR as the percentage of cells expressing ICAM-1 increased ($53.98\% \pm 2.38$ for control mice vs. $63.13\% \pm 3.25$ for SDR mice, $p=0.053$) but the fluorescence intensity measured by geometric mean of ICAM-1 was unaffected by SDR (141.17 ± 20.04 for control mice vs. 196.18 ± 32.25 for SDR mice, $p=0.186$) (Figure 5F). These data indicate that SDR increased the percentage of endothelial cells expressing P- and E-selectin and increased the level of P- and E-selectin expression per CD31+ cell. ICAM-1 expression is being regulated by an increase in the percentage of CD31+ cells expressing the adhesion molecule.

Discussion

We observed a significant increase in lung inflammation after six cycles of social disruption stress. Greater numbers of neutrophils and monocytes were present in the lungs after SDR and a greater percentage of neutrophils were in an activated state compared to those in control lungs. The increase in inflammatory cells was not a result of increased vasculature permeability but likely a result of increased levels of the chemokines KC, MIP2 and MCP-1, as well as increased expression of adhesions molecules P-selectin, E-selectin, and ICAM-1 within the lungs.

Previous reports demonstrate an enhanced inflammatory response after both social disruption stress and acute restraint stress. After a short period of restraint stress, increased numbers of neutrophils, monocytes, NK cells, and T cells accumulated in a surgical sponge implanted in a mouse (Viswanathan and Dhabhar 2005). Six cycles of SDR increased the amount of neutrophils and monocytes in the bone marrow, blood, and spleen (Engler et al., 2004). Data support the hypothesis that SDR induces the release of these cells from the bone marrow and their accumulation in the spleen after SDR. Additionally, the release of immune cells into the circulation simulates myelopoiesis, increasing the number of monocytes and neutrophils in the bone marrow (Engler et al., 2004). Interestingly, we report that these immune cells also accumulate in the lungs after six cycles of SDR. Previous reports evaluating the effects of SDR on immune cell populations used the RB6-8C5 antibody to define the neutrophil population and antibodies directed against CD11b (Mac-1) to define the monocyte/macrophage population (Engler et al., 2004; Stark et al., 2001). Clone RB6-8C5 is a well-published antibody directed against granulocyte receptor (Gr-1), which recognizes both Ly6G and Ly6C antigens (Daley et al., 2008). Ly6G, a granulocyte surface marker, is the major antigen detected by RB6-8C5 (Fleming et al., 1993); however, RB6-8C5 also detects Ly6C which is expressed by neutrophils and subsets of monocytes, macrophages, and lymphocytes (Daley et al., 2008; Jutila et al., 1988). Recently, an antibody directly targeting Ly6G (Clone 1A8) has been shown to deplete only neutrophils and to not affect blood monocyte populations *in vivo* (Daley et al., 2008).

Another well-characterized cell surface marker, CD11b (Mac-1) is a β_2 integrin that is expressed on both neutrophils and monocytes and aids in adhesion to vascular endothelium (Carlos and Harlan 1994). Therefore to further characterize and define the populations of cells affected by SDR, we chose to dually stain for Ly6G and CD11b. We defined neutrophils as Ly6G+ cells and monocytes as Ly6G-CD11b+ cells and observed increases in both populations of cells. The increase in neutrophils was much greater and confirmed by morphological analysis. We found that neutrophils were the main cells attributing to the lung inflammation observed during the social disruption paradigm.

Further, we observed an increased percentage of Ly6G+ cells expressing CD11b, indicating that in addition to increasing neutrophil numbers, SDR increased the activation state of neutrophils present in the lungs. Interestingly, clinical data supports these observations, as acute stressors, such as public speaking, have been shown to induce the expression of CD11b (Mac-1) (Mills and Dimsdale 1996; Redwine et al., 2003). These observations suggest that neutrophils are primed by SDR to respond much quicker to a secondary stimulus.

To elucidate the mechanism by which SDR induced lung inflammation, we first investigated the vasculature permeability in the lungs by injecting mice intravenously with Evans blue dye, which binds to albumin present in sera. If the endothelial lining of the blood vessel wall is damaged, the dye will leak into the lung interstitium (Nagy et al., 2008). We observed no change in vascular permeability after SDR, indicating that the inflammation observed after SDR was not a result of vascular leakage. Furthermore, while SDR causes an inflammatory response, it does not appear to directly injuring the lung vasculature. Therefore we believe that SDR is inducing a priming effect that could be either beneficial or detrimental to the animal depending in part on the nature of a subsequent immune challenge. Previous evidence supports this hypothesis as SDR mice infected with *Escherichia coli* display enhanced clearance of bacteria, but when SDR mice are given a LPS challenge, mortality is increased (Avitsur et al., 2006; Quan et al., 2001).

