

---

# Effects of endogenous glucocorticoids on allergic inflammation and T<sub>H</sub>1/T<sub>H</sub>2 balance in airway allergic disease

Suqin Zhang, PhD\*; Ziyin Shen, MD\*; Guorang Hu, PhD†; Runhong Liu, PhD\*; and Xinmin Zhang, PhD\*

---

**Background:** Glucocorticoids play an important role in modulating allergic inflammation and immune response. However, little is known about the role of endogenous glucocorticoids in airway allergic disease.

**Objective:** To investigate the effects of endogenous glucocorticoids on regulating allergic inflammation and T<sub>H</sub>1/T<sub>H</sub>2 balance in an airway allergic murine model.

**Methods:** An ovalbumin-sensitized murine model was established by intraperitoneal injection sensitization and intranasal challenge with ovalbumin. Glucocorticoid release was inhibited by administration of metyrapone, and the peripheral glucocorticoid receptors were blocked by administration of RU486. The numbers of eosinophils in the lung, peripheral blood, and bone marrow were quantified. The changes in T<sub>H</sub>2/T<sub>H</sub>1 cells were investigated by flow cytometry, and their cytokines were tested by enzyme-linked immunosorbent assay, including interleukin 4, interleukin 5, and interferon  $\gamma$ , in the supernatant of the spleen cell culture.

**Results:** Inhibition of endogenous glucocorticoids caused more sneezing and further increased eosinophil counts in the peripheral blood and bone marrow of the sensitized mice. However, by inhibition of endogenous glucocorticoids, the interferon  $\gamma$  levels were upregulated, the interleukin 4 and 5 levels were down-regulated, and the ratio of T<sub>H</sub>2/T<sub>H</sub>1 cells decreased significantly, indicating a shift to a T<sub>H</sub>1-predominant immune response in sensitized mice.

**Conclusions:** Our findings suggest that endogenous glucocorticoids play an important role in abating allergic inflammatory reaction and modulating the T<sub>H</sub>1/T<sub>H</sub>2 balance in an airway allergic murine model. Inhibition of endogenous glucocorticoids resulted in a shift to T<sub>H</sub>1 predominance, but that did not attenuate the severity of the allergic inflammatory reaction.

*Ann Allergy Asthma Immunol.* 2009;103:525–534.

## INTRODUCTION

Airway allergic diseases, such as allergic rhinitis and asthma, are characterized by a similar inflammatory process in which eosinophils have been shown to play a central role in the pathogenesis and clinical expression of the disease.<sup>1–3</sup> The pathologic processes that occur in the nasal mucosa with allergic rhinitis and in the lower airway with asthma are believed to reflect common manifestations of a generalized inflammatory disorder, which is both a local and systemic inflammatory process.<sup>4,5</sup>

The cascade of inflammatory reactions related to airway allergy depends on various participating cells for which T lymphocytes act as the main regulators.<sup>6</sup> It was previously proposed that T<sub>H</sub>1/T<sub>H</sub>2 imbalance of the immune system resulted in the clinical expression of allergic rhinitis and asthma. The T<sub>H</sub>1/T<sub>H</sub>2 hypothesis proposed by Cher and Mosmann<sup>7</sup> and Mosmann and Coffman<sup>8</sup> in 1986 was as follows: 2 very different subsets of T<sub>H</sub> cells exist, distinguished by

different sets of cytokines secreted after activation and, as a consequence, mediating very different regulatory and effector functions. Accumulating evidence indicates that T<sub>H</sub>2 cells are associated with regulation of allergic responses and mucosal inflammation. T<sub>H</sub>2 cells produce a characteristic pattern of cytokines, and specific interest is being paid to interleukin (IL) 4, which is known to stimulate production of IgE,<sup>9</sup> and IL-5, which is known to recruit and activate eosinophils.<sup>10</sup> In contrast, T<sub>H</sub> subsets, whose characteristic cytokine is interferon  $\gamma$  (IFN- $\gamma$ ), are involved in mediating classic cell-mediated immune responses, such as delayed-type hypersensitivity.<sup>6</sup> T<sub>H</sub> lymphocyte subsets counterregulate each other through the actions of selected cytokines.<sup>11</sup>

Glucocorticoids are important hormones involved in modulating allergic inflammatory responses and, as drugs, form the basis of the clinical treatment of respiratory tract allergy. They have inhibitory effects on the accumulation and activity of eosinophils, lymphocytes, macrophages, and other inflammatory cells. Also, glucocorticoids regulate a wide variety of immune-related genes and immune cell expression and function. The immune system has for many years been known to be influenced by glucocorticoids, which in general suppress maturation, differentiation, and proliferation of immune cells involved in all aspects of immunity, including innate T-cell and B-cell function, and chronic allergic reactions.<sup>12</sup> Exten-

---

**Affiliations:** \* Institute of Integrated Chinese and Western Medicine, Huashan Hospital, Fudan University; Shanghai, China; † Department of Medicine, University of Sydney, Sydney, Australia.

