Social stress induces glucocorticoid resistance in macrophages

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1Neuroscience Graduate Studies Program, Sections of 2Oral Biology and 5Health Services Research, 3Department of Molecular Virology, Immunology and Medical Genetics, 4The Institute for Behavioral Medicine Research, The Ohio State University Health Sciences Center, Columbus, Ohio 43218

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Stark, Jennifer L., Ronit Avitsur, David A. Padgett, Kim A. Campbell, F. Michael Beck, and John F. Sheridan. Social stress induces glucocorticoid resistance in macrophages. Am J Physiol Regulatory Integrative Comp Physiol 280: R1799–R1805, 2001.—Stress-induced levels of plasma glucocorticoid hormones are known to modulate leukocyte function. These experiments examined the effects of a social stressor on the responsiveness of peripheral immune cells. Male mice experienced six evening cycles of social disruption (SDR), in which an aggressive male intruder was placed into their home cage for 2 h. Although circulating corticosterone was elevated in SDR mice, they had enlarged spleens and increased numbers of splenic leukocytes. Splenocytes from SDR and control mice were cultured with lipopolysaccharide and corticosterone. Cells from SDR mice exhibited decreased sensitivity to the antiproliferative effects of corticosterone, suggesting that the peripheral immune cells were resistant to glucocorticoids. In addition, SDR cells produced more interleukin (IL)-6. To determine which cell population was affected, we used antibody-labeled magnetic beads to deplete splenocyte suspensions of B cells or macrophages. Depletion of macrophages from SDR cultures, but not depletion of B cells, abolished both the corticosterone resistance and enhanced IL-6 secretion. These findings demonstrate that a psychosocial stressor induced glucocorticoid resistance in mouse splenic macrophages.

interleukin-6; corticosterone resistance; lipopolysaccharide; spleen; mice

Social stress is known to impact regulation of the immune system and may alter an organism’s response to infectious challenges. Various rodent models demonstrate that social stress can induce changes in the immune system, such as altered leukocyte subset populations (6, 11, 27), decreased mitogen-induced proliferation (9), decreased cytokine production (9), and decreased antibody production (6, 12). These functional changes may underlie the findings that aggressive interactions among mice slowed clearance of a parasitic infection (3) and increased the probability of herpes simplex virus reactivation (22). However, a number of studies found that social conflict in rodents does not have global inhibitory effects on immune function. Klein et al. (15) noted that chronic social stress in rats did not impair natural killer cell activity, splenocyte proliferation, or antibody production. Other studies have reported an enhancement of mitogen-induced T cell proliferation (6) and splenocyte phagocytosis (18) after social stress.

Stress-induced increases in glucocorticoid hormones, such as corticosterone in rodents, mediate many of the suppressive effects of stress on the immune system. Type II glucocorticoid receptors are expressed in cells of lymphoid tissues, such as the spleen and thymus (16, 26), and ligand binding to these receptors has a number of inhibitory effects. In particular, expression of proinflammatory cytokines and cell adhesion molecules is reduced (1, 7). These changes have functional implications for proliferative responses, cell survival, and leukocyte trafficking patterns during tissue damage or infectious challenge. However, endogenous glucocorticoids also play a beneficial role in limiting inflammatory responses. It has been noted that during an infection, activated immune cells secrete proinflammatory cytokines, which stimulate the release of systemic glucocorticoids (5, 24). The triggering of this regulatory loop during inflammatory processes provides important control over the immune system.

The development of glucocorticoid resistance in immune cells, which prevents response to negative hormonal feedback, may lead to uncontrolled inflammation (10). Previous studies from our laboratory demonstrated that although social stress elevated circulating corticosterone levels in male mice, lung inflammation was enhanced during infection with influenza A virus. This hyperinflammatory response in stressed animals increased the likelihood of respiratory distress and mortality compared with infected controls (D. A. Padgett, J. L. Stark, N. Quan, and J. F. Sheridan; unpublished observations). In the current experiments, we tested the hypothesis that social
stress induces leukocytes to become hyposensitive to corticosterone. Over the course of 1 wk, male mice were exposed to six evening cycles of social disruption (SDR). For each cycle of SDR, an aggressive intruder was placed into a cage of male mice for 2 h, resulting in the attack and defeat of the residents. We report that when splenocytes from control and SDR mice were stimulated with lipopolysaccharide (LPS), cells from the stressed animals were resistant to the inhibitory actions of corticosterone on cell proliferation and interleukin (IL)-6 production. Removal of macrophages from the splenocyte suspension, but not the removal of B cells, abolished the corticosterone resistance. These results suggest that SDR alters the functional responses of splenic macrophages by making them corticosterone resistant.

