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Social stress enhances IL-1 β and TNF- α production by *Porphyromonas gingivalis* lipopolysaccharide-stimulated CD11b + cells

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Abstract

Psychological stress is associated with an increased expression of markers of peripheral inflammation, and there is a growing literature describing a link between periodontal pathogens and systemic inflammation. The hypothesis of the present work is that exposing mice to the social stressor, called social disruption (SDR), would enhance the inflammatory response to lipopolysaccharide (LPS) derived from the oral pathogen, *Porphyromonas gingivalis*. Mice were exposed to SDR for 2 hrs per day on 6 consecutive days. On the morning following the last cycle of SDR, mice were tested for anxiety-like behavior in the open field test and novel object test. The mice were sacrificed the following day and their spleens harvested. Spleen cells were stimulated with LPS derived from *P. gingivalis* in the absence or presence of increasing doses of corticosterone. Social disruption resulted in anxiety-like behavior, and the production of IL-1 β and TNF- α was significantly higher in spleen cells from mice exposed to SDR in comparison to levels from non-stressed control mice. In addition, the viability of spleen cells from mice exposed to SDR was significantly greater than the viability of cells from non-stressed control mice, even in the presence of high doses of corticosterone. The use of cultures enriched for CD11b+ cells indicated that the stressor was affecting the activity of splenic myeloid cells. This study demonstrates that social stress enhances the inflammatory response to an oral pathogen and could provide a critical clue in the reported associations between stress, inflammation, and oral pathogens.

Keywords

Stress; *Porphyromonas gingivalis*; macrophage; cytokine; inflammation; social defeat; neophobia; anxiety; novel object test; open field test

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Introduction

The body responds to tissue damage and infection by mounting an inflammatory response that serves to protect the affected tissue from further insult. If unresolved, however, inflammation can have consequences such as causing extensive damage to healthy tissue near the site of the original insult or even in other distal organ systems. For example, a growing body of literature is beginning to validate a link between chronic periodontitis, caused by unresolved inflammation that is initially localized in the gingival epithelium and underlying connective tissue [1], and an increased risk of coronary artery disease where inflammation appears to be the main culprit [2-4]. Interestingly, these same diseases have been associated with the stress response. Periodontal symptoms can be exacerbated during periods of stress, and stress can increase the risk of developing coronary disease [5]. As a result, a thorough understanding of the many ways through which inflammation can be regulated is imperative for learning how to control and to treat inflammatory diseases.

Porphyromonas gingivalis is an oral bacterial pathogen that is consistently associated with the development of chronic periodontitis. Although *P. gingivalis* possesses virulence factors that alone can induce tissue damage, such as Arg-X and Lys-X specific extracellular cysteine proteinases [6], the accumulation of inflammatory cytokines is a major contributor to the breakdown of periodontal tissues. The production of inflammatory cytokines is initiated when pattern recognition receptors, such as toll-like receptors (TLR) 2, 4, and 5 bind to and are activated by pathogen associated molecular patterns (PAMPs) such as lipoproteins, lipopolysaccharide (LPS), or fimbriae [7,8]. While there is some debate as to whether *P. gingivalis* derived LPS activates both TLR2 and TLR4, several studies indicate that both receptors can be stimulated by highly purified LPS or lipid A molecules, and by stimulation with the intact bacterium [9-11]. The cytokines that are produced upon TLR ligation are aimed at enhancing the immune response to ultimately eradicate the pathogen. However, when they are produced in excess, inflammatory cytokines will also facilitate the degradation of host tissue. For example, it is well known that cytokines like IL-1 β and TNF- α are key players in tissue destruction and bone resorption during experimental periodontitis in monkeys; blocking these cytokines significantly reduced disease progression [12,13]. As such, tissue damage during periodontitis is greatest when cytokine levels are highest.

As infection with *P. gingivalis* progresses in the oral cavity, the inflammatory response can result in ulceration and enhanced vascular permeability at the loci of infection. As a result, the infectious bacteria can enter into the bloodstream to cause a transient bacteremia. Although the bacteria have been found in coronary plaques [14,15], colonization of systemic organs is not necessary for this chronic oral disease to have systemic effects. Cytokine producing cells, such as CD11b+ macrophages in reticuloendothelial organs (i.e., the spleen, liver, and lungs), are capable of producing high levels of inflammatory cytokines upon encountering *P. gingivalis* or its lipopolysaccharide (LPS). In fact, patients with periodontal disease often have higher systemic levels of C-reactive protein (CRP), IL-6, IL-1, and TNF- α [16]. And, these inflammatory mediators are known to be involved in the development and progression of many systemic diseases. If the stress response is able to enhance the production of these inflammatory mediators, it could have a significant impact on systemic health.

The field of PsychoNeuroImmunology (PNI) has clearly shown that an individual's emotional state or exposure to psychological stressors can significantly affect the immune response [17]. Most studies have focused on the ability of stressors to suppress the immune response, and many of the mechanisms through which this occurs are already known. In general, suppression of immunity is due to the anti-inflammatory effects of adrenal glucocorticoid (GC) hormones, such as corticosterone in rodents or cortisol in humans [18,19]. Ligation of GC

receptors on mononuclear cells suppresses the expression of cytokines, chemokines, and adhesion molecules in part through a negative regulation of NF- κ B activation and function [20,21]. After exposure to the social stressor SDR, the GC receptor is no longer able to translocate to the nucleus of macrophages [22]. This renders the cells resistant to the suppressive effects of GCs and results in increased cell viability, even when high levels of corticosterone are added to *ex vivo* cultures [23,24]. In addition, the production of IL-1 α/β , TNF- α , and IL-6 is significantly increased in macrophages from mice exposed to SDR, in comparison to the production by macrophages from non-stressed home cage control mice [25-28]. It is not known whether this enhanced cytokine production only occurs when the macrophages are stimulated with LPS derived from enteric Gram-negative bacteria, such as *E. coli*, or whether the enhanced cytokine production will also occur when the cells are stimulated with the LPS from an oral Gram-negative bacterium that has been associated with systemic inflammation (i.e., *P. gingivalis*). The purpose of this study was to test the hypothesis that exposure to a social stressor would result in increased production of IL-1 β and TNF- α by macrophages stimulated with the LPS from *P. gingivalis*. If true, the findings could help to explain the interrelatedness of stress, inflammation, and oral pathogens.