**Disclosures:** Authors have nothing to disclose.

Received for publication May 5, 2009; Received in revised form June 30, 2009; Accepted for publication July 12, 2009.

sive research has shown the pharmacologic effects of glucocorticoids on many aspects of immune cell function.<sup>13,14</sup>

Endogenous glucocorticoids are synthesized in the adrenal glands as a response to stress and are regulated by the hypothalamic-pituitary-adrenal (HPA) axis.<sup>15</sup> Some studies have suggested that endogenous glucocorticoids can reduce the severity of experimental allergic reactions in both humans and animals. For example, treatment of dogs with metyrapone, a drug that blocks the adrenal synthesis of active glucocorticoids, increases the incidence and severity of late-phase reactions after antigen challenge in the lung and skin.<sup>16</sup> Cutaneous antigen challenge studies in humans have shown that late-phase reactions are more pronounced when the challenge is performed late in the day, when endogenous glucocorticoid levels are low.<sup>17</sup> Asthmatic patients whose forced expiratory volume in 1 second recovered rapidly, who had higher baseline levels of cortisol, and who displayed a late-phase reaction had lower levels of cortisol during the late-phase period.<sup>18</sup> The development of upper and lower respiratory tract allergy may thus be driven by defects in the equilibrium of the HPA axis. However, little is known about the effects of endogenous glucocorticoids on the pathophysiologic processes of airway allergic disease.

To investigate the significance of endogenous glucocorticoids, we used metyrapone and RU486 to block both the central and peripheral effects of glucocorticoids in an airway allergic murine model. Inhibition of glucocorticoid release is caused by metyrapone, an inhibitor of 11 $\beta$ -hydroxysteroid dehydrogenase type 1, which catalyzes 11 $\beta$ -hydroxylation in the synthesis of corticosterone. The peripheral inhibition of the effects of glucocorticoids is achieved with RU486, a potent glucocorticoid receptor antagonist known to block the ability of glucocorticoids. We studied eosinophils in the lung, peripheral blood, and bone marrow, which are clinically significant for allergy, and T<sub>H</sub>1/T<sub>H</sub>2 cells and their cytokines, such as IL-4, IL-5, and IFN- $\gamma$ , which are important factors in regulating allergic reaction.

## MATERIALS AND METHODS

### Animals

Specific pathogen-free, male, BALB/c mice were obtained from the animal center of Fudan University. The animals weighed 18 to 22 g at the initiation of sensitization. They were allowed food and water ad libitum and kept in an air-conditioned room on a 12-hour light-dark cycle. All of the experiments were approved by the regional committee for animal ethics.

### Reagents

Albumin from chicken egg white (ovalbumin grade V), aluminum hydroxide gel, RU486, and metyrapone were purchased from Sigma (St Louis, Missouri). IL-4, IL-5, and IFN- $\gamma$  mouse enzyme-linked immunosorbent assay kits were purchased from Biosource (Camarillo, California). A corticosterone enzyme immunoassay kit was purchased from Cayman Chemical Company (Ann Arbor, Michigan). Fluorescein isothiocyanate anti-mouse CD4 antibody, phycoerythrin (PE) anti-mouse IL-4 antibody, and PE anti-mouse IFN- $\gamma$  antibody were purchased from Biotrend (San Diego, California).

### Sensitization and Challenge

The mice were randomly allocated into 3 groups as follows: control group, ovalbumin group, and metyrapone-RU486 group. There were 10 mice in each protocol group. The sensitization and challenge protocol is shown in Figure 1. The mice in the ovalbumin group and the metyrapone-RU486 group were sensitized by administering 100  $\mu$ g of ovalbumin suspended in 13 mg/mL of aluminum hydroxide gel. A total of 0.3 mL of the suspension was intraperitoneally injected on day 0 and followed by a booster injection on day 7 with the same amount of the identical reagents. From day 18 to day 27, the mice were challenged by intranasal instillation of 100  $\mu$ g of ovalbumin (suspended in 20  $\mu$ L of saline dripped into the nostrils using a micropipette). In the same period (days 18–27), the mice in the metyrapone-RU486 group were pre-treated with RU486 (20 mg/kg), dissolved in 0.5% carboxymethyl cellulose (suspended in saline) 12 hours before the

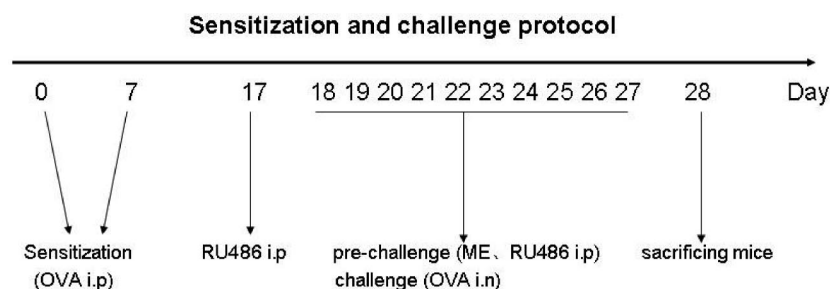


Figure 1. Experimental protocol. Mice were sensitized intraperitoneally (i.p.) with ovalbumin (OVA) (100  $\mu$ g per mouse) twice at weekly intervals and then challenged intranasally (i.n.) from day 18 to day 27. RU486 (20 mg/kg) was injected (i.p.) 12 hours before the first challenge and 1.5 hours before each challenge, and metyrapone (ME) (30 mg/kg) was injected (i.p.) 2 hours before each challenge procedure. The mice in the control group were sham sensitized and challenged. The mice in the control and ovalbumin groups were injected (i.p.) with saline before each challenge procedure.