METHODS

Animals. Male C57BL/6 mice were obtained from Charles River (Wilmington, MA) and were housed in a facility accredited by the American Association for Accreditation of Laboratory Animal Care. All animals were given free access to food and water and were maintained on a 12:12-h light-dark cycle (lights on at 6:00 AM). Experimental animals were received at 6–8-wk old, housed 5 per cage, and allowed to acclimate for at least 1 wk before any experimental procedures began. Intruders for the social disruption stress were individually housed after being purchased as retired breeders. Male mice that were isolated from their cagemates for aggressive behavior were also used as intruders.

Social disruption stress. Cages of 4–5 mice were randomly assigned as either control or SDR groups. Control mice remained undisturbed in their home cages. Stressed mice experienced six SDR events over the course of 1 wk: three nightly cycles, one night off, and three more cycles. One cycle of stress involved introducing an aggressive intruder into the home cage for 2 h (5:00–7:00 PM). Behavior was observed to ensure that the intruder attacked the residents and that the residents showed submissive postures. If the intruder did not attack or was attacked by any of the residents, he was replaced with a new intruder. The health status of the animals was checked each morning during the experiment. Approximately 15–20% of the SDR mice received visible back wounds, which were then washed twice in PBS containing 1% FBS and fixated with 1% paraformaldehyde. All antisera were obtained from BD PharMingen (San Diego, CA), and labeled monoclonal antibodies (or the appropriate isotype controls) were purchased from Sigma (C-2505) and diluted in supplemented RPMI with 0.2% ethanol.

Corticosterone radioimmunoassay. Blood samples were collected from the retroorbital plexus, and plasma was frozen at −70°C until analysis. Corticosterone was quantified using the double antibody rat corticosterone kit (ICN Biomedicals, Costa Mesa, CA) according to the manufacturer's instructions. Intra-assay and interassay variability for the kit is 7%. The detection limit of the assay was 25 ng/ml.

Corticosterone sensitivity assay. Spleens from each group of animals were pooled in ice-cold Hanks' balanced salt solution (HBSS) and washed between glass slides to obtain single cell suspensions. Red blood cells were eliminated by adding 2 ml of lysis buffer (0.16 M NH₄Cl, 10 mM KHCO₃, and 0.13 mM EDTA) for 2 min, followed by one wash with HBSS/10% heat-inactivated fetal bovine serum (FBS). Each cell pellet was resuspended in HBSS, filtered through a sterile 70-μm nylon cell strainer to remove debris, and washed a final time in HBSS. Viable mononuclear cells were counted using trypan blue dye exclusion, and samples were resuspended (2.5 × 10⁶ cells/ml) in supplemented RPMI medium (10% heat-inactivated FBS, 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). LPS (Sigma-L-2630), which is a component of gram-negative bacteria that stimulates B cells and macrophages, was added at a concentration of 1 μg/ml for mitogen stimulation. To test the sensitivity of cells to inhibition by glucocorticoids, we treated aliquots from each cell suspension with corticosterone over a dose range of 0.005–5 μM. Corticosterone was obtained commercially from Sigma (C-2505) and diluted in supplemented RPMI with 0.2% ethanol. Cell suspensions were added in triplicate to flat-bottom 96-well plates at a volume of 100 μl/well for cell proliferation assays and 200 μl/well for cytokine analysis, and culture plates were incubated at 37°C in 5% CO₂. At the end of incubation, cell supernatants were harvested and frozen at −20°C for cytokine analysis. Other cultures were incubated for 48 h, at which time the cell proliferation/viability assay was performed.

