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Repeated Allergen Exposure in A/J Mice Causes Steroid-Insensitive Asthma via a Defect in Glucocorticoid Receptor Bioavailability

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The importance of developing new animal models to assess the pathogenesis of glucocorticoid (GC)-insensitive asthma has been stressed. Because of the asthma-prone background of A/J mice, we hypothesized that asthma changes in these animals would be or become resistant to GCs under repeated exposures to an allergen. A/J mice were challenged with OVA for 2 or 4 consecutive d, starting on day 19 postsensitization. Oral dexamethasone or inhaled budesonide were given 1 h before challenge, and analyses were done 24 h after the last challenge. Airway hyperreactivity, leukocyte infiltration, tissue remodeling, and cytokine levels as well as phosphorylated GC receptor (p-GCR), p-GATA-3, p-p38, MAPK phosphatase-1 (MKP-1), and GC-induced leucine zipper (GILZ) levels were assessed. A/J mice subjected to two daily consecutive challenges reacted with airway hyperreactivity, subepithelial fibrosis, and marked accumulation of eosinophils in both bronchoalveolar lavage fluid and peribronchial space, all of which were clearly sensitive to dexamethasone and budesonide. Conversely, under four provocations, most of these changes were steroid resistant. A significant reduction in p-GCR/GCR ratio following 4- but not 2-d treatment was observed, as compared with untreated positive control. Accordingly, steroid efficacy to transactivate MKP-1 and GILZ and to downregulate p-p38, p-GATA-3 as well as proinflammatory cytokine levels was also seen after two but not four provocations. In conclusion, we report that repeated allergen exposure causes GC-insensitive asthma in A/J mice in a mechanism associated with decrease in GCR availability and subsequent loss of steroid capacity to modulate pivotal regulatory proteins, such as GATA-3, p-p38, MKP-1, and GILZ. *The Journal of Immunology*, 2018, 201: 000–000.

Asthma is a chronic pulmonary disease that affects millions of people globally, 55% of them having inadequate control of their symptoms despite recognized advances in currently available therapy. There are ~10% of moderate-to-severe asthmatics responding poorly to standard asthma treatments (1–4). Some triggers of asthma, like allergens, usually mount a type 2 inflammatory process, orchestrated by CD4 lymphocytes and eosinophils, and respond to inhaled glucocorticoids (GCs) (1, 5). There are also uncontrolled asthmatics exhibiting persistent airway eosinophilia, despite continued use of systemic synthetic steroids (6). Other triggers, including cigarette smoke or viruses, do not induce a typical type 2 inflammatory response, promoting instead a “type 2, low asthma” profile,

characterized by neutrophilic infiltration and resistance to steroid therapy (1, 7). An estimated 50% of asthma cases are related to eosinophilic airway inflammation, with the rest being linked to neutrophilic airway inflammation (8).

The impairment of GC responsiveness is multifactorial, being associated with overexpression of proinflammatory cytokines and functional changes in GC receptor (GCR), including mutations and impaired phosphorylation, among others (9, 10). However, the molecular mechanism underlying steroid resistance remains poorly understood (5, 11, 12). It is hoped that the development of short-term animal models that more properly mimic GC refractoriness and the hallmark features of asthma will be helpful in the identification of associated mechanisms and novel therapies (13–15). In this article, we report the development of a short-term murine model of asthma, in which actively sensitized A/J mice, exposed to four consecutive daily allergen provocations, reacted with airway hyperreactivity (AHR), subepithelial fibrosis, and tissue eosinophil accumulation, all of which were clearly resistant to GC treatment. The model also enabled us to demonstrate that the state of steroid resistance observed in A/J mice is strain specific and correlates with the steroid incapacity to downregulate the expression of GATA-3 and p-p38 and to upregulate MAPK phosphatase-1 (MKP-1) and GC-induced leucine zipper (GILZ), in a mechanism probably associated with a defect in the GCR signaling.

Materials and Methods

Male A/J and BALB/c mice were obtained from the Oswaldo Cruz Foundation Breeding Center. The Committee on Use of Laboratory Animals of the Oswaldo Cruz Foundation (license number, CEUA L-034/09; Rio de Janeiro, Brazil) approved all protocols and experimental procedures involving animals.

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Abbreviations used in this article: AHR, airway hyperreactivity; BAL, bronchoalveolar lavage; GC, glucocorticoid; GCR, GC receptor; GILZ, GC-induced leucine zipper; MKP-1, MAPK phosphatase-1; Penh, enhanced pause.

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Immunization and Ag challenge

Mice were immunized s.c. on day 0 and boosted 14 d later with 50 µg of OVA (Grade V; Sigma-Aldrich, Milwaukee, WI) adsorbed to 5 mg of Al (OH)₃ in 200 µl of 0.9% NaCl sterile (saline). Intranasal OVA challenges (25 µg/25 µl in saline) were administered using two different protocols under halothane volatile anesthesia (Cristália, São Paulo, Brazil): groups of OVA-immunized, challenged on days 19 and 20 (Fig. 1A) or on days 19, 20, 21, and 22 (Fig. 1B). Control mice were immunized with OVA and challenged with saline using the same protocol described above.

Treatment with dexamethasone and budesonide

Just 1 h before each allergen provocation, mice received two or four daily oral administrations of dexamethasone (3 mg/kg) (dexamethasone 21-phosphate; Sigma-Aldrich) (16) or vehicle (saline) by gavage as indicated (Figs. 1, 4). In another experiment, mice were placed in a plexiglass chamber (45 × 28 × 29 cm) and treated by exposure to an aerosol of budesonide solution (7.5 mg/ml; compound in 40% DMSO, 42% methanol, and 18% water) (Sigma-Aldrich) as previously described (17) or an equal volume of vehicle for 30 min using an air-driven nebulizer (model NS I-210/12; Indústria de Aparelhos Biomédicos, Paulínia, Brazil) at a flow rate of 10 l/min.

Airway responsiveness to methacholine

Noninvasive and invasive assessment of airway obstruction and AHR were carried out using barometric whole-body plethysmography (Buxco Research System, Wilmington, NC). We measured the enhanced pause (Penh) responses in conscious, spontaneously breathing mice following PBS and methacholine provocations (3 and 6 mg/ml) as previously described (18). In another setting of experiments, transpulmonary resistance was also assessed 24 h after the last OVA challenge by using invasive whole-body plethysmography. Mice were anesthetized with sodium pentobarbital (Nembutal) (60 mg/kg, i.p.) and subjected to endotracheal intubation for AHR assessment in a FinePointe Buxco Platform (Buxco Electronics, Sharon, CT). The assessment was carried out in mechanically ventilated mice under neuromuscular blockade induced by pancuronium bromide (1 mg/kg, i.p.). Changes in lung resistance (centimeters H₂O per milliliter per second) were taken at baseline after aerosolized PBS and after methacholine (3, 9, and 27 mg/ml) as previously reported (19).