first challenge and 1.5 hours before each challenge. The time and dose were known to block glucocorticoid action,<sup>19</sup> and it has been reported that the dose of RU486 administered (20 mg/kg) is sufficient to block glucocorticoid receptor signaling.<sup>20–22</sup> Metyrapone was used at a dose of 30 mg/kg (dissolved in saline) through intraperitoneal injection with 0.2 mL of the solution 2 hours before each challenge. The doses of metyrapone and the delivered route were mainly based on the instructions of Georen et al<sup>23</sup> and De Bie et al,<sup>24</sup> with little modification. The mice in the control group were sham sensitized and sham challenged, receiving only 0.3 mL of saline for intraperitoneal injection and 20  $\mu$ L of saline for nasal dripping. The mice in the control and the ovalbumin groups were pretreated by intraperitoneal injection with 0.2 mL of saline 2 hours before each challenge.

#### *Nasal Symptoms*

Nasal symptoms were evaluated for each mouse in each group on day 27 by counting the number of sneezes and nasal rubs (nasal itching) for 3 minutes immediately after either ovalbumin or saline intranasal challenge.

#### *Blood Sample Collection and Measurement of Serum Corticosterone Levels*

The mice were studied 24 hours after the final challenge (on day 28). Mice from each respective group were anesthetized with sodium pentobarbital in a rotating pattern (control group, ovalbumin group, metyrapone-RU486 group) so that 1 mouse from a group was anesthetized and then 1 mouse from the next group was anesthetized and so on. After the mice were anesthetized, blood samples were obtained by cardiac puncture. All blood samples were obtained between 8 and 9 AM and were centrifuged at 1,500 rpm for 10 minutes and the serum were stored at  $-80^{\circ}\text{C}$  until assay. Corticosterone levels were determined via a corticosterone enzyme immunoassay kit.

#### *Eosinophils in Peripheral Blood*

Blood samples were obtained as described herein, and the blood smears were prepared in duplicates. Total white blood cells were counted in heparinized blood, and eosinophils counts were performed on the blood smears using May-Grünwald-Giemsa stain based on morphologic and histologic criteria.

#### *Eosinophils in Bone Marrow Cell Flush*

Bone marrow cells were collected by carefully flushing 500  $\mu$ L of saline via a needle from the right femur (end-opened) of each mouse. The bone marrow cell flush fluid was collected into chilled tubes and centrifuged at 1,000 rpm for 10 minutes at  $4^{\circ}\text{C}$ , and then the cell pellets were smeared onto a slide, air-dried, and stained with May-Grünwald-Giemsa stain. A total of 300 cells were counted in each slide and categorized according to the standard morphologic criteria.

#### *Tissue Collection and Histologic Examination*

The left lobes of the lungs were surgically removed and fixed in 4% buffered formaldehyde. They were then embedded in

paraffin, sectioned, and stained with hematoxylin-eosin. Histologic findings, such as peribronchial and perivascular inflammatory cells infiltration, were graded semiquantitatively by an independent observer under the microscope with a focus on inflammatory cells (mainly eosinophils), using a scoring criteria from 0 to 3+ (0, no cell infiltration; 1+, few; 2+, moderate; and 3+, abundant).

#### *Spleen Cell Culture and Quantification of Cytokines*

Spleen cells were isolated from mice and suspended in the complete culture medium (RPMI 1640 containing 10% fetal bovine serum, 100 U/mL of penicillin, 100 U/mL of streptomycin, and 1% glutamine). The cells ( $2 \times 10^6$  per well) were cultured in 24-well plates in the presence of either ovalbumin (100  $\mu\text{g/mL}$ ) or concanavalin (10  $\mu\text{g/mL}$ ). The supernatants were collected after a 72-hour culture. The levels of IL-4, IL-5, and IFN- $\gamma$  in the supernatants were determined by enzyme-linked immunosorbent assay according to the manufacturer's protocol. Both the standards and samples were run in duplicate. The minimum detectable dose of mouse IL-4 is less than 5 pg/mL. The minimum detectable dose of mouse IL-5 is less than 3 pg/mL. The minimum detectable dose of mouse IFN- $\gamma$  is less than 1 pg/mL.