Cell proliferation/viability assay. The CellTiter 96 aqueous nonradioactive proliferation assay was purchased from Promega (G5430; Madison, WI). The tetrazolium substrate solution was prepared according to the instructions, and 20 μl was added to each well of the 96-well plates. The dehydrogenase enzymes in metabolically active cells convert this substrate to formazan, producing a brown precipitate. The plates were incubated at 37°C in 5% CO₂ for 3 h, and the resulting color changes were quantified by obtaining optical density (OD) readings at 490 nm on an ELISA plate reader. To account for differences in background activity of cells, we subtracted the mean OD of three RPMI wells for a given treatment from each of the corresponding LPS-stimulated values.

Splenocyte depletion. Splenocyte suspensions were prepared and counted as described above. Aliquots of control and SDR cells were set aside (original cell fractions) for the corticosterone sensitivity assay and for phenotypic analysis by flow cytometry. B cells or macrophages were removed from the suspensions using anti-CD19 or anti-CD11b magnetic microbeads (Miltenyi Biotec, Auburn, CA) with minor modification of the manufacturer’s protocol. For each 10⁷ cells to be depleted, 15 μl of microbeads was added. The cell suspensions were mixed and incubated at 4°C for 20 min. Cells were washed, and suspensions were loaded into 30-μm filters and magnetic cell sorting separation columns to remove the magnetically labeled cells. Aliquots of the depleted fractions were saved for phenotypic analysis by flow cytometry. The original and depleted cell fractions were cultured for assessment of corticosterone sensitivity, as described above.

Flow cytometric analysis. Single cell suspensions (1 × 10⁶ cells per sample) were incubated with 1 μg of fluorescently labeled monoclonal antibodies (or the appropriate isotype controls) after a 20-min blocking step with 30% mouse serum. Antibody labeling was performed at 4°C for 30 min. The cells were then washed twice in PBS containing 1% FBS and 0.09% NaN₃ and fixed with 1% paraformaldehyde. All antibodies were obtained from BD PharMingen (San Diego, CA), including PE-labeled anti-CD19, FITC-labeled anti-CD11b, FITC-labeled anti-CD3, PE-conjugated rat IgG₂a, FITC-conjugated rat IgG₂a, and FITC-conjugated hamster IgG. Samples were analyzed on an Epics XL flow cytometer (Coulter, Costa Mesa, CA) according to the manufacturer’s instructions, and 20 μl of microbeads was added. The cell suspensions were incubated at 37°C in 5% CO₂ for 20°C for cytokine analysis. Other cultures were incubated for 48 h, at which time the cell proliferation/viability assay was performed.
Hialeah, FL). Forward and right-angle light scatter was used to create a gate that included both lymphocyte and monocyte/macrophage populations. A total of 10,000 events was analyzed for each sample to assess phenotypic marker expression levels.

**IL-6 ELISA.** IL-6 concentrations in supernatants from cultured splenocytes were measured by sandwich ELISA with monoclonal antibodies and a standard protocol from BD PharMingen. Plates were coated with 50 μl of 2 μg/ml anti-IL-6 (clone MP5–20F3) overnight at 4°C then blocked with PBS/10% FBS for 2 h at room temperature. After 2 washes with PBS-Tween, 50 μl of standards and supernatant samples were added and incubated overnight at 4°C. Plates were washed four times before 100 μl/well of 1 μg/ml biotinylated anti-IL-6 (clone MP5–32C11) was added. After 45 min at room temperature, plates were washed six times with PBS-Tween. After addition of 100 μl/well of a 1:1,000 dilution of avidin-peroxidase (Vector, Burlingame, CA), plates were incubated at room temperature for 30 min. After eight washes, 100 μl of 0.2% 3,3’-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) DiIodinium salt substrate (#1888, Sigma) was added per well, and the enzymatic reaction was allowed to develop at room temperature. OD was measured at 405 nm on an ELISA plate reader, and IL-6 concentrations were quantified by comparison to standard curves generated by serial dilutions of recombinant IL-6 (BD PharMingen). For statistical purposes, a sample falling below the detection limit of the ELISA was assigned the value corresponding to the sensitivity of the assay. Intra-assay variability for this ELISA was 6%, and interassay variability was 9%.