Measurement of bronchoalveolar lavage fluid and pulmonary inflammation

Mice were killed (sodium pentobarbital, 500 mg/kg, i.p.) 24 h after the last challenge, and bronchoalveolar lavage (BAL) was performed as previously described (15). Total leukocyte cells were enumerated in a Neubauer chamber, and differential cell counts were carried out on May-Grunwald-Giemsa-stained cytospin preparations. Histological sections were fixed and embedded in Paraplast media (Sigma-Aldrich), and 3 µm-thick sections were stained with Llewellyn's Sirius Red (Direct Red 80, C.I 35780; Sigma-Aldrich) (20, 21). Eosinophil and neutrophil infiltrates were evaluated around the airway as well as between the bronchial epithelium and adventitia through an integrating eyepiece (10⁴ µm² of total area). Determinations were made in eight randomly selected fields at a magnification of ×1000 and expressed as eosinophils and neutrophils per unit area (square micrometers) (22).

Measurement of subepithelial fibrosis

Gömöri trichrome (Trichrome Stain LG Solution; Sigma-Aldrich) was used for assessment of subepithelial fibrosis as reported (23). The area of peribronchial trichrome staining (i.e., between the alveolar septum and airway epithelium of 10 distal airways by lung section) was outlined and quantified for the total deposition of the extracellular matrix (including collagen fibers). The evaluation was made in an image analyzer system (Image-Pro Plus Media Cybernetics, version 4.1) using digitalized images from a light microscope at a magnification of ×400. The mean of results was obtained from eight animals and expressed as extracellular matrix deposition area (square micrometers).

Quantification of cytokines and chemokines

Lung tissue was homogenized on ice using a Tissue Tearor (Omni µH homogenizer) for 30 s in 1 ml of PBS containing 0.05% Triton X-100 and a protease inhibitor mixture (Hoffmann-La Roche, Basel, Switzerland). The resulting supernatants were isolated after centrifugation (10,000 × g, 15 min, 4°C), and the samples were quantified using commercially available DuoSet kits for IL-4, IL-13, IL-17A, IL-25, eotaxin-1/CCL-11, and KC/CXCL-1 (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions.

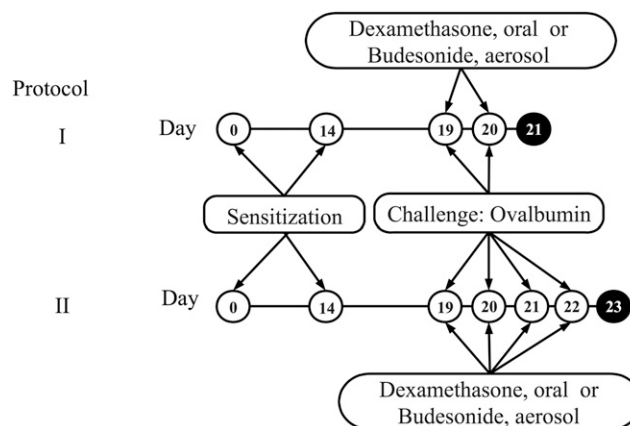


FIGURE 1. Schematic overview of study protocols for induction of allergic airway inflammation and treatments in mice. An acute airway inflammation model was used with mice being subjected to two (protocol I) or four daily allergen provocations (protocol II). The animals were treated with oral dexamethasone (3 mg/kg) or nebulized budesonide (7.5 mg/ml, 30 min) 1 h before OVA challenge.

Nuclear protein extraction

Nuclear proteins were obtained through mouse lung tissue extraction according to the manufacturer's instructions (Active Motif, Rixensart, Belgium). The nuclear-rich fraction was used for measurement of MKP-1 (sc-1199; Santa Cruz Biotechnology).

Western blotting analyses

The lung whole-cell extracts were evaluated using Western blot analysis (14, 15). The samples with equal protein concentration (50 µg) were separated on 10% NaDodSO₄-PAGE and electroblotted onto nitrocellulose membranes. Nonspecific binding was blocked with 5% BSA in Tris-buffered solution containing 0.1% Tween 20 for 2 h at room temperature. Membranes were incubated overnight with the primary Ab p-GATA-3 (ab61052; Abcam, Cambridge, U.K.), GATA-3 (sc-47778; Santa Cruz Biotechnology), p-GCR (p-Ser²²⁶) (SAB4503874; Sigma-Aldrich), GCR (sc-1004; Santa Cruz Biotechnology), β-actin (sc-47778; Santa Cruz Biotechnology), α-tubulin (no. 2144; Cell Signaling Technology), GILZ (sc-133216; Santa Cruz Biotechnology), and p-p38 (no. 4515S; Cell Signaling Technology). After washing with Tris-buffered solution, membranes were incubated with HRP-conjugated anti-mouse IgG secondary Ab (no. HAF007; R&D Systems) or HRP-conjugated anti-rabbit IgG secondary Ab (no. HAF008; R&D Systems) for 1 h at room temperature. After three washes of membranes with Tris-buffered solution (10 min each), protein bands were detected using an electrochemiluminescence plus Western blotting detection system with subsequent exposure to x-ray film (Kodak, Rochester, NY). Western blotting images were analyzed by densitometry using ImageJ 1.3 software (National Institutes of Health).

Statistical analysis

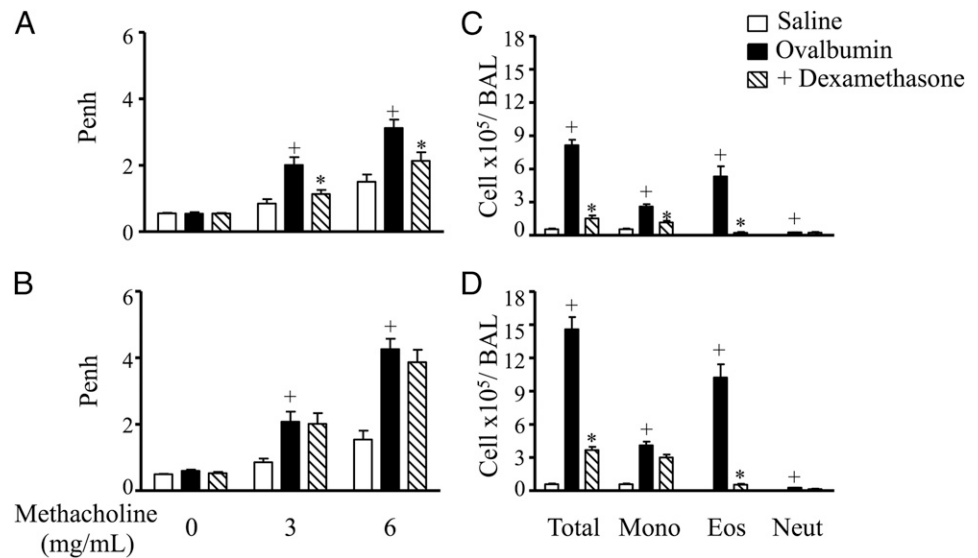
The results were expressed as mean ± SEM and statistically assessed by ANOVA, followed by the Newman-Keuls Student test. The *p* values of 0.05 or less were considered significant.