#### *Flow Cytometric Analysis*

The isolated spleen cells were washed with 0.1% bovine serum albumin-phosphate-buffered saline and subsequently incubated in fluorescein isothiocyanate-conjugated anti-mouse CD4 antibody for 30 minutes at room temperature. After staining, they were washed once with 2 mL of cell-wash solution and then fixed for 15 minutes. Then permeabilization of the spleen cells was performed by adding 1.5 mL of penetrant and incubated in the dark for 15 minutes. After centrifugation and removal of the supernatants, the cells were incubated with PE-conjugated anti-mouse IL-4 antibody or PE-conjugated anti-mouse IFN- $\gamma$  antibody in the dark for 30 minutes and then washed twice with 2 mL of the cell-wash solution and fixed with 350  $\mu$ L of fixative. The flow cytometric evaluation with FACS Caliber Flow Cytometry (Becton Dickinson, Franklin Lakes, New Jersey) was performed within 24 hours. Acquisition and analysis were performed with the Cell Quest software package (Becton Dickinson).

#### *Statistical Analysis*

Results are expressed as mean (SEM). The *t* test was used to determine significant differences among the study groups.  $P < .05$  was considered statistically significant.

## **RESULTS**

#### *Nasal Symptoms*

In both the ovalbumin and metyrapone-RU486 groups, sensitization with ovalbumin significantly increased the numbers of nasal rubbing and sneezing episodes compared with the control group. In addition, inhibition of endogenous glucocorticoids resulted in more sneezing compared with the ovalbumin group mice (Fig 2).

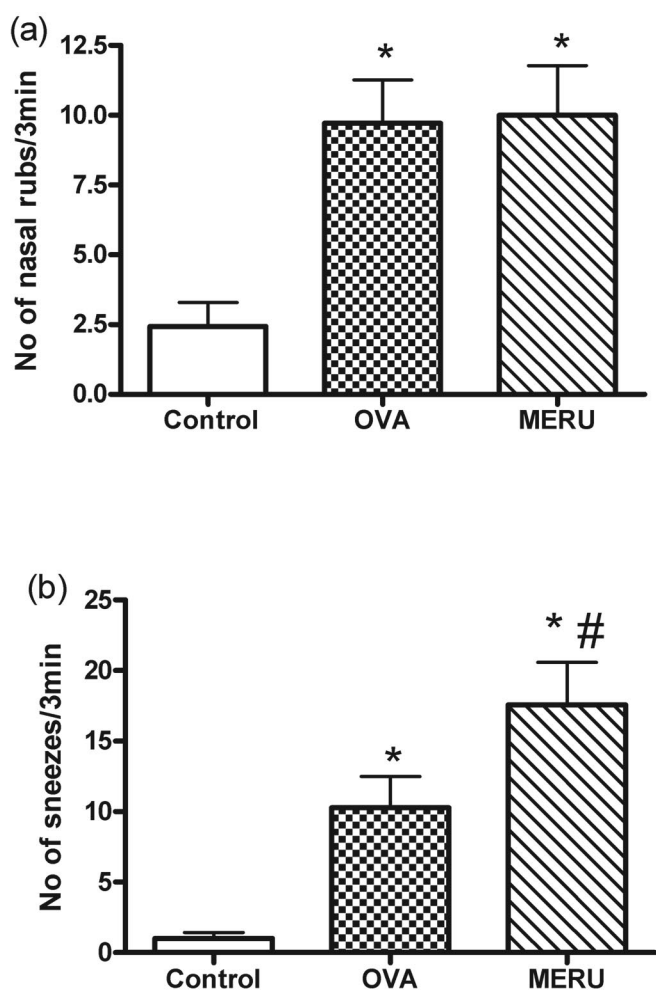


Figure 2. Nasal symptoms. Nasal symptoms evaluated immediately after either ovalbumin (OVA) or saline intranasal challenge on day 27 of the sensitization protocol. a, Nasal rubs; b, sneezes. \* $P < .05$  compared with those in the control group. # $P < .05$  compared with those in the OVA group. MERU indicates metyrapone-RU486.

#### Lung Histopathologic Findings

The lung pathologic findings (Fig 3 and Fig 4) demonstrated that the sensitized mice exhibited a significant increase in inflammatory cell (mainly eosinophil) infiltration compared with the control group. However, no significant difference was found in inflammatory cell infiltration into lungs between the ovalbumin and metyrapone-RU486 group mice.

#### Eosinophils in Peripheral Blood

As shown in Figure 4b, there was a significant increase in eosinophils in the peripheral blood in the sensitized groups compared with the control group. In addition, inhibition of endogenous glucocorticoids with metyrapone and RU486 produced a further increase in the numbers of eosinophils in the peripheral blood.

#### Percentage of Eosinophils in the Bone Marrow

The percentage of eosinophils in the bone marrow as a function of total cells is shown in Figure 4c. We found an increase in eosinophils in the bone marrow of the sensitized group compared with the control group. Inhibition of endogenous glucocorticoids with metyrapone and RU486 increased the inflammatory eosinophil response in the bone marrow compared with the ovalbumin sensitized group and the control group.