Materials and Methods

Mice

Male CD-1 mice (aged 6–8 weeks) were purchased from Charles River Laboratories (Hollister, CA) and allowed to acclimate to the animal facility for at least 1 week prior to experimentation. Mice were kept in an AAALAC approved vivarium with food and water available *ad libitum*. The lights were maintained on a 12:12 hr light:dark schedule with lights on at 0600. All procedures were approved by The Ohio State University's Animal Care and Use Committee.

Social Disruption

The social stressor, Social Disruption (SDR), entails agonistic interactions between resident mice and an intruder mouse and has been extensively described (see [23,24,29-33]). The intruder mice were separated from the rest of the colony after observed aggressiveness toward cage mates, and were then housed individually for > one month prior to the start of this experiment. This aggressor was placed into the cage of 3–5 resident mice for 2 hrs during the transition between the inactive/light cycle and the active/dark cycle (i.e., 4:30 – 6:30 pm). During this 2 hr period, interactions were monitored to ensure that the aggressor repeatedly attacked and defeated all of the resident mice in the cage. At the end of the 2 hr cycle, the aggressor was removed from the cage and the residents were left undisturbed until the following evening when SDR was repeated. This was repeated for a total of 6 evenings such that the resident mice in the cage (which are the experimental subjects) were exposed to six, two-hour cycles of SDR.

Behavioral Testing

Behavioral testing was conducted on the morning immediately following the sixth SDR session. Two tests of anxiety-like behavior were used in separate groups of mice, the open field test and the novel object test of neophobia [34]. An automated system using optical sensors recorded the horizontal and vertical movements of each mouse (AccuScan Instruments, Columbus, OH). The test apparatus consisted of a 30 $\times$ 30 $\times$ 25 cm Plexiglass box. VersaMap software was used to divide the field into two zones: the perimeter, which was the space within 5 cm of the side walls, and the center of the open field, which was the remaining 20 $\times$ 20 cm area not adjacent to the side walls. Mice expressing anxiety-like behavior tend to spend less time in the center of the open field and also tend to locomote near the walls of the apparatus (thigmotaxis). These effects are reversed by anxiolytic compounds [35].

To test for neophobia, an identical open field apparatus was used. VersaMap software was used to divide the field into two equally sized (30 × 15 cm) zones: proximal to and distal from a novel object. The novel object was a blue plastic cap from a 50 ml centrifuge tube. Thus, in order to be considered near the novel object, the mouse had to be on the same side of the open field as the novel object. The test apparatus was cleaned with water-dampened cloths between subjects. Observations for both tests lasted 5 minutes.

Splenocyte Isolation

The spleens from individual mice were removed the morning following behavioral testing, placed into 5 ml of ice-cold Hanks' Buffered Saline Solution (HBSS), and macerated with a stomacher machine (Stomacher 80 Biomaster, Seward, UK). The resultant cell suspension was washed by centrifuging at $600 \times g$ for 10 min at 4°C and red blood cells lysed with red blood cell lysis buffer (0.16M NH_4Cl , 10mM KHCO_3 , and 0.13mM EDTA). Debris was removed from the suspension via a 70 μm nylon mesh filter and after washing, the cells were then resuspended in CTLL RPMI 1640 (containing 0.075% sodium bicarbonate, 10 mM HEPES buffer, 100 U/ml penicillin G, 100 $\mu\text{g}/\text{ml}$ 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 Z2 particle counter (Beckman Coulter Inc., Fullerton, CA) and adjusted to a concentration of 5×10^6 cells/ml.

CD11b+ Cell Enrichment

In experiments in which CD11b+ cells were either enriched or depleted, 12 μl of anti-CD11b magnetic microbeads (Miltenyi Biotec, Auburn, CA) were added per 10^7 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 2mM EDTA. After washing, the cells were filtered through a 70 μm filter prior to loading 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 an additional wash step, the cells were counted and resuspended at a concentration of 5×10^6 cells/ml in CTLL RPMI with 10% heat-inactivated FBS.

Flow Cytometry

Flow cytometry was used to determine the purity of the enriched CD11b+ cell population. A total of 2.5×10^5 CD11b+ enriched splenocytes were incubated at 4°C for 45 min with FITC-conjugated anti-mouse Gr-1/Ly-6G (clone RB6-8C5) and APC-conjugated anti-mouse CD11b/Mac-1 (clone M1/70) (BD Pharmingen, San Diego, CA). Antibody labeling was performed by a standard lyse-wash procedure using FACS lysing solution (BD Immunocytometry Systems, San Jose, CA) and supplemented PBS (Dulbecco's PBS without calcium or magnesium, 2% FBS, 0.1% NaN_3). A total of ten thousand cells from each sample were analyzed on a dual-laser flow cytometer (FACSCalibur, BD Immunocytometry Systems) using CellQuest Pro (Version 4.0.2) and Attractions (Version 3.1.0) software. Matched isotype controls were used for all antibodies to set positive and negative staining criteria.

