

Social Stress Enhances Allergen-Induced Airway Inflammation in Mice and Inhibits Corticosteroid Responsiveness of Cytokine Production¹

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Chronic psychosocial stress exacerbates asthma, but the underlying mechanisms remain poorly understood. We hypothesized that psychosocial stress aggravates allergic airway inflammation by altering innate immune cell function. The effects of stress on airway inflammation, lung function, and glucocorticoid responsiveness were studied in a novel in vivo murine model of combined social disruption stress and allergic sensitization. The effects of corticosterone were assessed on cytokine profile and glucocorticoid receptor activation in LPS-stimulated spleen cell cultures in vitro. Airway inflammation resolved 48 h after a single allergen provocation in sensitized control mice, but not in animals that were repeatedly exposed to stress before allergen challenge. The enhanced eosinophilic airway inflammation 48 h after allergen challenge in these mice was associated with increased levels of IL-5, GM-CSF, IgG1, thymus-activated and regulatory chemokine, TNF- α , and IL-6 in the airways and a diminished inhibition of these mediators by corticosterone in LPS-stimulated splenocyte cultures in vitro. Stress-induced reduction of the corticosteroid effects paralleled increased p65 expression and a decreased DNA-binding capability of the glucocorticoid receptor in vitro. Furthermore, glucocorticoid receptor mRNA and protein expression in the lungs of mice exposed to both stress and allergen was markedly reduced in comparison with that in either condition alone or in naive mice. Thus, exposure to repeated social stress before allergen inhalation enhances and prolongs airway inflammation and alters corticosterone responsiveness. We speculate that these effects were mediated at least in part by impaired glucocorticoid receptor expression and function. *The Journal of Immunology*, 2009, 182: 7888–7896.

Psychological stress can modulate immune and inflammatory cell function through neural and hormonal pathways that link the autonomic nervous and the immune systems (1–9). Physical and psychological stressors were shown to activate the hypothalamic-pituitary-adrenal axis and increase levels of circulating glucocorticoid hormones. Endogenous circulating glucocorticoids in turn significantly modulate immune cell differentiation, distribution, and function, and regulate many aspects of the cross-talk between innate and adaptive immune systems (10–12). This mechanism is particularly important in the lung, where a pathogen- and inflammation-free environment is maintained by a finely orchestrated innate immune system. Stress-induced increase in corticosteroid plasma levels, for example, inhibits the IL-12/IFN- γ regulatory axis, eliciting an immune-suppressed state. Interestingly, despite the increased circulating corticosteroid and catecholamine levels, chronic psychosocial stress has been long considered a major cause of exacerbation of allergic airway in-

flammation (1, 2, 5, 7, 8, 13–16). Experimental studies showed that asthma patients have increased bronchoconstriction after distressful experiences (3), listening to stressful interactions (17), or exposure to asthma-related visual brain stimulation (13). Asthma is exacerbated in depressed and anxious patients who have low social support (18) and in children during school examinations (19).

Although the underlying mechanisms are not clear, there are indications that altered innate immune cell function may play a role. We have previously reported that LPS-stimulated splenocytes released more TNF- α and IL-6 in mice exposed to social stress than in control animals, and that these cells were less sensitive to the inhibitory effects of glucocorticoids (20).

Glucocorticoids activate intracellular receptors that are ubiquitously found in most cells (11, 21). The activated cytosolic glucocorticoid receptor (GR)³ translocates to the nucleus and acts as a ligand-dependent transcription factor that modulates the expression of glucocorticoid-responsive genes or modifies the activity of other transcription factors such as NF- κ B (10, 21). Recent studies in mice from our laboratories and others suggested that repeated social defeat may disrupt the ability of glucocorticoids to suppress inflammation (22–30). Although the exact mechanism of the impaired glucocorticoid action remains unknown, there was a decreased nuclear translocation of the GR in LPS-stimulated splenocytes in vitro, from mice that were repeatedly exposed to social stress (23, 28).

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³ Abbreviations used in this paper: GR, glucocorticoid receptor; *Af*, *Aspergillus fumigatus*; BAL, bronchoalveolar lavage; Ct, cycle threshold; GRE, glucocorticoid response element; HPA, hypothalamic-pituitary-adrenal; MCh, methacholine; Penh, enhanced pause; SDR, social disruption stress; TARC, thymus-activated and regulatory chemokine.

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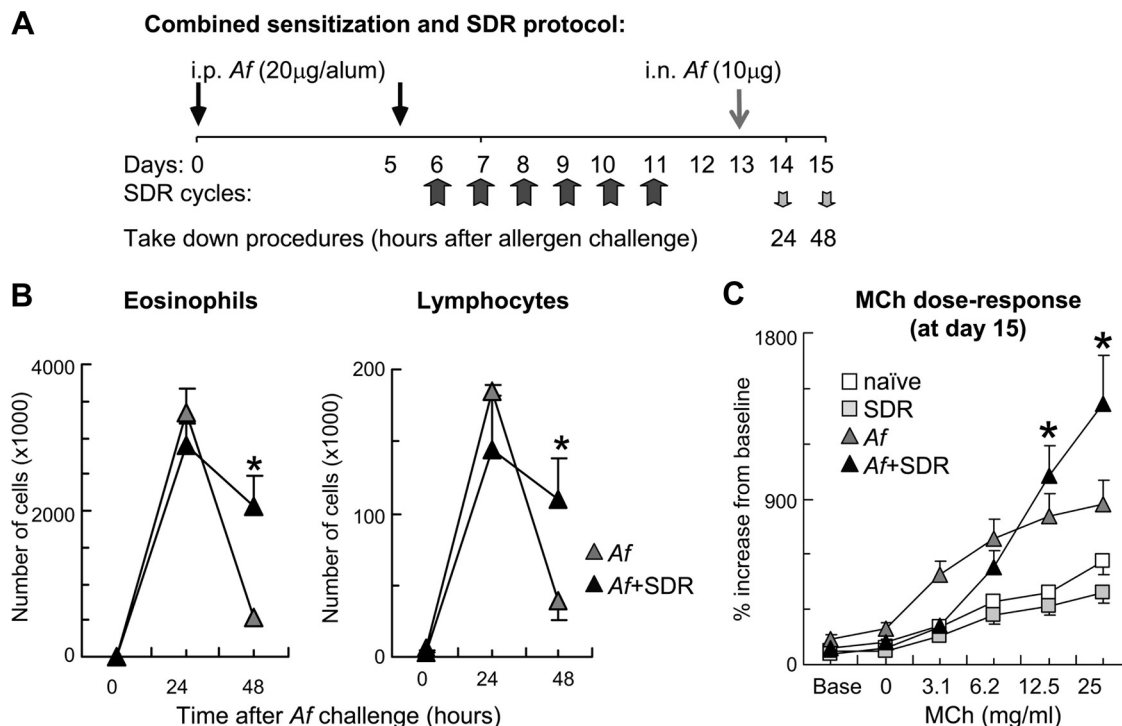