Results

GC efficacy on AHR reduces by increasing the number of allergen provocations in A/J mice

Under protocol I (Fig. 1), sensitized A/J mice reacted with increased AHR, as attested by noninvasive methods (Fig. 2A), accompanied by increased leukocyte accumulation in the BAL effluent, particularly eosinophils (Fig. 2C), as compared with negative controls. Under protocol II (Fig. 1), when sensitized mice received four instead of two consecutive allergen challenges, AHR appeared increased at the higher dose of aerosolized methacholine (6 mg/ml) (Fig. 2B), and leukocyte accumulation in the bronchoalveolar space almost doubled,

FIGURE 2. Effect of treatment with dexamethasone on AHR measured noninvasively (Penh response) (**A** and **B**) and bronchoalveolar accumulation of leukocytes (**C** and **D**) induced by two (protocol I) (**A** and **C**) or four (protocol II) (**B** and **D**) daily allergen provocations by OVA in sensitized mice. Changes were measured 24 h after the last OVA provocation. Data are expressed as mean $\pm$ SEM ($n = 7$ for all groups). $^+p < 0.05$ as compared with saline group, $^*p < 0.05$ as compared with OVA-challenged group.



mainly concerning eosinophils (Fig. 2D). Notably, oral dexamethasone pretreatment (3 mg/kg), 1 h before provocations, clearly inhibited allergen-induced AHR observed under protocol I (Fig. 2A) but failed to modify the response in mice subjected to protocol II (Fig. 2B). In contrast, this treatment prevented eosinophil accumulation in the BAL effluent irrespective of the protocol employed (Fig. 2C, 2D). Similar to the AHR readouts, dexamethasone significantly reduced mononuclear cell numbers only in the mice subjected to protocol I (Fig. 2C, 2D).

In an attempt to double-check the state of steroid refractoriness observed for readouts of AHR, we mounted a new set of

experiments in which changes in lung resistance were measured invasively in anesthetized and mechanically ventilated animals, instead of using noninvasive whole-body plethysmography (Penh measurements), as in the first assay. Under this condition, allergen-induced AHR in sensitized A/J mice was inhibited by either orally administered dexamethasone (Fig. 3A) or nebulized budesonide (Fig. 3C) in animals subjected to protocol I (two OVA challenges) but not to those subjected to protocol II (four OVA challenges), as shown in Fig. 3B and 3D, respectively. Notably, BALB/c mice subjected to protocol II were clearly sensitive to dexamethasone (Fig. 3E).

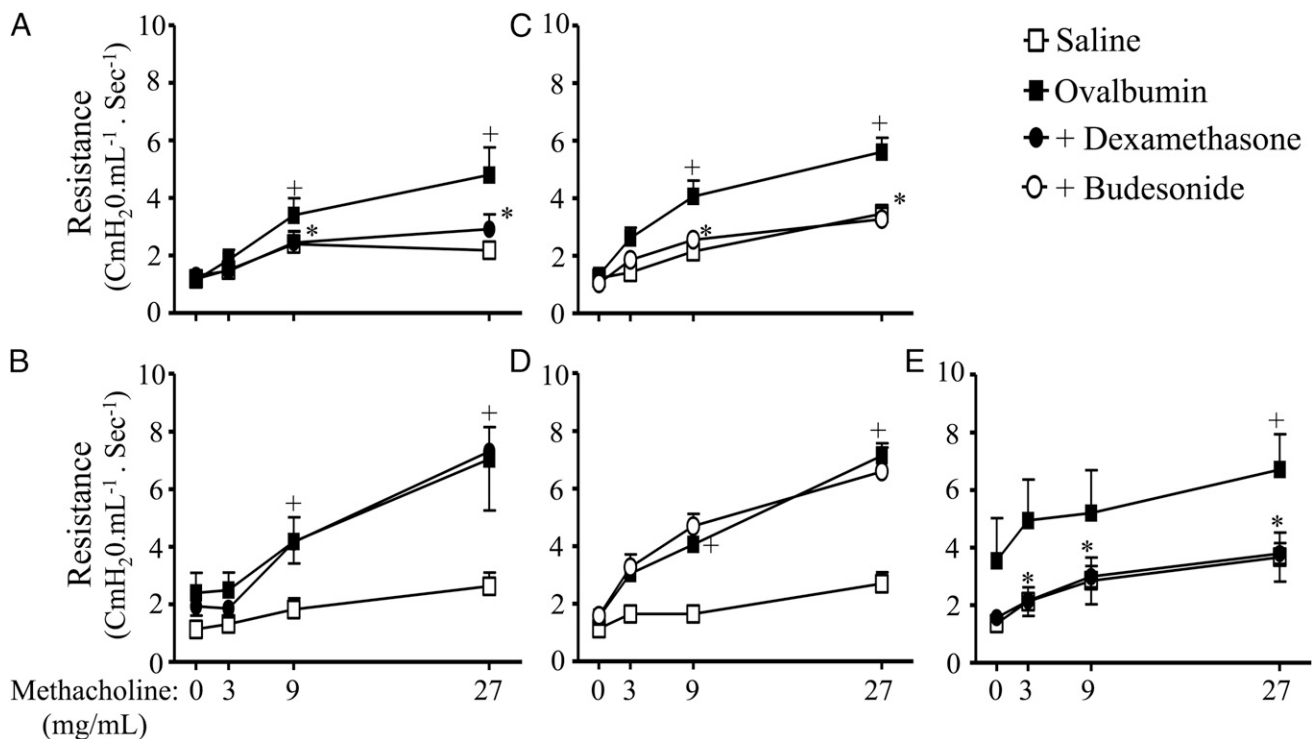


FIGURE 3. Effect of treatment with dexamethasone (**A** and **B**) or budesonide (**C** and **D**) on AHR, measured invasively, following protocol I (**A** and **C**) or protocol II (**B**, **D**, and **E**) daily OVA provocations in sensitized A/J mice (**A**–**D**) or BALB/c mice (**E**). Airway responsiveness was measured by changes in transpulmonary resistance induced by increasing concentrations of aerosolized methacholine 24 h after the last allergen provocation. Data are expressed as mean $\pm$ SEM ($n = 9$ for all groups). $^+p < 0.05$ as compared with saline group, $^*p < 0.05$ as compared with OVA-challenged group.

While trying to determine how the use of prophylactic intervention in this model of actively sensitized A/J mice compares with that when the steroid is given therapeutically, we initiated the steroid treatment after subjecting sensitized mice to two consecutive daily OVA provocations (Fig. 4A). We found that the interventional treatment, given 1 h before the third and fourth allergen challenges, failed to modify allergen-induced AHR (Fig. 4B), suggesting that it is essential to prophylactically give the steroid in this model.

Dexamethasone efficacy upon lung histological changes is lost by increasing the number of allergen provocations in A/J mice

Lung sections stained with Sirius Red revealed an extensive inflammatory cell infiltration around the bronchioles, composed mostly by eosinophils and neutrophils, following protocol I (Fig. 5B) when compared with lungs of the negative control group (sham-challenged mice) (Fig. 5A). The cellular infiltrative response was even higher when animals were given four instead of two allergen provocations (protocol II), as pointed out by the representative lung sections of negative (Fig. 5D) and positive control groups (OVA-challenged mice) (Fig. 5E), as well as by the quantifications of eosinophil (Fig. 5G) and neutrophil numbers (Fig. 5H) in the peribronchial area. Notably, oral pretreatment with dexamethasone (3 mg/kg) inhibited eosinophil infiltration following two but not four allergen provocations under conditions in which increased neutrophil numbers remained unaltered within two (Fig. 5C) and four provocations (Fig. 5F). Quantitative data on eosinophil and neutrophil enumerations following dexamethasone were also shown in Fig. 5G and 5H, respectively.