Glucocorticoid Insensitivity Assay

Glucocorticoid (GC) insensitivity was assessed as described previously [23,33]. Briefly, triplicate samples were cultured at 2.5×10^5 cells per well in flat-bottom 96-well tissue culture plates in complete RPMI/10% FBS. Cultures were stimulated with 10 $\mu\text{g}/\text{ml}$ or 1 $\mu\text{g}/\text{ml}$ (for CD11b+ enriched cultures) of *P. gingivalis* LPS (InvivoGen, San Diego, CA). The dose of LPS was chosen based on dose response curves (data not shown), and reflects a dose of LPS that elicits cytokine responses that are approximately 1/2 of maximal responses. The doses of LPS used also reflect doses used by others [36-38]. Corticosterone was also added to the

cultures (dose range 0.005 – 5 μ M) that were incubated for 48 hr at 37° C and 5% CO₂. Cell viability was measured with a tetrazolium substrate solution (Cell Titer 96 non-radioactive proliferation kit, Promega, Madison, WI), and read at 490 nm by an ELISA plate reader. Cell viability was expressed as the mean optical density (OD) of each LPS-stimulated sample, minus OD of non-stimulated samples treated with the same corticosterone concentration. Viable cells bioreduce the tetrazolium substrate to a colored formazan product. Thus, higher OD indicates higher cell survival.

Assessment of Cytokine Production

To determine the ability of the cells to produce cytokines, spleen cell cultures, or enriched/depleted CD11b⁺ spleen cell cultures, were cultured with 10 μ g/ml or 1 μ g/ml of *P. gingivalis* LPS (respectively) for 18 hrs in culture. The cytokines IL-1 β and TNF- α were assessed in the supernatants with the BD Opt EIA ELISA kits as per manufacturer instructions (BD Pharmingen, San Diego, CA). Optical density was measured and converted to cytokine levels in pg/ml using a standard curve.

Statistics

Differences between stressed and non-stressed groups were determined using independent measures t tests (i.e., for tests of differences in spleen mass and spleen cell numbers) or analysis of variance (ANOVA). A two factor ANOVA was used to assess cytokine production by splenic CD11b⁺ cells with group (i.e., HCC vs. SDR) and cell type (i.e., CD11b⁺ vs. CD11b[−]) as the two between subjects factors. A mixed factor ANOVA was used when corticosterone was added to the cultures to assess cell viability and cytokine production. In this case the between subjects factor was the group (i.e., HCC vs. SDR) and the corticosterone concentration was the repeated factor. Post hoc testing was accomplished with t tests utilizing Bonferroni correction. In all cases, the level of significance was set at an $\alpha = .05$ and was conducted using SPSS software (version 15.0).

Results

Exposure to SDR Changed Behavioral Responses to an Open Field and to a Novel Object

In concordance with previous studies, exposure to the social stressor, SDR, significantly affected behavior in the open field test. Mice exposed to SDR spent significantly less time in the center of the open field than did the non-stressed control mice ($t(16)=2.99$, $p < .01$; Fig. 1A). This reduced amount of time was not related to a reduction in overall activity, since SDR did not affect overall locomotor activity as assessed by the total distance traveled ($t(16)=0.49$, $p > .05$, not significant; Fig. 1B). In addition to affecting behavior in the open field, exposure to SDR reduced the amount of time mice spent in close proximity to a novel object ($F(1,16) = 16.26$, $p < .01$; Fig. 1C). This difference, however, depended upon the amount of time the animals were in the test apparatus, as signified by a significant group $\times$ time interaction ($F(4, 16) = 5.11$, $p < .01$). Post-hoc testing indicated that SDR mice spent less time near the novel object during the first three minutes of testing ($p < .05$). However, by the fourth minute of testing, the SDR mice spent as much time near the novel object as did the non-stressed HCC mice (Fig. 1C).

Viability and Cytokine Production of *P. gingivalis* LPS-stimulated Splenocytes

Exposure to SDR also resulted in a significant increase in spleen mass ($t(7) = 5.67$, $p < .001$) (Fig. 2A) that was in part due to a significant increase in CD11b⁺ myeloid cells, including monocytes/macrophages ($t(7) = 3.45$, $p < .05$) (Fig. 2B) and neutrophils ($t(7) = 3.32$, $p < .05$) (Fig. 2C). The increase in splenic CD11b⁺ cells was associated with enhanced spleen cell viability. Viability was significantly higher in spleen cells from mice exposed to SDR, in

comparison to viability of splenocytes from non-stressed control mice 48 h after *P. gingivalis* LPS-stimulation (main effect for group: $F(1, 8) = 5.00$, $p = .05$) (Fig. 3). This effect, however, was dependent upon the dose of corticosterone in the culture (as indicated by a significant interaction ($F(5, 40) = 4.89$, $p < .001$) and was primarily driven by significantly increased viability in the splenocytes from mice exposed to SDR when 0.05, 0.5, or 5 μM corticosterone was added ($p < .05$) (Fig. 3).

In addition to cell viability, cytokine production by spleen cells from mice exposed to SDR, as well as non-stressed control mice, was evaluated 18 hrs after stimulation with LPS derived from *P. gingivalis*. In cells from both non-stressed HCC mice and SDR mice, cytokine production was significantly reduced by *in vitro* treatment with corticosterone (TNF- α : $F(5, 40) = 41.84$, $p < .001$; IL-1 β : $F(5, 50) = 37.48$, $p < .001$). However, in both cases, there was a significant main effect for the group indicating that TNF- α ($F(1, 8) = 71.77$, $p < .001$) and IL-1 β ($F(1, 10) = 30.44$, $p < .001$) were produced in significantly higher levels by cells from mice exposed to SDR across all doses of corticosterone (Fig. 4).

CD11b+ Cell Enrichment

To determine whether the effects of the social stressor, SDR, were due to effects on CD11b+ cells, splenocyte suspensions were enriched for CD11b+ cells prior to being stimulated with *P. gingivalis* LPS. Cell viability was similar in the CD11b+ cell depleted cultures from mice exposed to SDR when compared to cultures from non-stressed HCC control mice across the different doses of corticosterone ($F(1, 8) = .74$, not significant; Fig. 5). In contrast, cultures from mice exposed to SDR that were enriched for CD11b+ cells had significantly increased viability in comparison to the non-stressed control mice ($F(1, 8) = 9.54$, $p < .05$; Fig. 5). This difference did not appear to be due to differences in viability at different corticosterone doses in the cultures, as evidenced by the lack of a significant interaction effect between the groups (HCC vs. SDR) and the corticosterone doses ($F(5, 40) = .59$, not significant).