FIGURE 1. Combination of allergic airway sensitization and SDR caused delayed resolution of the inflammatory airway response and enhancement of allergic airway hyperresponsiveness at high MCh concentrations. **A**, In a combined protocol for allergic sensitization and SDR, mice received i.p. Af in alum on days 0 and 5, followed by repeated SDR for 2 h on days 6–11. Mice received intranasal (i.n.) allergen challenge on day 13 and were sacrificed either 24 or 48 h later. Control naive and SDR mice were sacrificed on day 15. **B**, Combination of allergic airway sensitization and SDR increased number of airway eosinophils and lymphocytes 48 h after Af challenge. BAL differential cell count was evaluated in Giemsa preparations. Naive and SDR mice did not receive allergen challenge. There was no difference between naive and SDR mice. **C**, Exposure to allergen and stress enhanced airway hyperresponsiveness at high MCh concentrations. Mice were studied 48 h after a single Af challenge. Mice received increasing doses of inhaled MCh. Lung function was assessed using noninvasive plethysmography and Penh. There was no difference between naive and SDR mice. Data are expressed as percentage of changes from baseline. Baseline Penh measurements were as follows: naive, 0.385 ± 0.050 ; SDR, 0.471 ± 0.028 ; Af, 0.924 ± 0.367 ; Af plus SDR, 0.614 ± 0.082 . **B** and **C**, Mean $\pm$ SEM of $n = 5$ –8 each; *, $p < 0.05$ (Af vs Af plus SDR).

To investigate the effects of psychosocial stress on the allergic airway response, we used a unique murine model that combined social disruption stress (SDR) and sensitization with an allergenic extract of *Aspergillus fumigatus* (Af). We hypothesized that social stress aggravates allergic airway inflammation by modifying innate immune cell production of cytokines, and that this effect is mediated by an altered corticosteroid action.

Materials and Methods

Mice

Male CD-1 mice were purchased from Charles River Laboratories. Mice that were studied for their stress and allergic responses (aged 6–8 wk) were allowed to acclimate to their new environment for 1 wk before experimentation and were maintained on a 12-h light/dark schedule (lights on at 6:00 a.m.) with food and water available ad libitum. All procedures were approved by the animal use and care committees at Ohio State University and University of Pennsylvania.

Social disruption model

SDR has been extensively used and has been previously described by our laboratory and others (26, 30, 31). Briefly, a retired large male breeder mouse (the intruder) was placed into the cage containing an established social hierarchy of three to five young male mice (the residents) for a 2-h confrontation that was repeated on 6 consecutive days. Disrupting an established social hierarchy by introducing an aggressive intruder results in intermale aggression and anxiety-like behavior. The confrontations were continuously monitored, and if severe fighting occurred, the aggressor was removed. The extent of anxiety that resulted from SDR was assessed using the open field test, as described previously (31). Mice in the control groups

were left undisturbed, with the exception of the handling associated with the vehicle or sensitization injections.

Allergic sensitization and challenge

Mice were sensitized by i.p. injection of 20 μ g of *Aspergillus fumigatus* (Af) extract (Hollister-Stier Laboratories) and 20 mg of $\text{Al}(\text{OH})_3$ (Imject Alum; Pierce) in PBS (100 μ l) on days 0 and 5 (32–34). Mice assigned to the stress condition were exposed to SDR on days 7–12. On day 13, all sensitized mice were intranasally challenged with 10 μ g of Af Ag in PBS (30 μ l) and then sacrificed on day 15. The following groups of mice were used: naive controls (no stress, no sensitization), SDR (SDR stress, no sensitization), Af (no stress, Af sensitization), and Af plus SDR (SDR stress, Af sensitization) (Figs. 1A and 2A).

Serum corticosterone RIA

Serum corticosterone was measured in duplicate, using a RIA kit (ImmuChem double Ab corticosterone ^{125}I RIA kit; MP Biomedicals), per manufacturer's instructions (27, 30). Blood was collected on day 15 (Fig. 1A) from the retroorbital plexus immediately after euthanasia, and serum was stored at -70°C until assay. The minimum sensitivity of the assay was 23.5 ng/ml.

Evaluation of airway hyperresponsiveness

Airway responsiveness to methacholine (MCh) was assessed in conscious, unrestrained, spontaneously breathing mice in a noninvasive whole body plethysmograph (Buxco Electronics), as described previously (32). The airway function of the animal was determined by a dimensionless parameter, enhanced pause (Penh), that reflects changes in box pressure from both the inspiration and expiration of the animal, along with the timing comparison of early and late expiration (pause). Each mouse received two baseline readings, followed by saline inhalation. Mice were then exposed