Gömöri trichrome staining of the lung sections revealed that a comparable increased deposit of extracellular matrix occurred in the lungs of A/J mice subjected to protocol I (Fig. 5J) or II (Fig. 5M) as compared with the respective negative controls (sham-challenged mice) (Fig. 5I, 5L). Administration of dexamethasone (3 mg/kg, oral) clearly decreased peribronchiolar fibrosis in the lungs of mice receiving two provocations (protocol I) (Fig. 5K) but not four allergen provocations (protocol II) (Fig. 5N). Quantitative data were shown in Fig. 5O.

GC efficacy upon cytokine and chemokine generation diminishes by increasing the number of allergen provocations in A/J mice

We then examined the effect of orally administered dexamethasone and nebulized budesonide on the lung generation of several cytokines and chemokines in A/J mice receiving two or four OVA provocations. We found that IL-4 (Fig. 6A, 6G), IL-13 (Fig. 6B, 6H), IL-17A (Fig. 6C, 6I), IL-25 (Fig. 6D, 6J), CCL11/eotaxin-1 (Fig. 6E, 6K), and CXCL1/KC (Fig. 6F, 6L) were increased in comparable levels in the lung tissue of OVA-challenged mice subjected to protocol I (white columns) or II (black columns) as compared with those of the respective negative control groups. In mice receiving two provocations (protocol I), dexamethasone inhibited the production of IL-4, IL-13, and CCL11/eotaxin-1 (Fig. 6A, 6B, 6E) but failed to alter the levels of IL-17A (Fig. 6C), IL-25 (Fig. 6D), and KC/CXCL-1 (Fig. 6F). Under the same condition (protocol I), budesonide inhibited the production of IL-4, IL-13, CCL11/eotaxin-1, and CXCL1/KC (Fig. 6G, 6H, 6K, 6L) but failed to alter the levels of IL-17A (Fig. 6I) and IL-25 (Fig. 6J). Concerning the mice receiving four allergen provocations (protocol II), treatment with either dexamethasone (Fig. 6A–F) or budesonide (Fig. 6G–L) clearly failed to alter allergen-induced elevations in the levels of proinflammatory cytokine and chemokines.

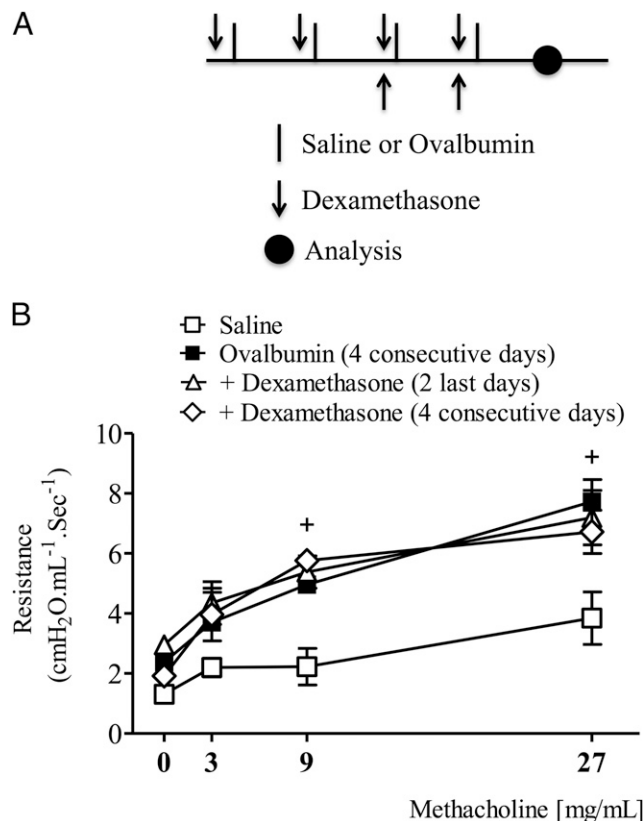


FIGURE 4. (A) The schematic protocol for induction of allergic airway inflammation and treatments. An acute airway inflammation model was used with mice being subjected to four daily allergen provocations (protocol II). Treatment with dexamethasone was carried out 1 h before each provocation or 1 h before the third and fourth provocations only. (B) The effect of dexamethasone (3 mg/kg, oral) on methacholine-induced bronchospasm (as attested by increased airway resistance) in sensitized mice challenge with saline or OVA in presence or absence of dexamethasone treatment. Data are expressed as mean $\pm$ SEM ($n = 4$ for all groups). $^+p < 0.05$ as compared with saline group.

GC resistance in A/J mice is related to decreased phosphorylation of GCR in lungs

While trying to better clarify the mechanism underlying the development of GC refractoriness, we performed Western blot analyses to investigate the impact of dexamethasone (3 mg/kg, oral) on levels of GCR and GCR phosphorylation. We noted that the m.w. of GCR and p-Ser²²⁶ GCR (p-GCR) and β -actin in lung extracts matched with predicted band sizes (Fig. 7A, 7B). When A/J mouse lung was collected 24 h after allergen exposure for 2 consecutive d (protocol I), the expression of GCR decreased significantly, which was prevented by dexamethasone given 1 h before allergen challenge (Fig. 7C, open columns). In contrast, steroid treatment failed to alter GCR expression in lung tissue samples from mice exposed to allergens for 4 consecutive d (protocol II) (Fig. 7C, closed columns). In parallel, we detected a significant elevation on GCR phosphorylation at Ser²²⁶ in the lung tissue homogenates in A/J mice subjected to both protocol I and II (Fig. 7D) 24 h after the last allergen provocation. As compared with untreated challenged mice, GC treatment led to a significant reduction in p-GCR/GCR ratios in the mice exposed to four provocations (closed columns) but not two allergen provocations (open columns), despite the tendency to reduce the p-GCR/GCR values shown by dexamethasone under protocol I.