Social disruption-enhanced cytokine production was also partly due to stress effects specifically on CD11b+ myeloid cells. In enriched CD11b+ cell cultures from the spleens of mice exposed to SDR, levels of both IL-1 β ($F(1, 16) = 9.49$, $p < .01$) and TNF- α ($F(1, 16) = 10.89$, $p < .01$) were significantly higher than in the CD11b+ cell cultures from the spleens of non-stressed control mice (Fig. 6). This stress-induced increase in IL-1 β only occurred in the cultures enriched with CD11b+ cells; IL-1 β levels were similar in the CD11b+ depleted cultures from stressed and non-stressed mice (Fig. 6). However, depleting cultures of CD11b+ cells did not affect the stress-induced increase in TNF- α levels (Fig. 6).

Discussion

Exposure to the social stressor, SDR, led to the development of anxiety-like behavior as evidenced by two widely used measures to assess anxiety in rodents (i.e., the open field test and the novel object/neophobia test) [39,40]. This is in concurrence with our previous study showing that SDR enhances anxiety like behavior in the open field and light dark preference test, with the current study adding neophobia to the list of SDR-induced anxiety-like behaviors. Several studies have shown that anxiety and anxiety-like behaviors are modulated by neural circuits within the bed nucleus of the stria terminalis, prefrontal cortex, hippocampus, amygdala, and hypothalamus (recently reviewed in [41]). Although it was not the focus of the current study, unpublished data indicate that exposure to the SDR stressor significantly increases neuronal activation in these same brain regions, as assessed by neuronal cfos staining (unpublished observations). This increase in SDR-induced neuronal activation is likely mediated by corticotrophin releasing hormone, since others have demonstrated that infusion of CRH into the BNST increases fear-potentiated startle [42], whereas infusion of the CRH antagonist, α -hCRH₉₋₄₁, blocked fear potentiated startle [42]. Studies have been planned to

determine the role of stressor-induced activation of these brain regions in the induction of SDR-induced anxiety-like behaviors.

Interestingly, depressive-like behavior has not been identified in mice exposed to SDR [43], suggesting that SDR enhances anxiety-like behavior, while having few, if any, effects on behaviors reflective of depression. In humans, enhanced anxiety has been linked to increased markers of inflammation, such as C-reactive protein and inflammatory cytokines, in otherwise healthy individuals [44]. And, it is becoming evident that systemic inflammation is linked to a variety of diseases, including diabetes, obesity, pre-term birth, stroke, and atherosclerotic cardiovascular disease [45]. Because infection with the periodontal pathogen, *P. gingivalis* is also associated with increased circulatory cytokines, we sought to determine whether exposing mice to SDR would further enhance the inflammatory response to this pathogen.

Social disruption is a well defined murine stressor that results in significant elevations in circulating levels of corticosterone as well as the development of anxiety-like behaviors [23, 24,43]. The mice do not habituate to this behavioral paradigm, because serum corticosterone levels tend to be higher after 6 cycles of SDR than they are after 1 cycle of SDR [23,24]. High levels of corticosterone can induce apoptosis in leukocytes, and as a result, many experimental murine stressors result in leukocytopenia in secondary lymphoid organs. During SDR, however, spleen mass is significantly increased (Fig. 2) due to a corresponding increase in CD11b+ cells of myeloid origin (primarily monocytes/macrophages and neutrophils) (Fig. 2). Earlier studies showed that these CD11b+ cells from the spleens of mice exposed to SDR remain viable even when high doses of the glucocorticoid (GC) corticosterone are added to the cultures [33]. Interestingly, this GC insensitivity was only evident when these cells were stimulated with LPS from the Gram-negative pathogen *E. coli* [22,46]. This study confirms and extends these previous observations to now include GC insensitivity after stimulation with LPS derived from the oral pathogen, *P. gingivalis*.

There are substantial differences between enterobacterial LPS and the LPS derived from *P. gingivalis*. For example, it is well known that the LPS derived from *P. gingivalis* is less pyrogenic than enterobacterial LPS, and results in comparatively less spleen cell proliferation and cytokine production [47]. These effects are thought to be in part due to the structure of the lipid A core of the *P. gingivalis* LPS molecule, which contains branched fatty acids of 15–17 residues; these structures were not found in enterobacterial lipid A molecules [48]. Moreover, *P. gingivalis* derived LPS stimulates TLR2 and has been reported to have antagonist or weak stimulatory effects on TLR4 [49]. This is in stark contrast to LPS derived from *E. coli*, which is known to strongly activate TLR4, with weaker effects on TLR2 [49]. Assessing the impact of *P. gingivalis* LPS on splenocyte activity is also likely to have translational importance. Routine oral hygiene and dental treatments are known to introduce bacteria into the bloodstream where they circulate and eventually reach circumventricular organs, such as the spleen [3,50]. Thus, it is likely that splenic macrophages come into contact with oral bacteria and their products. If hyper-reactive, these macrophages would have the potential to significantly impact systemic health.

Social disruption had the additional effect of enhancing cytokine production upon stimulation with *P. gingivalis* (Fig. 4 and 6). Splenic CD11b+ cells from mice exposed to SDR produced significantly higher levels of IL-1 β and TNF- α when stimulated with *P. gingivalis* LPS (Fig. 6). While the CD11b+ cells were responsible for the stress-induced increase in IL-1 β , TNF- α levels were still increased in the cultures depleted of CD11b+ cells. It is not immediately clear which additional cell type was affected by the stressor, but a recent study has demonstrated that the SDR stressor increases the reactivity of CD11c+ dendritic cells to *E. coli* LPS [51]. Thus, it is likely that the increased TNF- α levels are due to an increased production by both CD11b+ cells and CD11c+ cells. This increase in GC insensitivity and cytokine production

does not appear to be due to the effects of behavioral testing on host physiology, because mice that were only exposed to SDR, and not to any behavioral testing, still showed a significant increase in *P. gingivalis* LPS-induced IL-1 β and TNF- α . No differences in GC insensitivity or cytokine production were observed between animals exposed to behavioral testing and those that were not tested (data not shown).