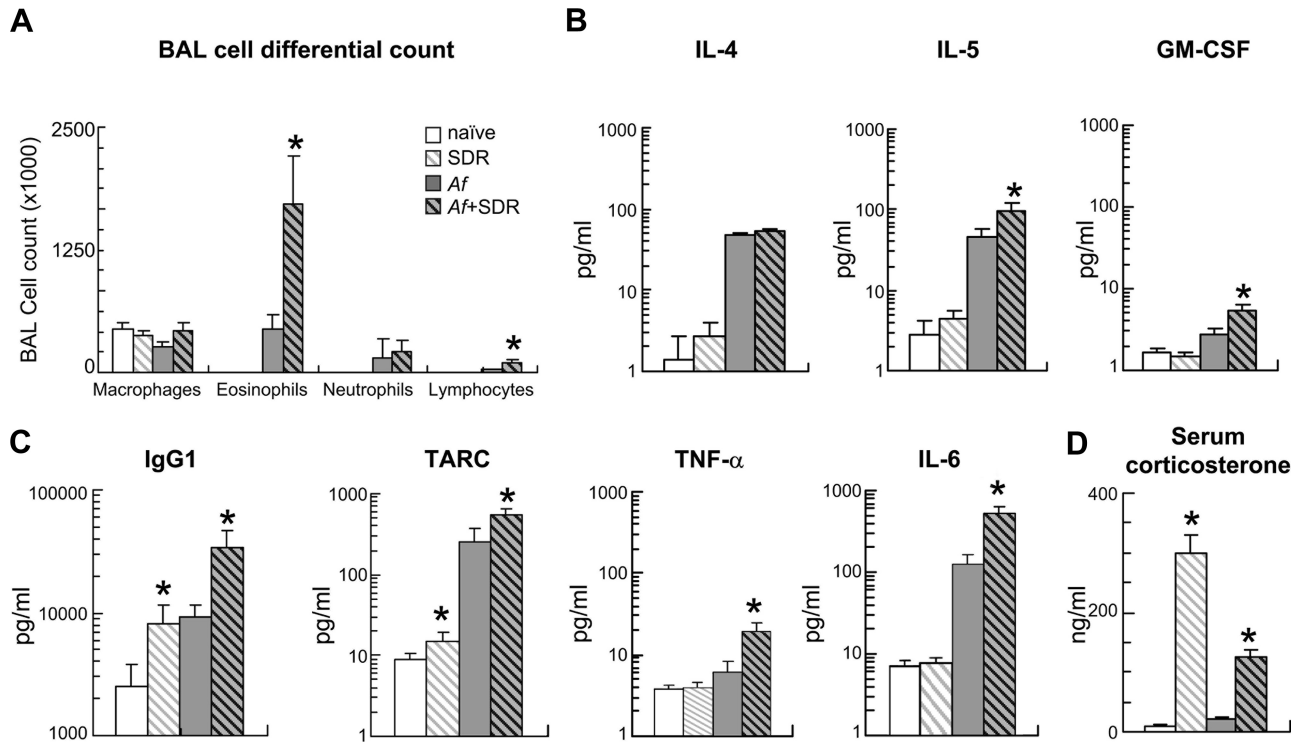


FIGURE 2. Mice were sensitized (i.p.) and challenged (intranasally) with *Af* extract, and were also exposed to social stress (SDR) daily between days 7 and 12, as indicated. Sensitized mice were studied 48 h after a single *Af* challenge (on day 15). Naive and SDR mice were also studied on day 15. **A**, BAL differential cell count was evaluated in Giemsa preparations. Stress and allergic sensitization enhanced numbers of eosinophils and lymphocytes, but not neutrophils and macrophages in the BAL fluid. There was no difference between naive and SDR mice. **B** and **C**, Chemokine, cytokine, and Ig levels in the BAL were measured in the same groups by SearchLight technology. Increased eosinophilia was paralleled by enhanced levels of the eosinopoietic IL-5 and GM-CSF (**B**), increased levels of the B cell-derived IgG1, and the innate immune cell-derived TARC, TNF- α , and IL-6 (**C**). **D**, Stress significantly increased serum corticosterone levels $p < 0.01$: SDR vs naive, *Af* or *Af* plus SDR. Blood for endocrine measures was taken on day 15. **A–D**, Mean $\pm$ SEM of $n = 8$ per group; *, $p < 0.05$ naive vs SDR and *Af* vs *Af* plus SDR; repeated measure ANOVA, followed by Tukey's honestly significant difference.

to aerosolized MCh at increasing doses of MCh, ranging from 3.1 to 25 mg/ml, for 1 min. Pulmonary function was averaged over the following 4-min period.

Bronchoalveolar lavage (BAL) analysis for differential cell count and cytokine, chemokine, and Ig levels

Lungs were lavaged with sterile PBS, as described previously (32, 34). Briefly, 0.75 ml of sterile PBS-containing protease inhibitors was injected and withdrawn twice through a tracheal canula by a 1-ml syringe. This procedure was repeated an additional two times with 1 ml of PBS each time. The withdrawn lavage volume (~2.5 ml) was collected and centrifuged at $400 \times g$ for 10 min at 4°C. The supernatant was aliquoted and frozen at -80°C until analysis. Total cell number in the BAL was determined by using a Z2 particle counter (Beckman Coulter). Differential cell counts were performed, as described previously (34).

Splenocyte survival assay

Glucocorticoid-induced cell death was quantified, as described previously (26, 30, 35). A single-cell splenocyte suspension was prepared, and triplicate samples were cultured (2.5×10^5 cells/well) in flat-bottom 96-well tissue culture plates. Cells were stimulated with 1 $\mu\text{g/ml}$ LPS and corticosterone (dose range 0.005–5 μM) for 48 h at 37°C and 5% CO_2 . Cell viability was measured with a tetrazolium substrate solution (Cell Titer 96 nonradioactive proliferation kit; Promega), and read at 490 nm by an ELISA plate reader. Cell viability was expressed as percentage of OD of the no-corticosterone control. The cell-free supernatant was collected at the end of the 48-h culture period and was kept at -80°C pending analysis.

Cytokine, chemokine, and Ig levels

The cell-free supernatant from the BAL and splenocyte cultures was investigated without concentration or dilution using SearchLight mouse multiplex arrays. These arrays were performed by the manufacturer (Pierce Biotechnology).

GR-DNA-binding activity

Splenocytes were cultured in the presence of 1×10^{-7} M dexamethasone to determine GR-binding activity in nuclear extracts. Nuclear extraction was performed, as described previously (36, 37). GR-binding activity was assessed by adding 10 μg of nuclear extract to TransAM GR kits, according to the manufacturer's instructions (Active Motif). The ODs of the plates were measured at 450 nm and expressed as a percentage change from untreated cells.

GR α Western blot analysis

A total of 50 μg of lung or 25 μg of spleen lysates was applied to Minigel and run and transferred in semidry systems under standard conditions. Anti-GR α (Affinity BioReagents; 0.1 $\mu\text{g/ml}$) or anti-GAPDH (Sigma-Aldrich; 1:50,000) Ab was added for 1 h at room temperature. Optimal sample concentration and Ab dilutions as well as incubation times were determined by pilot experiments. Secondary Ab (HRP-conjugated goat anti-rabbit Ab; Chemicon International; diluted at 1/100) was added for 1 h at room temperature. For GAPDH Chemicon rabbit anti-mouse HRP was used at 1:10,000. We used the ECL plus Western detection system (Amersham) and Gel-Pro Analyzer 6.0. GR bands were normalized to GAPDH.