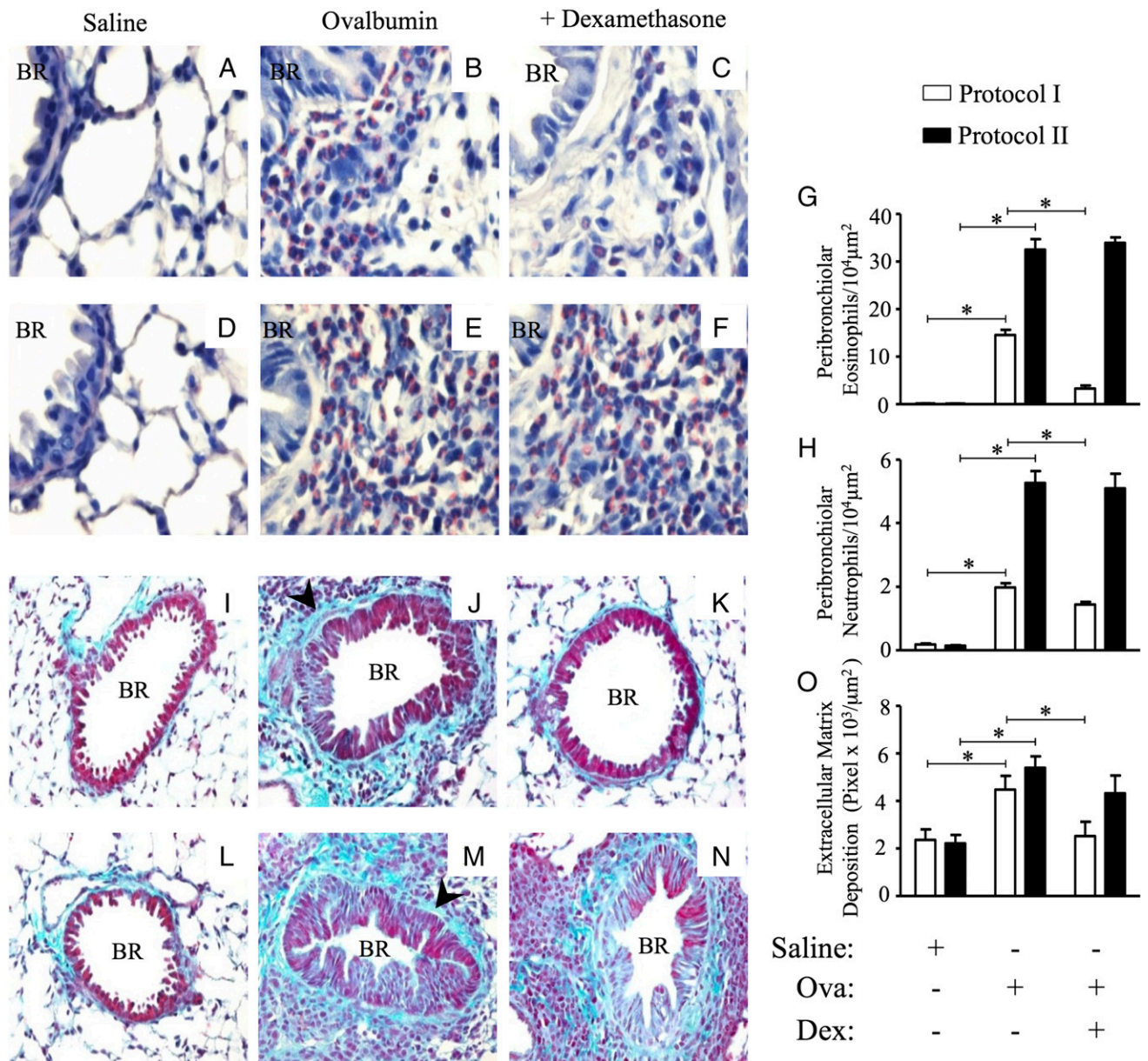


FIGURE 5. Effect of dexamethasone on allergen-induced leukocyte infiltration and peribronchiolar fibrosis in A/J mice receiving two allergen provocations (protocol I) [(A–C) and (I–K), respectively] or four allergen provocations (protocol II) [(D–F) and (L–N), respectively]. Photomicrographs were taken of representative airways stained by Sirius Red [(A–F), original magnification $\times 1000$] or Gömöri trichrome [(I–N), original magnification $\times 400$]. Quantitative data for eosinophils, neutrophils, and fibrotic changes are shown in (G), (H), and (O), respectively. Arrows indicate peribronchiolar fibrosis. Data are expressed as mean $\pm$ SEM ($n = 7$ for all groups). * $p < 0.05$. BR, bronchioles.

GC effect on the lung expression of p-GATA-3 and p-p38 reduces by increasing the number of allergen provocations in A/J mice

Western blot analyses were also performed to investigate the impact of treatments on the levels of p-GATA-3 and p-p38 in the lung tissue of sensitized and challenged A/J mice. In mouse lung collected 24 h after 2 or 4 consecutive d of allergen exposure, there was a progressive increase in p-GATA-3/GATA-3 ratios, which was significantly inhibited by dexamethasone pretreatment when the animals were subjected to two (Fig. 8A) but not four allergen provocations (Fig. 8B). Quantitative data are shown in Fig. 8C.

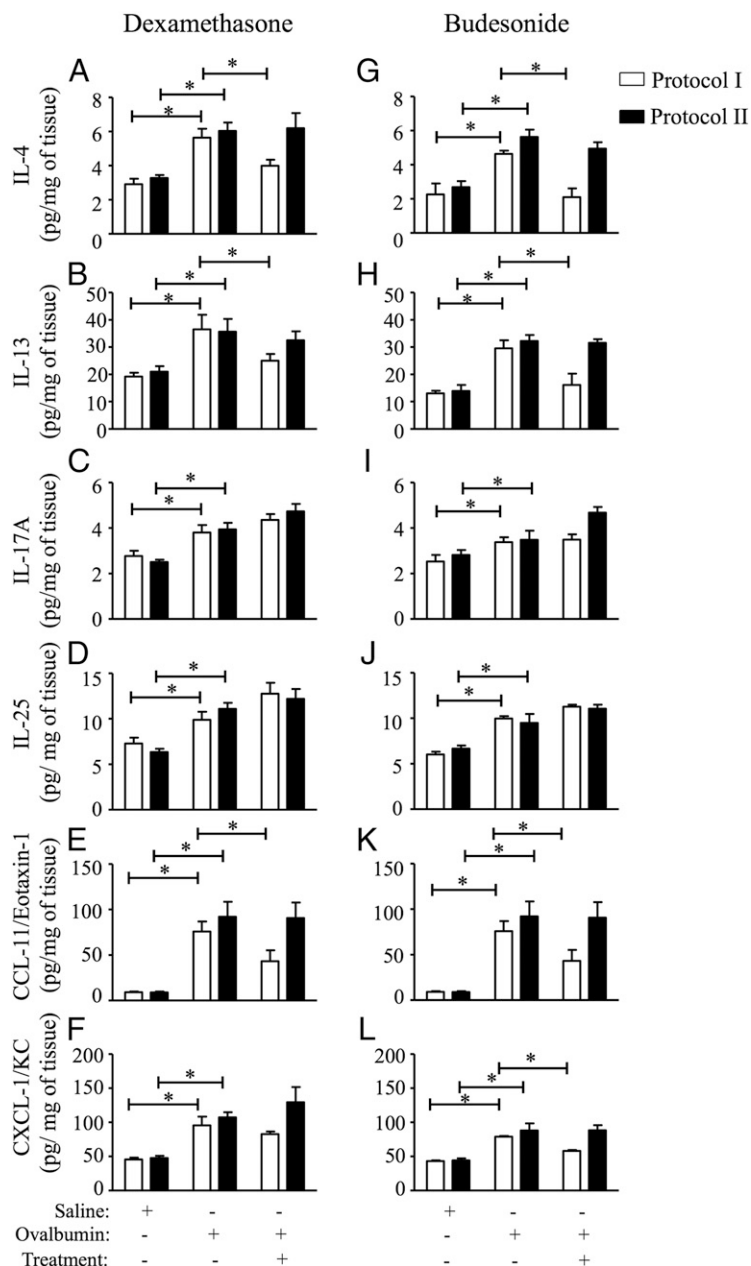
Consistently, similar findings were obtained concerning p-p38. Progressive increases in p-p38 levels were detected in mice receiving two (Fig. 8D) and four allergen provocations (Fig. 8E),

with quantitative data being shown in Fig. 8F. Pretreatment with dexamethasone inhibited the expression of p-p38 in animals receiving two but not four allergen provocations (Fig. 8D–F).