#### Cytokines IL-4, IL-5, and IFN- $\gamma$ in the Spleen Cell Culture Supernatants

To evaluate the ability of splenocytes to produce type 1 or type 2 cytokines, the spleen cells from mice of the 3 different groups were stimulated with either concanavalin or the immunizing antigen ovalbumin for 72 hours. The supernatants were collected and assayed for cytokines. As shown in Figure 5, compared with the control group, IFN- $\gamma$  levels in the ovalbumin group were much lower, especially after being stimulated by concanavalin, resulting in  $T_H2$  response priority in the ovalbumin group. When the effects of endogenous glucocorticoids were inhibited with metyrapone and RU486, the production of IFN- $\gamma$  increased significantly, whereas the levels of IL-4 and IL-5 decreased significantly compared with

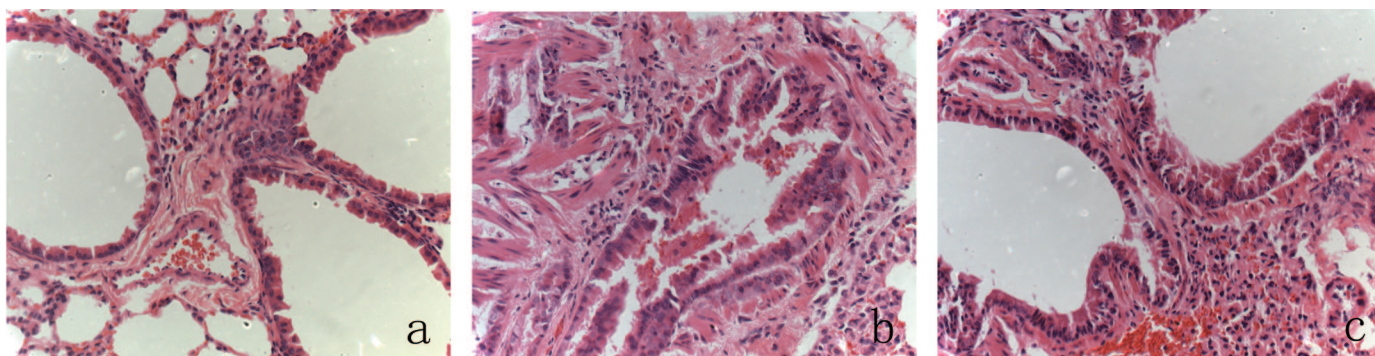


Figure 3. Histologic analysis of lung sections. The photomicrographs show hematoxylin-eosin-stained sections of lung tissue (original magnification  $\times 400$ ). a, Saline-challenged control mice; b, ovalbumin-challenged mice; and c, ovalbumin-challenged mice pretreated with metyrapone and RU486.