Real-time PCR

A relative quantitation of lung GR (nuclear receptor 3c1) mRNA expression was performed by real-time PCR using TaqMan gene expression assay (Mm00433832) and TaqMan Universal PCR Master Mix (Applied Biosystems), according to the manufacturer's instructions. In brief, total RNA (four samples per time point) was reverse transcribed using Superscript III First Strand Synthesis System (Invitrogen). Approximately 100 ng of cDNA was used per singleplex PCR in a total volume of 20 μl . Cycling was performed on an ABI SDS-7000 and ABI SDS-7500, respectively, with an initial denaturation step at 95°C for 10 min and 40 cycles of 15 s at 95°C and 1 min at 60°C . Every sample was run in triplicate, and β_2 -microglobulin (Mm00437762) was used as endogenous control. The

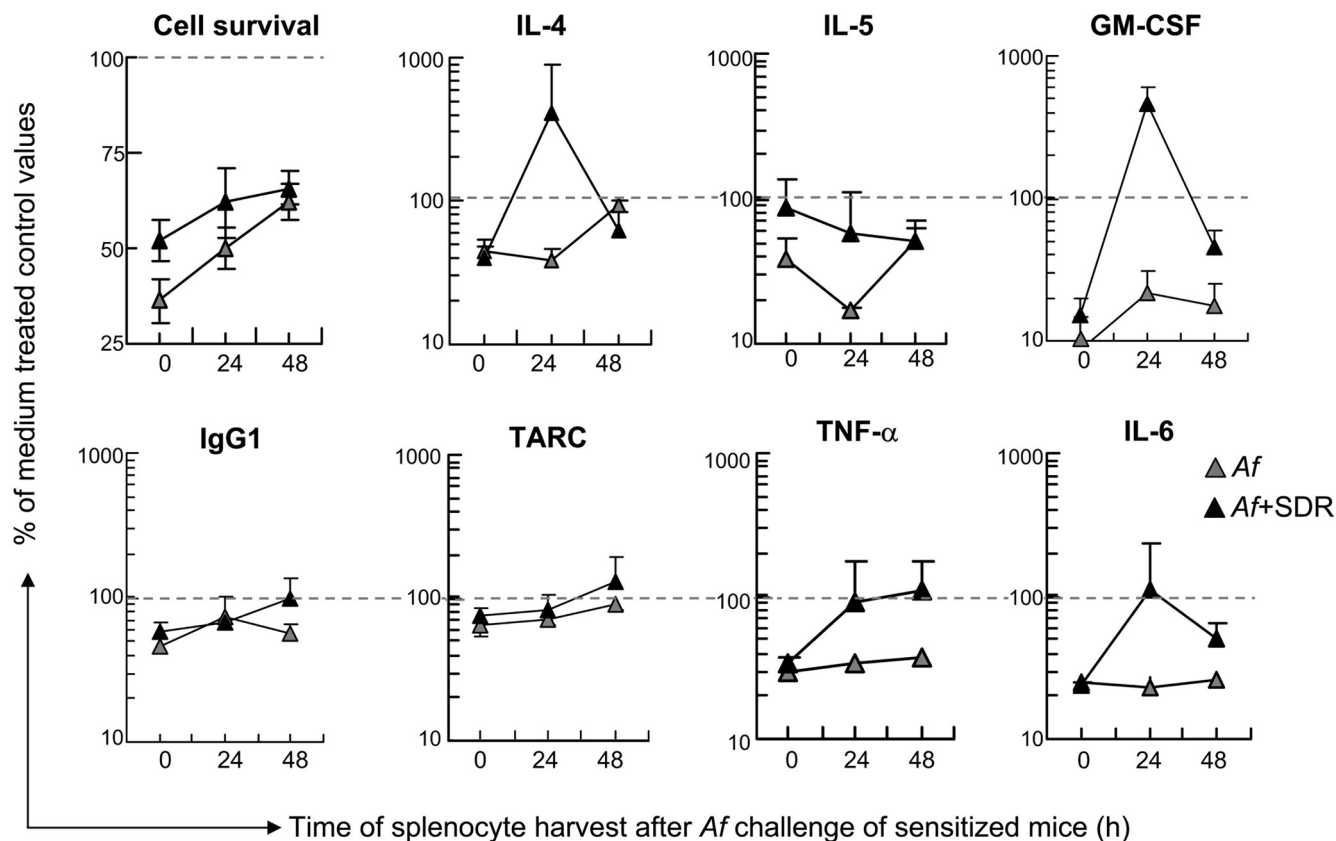


FIGURE 3. Combination of stress and allergen challenge attenuated the time-dependent inhibitory effect of corticosterone on splenocytes. Spleens were harvested before (0 h) and 24 and 48 h after *Af* challenge. Splenocytes were cultured in the presence of LPS (1 μ g/ml) and 5 μ M corticosterone for 48 h. Cell viability (top left panel) was determined using a colorimetric assay. Cytokine, chemokine, and Ig levels in the supernatant were measured by SearchLight technology. Data are presented as the percentage of cell viability or cytokine, chemokine, and Ig concentration of control values measured in the absence of corticosterone, marked by dashed lines (mean $\pm$ SEM of $n = 5$ –8 per group).

comparative cycle threshold (Ct) method was used to analyze the results using ABI PRISM(R) 7900 SDS software. Results are expressed as fold difference (with range incorporating the SD of the $\Delta\Delta$ Ct value into the fold difference calculation) relative to the naive controls.

Results

Social stress inhibited resolution of allergen-induced airway inflammation

Previously, we showed that SDR induced significant alterations in the function of splenic leukocytes (26, 30, 31, 38). To investigate whether immune alterations also occur in the lung in response to repeated stress, we combined mouse models of allergen-induced AHR and SDR, as illustrated in Fig. 1A. The cellular and cytokine profiles in the BAL of mice exposed to *Af* and SDR were assessed. The behavioral effects of social stress exposure were confirmed using open field testing. SDR markedly reduced the amount of time the mice spent in the center of the open field (an indication of increased anxiety-like behavior) (31), whereas allergen exposure alone had no effect (data not shown). *Af* challenge of sensitized mice induced influx of inflammatory cells into the airways that peaked 24 h and partially resolved 48 h after *Af* exposure. Clearance of eosinophils and lymphocytes from the airways was markedly impaired in mice that were exposed to stress before allergen challenge (Fig. 1B). Significantly greater numbers of eosinophils and lymphocytes in the BAL fluid were observed in stress- and allergen-exposed mice in comparison with that obtained with allergen alone ($p < 0.05$; Fig. 1B).