GC-induced transactivation of MKP-1 and GILZ in the lungs reduced by increasing the number of allergen provocations in A/J mice

After exposure of A/J mice to allergen for 2 or 4 consecutive d, we examined the impact of dexamethasone upon MKP-1 and GILZ protein levels in the lung extracts. In comparison with the negative control groups, there were no changes in the lung tissue levels of MKP-1 following either two (Fig. 9A) or four allergen provocations (Fig. 9B), with quantitative data being shown in Fig. 9C. As shown in Fig. 9A and 9B, dexamethasone led to an increase in MKP-1 levels following 2 or 4 consecutive d of allergen exposure,

FIGURE 6. Effect of oral dexamethasone (**A–F**) or nebulized budesonide (**G–L**) on allergen-induced lung tissue production of IL-4 (**A** and **G**), IL-13 (**B** and **H**), IL-17A (**C** and **I**), IL-25 (**D** and **J**), CCL-11/Eotaxin-1 (**E** and **K**), and CXCL-1/KC (**F** and **L**) in mice receiving two allergen provocations (protocol I) (white columns) or four allergen provocations (protocol II) (black columns). Data are expressed as mean $\pm$ SEM ($n = 7$ for all groups). * $p < 0.05$.



but the response was clearly more pronounced in lung homogenates from the former (Fig. 9A, 9C) than from the latter group (Fig. 9B, 9C).

Finally, in comparison with the negative control groups, there were no changes in the lung tissue levels of GILZ following either two (Fig. 9D) or four allergen provocations (Fig. 9E), with quantitative data being shown in Fig. 9F. Furthermore, dexamethasone treatment clearly increased GILZ protein levels following two (Fig. 9D, 9F) but not 4 consecutive d of allergen exposure (Fig. 9E, 9F).

Discussion

Substantial advances in the understanding of pathogenic mechanisms underlying GC resistance have been reached using in vitro assays (9, 18–20). However, the complexity of this condition seems to require more in vivo studies, which is not a trivial task given the difficulty to achieve, in the diversity of animal models available, studies really able to replicate the pivotal aspects of GC-resistant asthma (21, 22). In this article, we provide evidence

that A/J mice, as subjected to the often-used OVA-based protocol to induce asthma-like changes in rodents, can evolve to a state of steroid resistance. After four daily repetitive intranasal instillations of OVA, starting on day 19 postsensitization, asthma features, such as AHR, eosinophilic infiltration, and peribronchiolar deposits of extracellular matrix, became refractory to oral dexamethasone or aerosolized budesonide. The model enabled us to demonstrate that the condition of steroid resistance observed in A/J mice is strain specific. Moreover, like in humans, it correlates with a limitation on GCR bioavailability and a consequent GC incapacity to efficiently upregulate the expression of MKP-1 and GILZ and to downregulate the phosphorylation of p38 and GATA-3.

Several clues advance the genetic asthma-prone background of the A/J mouse. For instance, when mice of strains A/J, BALB/c, C57BL/6, and others were subjected to repeated allergen intranasal instillations chronically, asthma pathological features persisted only in A/J mice (23). Accordingly, A/J mice also presented the highest amount of collagen, elastic fiber, and α -actin in

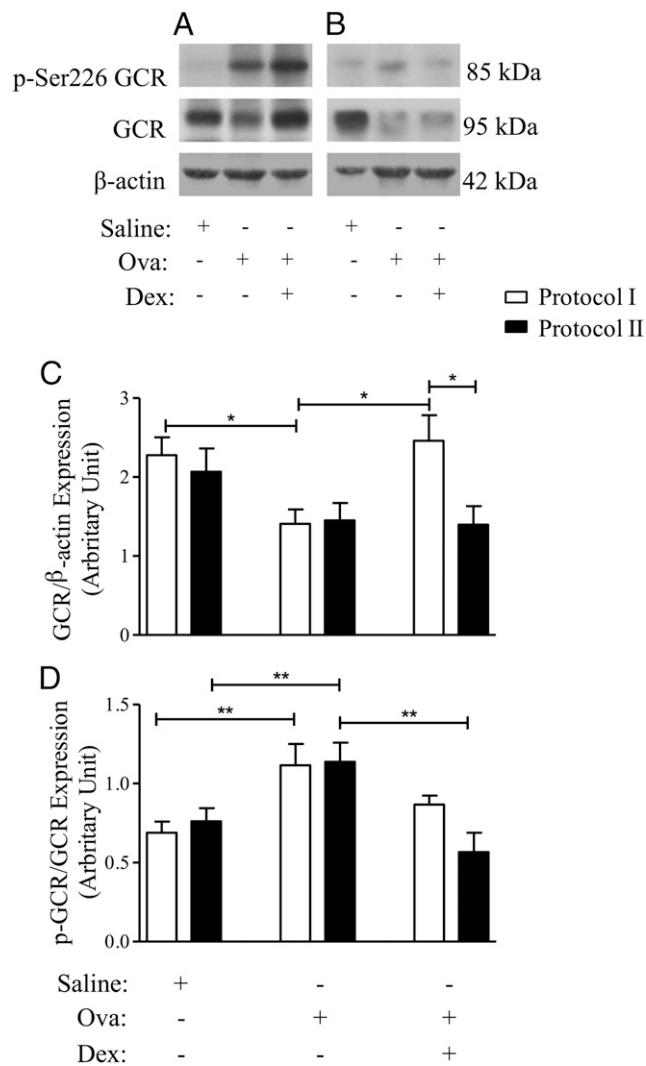


FIGURE 7. Western blot analysis of dexamethasone-induced GCR expression and phosphorylation in lung tissue following two (protocol I) (A, C, and D) or four (protocol II) (B–D) daily OVA provocations in sensitized A/J mice. Changes were measured 24 h after the last OVA provocation in whole-lung extract. Intensity of GCR and p-Ser²²⁶ GCR were quantified by densitometry and expressed as relative intensity. All results were expressed as ratios with GCR or β-actin (loading control). Data are expressed as mean ± SEM ($n = 6$ for all groups). * $p < 0.05$, ** $p < 0.01$.

distal airways and lung parenchyma and the highest levels of AHR (24). Notably, only A/J mice did not develop T cell tolerance when challenged chronically (23, 24). Moreover, A/J mice possess an innate AHR that is genetically controlled (25, 26) and occurs independently of an adaptive immune response (27). Considering the fact that the native GC hormone is an effective anti-inflammatory agent (28), we hypothesized that A/J mice might possess a natural inclination to express GC insensitivity under repeated allergen provocation.

While trying to establish a short-term model of steroid-resistant asthma in A/J mice, we screened OVA-sensitized animals subjected to a daily intranasal instillation of OVA for 2 or 4 consecutive d. We noted that two allergen provocations (protocol I) were sufficient for mounting AHR and peribronchiolar fibrotic response, in line with our previously reported findings (13, 14). Such changes were underlined by marked leukocyte accumulation in the BAL fluid, which was mainly accounted for by elevations in eosinophil and mononuclear cell numbers, with

mild changes in neutrophil counts. Histological evaluations also revealed a dominant eosinophilic inflammatory infiltrate in the peribronchiolar zone, although significant augmentation in neutrophil levels were also observed. It is worth noting that all these changes were clearly responsive to dexamethasone given prophylactically 1 h before each OVA provocation.