Both IL-1 β and TNF- α are known to be important in the development of oral inflammatory diseases like gingivitis and periodontitis, and are found in high levels in periodontal patients at the site of active tissue breakdown [52,53]. These cytokines have many effects on gingival tissue, such as degradation of connective tissue matrix via an increase in matrix metalloproteinases, and activation of osteoblastic/osteoclastic responses via destruction of the balance between physiological repair and remodeling [54]. Elevated cytokine levels, however, are not limited to the infected gingiva, and people with chronic periodontitis have been found to have increased levels of cytokines in the circulation, including IL-1 β and TNF- α [55]. These circulatory cytokines are not likely the result of “spillover” from the infected gingiva, but rather are likely to be derived from peripheral cytokine producing cells, such as CD11b+ macrophages. Oral bacteria and bacterial derived products can enter into the bloodstream through standard oral hygiene and treatments [56] as well as through pathogen-induced ulceration and vascular permeability in the gingiva [45]. As they circulate through the body, the microbial products are recognized by cells of the reticuloendothelial macrophages and other innate immune cells. These cells are able to respond to *P. gingivalis* and *P. gingivalis*-associated products, by producing a variety of inflammatory mediators, including inflammatory cytokines. These inflammatory mediators are thought to be the link between oral inflammatory diseases and systemic diseases. Our results indicate that exposure to a stressor can further enhance the production of inflammatory cytokines when these macrophages are stimulated with *P. gingivalis* LPS.

The mechanisms through which SDR enhances cytokine production have not been completely defined, but likely involve SDR-induced increases in TLRs. Toll-like receptor 4 is traditionally thought of as the receptor for LPS which is found in abundance on Gram-negative bacteria, whereas TLR2 is generally thought of as the receptor for peptidoglycans abundantly found on Gram-positive bacteria. However, both TLR2 and TLR4 can bind to additional pathogen associated molecules. The activation of specific TLRs by *P. gingivalis* LPS has been controversial, but most results indicate that *P. gingivalis* LPS or the lipid A core of the LPS molecule can activate TLR2, with weaker stimulatory effects on TLR4 [10,11,49]. Importantly, macrophages from the spleens of mice exposed to SDR express significantly higher levels of both TLR2 and TLR4 [31], with studies showing that stimulation of TLRs is necessary for the effects of SDR to manifest [22,46]. Ligation of TLRs induces the activation of signaling cascades that culminate in the activation of transcription factors, such as NF- κ B. Because both IL-1 β and TNF- α are under the transcriptional control of NF- κ B, it is likely that exposure to SDR enhanced cytokine production by increasing TLR-driven NF- κ B expression. This hypothesis is consistent with previous results that showed that NF- κ B levels are higher in nuclear fractions of cells from mice exposed to SDR after stimulation with *E. coli* LPS [22].

Stressor-induced dysregulation of macrophage activity can have wide ranging effects on health. Although the increased reactivity of macrophages to microbial products results in excessive production of inflammatory mediators, early studies showed that the stress response can also significantly increase the microbicidal activity of splenic and peritoneal macrophages [31,42, 57,58], possibly through adrenergic receptor signaling [59]. These findings are intuitive from an evolutionary standpoint, given that the stress response developed to respond to stressors primarily involving stimuli that could result in physical damage, as well as possible bacterial contamination. Thus, natural selection would favor animals that could survive not only physiological insult, but also those that are able to control bacteria at the wound site.

Macrophages (and other innate immune cells, such as dendritic cells), are critical to the initial innate immune response to injury and infection and also help to shape the adaptive immune response. As a result, stressor-induced changes to macrophage functioning can alter adaptive immunity, and not just the innate inflammatory response. This assertion is supported by previous studies that indicate that these stressor-induced effects on macrophages can either enhance or suppress adaptive immunity. For example, exposing BALB/c mice to a restraint stressor prior to immunizing with keyhole limpet hemocyanin significantly increased the number of IgM and IgG producing cells in the spleen. Depleting the animals of macrophages abrogated this effect [60]. Stressor-induced effects on macrophages may also suppress certain components of adaptive immunity. For example, Fleshner et al. (1995) showed that stressor-induced suppression of lymphocyte proliferation in mixed lymphocyte whole blood assays was due to the activity of macrophages [61]. Similar results by Coussons-Read et al. (1994) indicated that this effect was due to a stressor-induced increase in macrophage production of nitric oxide [62]. Thus, it is evident that dysregulation of macrophage function can have wide ranging effects on the immune response.

Glucocorticoid hormones are important regulators of immune activity that normally serve to suppress the inflammatory response. But, psychological stress and social factors have been associated with heightened circulating levels of cytokines and other markers of inflammation. Human studies using gene chip analyses of peripheral blood leukocytes have found an underrepresentation of genes that are activated by glucocorticoids, and an overrepresentation of genes regulated by NF- κ B, which are normally repressed by glucocorticoids [63,64]. This pattern of gene expression, along with our studies in laboratory animals, suggests that periods of psychological stress are associated with an inflammatory profile that is not able to be controlled by endogenous glucocorticoids. The current study indicates that this stress-induced cytokine production by peripheral inflammatory cells can be further enhanced by stimulation with an oral microbe capable of inducing peripheral inflammation, and points to a potential mechanism through which oral inflammatory diseases, stress, and systemic health may be linked.