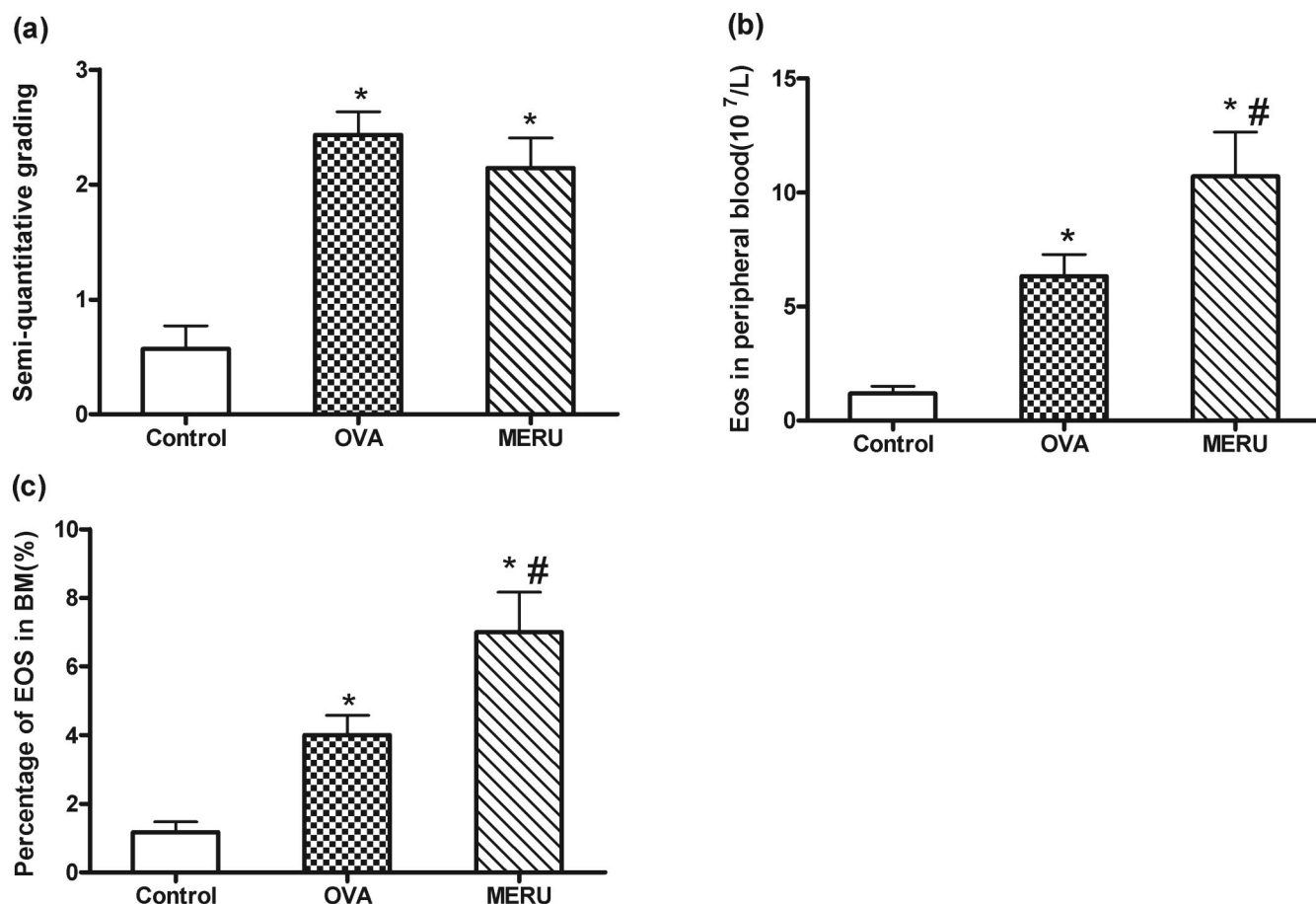


Figure 4. The eosinophils (EOS) in the lung, peripheral blood, and bone marrow (BM). a, The lung histology grading scores of inflammatory cells (mainly eosinophils) after ovalbumin (OVA) sensitization and challenge. b, EOS in the peripheral blood 24 hours after the last intranasal challenge. c, Percentage of EOS in the BM after OVA sensitization and challenge. \* $P < .05$  compared with those in the control group. # $P < .05$  compared with those in the OVA group. MERU indicates metyrapone-RU486.

the ovalbumin group. Therefore, the balance of  $T_H1/T_H2$  in the metyrapone-RU486 group shifted to  $T_H1$  predominance.

#### Percentage of $T_H1$ or $T_H2$ Cells and the Ratio of $T_H2/T_H1$ Cells

As shown in Figure 6, the percentages of  $T_H2$  cells in the ovalbumin group and the metyrapone-RU486 group were significantly higher than that of the control group. The percentage of  $T_H1$  cells in the ovalbumin group was equivalent to that of the control group, but the percentage of  $T_H1$  cells in the metyrapone-RU486 group increased significantly compared with the control group and the ovalbumin group. The ratio of  $T_H2/T_H1$  cells in the ovalbumin group was significantly higher than the control group, indicating a predominant  $T_H2$  response. However, inhibition of endogenous glucocorticoids with metyrapone and RU486 produced a shift of  $T_H1$  predominance with a significant decrement of the ratio of  $T_H2/T_H1$  cells compared with the ovalbumin group and the control group.

#### Serum Corticosterone Levels

As shown in Figure 7, the serum corticosterone levels in the metyrapone-RU486 group were much lower compared with those in the control group and the ovalbumin group.

#### DISCUSSION

In this study we examined the role of endogenous glucocorticoids in the process of airway allergic disease. We found that inhibiting the effects of endogenous glucocorticoids with metyrapone and RU486 before the allergen challenge resulted in augmented eosinophils in the bone marrow and peripheral blood compared with the ovalbumin group.

An impaired HPA axis response has been shown to lead to an increased sensibility to inflammation induction in rat models of chronic inflammatory diseases.<sup>25,26</sup> In some studies a defective HPA axis response has been proposed in patients with airway allergic diseases, including allergic rhinitis and asthma.<sup>27-31</sup> Peebles et al<sup>18</sup> found that those