**Statistical analysis.** Mean differences in LPS-stimulated proliferation and IL-6 concentration were assessed with ANOVA. The independent variables were treatment group, consisting of two levels (control and SDR), and corticosterone concentration with repeated measures, consisting of six levels (0, 0.005, 0.05, 0.1, 0.5, and 5 μM). A log transformation was necessary to stabilize the variance in the IL-6 concentration data. Post hoc testing was done via the Tukey procedure. In addition, independent t-tests were used to analyze the differences in plasma corticosterone concentration after the final cycle of stress, in spleen mononuclear cell counts, and in splenocyte phenotype percentages. Results were considered significant if \( P < 0.05 \).

**RESULTS**

To examine the effects of social disruption stress on splenic leukocytes, we exposed male C57BL/6 mice to six 2-h evening cycles with an aggressive intruder mouse. Compared with home-cage controls, mice that experienced SDR showed significantly elevated levels of plasma corticosterone at 7:00 PM, immediately after the final cycle of stress (means ± SE was 275 ± 29 ng/ml vs. 451 ± 38 ng/ml, respectively; \( P < 0.05 \)). Despite exposure to high concentrations of corticosterone in vivo, SDR mice exhibited splenomegaly after 1 wk of social stress. The average number of mononuclear cells from the spleen of a stressed mouse was 3.5-fold greater than the cell count of a control spleen (means ± SE was 4.9 × 10^7 ± 0.9 × 10^7 vs. 1.4 × 10^7 ± 0.17, respectively; \( P < 0.05 \)). Flow cytometric analysis of spleen cell phenotypes from four experiments showed that the mean percentage of T cells, B cells, and macrophages was not significantly different between control and SDR mice (means ± SE for T cells: 36% ± 5 vs. 25% ± 2, B cells: 51% ± 5 vs. 51% ± 3, macrophages: 10% ± 2 vs. 14% ± 2; \( P > 0.05 \) for all cell types).

To test if the splenocytes from stressed animals showed impaired responsiveness to corticosterone, we cultured cells from control and SDR mice with 1 μg/ml of the mitogen LPS along with corticosterone. After 48 h of incubation, cell proliferation/viability was measured using the CellTiter 96 aqueous nonradioactive proliferation assay. Corticosterone decreased the proliferative response of control splenocytes in a dose-dependent fashion (Fig. 1A). Splenocytes from SDR mice exhibited reduced sensitivity to inhibition by corticosterone. Proliferation of control cells was signifi-
spleen cell suspensions showed corticosterone resistance, similar to the results seen in Fig. 1. B cells were depleted from the cell suspensions using anti-CD19 labeled magnetic beads. Because B cells proliferate in response to LPS, proliferation was lower in both groups after their removal (Fig. 2A). However, the response of these cultures to corticosterone was similar to that of the original spleenocyte cultures. Cells from control mice were inhibited in a dose-dependent manner, whereas cells from SDR mice were completely insensitive to inhibition. Phenotypic analysis of the remaining cell fractions from the control and SDR groups confirmed that the CD19-positive populations were similarly diminished, by 75 and 80%, respectively (Table 1).

The fact that B cell depletion did not eliminate the corticosterone resistance in LPS-stimulated SDR cultures suggested that the macrophage was the affected cell type. To test this possibility, we removed macrophages from the splenocyte suspensions with anti-CD11b coated magnetic beads. After depletion, the SDR effect of decreased sensitivity to corticosterone was abolished (Fig. 2B). Flow cytometric analysis of the postdepletion cell suspensions showed that CD11b-positive cells were decreased by 80% in the control and 71% in the SDR suspensions (Table 1). These results indicate that the CD11b-positive cell population in SDR spleens was necessary for the corticosterone resistance.