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References

1. Pihlstrom BL, Michalowicz BS, Johnson NW. Periodontal diseases. *Lancet* 2005;366:1809–1820. [PubMed: 16298220]
2. Gibson FC III, Genco CA. Porphyromonas gingivalis mediated periodontal disease and atherosclerosis: disparate diseases with commonalities in pathogenesis through TLRs. *Curr. Pharm. Des* 2007;13:3665–3675. [PubMed: 18220804]
3. Gibson FC III, Ukai T, Genco CA. Engagement of specific innate immune signaling pathways during Porphyromonas gingivalis induced chronic inflammation and atherosclerosis. *Front Biosci* 2008;13:2041–2059. [PubMed: 17981690]
4. Paoletti R, Gotto AM Jr, Hajjar DP. Inflammation in atherosclerosis and implications for therapy. *Circulation* 2004;109:III20–III26. [PubMed: 15198962]
5. Dimsdale JE. Psychological stress and cardiovascular disease. *J. Am. Coll. Cardiol* 2008;51:1237–1246. [PubMed: 18371552]
6. Potempa J, Sroka A, Imamura T, Travis J. Gingipains, the major cysteine proteinases and virulence factors of Porphyromonas gingivalis: structure, function and assembly of multidomain protein complexes. *Curr. Protein Pept. Sci* 2003;4:397–407. [PubMed: 14683426]

7. McGettrick AF, O'Neill LA. Toll-like receptors: key activators of leucocytes and regulator of haematopoiesis. *Br. J. Haematol* 2007;139:185–193. [PubMed: 17897294]
8. Doyle SL, O'Neill LA. Toll-like receptors: from the discovery of NF κ B to new insights into transcriptional regulations in innate immunity. *Biochem. Pharmacol* 2006;72:1102–1113. [PubMed: 16930560]
9. Tabeta K, Yamazaki K, Akashi S, et al. Toll-like receptors confer responsiveness to lipopolysaccharide from *Porphyromonas gingivalis* in human gingival fibroblasts. *Infect. Immun* 2000;68:3731–3735. [PubMed: 10816537]
10. Darveau RP, Pham TT, Lemley K, et al. *Porphyromonas gingivalis* lipopolysaccharide contains multiple lipid A species that functionally interact with both toll-like receptors 2 and 4. *Infect. Immun* 2004;72:5041–5051. [PubMed: 15321997]
11. Zhou Q, Desta T, Fenton M, Graves DT, Amar S. Cytokine profiling of macrophages exposed to *Porphyromonas gingivalis*, its lipopolysaccharide, or its FimA protein. *Infect. Immun* 2005;73:935–943. [PubMed: 15664935]
12. Assuma R, Oates T, Cochran D, Amar S, Graves DT. IL-1 and TNF antagonists inhibit the inflammatory response and bone loss in experimental periodontitis. *J. Immunol* 1998;160:403–409. [PubMed: 9551997]
13. Rowland RW. Necrotizing ulcerative gingivitis. *Ann. Periodontol* 1999;4:65–73. [PubMed: 10863376]
14. Nakano K, Nemoto H, Nomura R, et al. Detection of oral bacteria in cardiovascular specimens. *Oral Microbiol. Immunol* 2009;24:64–68. [PubMed: 19121072]
15. Zaremba M, Gorska R, Suwalski P, Kowalski J. Evaluation of the incidence of periodontitis-associated bacteria in the atherosclerotic plaque of coronary blood vessels. *J. Periodontol* 2007;78:322–327. [PubMed: 17274722]
16. Pussinen PJ, Paju S, Mantyla P, Sorsa T. Serum microbial- and host-derived markers of periodontal diseases: a review. *Curr. Med. Chem* 2007;14:2402–2412. [PubMed: 17896988]
17. Ader, R. *Psychoneuroimmunology*. Vol. 4th ed. Elsevier/Academic Press; Amsterdam: 2007.
18. Sternberg EM. Neural regulation of innate immunity: a coordinated nonspecific host response to pathogens. *Nat. Rev. Immunol* 2006;6:318–328. [PubMed: 16557263]
19. Padgett DA, Glaser R. How stress influences the immune response. *Trends Immunol* 2003;24:444–448. [PubMed: 12909458]
20. Almawi WY, Melemedjian OK. Negative regulation of nuclear factor- κ B activation and function by glucocorticoids. *J. Mol. Endocrinol* 2002;28:69–78. [PubMed: 11932204]
21. Werb Z, Foley R, Munck A. Interaction of glucocorticoids with macrophages. Identification of glucocorticoid receptors in monocytes and macrophages. *J. Exp. Med* 1978;147:1684–1694. [PubMed: 681878]
22. Quan N, Avitsur R, Stark JL, et al. Molecular mechanisms of glucocorticoid resistance in splenocytes of socially stressed male mice. *J. Neuroimmunol* 2003;137:51–58. [PubMed: 12667647]
23. Bailey MT, Avitsur R, Engler H, Padgett DA, Sheridan JF. Physical defeat reduces the sensitivity of murine splenocytes to the suppressive effects of corticosterone. *Brain Behav. Immun* 2004;18:416–424. [PubMed: 15265534]
24. Engler H, Engler A, Bailey MT, Sheridan JF. Tissue-specific alterations in the glucocorticoid sensitivity of immune cells following repeated social defeat in mice. *J. Neuroimmunol* 2005;163:110–119. [PubMed: 15885313]
25. Avitsur R, Stark JL, Dhabhar FS, Sheridan JF. Social stress alters splenocyte phenotype and function. *J. Neuroimmunol* 2002;132:66–71. [PubMed: 12417435]
26. Avitsur R, Kavelaars A, Heijnen C, Sheridan JF. Social stress and the regulation of tumor necrosis factor- α secretion. *Brain Behav. Immun* 2005;19:311–317. [PubMed: 15944070]
27. Engler H, Bailey MT, Engler A, Stiner-Jones LM, Quan N, Sheridan JF. Interleukin-1 receptor type 1-deficient mice fail to develop social stress-associated glucocorticoid resistance in the spleen. *Psychoneuroendocrinology* 2008;33:108–117. [PubMed: 18037570]
28. Stark JL, Avitsur R, Hunzeker J, Padgett DA, Sheridan JF. Interleukin-6 and the development of social disruption-induced glucocorticoid resistance. *J. Neuroimmunol* 2002;124:9–15. [PubMed: 11958817]