We have previously performed extensive kinetic studies on airway function 1, 6, 12, 24, 48, 72, and 96 h after a single *Af* chal-

lenge and found that both baseline Penh and MCh responsiveness reached a peak 24 h after *Af* inhalation that was subsequently resolved (32). Although it is possible that stress affects AHR at earlier time points, detailed kinetic studies on the effects of stress on airway physiology exceeded the scope of the current paper. The main goal in this study was to investigate whether the prolonged airway eosinophilia we observed 48 h after *Af* was associated with increased AHR. Although Penh in the *Af* plus SDR group at low doses (i.e., 3.1 mg/ml) was lower than in the *Af* group, there was a significantly enhanced AHR to inhaled MCh at 12.5 and 25 mg/ml, as assessed by whole body plethysmography in the *Af* plus SDR group ($p < 0.05$; Fig. 1C).

Despite increased serum corticosterone levels, combination of SDR and allergen challenge enhanced airway inflammation 48 h after allergen challenge

Differential cell counts 48 h after *Af* challenge showed that stress exposure of *Af*-sensitized and -challenged mice significantly enhanced the numbers of eosinophils and lymphocytes, but not neutrophils and macrophages in the BAL fluid (Fig. 2A). Social stress by itself had no effect on leukocyte trafficking into the BAL fluid because the total and differential cell counts were similar between naive controls and the SDR mice. The increased eosinophilia paralleled enhanced levels of the T cell-derived eosinopoietic IL-5 and GM-CSF (Fig. 2B).

SDR and allergen markedly amplified IgG1 and the chemokine, thymus-activated and regulatory chemokine (TARC) levels in the BAL fluid as compared with that of either exposure alone. It is

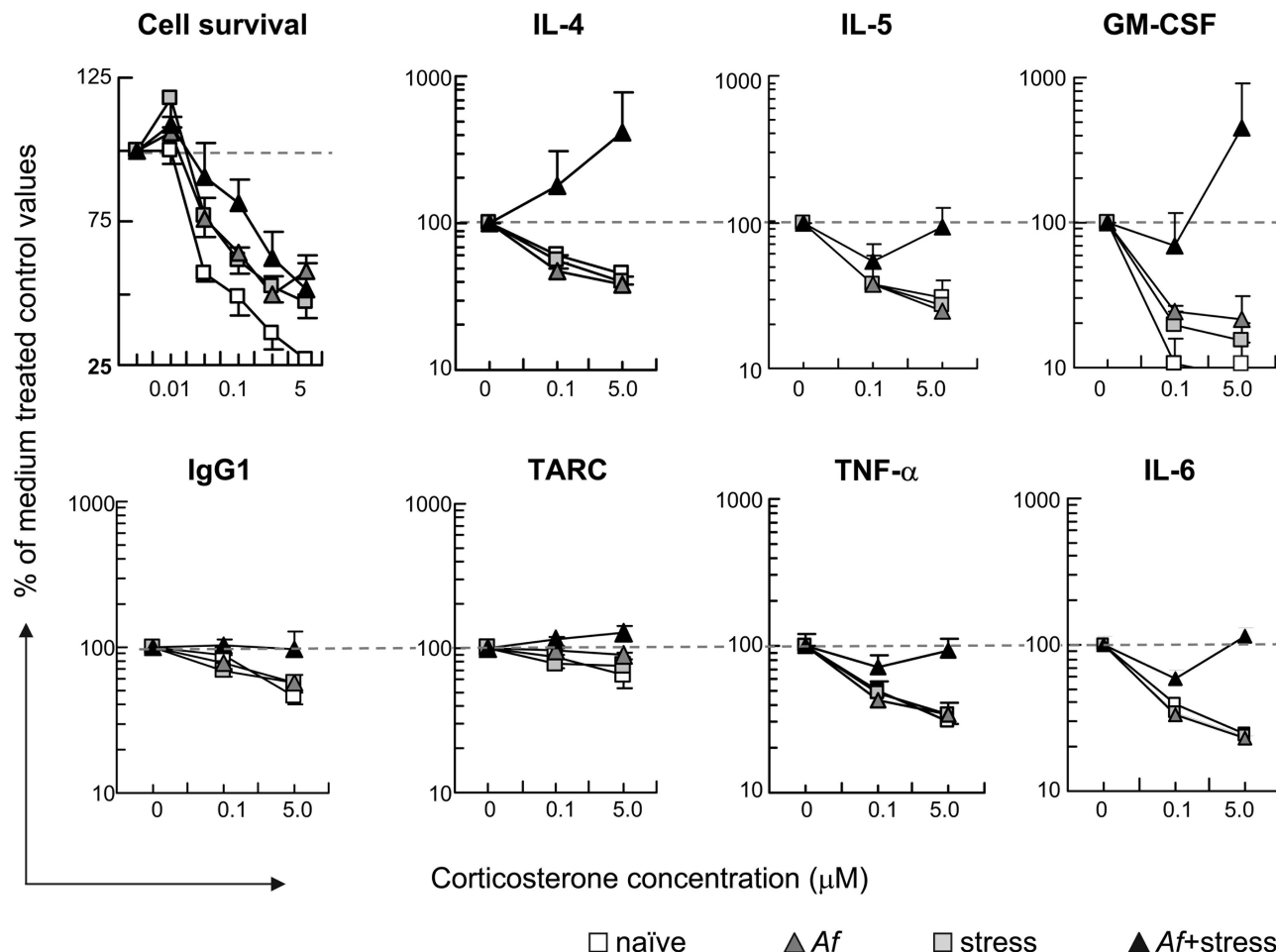


FIGURE 4. Combination of stress and allergen challenge abolished the dose-dependent inhibitory effects of corticosterone on LPS-stimulated splenocytes. Spleens were harvested from naïve ($\square$) or SDR controls ($\blacksquare$), and from *Af*-sensitized mice 24 h after *Af* challenge (*Af*, gray triangles; *Af* plus SDR, $\blacktriangle$). Splenocytes were cultured in the presence of LPS ($1 \mu\text{g/ml}$) and 0, 0.01, 0.05, 0.1, 0.5, or $5 \mu\text{M}$ corticosterone for 48 h. Cell viability (top left panel) was determined using a colorimetric assay. Cytokine, chemokine, and Ig levels in the supernatant were measured by SearchLight technology in each group. Data are presented as the percentage of cell viability or cytokine, chemokine, and Ig concentration of control values measured in the absence of corticosterone, marked by dashed lines (mean $\pm$ SEM of $n = 5$ –8 per group).