In addition to inhibiting proliferation, glucocorticoids are known to decrease cytokine production by immune cells (1). Therefore, the proinflammatory cytokine IL-6 was measured in supernatants of control and SDR cell cultures after 18 h of treatment with medium or LPS in the presence of corticosterone. Without mitogen, there was no detectable cytokine in the cell supernatants. With LPS stimulation, IL-6 production by SDR splenocytes was elevated in the basal condition (no corticosterone added) and in the presence of all concentrations of corticosterone, compared with cells from control mice (Fig. 3A, $P < 0.05$). Although the addition of corticosterone to the cells reduced IL-6 production in both groups, the cytokine level in SDR

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**Table 1. Flow cytometric analysis of splenocyte phenotypic markers**

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<thead>
<tr>
<th>CD19 Depletion Study</th>
<th>CD11b Depletion Study</th>
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<tr>
<td>Control</td>
<td>SDR</td>
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<tr>
<td>Control</td>
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<td>CD19, %</td>
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<td>Predepletion</td>
<td>54 44</td>
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<td>Postdepletion</td>
<td>15 9</td>
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<tr>
<td>CD11b, %</td>
<td></td>
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<tr>
<td>Predepletion</td>
<td>4 10</td>
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<tr>
<td>Postdepletion</td>
<td>7 18</td>
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CD19 depletion study data correspond to data in Fig. 2A. CD11b depletion study data correspond to data in Figs. 2B and 3B. Predepletion percentages for both CD19 and CD11b are derived from analysis of the original splenocyte suspensions. Postdepletion percentages for both CD19 and CD11b are derived from analysis of the splenocyte suspensions after treatment with anti-CD19 or anti-CD11b magnetic beads.
cultures treated with the highest dose of corticosterone was equivalent to the baseline level in untreated control cultures. To determine if macrophages were responsible for the enhanced IL-6 secretion by splenocytes from SDR mice, we measured cytokine levels in supernatants of CD11b-depleted cultures. Removal of macrophages from the control suspension virtually eliminated the production of IL-6 (Fig. 3B). Low levels of IL-6 were detected in cultures from stressed mice and were likely due to the presence of residual macrophages following the depletion (Table 1).

DISCUSSION

Using an in vitro corticosterone sensitivity assay, these studies showed that LPS-stimulated splenocytes from socially disrupted mice were resistant to the immunosuppressive effects of glucocorticoids. Selective cell depletion studies suggested that the decreased sensitivity to corticosterone occurred specifically in the macrophage population and not in the B cell population. These results were obtained with the Promega CellTiter proliferation assay to measure splenocyte responses to mitogen stimulation. Because the substrate in the assay is converted by all living cells, processes (other than proliferation) that alter the cell numbers in culture, such as apoptosis or necrosis, would also affect the results. Therefore, this assay may actually be a measure of the net effects of SDR and corticosterone on splenocyte proliferation and viability. Because macrophages do not typically proliferate in culture, it may be that the macrophages from SDR mice were resistant to the apoptotic effects of corticosterone. Similar to our results with social disruption, other studies using rodent models of social stress have shown splenomegaly in subordinate animals (14, 25, 29) or a lack of immunosuppressive effects (6, 15, 18). None of these studies, however, assessed the effect of glucocorticoids on leukocytes. In humans, the chronic psychosocial stress of caring for a dementia patient has been associated with reduced lymphocyte sensitivity to glucocorticoids when stimulated in vitro with a T cell mitogen (4). In our model system, corticosterone resistance has not been seen reliably in SDR splenocytes treated with concanavalin A to stimulate T cells (data not shown). Therefore, in this murine model, the changes seem to be primarily in the macrophage population.

The development of glucocorticoid resistance in macrophages of defeated mice may be related to the generation of innate inflammatory responses. When mice are attacked by an intruder during social disruption stress, they are likely to receive bite wounds. In recent studies from our laboratory, splenocytes from individual SDR mice showed very different responses to corticosterone. Cells from some animals were suppressed by corticosterone, similar to control cells, whereas cells from other animals were completely insensitive. The phenomenon of glucocorticoid resistance was most evident in mice that were subordinate and received severe bite wounds (1a). In addition, the resistant animals had the largest spleens. These findings suggest that corticosterone insensitivity may develop in splenic macrophages as a result of the inflammatory response to tissue damage occurring during social disruption.