29. Avitsur R, Stark JL, Sheridan JF. Social stress induces glucocorticoid resistance in subordinate animals. *Horm. Behav* 2001;39:247–257. [PubMed: 11374910]
30. Avitsur R, Stark JL, Dhabhar FS, Padgett DA, Sheridan JF. Social disruption-induced glucocorticoid resistance: kinetics and site specificity. *J. Neuroimmunol* 2002;124:54–61. [PubMed: 11958822]
31. Bailey MT, Engler H, Powell ND, Padgett DA, Sheridan JF. Repeated social defeat increases the bactericidal activity of splenic macrophages through a Toll-like receptor-dependent pathway. *Am. J. Physiol Regul. Integr. Comp Physiol* 2007;293:R1180–R1190. [PubMed: 17596326]
32. Engler H, Bailey MT, Engler A, Sheridan JF. Effects of repeated social stress on leukocyte distribution in bone marrow, peripheral blood and spleen. *J. Neuroimmunol* 2004;148:106–115. [PubMed: 14975591]
33. Stark JL, Avitsur R, Padgett DA, Campbell KA, Beck FM, Sheridan JF. Social stress induces glucocorticoid resistance in macrophages. *Am. J. Physiol Regul. Integr. Comp Physiol* 2001;280:R1799–R1805. [PubMed: 11353685]
34. Weiss SM, Lightowler S, Stanhope KJ, Kennett GA, Dourish CT. Measurement of anxiety in transgenic mice. *Rev. Neurosci* 2000;11:59–74. [PubMed: 10716656]
35. Dulawa SC, Holick KA, Gundersen B, Hen R. Effects of chronic fluoxetine in animal models of anxiety and depression. *Neuropsychopharmacology* 2004;29:1321–1330. [PubMed: 15085085]
36. Park HJ, Jeong SK, Kim SR, et al. Resveratrol inhibits *Porphyromonas gingivalis* lipopolysaccharide-induced endothelial adhesion molecule expression by suppressing NF-kappaB activation. *Arch. Pharm. Res* 2009;32:583–591. [PubMed: 19407977]
37. Domon H, Honda T, Oda T, Yoshie H, Yamazaki K. Early and preferential induction of IL-1 receptor-associated kinase-M in THP-1 cells by LPS derived from *Porphyromonas gingivalis*. *J. Leukoc. Biol* 2008;83:672–679. [PubMed: 18156187]
38. Zhou Q, Amar S. Identification of signaling pathways in macrophage exposed to *Porphyromonas gingivalis* or to its purified cell wall components. *J. Immunol* 2007;179:7777–7790. [PubMed: 18025224]
39. Crawley JN. Behavioral phenotyping of transgenic and knockout mice: experimental design and evaluation of general health, sensory functions, motor abilities, and specific behavioral tests. *Brain Res* 1999;835:18–26. [PubMed: 10448192]
40. File SE. Factors controlling measures of anxiety and responses to novelty in the mouse. *Behav. Brain Res* 2001;125:151–157. [PubMed: 11682106]
41. Roozendaal B, McEwen BS, Chattarji S. Stress, memory and the amygdala. *Nat. Rev. Neurosci* 2009;10:423–433. [PubMed: 19469026]
42. Lee Y, Davis M. Role of the hippocampus, the bed nucleus of the stria terminalis, and the amygdala in the excitatory effect of corticotropin-releasing hormone on the acoustic startle reflex. *J. Neurosci* 1997;17:6434–6446. [PubMed: 9236251]
43. Kinsey SG, Bailey MT, Sheridan JF, Padgett DA, Avitsur R. Repeated social defeat causes increased anxiety-like behavior and alters splenocyte function in C57BL/6 and CD-1 mice. *Brain Behav. Immun* 2007;21:458–466. [PubMed: 17178210]
44. Pitsavos C, Panagiotakos DB, Papageorgiou C, Tsetsekou E, Soldatos C, Stefanadis C. Anxiety in relation to inflammation and coagulation markers, among healthy adults: the ATTICA study. *Atherosclerosis* 2006;185:320–326. [PubMed: 16005881]
45. Gibson FC III, Ukai T, Genco CA. Engagement of specific innate immune signaling pathways during *Porphyromonas gingivalis* induced chronic inflammation and atherosclerosis. *Front Biosci* 2008;13:2041–2059. [PubMed: 17981690]
46. Avitsur R, Padgett DA, Dhabhar FS, et al. Expression of glucocorticoid resistance following social stress requires a second signal. *J. Leukoc. Biol* 2003;74:507–513. [PubMed: 12960258]
47. Nair BC, Mayberry WR, Dziak R, Chen PB, Levine MJ, Hausmann E. Biological effects of a purified lipopolysaccharide from *Bacteroides gingivalis*. *J. Periodontal Res* 1983;18:40–49. [PubMed: 6222173]
48. Kumada H, Haishima Y, Umemoto T, Tanamoto K. Structural study on the free lipid A isolated from lipopolysaccharide of *Porphyromonas gingivalis*. *J. Bacteriol* 1995;177:2098–2106. [PubMed: 7721702]