noteworthy that SDR by itself significantly increased IgG1 and TARC in the BAL without allergen exposure, suggesting that stress can skew the immune milieu in the lung through innate mechanisms. For this reason, TNF- α and IL-6 that are typically produced by alveolar macrophages and activated dendritic cells were also studied. Indeed, both cytokines were consistently elevated in the BAL of mice that received a combination of *Af* and social stress (Fig. 2C), indicating involvement of the innate immune system. Furthermore, SDR by itself elicited proinflammatory changes not only in the lung, but also in the spleen of mice. We measured an increased splenic mass and heightened translocation of NF- κ B (p65) in the nuclear extract from splenic mononuclear cells together with an increased ability to produce TARC and IgG1 upon LPS stimulation (data not shown).

Proinflammatory mediator release in the SDR mice occurred in the presence of a ~ 30 -fold increase of the circulating corticosterone levels (Fig. 2D, gray/whited hatched bar). Although there was less corticosterone in the *Af* plus SDR group (gray/black hatched bar; $p < 0.05$ vs SDR), it was still considerably (~ 15 -fold) greater than in the nonstressed mice. Mice in the *Af*-alone group (gray bar) did not have elevated corticosterone, indicating that handling and manipulation associated with sensitization and challenge or with the euthanasia did not affect corticosterone release.

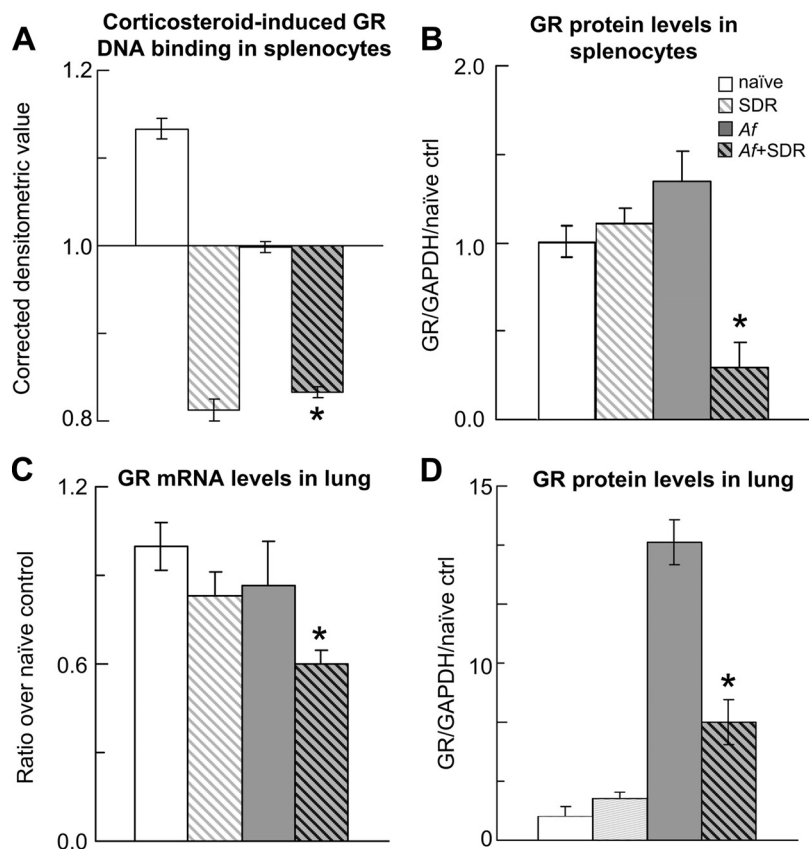
Our results raised the possibility that social stress reduces the inhibitory effects of corticosteroids on innate immune function in allergen-challenged mice. To address this hypothesis, corticosteroid responsiveness of the innate immune cells was investigated in LPS-stimulated splenocytes.

Stress and allergen exposure reduced the inhibitory effects of corticosterone

In vitro treatment with corticosterone ($5 \mu\text{M}$) significantly inhibited survival of cells as well as release of cytokines, chemokines, and IgG1 in splenocytes derived from mice before allergen challenge (0 h), as shown in Fig. 3 (gray triangles). After *Af* challenge, however, particularly in mice exposed to both stress and *Af* ($\blacktriangle$), there was a time-related reversal of the inhibitory effects.

Splenocyte function, including the release of cytokines, should normally be significantly inhibited by corticosteroids. Such dose-dependent inhibition is clearly shown in Fig. 4 in the cells derived from *Af*-only-treated mice (gray triangles). However, in splenocytes derived from the *Af* plus SDR group, the inhibitory effects of corticosterone were completely abolished, and on IL-4, GM-CSF, and TARC it appeared to be even reversed: instead of inhibition, corticosterone stimulated release of these cytokines in cells from the *Af* plus SDR group. These results supported the hypothesis that

FIGURE 5. To study the effects of stress and asthma on function and expression of the GR, splenocytes and whole lung tissue were investigated. Naive mice (□) and mice subjected to stress (SDR, ▨), allergen (Af, ▩), or allergen and stress (Af + SDR, gray hatched bars) were studied. On day 15 (48 h after Af challenge), spleens for GR nuclear translocation and lungs for GR mRNA and protein expression were harvested. *A*, SDR inhibited nuclear translocation and GRE binding of the GR. Splenocytes were isolated and incubated in the presence of dexamethasone (10^{-7} M). GR nuclear translocation was quantitated by an ELISA-based TransAM GR kit with GRE-coated wells. Mean $\pm$ SD of $n = 3$ (duplicate experiments). Densitometric data are corrected for values obtained from HeLa cell-positive controls. *B* and *D*, Combination of Af and SDR reduced expression of GR protein. Total protein was isolated from spleens (*B*) and lung tissue (*D*). Western blot analysis was performed using an anti-mouse GR α mAb. Expression was assessed using densitometric analysis and GAPDH as control. Mean $\pm$ SEM of $n = 6$ (duplicate experiment). *C*, Combination of SDR and Af reduced expression of GR mRNA in the lung tissue. Total RNA was isolated from lungs of mice. RT-PCR and real-time PCR were performed. β_2 -macroglobulin mRNA was used as control. Data were analyzed using the $\Delta\Delta C_t$ method. Mean $\pm$ SEM of $n = 4-6$ (duplicate experiments). *A-D*, *, $p < 0.05$, Af vs Af plus SDR.



corticosteroid responsiveness of immune cells (i.e., susceptibility to inhibition) in the spleen of mice that were subjected to Af plus SDR is significantly impaired. The combination of stress and Af appeared synergistic in reversing the inhibitory effects of corticosterone.