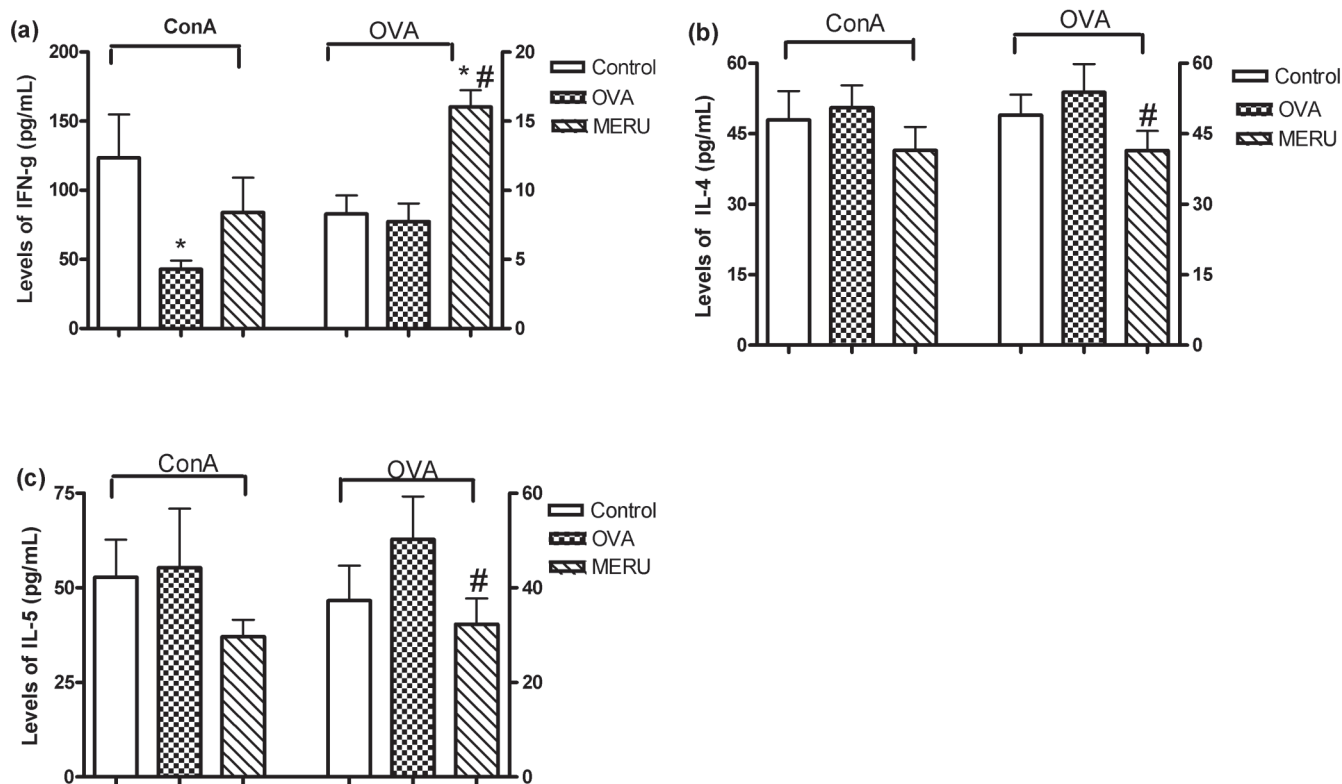


Figure 5. Quantification of cytokines interferon  $\gamma$  (IFN- $\gamma$ ), interleukin 4 (IL-4), and interleukin 5 (IL-5). The spleen cells were isolated from the mice of 3 different groups and cultured in 24-well plates in the presence of either ovalbumin (OVA) (100  $\mu$ g/mL) or concanavalin (ConA) (10  $\mu$ g/mL), respectively. The levels of IFN- $\gamma$ , IL-4, and IL-5 (a-c) in the supernatants were determined by enzyme-linked immunosorbent assay. \* $P$  < .05 compared with those in the control group. # $P$  < .05 compared with those in the OVA group. MERU indicates metyrapone-RU486.

asthmatic patients presenting with a late-phase reaction had lower levels of cortisol during the late-phase period.<sup>18</sup> Therefore, we hypothesized that the attenuated response of endogenous glucocorticoids participates in an accelerated expression of allergic disease. This idea is also corroborated by our present results, which show that inhibition of endogenous glucocorticoids resulted in more severe systemic inflammation and more sneezing in sensitized mice, even though no significant difference of inflammatory cell infiltration in the lung tissues between the inhibition and noninhibition of endogenous glucocorticoids was found in our study. Several lines of evidence have demonstrated enhancement of the local airway allergic inflammatory response by decreased endogenous glucocorticoids. For example, the study by De Bie et al<sup>24</sup> showed increasing eosinophilia in bronchoalveolar lavage fluid after ovalbumin challenge and glucocorticoid inhibition with metyrapone. Silverman et al<sup>32</sup> found that serum corticosterone levels of corticotropin-releasing hormone knockout mice decreased, which might account for the allergen-induced airway inflammation and lung mechanical dysfunction in mice. Taken together, in parallel with the well-established therapeutic benefit of exogenously administered glucocor-

ticoids in the treatment of airway allergic disease, endogenous glucocorticoids also play an important role in attenuating the allergic inflammation elicited by allergen exposure. Because of the impaired HPA axis response in the airway allergic disease, the insufficient increase of endogenous glucocorticoids after exposure to allergen may contribute to the progression of allergic disease and inflammatory response. The use of exogenous glucocorticoids might compensate for the insufficient endogenous glucocorticoids to inhibit the allergic inflammation. These findings suggest that enhancement of the HPA axis function may be a good alternative approach to the treatment of airway allergic diseases. The reasons for the difference in the local airway inflammatory reaction between inhibition and noninhibition of endogenous glucocorticoids may be due to different sensitization or challenge protocols. However, there may be other possible mechanisms causing the difference in eosinophil infiltration in the lungs of sensitized mice in the glucocorticoid-inhibition and noninhibition groups that warrant further investigation.