Tissue-specific glucocorticoid hyposensitivity is commonly caused by downregulation or decreased affinity of receptors (2), although it is not clear what might induce such changes in immune cells of SDR mice. The fact that some studies report immunosuppressive effects after social stress (6, 9, 12) suggests that not all social interaction paradigms promote the development of glucocorticoid resistance. In addition to the incidence of wounding, many factors may be important determinants, such as the species or strain of the animals and the chronicity or intensity of the stressor. It is possible that hormones released specifically during aggressive interactions affect the activation state...
or glucocorticoid sensitivity of macrophages. Alternatively, the change in cells from SDR mice may be due to increases in systemic or local cytokine concentrations as a result of the stress or an immune response to bite wounds. A combination of the cytokines IL-1β, tumor necrosis factor-α (TNF-α), and IL-6 has been reported to modulate glucocorticoid receptor responsiveness in a monocytic cell line (23). In addition, IL-1α has been found to inhibit nuclear translocation of the glucocorticoid receptor and prevent glucocorticoid receptor-mediated gene transcription in a mouse fibroblast cell line (19).

In addition to exhibiting insensitivity to the immunosuppressive effects of corticosterone, LPS-stimulated splenic macrophages from SDR mice produced more IL-6 than control cells. Statistical analysis of four separate SDR experiments did not show differences in the percentage of CD11b-positive cells between control and SDR spleens, suggesting that the macrophages from SDR mice may have been transcriptionally more active. Production of other proinflammatory cytokines did not appear to be enhanced, as levels of TNF-α and IL-1α were low to undetectable for both groups (data not shown). It is possible that IL-6 production is preferentially induced after SDR. Increased local or systemic levels of IL-6 have been associated with various autoimmune and inflammatory conditions in humans and animal disease models (13). However, it is not clear whether this cytokine always has proinflammatory actions, because it induces the secretion of glucocorticoids and acute-phase proteins, both of which have immunosuppressive activity (28).

Because of their numerous inhibitory effects on leukocytes, glucocorticoids are frequently used to treat patients suffering from inflammatory conditions such as asthma, rheumatoid arthritis, and leukemia. Although this therapy is usually effective, glucocorticoid-resistant asthmatics fail to respond to high doses of steroids. This resistance has been attributed to abnormalities in monocytes (30) and T cells (8). Furthermore, decreased sensitivity to glucocorticoids has been noted in immune cells of depressed (17) and HIV-infected (20) individuals. Further investigation, using animal models such as social disruption stress, of the mechanisms behind the development of glucocorticoid resistance may advance our understanding of disease.

**Perspectives**

Stress-induced increases in glucocorticoids are known to suppress the expression of genes required for recruitment of leukocytes to sites of infection or tissue damage (7). This can impair the immune response to the challenge. For example, it has been observed that restraint stress slows the healing of experimental cutaneous wounds in mice and that this effect is mediated by corticosterone (21). However, in addition to increasing plasma corticosterone, social stress in mice can result in fighting-induced wounds. Therefore, glucocorticoid resistance may be a mechanism to preserve the wound healing response in animals that receive injuries during aggressive social interactions. The tendency of macrophages from SDR mice to produce high levels of the B lymphocyte growth factor IL-6 might be important for generating a strong antibody response should an animal’s fighting-induced wounds become infected. In addition to affecting the innate inflammatory response, the interaction of resistant macrophages with T and B cells may also impact antigen-specific immune responses. Enhanced macrophage function (increased production of IL-6) may therefore explain the excessive inflammatory responses observed in socially stressed mice that were infected with influenza virus (D. A. Padgett, J. L. Stark, N. Quan, and J. F. Sheridan; unpublished observations). Although the development of glucocorticoid resistance may be adaptive for healing cutaneous injuries, it may be maladaptive if the resistant animal has a tendency toward autoimmune disease or is exposed to an infectious challenge.

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