Stress and allergen exposure inhibits the function and expression of the GR

To determine whether the stress-reduced glucocorticoid inhibition was due to an impaired function of the GR, binding to glucocorticoid response elements (GRE) in nuclear fractions from cells of naive, SDR, Af, and Af plus SDR mice was studied. Samples were stimulated with dexamethasone for 2 h. The ratio of GRE-bound GR in the nuclear extract of cells was significantly less in the SDR-, Af-, or Af plus SDR-exposed groups upon dexamethasone stimulation in comparison with the naive control group, as shown in Fig. 5A. However, there was no difference between the SDR and the Af plus SDR groups. We therefore also studied GR protein expression in splenocytes of the same groups. Using an anti-mouse GR α mAb, we found that GR expression in splenocytes of the Af plus SDR mice was significantly lower than in the Af group (Fig. 5B).

To confirm the effects of Af plus SDR on GR expression locally in the lung, we studied GR gene activation in the whole lung tissue of mice. Real-time PCR showed that Af plus SDR significantly inhibited GR mRNA expression in the lung (Fig. 5C). Furthermore, Western blot analysis of lung extracts from the mice showed that sensitization and challenge with Af significantly increased GR expression compared with the non-Af-exposed mice. However, this increase was markedly attenuated in the Af plus SDR group (Fig. 5D). These data indicated that the impaired resolution of the airway proinflammatory changes 48 h after allergen challenge in

stress-exposed mice was associated with a reduction of GR protein expression in the lung.

Discussion

This study demonstrates that social stress prolonged the airway inflammatory response to allergen challenge in mice. The enhanced inflammatory changes included MCh hyperresponsiveness at high concentrations, increased eosinophil and lymphocyte count, and elevated levels of cytokines, chemokines, and Igs, reflecting activation of both the innate and adaptive immune system. Importantly, heightened inflammation occurred in the presence of elevated serum corticosterone in mice exposed to social stress and allergen inhalation, suggesting a diminished inhibitory action of endogenous corticosteroids. Indeed, the combination of stress and allergen exposure profoundly altered the *in vitro* corticosteroid responsiveness of LPS-stimulated splenocytes and inhibited GR nuclear translocation. Furthermore, social stress significantly diminished GR expression in the spleen as well as in the lung of mice sensitized and challenged with Af. Based on these data, we propose that repeated exposure to social stress skews the immune status toward a Th2 type in the airways and predisposes to a persistent and amplified inflammation after allergen challenge. These changes, in part, may be due to endogenous corticosteroid insensitivity, mediated by decreased function and expression of the GR.

Although stress may impair asthma control (1, 2, 5, 7, 8, 13–16), little evidence suggests that stress directly modulates immune function in the disease. In this study, we describe a unique murine model in which social stress and allergen exposure provides a platform to investigate the psycho-endocrine-immunological axis that regulates airway inflammation. Social disruption, a well-characterized psychological stressor, has been used by our laboratory and others to assess the impact of a seminatural stressor on immune

function (35, 39–42). In this model, a previously established social hierarchy of a stable cohort of younger male mice is disrupted by a large, aggressive male that confronts and repeatedly defeats the cage residents. This repeated defeat leads to physiological and behavioral responses (collectively referred to as the stress response), such as development of anxiety-like behaviors (31) and glucocorticoid release (22, 43). Endogenous glucocorticoid levels can regulate the development, distribution, and function of immune and structural cells (10–12), but the effects of stress-induced glucocorticoid release on the pulmonary immune function have not been studied in detail.

Levels of the Ig IgG1 and TARC (CCL17), a chemokine produced by dendritic cells and macrophages and responsible for attracting Th2 lymphocytes and activated dendritic cells (44), were enhanced in mice exposed to SDR alone. Both IgG1 (45) and TARC (44) are under the regulation of IL-4/IL-13 and are part of the Th2 immune response. Because these cytokine/chemokine and Ig changes occurred without the presence of antigenic stimulation, we hypothesized that alterations in innate immune cell function played an important role. TNF- α and IL-6 are prominently involved in allergen-induced inflammation, but are also relevant to LPS-stimulated innate immune response. Therefore, these cytokines are highly suitable to assess the activation state of innate immune cells both in the lung (in vivo) and in the spleen (in vitro). Activation of dendritic cells and macrophages (46) was confirmed by consistently elevated TNF- α and IL-6 in mice that received Af plus SDR.

Because TARC and IgG1 are characteristic players of a Th2-type immune response, we also raised the question whether animals exposed to repeated social stress would have an increased susceptibility to developing allergic airway inflammation (32–34, 47). Indeed, combined exposure to allergen and stress significantly enhanced the numbers of eosinophils and lymphocytes in the airways. These cells interact during the allergic airway response through the T lymphocyte-derived IL-4, IL-5, and GM-CSF, which are essential for promoting allergen-induced tissue eosinophilia. The genes for these cytokines are located in a cluster of chromosome 5q and are all sensitive to corticosteroid regulation. The enhanced airway eosinophilia 48 h after allergen challenge was paralleled by increased release of the eosinopoietic IL-5 and GM-CSF, as well as IgG1 and TARC in the airways of mice exposed to both stress and allergen challenge.

Although ours is the first study that investigated the effects of SDR, stress-induced enhancement of allergic airway inflammation has been noted in different models. In rats, the authors showed an increased cellular influx into inflamed paws and airways in stressed animals (48). In a murine model of ultrasound stress and OVA-induced allergic airway sensitization, stressed mice displayed not only increased cell number, but also heightened release of IL-4, IL-5 (49), TNF- α (50), and eotaxin (51) in comparison with nonstressed mice. Using a different sound stress model on pregnant BALB/c mice, Pincus-Knackstedt et al. (52) showed that maternal stress increased susceptibility of the offspring to allergic sensitization to OVA.