Next, we investigated the effects of SDR on sera and lung levels of proinflammatory cytokines and chemokines responsible for neutrophil and monocyte recruitment. Three major pro-inflammatory cytokines, tumor necrosis factor α (TNF- α), IL-1 β , and IL-6, have been shown to be altered during social disruption stress. Elevated sera levels of IL-6 and IL-1 β , as well as elevated mRNA levels IL-1 β in the spleen and liver, have been detected immediately after six cycles of SDR (Engler et al., 2008; Snyder and Unanue 1982). In mice challenged with the bacterial endotoxin lipopolysaccharide (LPS), SDR increases the mRNA production of TNF- α and IL-1 β in the lungs of mice (Quan et al., 2001). We observed a significant increase in IL-1 β lung mRNA and protein expression but no change in sera levels. Conversely, IL-6 was unchanged in the lung but increased significantly in the sera. These data indicate that SDR alone is inducing a pro-inflammatory environment in the lungs but that the effect of SDR on IL-6 production is systemic.

KC/CXCL1 and MIP2/CXCL2 are chemokines implicated in the recruitment of neutrophils to sites of inflammation by binding to CXCR2, the mouse homolog of the IL-8 receptor (Yoshimura 2007). MCP-1/CCL2 is responsible for monocyte chemotaxis (Maus et al., 2001). We observed marked increases in both sera and lung protein levels of KC and MIP-2 after two cycles of SDR, prior to previously observed neutrophil accumulation in the blood and our data of neutrophil accumulation in the lungs after four and six cycles of SDR (Engler et al., 2004). However we did not observe increased levels of KC or MIP-2 RNA in the lungs. There are many possible explanations for changes in protein levels that do not correlate with changes in RNA expression. In this set of experiments, the protein and mRNA levels were measured at the same time point, immediately after 2 cycles of SDR. One would expect the mRNA levels to peak earlier than the protein levels. Furthermore, it is possible that KC and

MIP-2 could be regulated post-translationally or by miRNAs, neither of which would be detected by Real time PCR. We did observe increased MCP-1 lung RNA and protein with SDR but no significant change in MCP-1 sera levels, indicating that monocyte recruitment may be a local effect. The high levels cytokines and chemokines observed after just two cycles of social disruption stress further supports the pro-inflammatory state induced by SDR.

In order for circulating neutrophils and monocytes to enter the lung, the cells must adhere to and pass between the endothelial cells lining the walls of blood vessels by extravasation. The process of extravasation begins with the expression of E- and P-selectins, which bind Sialyl Lewis x (SLe^x) that is present on leukocytes (Fabbri et al., 1999). These bonds work to slow down the leukocytes to allow the formation of tight adhesions between ICAM-1 and the integrins, LFA-1(CD11a/CD18) or Mac-1 (CD11b/CD18). Tight adhesions arrest the motion of the rolling leukocytes and allow the cells to diapedes through the vascular wall and into the tissue (Carlos and Harlan 1994). P-selectin is stored in cytoplasmic granules termed Weibel-Palade bodies, allowing it to be quickly mobilized to the external plasma membrane (McEver et al., 1989). Therefore, it is reasonable that SDR did not affect P-selectin mRNA levels, as the protein is not transcriptionally regulated. In contrast, SDR induced a significant elevation in E-selectin mRNA levels after two cycles, followed by increased ICAM-1 mRNA after four cycles of SDR. Using flow cytometry of lung endothelial cells after six cycles of SDR, we found increased surface expression for P-selectin, E-selectin and ICAM-1. Interestingly, IL-1 β can induce neutrophil and monocyte recruitment by up-regulating the expression of E-selectin and ICAM-1 and the enhanced levels of IL-1 β that we observed in the lungs coincide with the increase in E-selectin and ICAM-1 mRNA expression (Wang et al., 1995). Furthermore, both KC and MIP-2 have been shown to regulate neutrophil recruitment through a P-selectin-dependent manner (Zhang et al., 2001), and MCP-1, as well as IL-8, have been shown to induce adherence of monocytes to vascular endothelium expressing E-selectin (Gerszten et al., 1999). Therefore, we propose that social disruption stress is inducing the secretion of pro-inflammatory cytokines and chemokines, thereby enhancing adhesion molecule expression and aiding in cellular recruitment to the lungs.