After four allergen provocations (protocol II), almost all these changes were exacerbated as compared with those observed in mice receiving two provocations, as should be expected. Under this condition, differing from BALB/c mice, the A/J mice were clearly resistant to the treatment with either oral dexamethasone or aerosolized budesonide in almost all parameters evaluated. Notably, although AHR, fibrotic response, and mononuclear cell accumulation in the BAL effluent remained unaltered, the recruitment of airway eosinophils was abolished, which might suggest a lack of causative relationship between eosinophils and other pathological changes observed in this model. Nevertheless, prior investigations have emphasized that eosinophil infiltration into the lung tissue, rather than eosinophils present in the airway lumen, can create a predisposition for developing AHR (29). Therefore, our observation that allergen-induced tissue eosinophil accumulation was responsive to GC following protocol I but not II supports the concept that persistent tissue eosinophilia does have a paramount importance in the mechanism of AHR and lung damage (30–33). Furthermore, it is also worth noting that after allergen exposure for 4 consecutive d, the interventional treatment with dexamethasone carried out the third and fourth day was clearly inactive, emphasizing the importance of prophylactic intervention compared with when the steroid is given therapeutically in the context of this model.

Being driven by a type 2 immune response is perhaps the most prominent aspect shared by mouse models of asthma and human asthma (34, 35). Adaptive T_H2 and T_H17 cells have been shown to be important sources of cytokines able to contribute to GC resistance. They are known to promote persistent eosinophil and neutrophil accumulation into the site of inflammation, respectively (36). We therefore explored whether these cytokines might be implicated in the state of steroid refractoriness observed in this model. We noted that following two or four OVA provocations, increased levels of IL-4, IL-13, IL-17A, and IL-25 as well as CCL11/eotaxin-1 and CXCL1/KC were found in lung tissue homogenates from A/J mice. Treatments with either oral dexamethasone or aerosolized budesonide failed to modify these changes in mice receiving four provocations (protocol II). Even in case of protocol I (animals receiving two OVA provocations), only IL-4, IL-13, and CCL11/eotaxin-1 were responsive to GC, which suggests that an uncontrolled expression of proinflammatory cytokines and chemokines might indeed contribute to the state of steroid refractoriness and persistent eosinophilic/neutrophilic infiltration in this model.

Persistent exposure of pivotal cell targets to certain cytokine combinations, including IL-2 plus IL-4 as well as TNF-α plus IFN-γ or IL-5, can promote tissue resistance to GC, which has been associated with negative modulation of GC binding, receptor dimerization, nuclear translocation, and other signaling defects (5, 37, 38). Interestingly, in a pediatric asthma cohort, cells recovered from poor GC responders displayed lower GCR protein levels as compared with good GC responders (39). In this study, when the GCR level in lung homogenates was assessed using Western blot assays, we observed that repeated exposure of allergens on 2 or 4 consecutive d downregulated GCR expression in samples from A/J mice, in line with a previous report (40). Moreover, the reduction in lung tissue GCR protein levels was clearly impaired by dexamethasone given 1 h before provocation in the mice exposed to

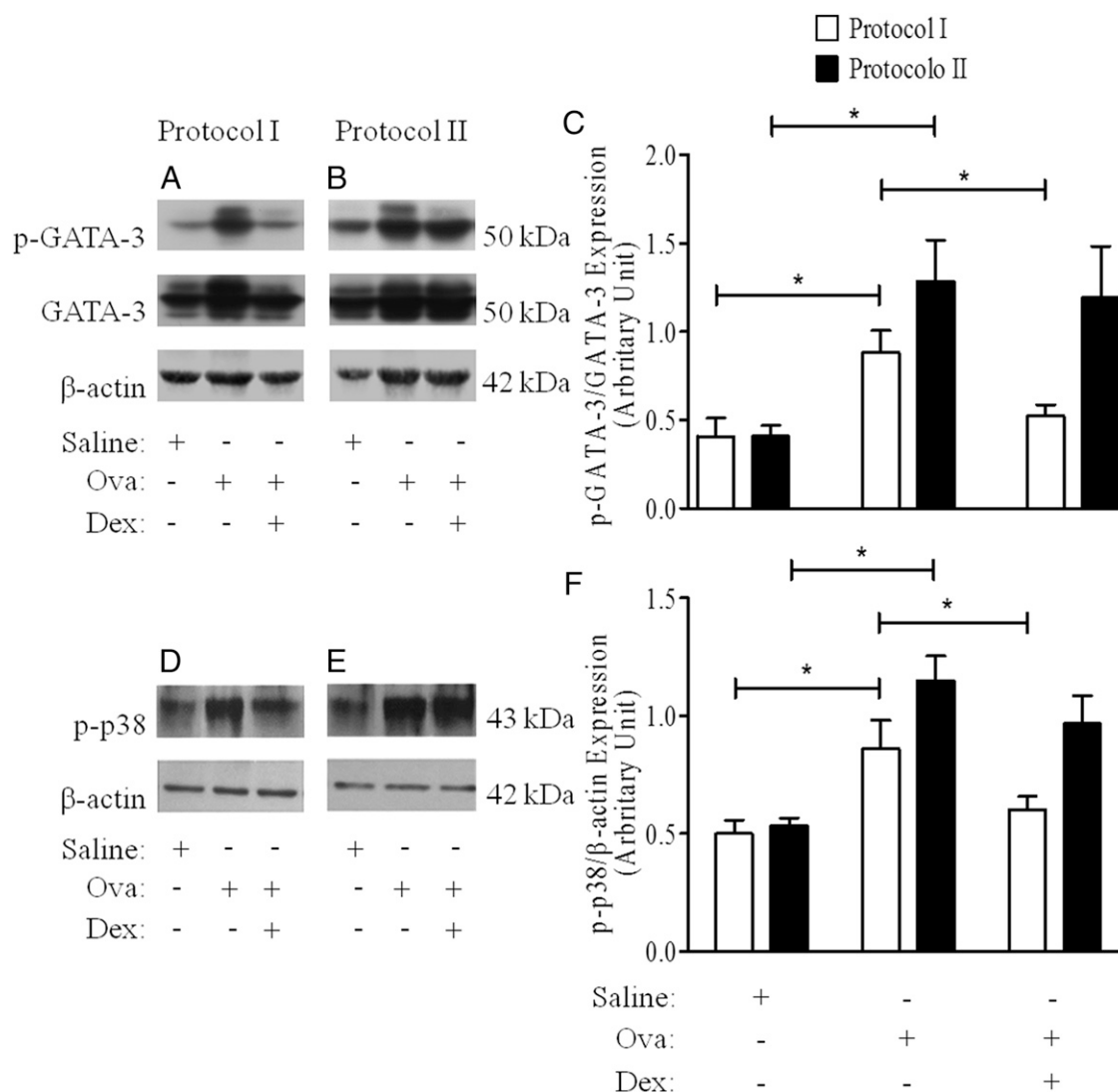


FIGURE 8. Effect of treatment with dexamethasone on p-GATA-3 (A–C) and p-p38 (D–F) expression in lung tissue from A/J mice receiving two allergen provocations (protocol I) (A and D) or four allergen provocations (protocol II) (B and E). Changes were measured in whole-lung extracts. Intensity of p-GATA-3 (C) and p-p38 (F) bands were quantified by densitometry and expressed as β -actin ratios (loading control). Results are shown as mean $\pm$ SEM ($n = 6$ for all groups). * $p < 0.05$.

allergen on 2 consecutive d, although it was refractory in those subjected to four provocations.