The underlying mechanisms of the enhancement of allergic airway inflammation by SDR are unclear. One of the earliest findings in various models of stress has been an acute atrophy of the immune organs and an increased susceptibility to viral or bacterial infections due to the high circulating levels of corticosteroids and a suppressed immune system (53, 54). Stress in the SDR model, however, was not immunosuppressive. We found increased spleen mass and enhanced NF- κ B p65 expression in the nuclear extract of the SDR mice (data not shown), suggesting activation (not suppression) of the innate immune cells and Th2-type immune medi-

ators. How SDR may differ from other models of stress needs further elucidation, but there are indications that both the duration and type of stressors applied can alter the effects of corticosteroids on the immune system. For instance, Okuyama et al. (55) found that in contrast to acute stress, chronic restraint stress significantly increased the number of eosinophils and lymphocytes as well as the amount of IL-4 and IL-5 released in BALB/c mice in response to allergen challenge, indicating that acute and chronic stress may affect the allergic airway response differentially. Opposing effects of short- and long-term stress on airway inflammation were also suggested in models of restraint and the forced swim test. Forsythe et al. (56) found that the inhibitory effects of short-term stress on airway inflammation were prevented by the GR antagonist, RU486, suggesting that acute release of endogenous corticosteroids may attenuate the inflammatory airway response. Corticotropin-releasing hormone knockout mice in a model of allergic airway sensitization responded with a significantly enhanced cellular inflammation to allergic sensitization compared with wild-type mice (57). Similarly, inhibition of the GR with RU-486 enhanced stress-induced eosinophilia in the bone marrow of allergen-sensitized animals in another study. Furthermore, inhibition of glucocorticoid release by metirapon also increased eosinophil numbers, suggesting that acute stress-induced endogenous corticosteroids may be important in attenuating immunological changes (58).

Interestingly, using a psychological stressor, Chida et al. (59) more recently showed that pretreatment of mice with RU486 before allergen challenge completely inhibited stress-induced exacerbation of airway inflammation, suggesting that increased levels of circulating corticosterone may be responsible for down-regulating glucocorticoid responsiveness in inflammatory cells, including eosinophil granulocytes. Steroid responsiveness is highly important during the resolution phase of the inflammatory response. For example, eosinophils were shown to have defective caspase-induced apoptosis in corticosteroid-resistant asthmatic individuals (60). Thus, low baseline levels of endogenous corticosterone may be sufficient to facilitate eosinophil clearance, whereas high levels seen in stressed mice may cause a steroid unresponsiveness in these cells. Comparison of our SDR stressor with the nonpsychological repeated restraint stress paradigm in our previous studies (using similar duration) also suggested differential effects on the immune system (61, 62). Although the hypothalamic-pituitary-adrenal (HPA) axis and the sympathetic nervous systems were similarly activated by the two stressors, repeated restraint resulted in a disruption of the circadian rhythm of the HPA axis and a prolonged elevation of circulating corticosterone with splenic hypotrophy, suppressed mononuclear cell function, and cytokine production (61). These responses were restored by blocking the glucocorticoid action with RU486 (63). In comparison, whereas repeated cycles of SDR elevated circulating corticosterone levels, it did not disrupt the circadian rhythm of the HPA axis, cause splenic hypotrophy, or suppress mononuclear cell activation (38). In addition, development of glucocorticoid resistance to inhibition of cytokine production by splenocytes (23) was seen in SDR, but not restraint stress (20). Taken together, exposure to sound (49–52) or psychological stressors (59) is more likely to predispose to an allergic immune response than restraint stress. Furthermore, whereas the acute stress response is immunosuppressive, after a prolonged or repeated stress exposure glucocorticoid unresponsiveness may develop. Our results corroborate these findings, and the in vitro splenocyte studies suggest the novel concept that stress and allergen are synergistic in reversing the immunosuppressive action of corticosteroids on proinflammatory cytokine production by immune cells.

Although the mechanisms of such synergistic effects between stress and the allergic immune response need further elucidation, it was previously shown that stress-induced disruption of the glucocorticoid action was associated with a failure of the GR to translocate to the nucleus (23, 28). In this study, we quantified GR binding to GRE in the nuclear fractions of splenocytes stimulated with dexamethasone (a steroid that binds both the glucocorticoid and mineralocorticoid receptor with high affinity). Stress by itself significantly inhibited GR binding to GRE, confirming our previous studies (23, 28), but additional *Af* exposure of the stressed mice did not enhance this effect. We therefore could not explain the synergistic reversal of corticosteroid action we saw on the LPS-stimulated cells from the *Af* plus SDR group. Because constitutive GR expression is essential for an adequate glucocorticoid action, we also investigated GR expression both in the spleen and the lung of mice. GR mRNA and protein expression was significantly inhibited in the *Af* plus SDR group in comparison with the *Af*-sensitized control mice. Although there are a number of pathways through which the corticosteroid effects can be changed, we speculate that an impaired nuclear translocation and DNA binding together with reduced GR mRNA and protein expression could be responsible for the observed alterations in corticosteroid responsiveness.

Our results raise a number of intriguing questions. For instance, decreases in GR mRNA could be due to reduced mRNA stability, suppression of promoter activation, or both (64). Furthermore, GR expression could be reduced by homologous ligand down-regulation (by GR agonists) in a number of tissues and cell types, but interestingly, not in T lymphocytes (64). Although we showed that innate immune cells are important, the cell types that are ultimately responsible for mediating the effects of corticosteroid insensitivity are yet to be identified. Expression of the GR falls under complex transcriptional and posttranslational regulation that can be significantly modified by inflammatory processes during the allergic airway response (65). Indeed, reduced GR expression was found in patients with asthma and chronic obstructive pulmonary disease with insensitivity to corticosteroid treatment (66) and is thought to be an important factor in glucocorticoid resistance. Finally, the expression and function of the human GR are somewhat different from that of the mouse. Specifically, the human GR α isoform can mutually trans-repress NF- κ B, a complex mechanism that could be important in mediating steroid resistance in the inflamed tissue (64, 66). Whether such interaction could be reproduced in mice is currently unclear.

Although previous reports indicated the importance of stress in induction of AHR and airway inflammation in mice (49, 50, 52, 55–59, 67), our study is the first demonstration that social stress enhanced the allergic airway response in association with innate immune cell corticosteroid insensitivity and impaired GR translocation and expression. These findings are significant because they may elucidate potential mechanisms of asthma exacerbation and diminished effectiveness of corticosteroid therapies.

Disclosures

The authors have no financial conflict of interest.

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