Many pulmonary diseases, including idiopathic pulmonary fibrosis (IPF), chronic obstructive pulmonary disease (COPD), acute respiratory distress syndrome (ARDS) and asthma, are characterized by the accumulation of inflammatory cells in the lungs (Barnes 2008; Bringardner et al., 2008; Gan et al., 2004; Ware and Matthay 2000). Investigations into the effects of stress on these disease states have been limited, with the greatest emphasis on asthma, where psychological stress has been shown to greatly exacerbate the symptoms of the disease (Wright et al., 1998). Recently, SDR administered prior to allergenic inhalation was shown to enhance and prolong airway inflammation in a mouse asthmatic model (Bailey et al., 2009). These data demonstrate the priming effects of SDR on the lung microenvironment and emphasize the need to further investigate the role of psychosocial stress in other diseases that are characterized by lung inflammation. Such research could lead to a greater understanding of the development of pulmonary disease and new therapeutic approaches that reinstate appropriate glucocorticoid regulation of inflammation in underlying disease states or immune challenges.

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Figure 1. Social disruption causes lung inflammation

Male C57BL/6 mice were subjected to social disruption (SDR) for 2 hours a night for six consecutive nights. Home cage control mice (Control) were left undisturbed in their cages. **A**) As previously reported, spleens removed from mice subjected to SDR were significantly larger than those in control mice ($p=0.008$) ($n=13$ for Control and 16 for SDR). **B**) Lungs were removed, insufflated, sectioned, and stained with Hematoxylin and Eosin (H&E). Pictures taken at 4 and 20X (inset) represent the inflammation observed in the lungs of mice subjected to SDR. **C**) At the time of sacrifice, the upper right lobe was flash frozen and later analyzed for myeloperoxidase (MPO) levels. Mice subjected to SDR had a 2-fold increase in the amount of MPO present in their lungs ($p=0.013$) ($n=8$ for Control and 5 for SDR).

Figure 2. Mice subjected to the social disruption paradigm have increased levels of neutrophils present in the lung

After two, four and six cycles of SDR, lungs were removed and digested using collagenase followed by mechanical disruption. **A–B**) A portion of the lung homogenate was cytopun and stained for morphology. Neutrophils (red arrows) were determined by cellular morphology and represented as a percent of immune cells counted. The percentage of immune cells that were neutrophils increased almost 2-fold after four and six cycles of SDR compared to control mice ($p=0.041$ and 0.006 respectively) ($n=6$ for Control and 5 for all other groups). **C–D**) Lung homogenate stained for the Ly6G antigen was analyzed for the % of positive cells in the gate shown. SDR caused a 2-fold increase in the % of Ly6G positive cells in the lungs ($p=0.001$) ($n=20$ for Control and 13 for SDR).

Figure 3. Social disruption enhances the activation of neutrophils and increases the percentage of monocytes in the lungs

After six cycles of SDR, lungs were processed as in Figure 2 and stained for flow cytometry with Ly6G, a neutrophil specific marker, and CD11b, a cell surface antigen expressed on monocytes and neutrophils whose expression is increased with cellular activation. **A–B**) Ly6G+ cells were gated as in Figure 2C and then histogram analysis was used to investigate CD11b expression on Ly6G+ cells. The percentage Ly6G+ cells expressing CD11b (Marker 1) was significantly increased from ~65% to 90% ($p=0.003$). **C**) The percentage of cells that were Ly6G-CD11b+, indicating a monocytic phenotype, increased 3% after SDR ($p=0.01$) ($n=20$ for Control and 13 for SDR).

Figure 4. SDR alters the sera and lung cytokine milieu

Mice were subjected to two cycles of SDR and sacrificed immediately after the final SDR cycle. Blood was removed from the mice via heart stick, followed by extraction of the lungs which were flash frozen. Protein was isolated from the left lung lobe by homogenation, followed by incubation in 2X PBS, and RNA was isolated from the remaining lung lobes and analyzed for expression by Real time PCR. Sera and lung supernatant were analyzed for protein levels by Bioplex (n=5 for Control and 8 for SDR).

Figure 5. Social disruption induces expression of the adhesion molecules P-selectin, E-selectin, and ICAM-1

A–C) RNA was isolated from lungs of mice and analyzed for adhesion molecule expression by Real time PCR. No change was observed in P-selectin mRNA levels. However, after two cycles SDR, E-selectin mRNA was significantly increased, followed by a significant increase in ICAM-1 mRNA after four cycles SDR ($p=0.008$ and $p=0.001$, respectively) ($n=13$ for Control and $n=5$ for all other groups). **D–F)** Single-cell lung suspensions were isolated from mice and dually stained with P-selectin, E-selectin, and CD31 or ICAM-1 and CD31. Endothelial cells were gated on CD31 expression, and then assessed for the percentage of positive cells and fluorescence intensity measured by geometric mean. Both P- and E-selectins had significant increases in the percentage of positive cells after six cycles of SDR and P-selectin displayed a shift in the geometric mean after six cycles SDR. Further the percentage of CD31+ cells expressing ICAM-1 was significantly increased after six cycles of SDR ($n=5$ for all groups).