Because a defect in GCR phosphorylation and signaling has been shown to underline the cytokine-induced resistance to GC-mediated human eosinophil apoptosis (9), we also compared levels of GCR phosphorylation in lung tissue samples with the distinct protocols explored in this article. We detected a significant elevation on GCR phosphorylation at Ser²²⁶ in the lung tissue homogenates in A/J mice subjected to both protocol I and II 24 h after the last allergen provocation. Notably, lung homogenates derived from mice prophylactically treated with dexamethasone during allergen exposure on 4 consecutive d displayed a significant reduction on the level of GCR phosphorylation at Ser²²⁶. Despite the tendency to decrease GCR phosphorylation, no significant changes were observed on lung samples from dexamethasone-responsive mice (exposed on 2 consecutive d to allergen). Because GC–GCR interaction in the cytoplasm results in phosphorylation and nuclear translocation of the hormone receptor complex to activate transcriptional regulatory functions (41), it is likely that an abnormality on the GC–GCR phosphorylation

mechanism might underlie the GC refractoriness evidenced in this model.

It is worth noting that persistent exposure of pivotal cell targets to certain inflammatory cytokines can also promote tissue resistance to GCs through phosphorylation of the GCR in its ligand-free form, which can be overcome by inhibition of p38 activity, emphasizing the putative role of p38-induced receptor phosphorylation in the case of steroid resistance invoked by cytokines (42). In this study, we also found a close correlation between dexamethasone efficacy in inhibiting allergen-induced pathological changes and the inhibitory impact of this treatment on the lung tissue levels of p-p38 and p-GATA-3, a transcription factor that has a critical role in CD4⁺ helper T cell-subset development and production of associated cytokines (5, 37, 43). In fact, administered 1 h before each provocation, dexamethasone clearly downregulated the phosphorylation of p38 and GATA-3 following two but not four consecutive OVA challenges in this model. Additionally, we demonstrated that the dexamethasone-induced elevation in MKP-1 and GILZ observed after two allergen challenges was clearly more pronounced than that noted after four challenges. The correlation

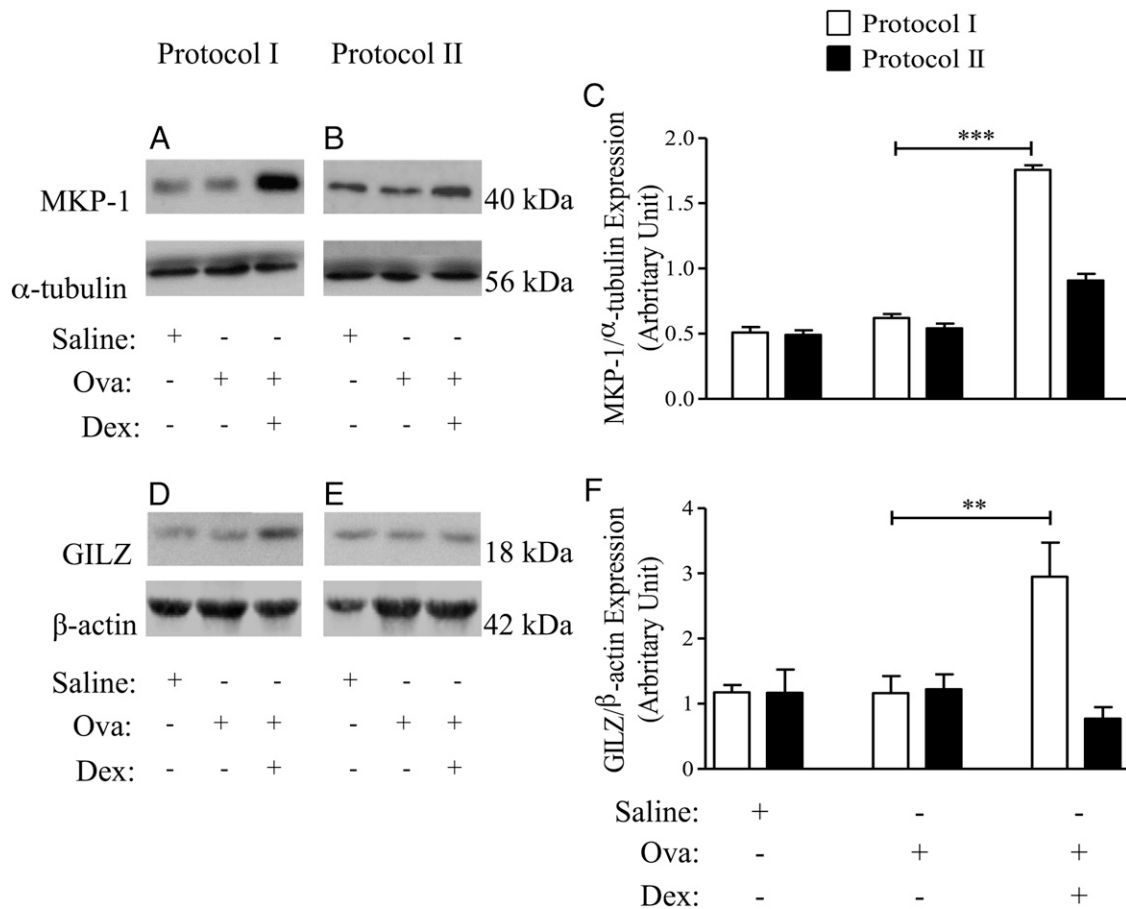


FIGURE 9. Effect of treatment with dexamethasone on the expression of GC-regulated proteins MKP-1 (**A–C**) and GILZ (**D–F**) in lung tissue following two (protocol I) (**A** and **D**) or four (protocol II) (**B** and **E**) daily OVA provocations in sensitized A/J mice. Changes in GILZ and MKP levels were measured 24 h after the last OVA provocation in whole-lung extracts and nuclear protein fraction, respectively. Intensity of MKP-1 and GILZ were quantified by densitometry and expressed as ratios with α-tubulin or β-actin (loading control), respectively. Data are shown as mean ± SEM ($n = 6$ for all groups). ** $p < 0.01$, *** $p < 0.001$.

among reduced GCR bioavailability, reduced transactivation of MKP-1 and GILZ, and increased levels of p-p38 and GATA-3 in lung samples from mice exposed to four OVA provocations reinforces the interpretation that there is indeed a causative association of these events with the state of GC insensitivity in the 4-d condition.

Thus, useful information concerning inflammatory, structural, and functional lung changes is obtainable using this short-term model of GC-resistant asthma. Further experiments might be essential to clarify the mechanism accounting for by the decrease in GCR bioavailability observed in this model.

In conclusion, these findings suggest that OVA-sensitized A/J mice evolve from a steroid-responsive to steroid-unresponsive eosinophilic inflammatory condition, depending on the number of allergen provocations. The refractoriness is strain specific and correlates with the loss of the steroid capacity to upregulate MKP-1 and GILZ and downregulate phosphorylated GATA-3 and p-p38 levels in the lungs.

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Disclosures

The authors have no financial conflicts of interest.

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