49. Diya Z, Lili C, Shenglai L, Zhiyuan G, Jie Y. Lipopolysaccharide (LPS) of *Porphyromonas gingivalis* induces IL-1 β , TNF- α and IL-6 production by THP-1 cells in a way different from that of *Escherichia coli* LPS. *Innate. Immun* 2008;14:99–107. [PubMed: 18713726]
50. Kinane DF, Peterson M, Stathopoulou PG. Environmental and other modifying factors of the periodontal diseases. *Periodontol* 2000;200640:107–119.
51. Powell ND, Bailey MT, Mays JW, et al. Repeated social defeat activates dendritic cells and enhances Toll-like receptor dependent cytokine secretion. *Brain Behav. Immun* 2009;23:225–231. [PubMed: 18848983]
52. Gamonal J, Sanz M, O'Connor A, et al. Delayed neutrophil apoptosis in chronic periodontitis patients. *J. Clin. Periodontol* 2003;30:616–623. [PubMed: 12834499]
53. Orozco A, Gemmell E, Bickel M, Seymour GJ. Interleukin-1 β , interleukin-12 and interleukin-18 levels in gingival fluid and serum of patients with gingivitis and periodontitis. *Oral Microbiol. Immunol* 2006;21:256–260. [PubMed: 16842511]
54. Graves DT, Cochran D. The contribution of interleukin-1 and tumor necrosis factor to periodontal tissue destruction. *J. Periodontol* 2003;74:391–401. [PubMed: 12710761]
55. Golub LM, Payne JB, Reinhardt RA, Nieman G. Can systemic diseases co-induce (not just exacerbate) periodontitis? A hypothetical “two-hit” model. *J. Dent. Res* 2006;85:102–105. [PubMed: 16434727]
56. Kinane DF, Riggio MP, Walker KF, MacKenzie D, Shearer B. Bacteraemia following periodontal procedures. *J. Clin. Periodontol* 2005;32:708–713. [PubMed: 15966875]
57. Fleshner M, Nguyen KT, Cotter CS, Watkins LR, Maier SF. Acute stressor exposure both suppresses acquired immunity and potentiates innate immunity. *Am. J. Physiol* 1998;275:R870–R878. [PubMed: 9728086]
58. Lyte M, Nelson SG, Thompson ML. Innate and adaptive immune responses in a social conflict paradigm. *Clin. Immunol. Immunopathol* 1990;57:137–147. [PubMed: 2394033]
59. Weatherby KE, Zwilling BS, Lafuse WP. Resistance of macrophages to *Mycobacterium avium* is induced by α 2-adrenergic stimulation. *Infect. Immun* 2003;71:22–29. [PubMed: 12496145]
60. Shanks N, Kusnecov AW. Differential immune reactivity to stress in BALB/cByJ and C57BL/6J mice: in vivo dependence on macrophages. *Physiol Behav* 1998;65:95–103. [PubMed: 9811371]
61. Fleshner M, Bellgrau D, Watkins LR, Laudenslager ML, Maier SF. Stress-induced reduction in the rat mixed lymphocyte reaction is due to macrophages and not to changes in T cell phenotypes. *J. Neuroimmunol* 1995;56:45–52. [PubMed: 7822481]
62. Coussons-Read ME, Maslonek KA, Fecho K, Perez L, Lysle DT. Evidence for the involvement of macrophage-derived nitric oxide in the modulation of immune status by a conditioned aversive stimulus. *J. Neuroimmunol* 1994;50:51–58. [PubMed: 8300858]
63. Cole SW, Hawkey LC, Arevalo JM, Sung CY, Rose RM, Cacioppo JT. Social regulation of gene expression in human leukocytes. *Genome Biol* 2007;8:R189. [PubMed: 17854483]
64. Miller GE, Chen E, Sze J, et al. A Functional Genomic Fingerprint of Chronic Stress in Humans: Blunted Glucocorticoid and Increased NF- κ B Signaling. *Biol. Psychiatry*. 2008

Open Field Test

Novel Object Test

Figure 1.

The social disruption stressor significantly increased anxiety-like behavior. Behavior was assessed in the open field, and mice exposed to SDR were found to spend less time in the center of the open field (Fig. 1A). This was not dependent upon a reduction in the overall locomotion of the mice, since mice exposed to SDR traveled as far as the non-stressed HCC mice while in the open field (Fig. 1B). Mice exposed to SDR also spent a significantly reduced amount of time on the same side of the open field as a novel object (Fig. 1C), especially during the first three minutes of testing. $n = 9$ mice per group. * $p < .05$ vs. non-stressed HCC mice.

Figure 2.

Exposure to social disruption significantly affects the cellular composition of the spleen. Spleens were removed from mice the morning following the last cycle of SDR, weighed (Fig. 2A), and macerated to create a single cell suspension. Flow cytometry was used to quantify the number of splenic monocytes/macrophages (Fig. 2B) and granulocytes (Fig. 2C). $n = 4 - 5$ per group. * $p < .05$ vs. non-stressed HCC mice.

Figure 3.

Cell viability was significantly enhanced after exposure to social disruption. 2.5×10^5 spleen cells were cultured with $10 \mu\text{g/ml}$ of *P. gingivalis* LPS with increasing doses of corticosterone for 48 h. Cell viability was assessed using a colorimetric assay, with data expressed as the optical density. $n = 5$ per group. * $p < .05$ vs. non-stressed HCC mice at the same corticosterone concentration.

Figure 4.

Cytokine production was significantly enhanced by exposure to social disruption. 5×10^5 spleen cells were culture with $10 \mu\text{g/ml}$ of *P. gingivalis* LPS with increasing doses of corticosterone for 18 h. Cytokine levels in the supernatants were determine via ELISA. For both IL-1 β and TNF- α , cytokine levels were higher in cultures containing splenocytes from stressed mice at all dose of corticosterone ($p < .05$). $n = 5$ per group.

Figure 5. Stress-induced increases in spleen cell viability were dependent upon CD11b+ myeloid cells. Splenic CD11b+ myeloid cells were enriched from spleen cell cultures and stimulated with 1 μg/ml of *P. gingivalis* LPS and increasing doses of corticosterone for 48 h prior to assessing cell viability. Data are the optical density of the assay. * p < .05 vs non-stressed HCC mice. n = 5 per group.

Figure 6.

Stress-induced increases in IL-1 β and TNF- α were partly due to CD11b+ myeloid cells. Splenic CD11b+ myeloid cells were enriched from spleen cell cultures and stimulated with 1 μ g/ml of *P. gingivalis* LPS for 18 h. Cytokine levels in the supernatants were assessed via ELISA and are expressed as pg/ml. n = 5 per group. * p < .05 vs non-stressed HCC mice.