In addition, airway allergic diseases are characterized by the imbalance of  $T_H1/T_H2$  responses associated with allergic inflammation. In the present study, we investigated the

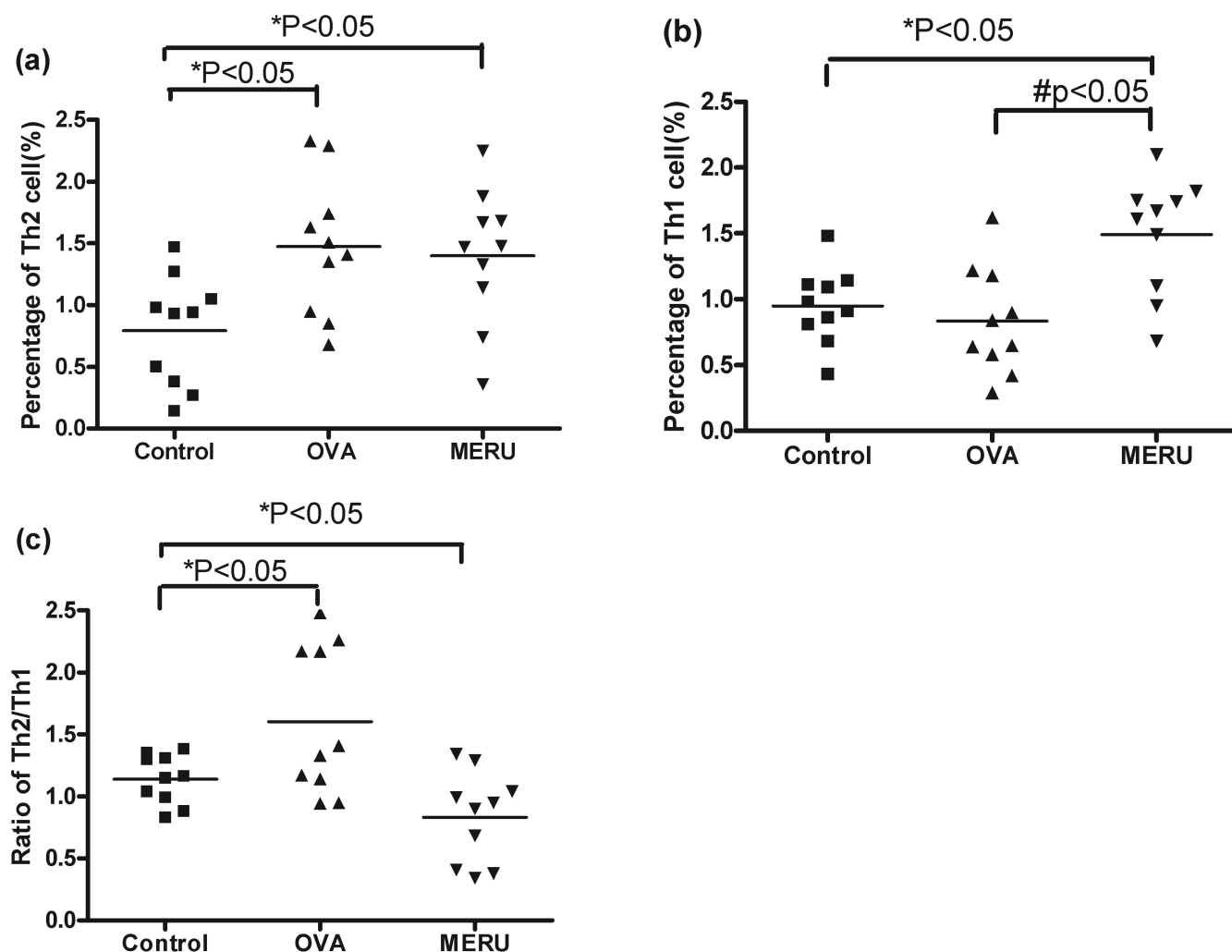


Figure 6. The percentage of  $T_H2$  cells,  $T_H1$  cells, and the ratio of  $T_H2/T_H1$  cells. The spleen cells were isolated from 3 different group mice, and the percentage of  $T_H2$  cells and  $T_H1$  cells was measured by flow cytometry. a, The percentage of  $T_H2$  cells; b, the percentage of  $T_H1$  cells; and c, the ratio of  $T_H2/T_H1$  cells. \* $P < .05$  compared with those in the control group. # $P < .05$  compared with those in the ovalbumin group. MERU indicates metyrapone-RU486.

role of endogenous glucocorticoids in regulating the immune response. We found that inhibition of the effects of endogenous glucocorticoids led to an upregulation of the  $IFN-\gamma$  levels, as well as a shift to  $T_H1$  predominance. Studies in the 1970s and 1980s revealed that lymphocyte proliferation and cytotoxicity and the secretion of tumor necrosis factor  $\alpha$ , IL-2, and  $IFN-\gamma$  were all inhibited by glucocorticoids. These observations, in the context of the broad clinical use of glucocorticoids, initially led to the conclusion that glucocorticoids were, in general, immunosuppressive. Recent evidence indicates, however, that systemic glucocorticoids cause selective suppression of the  $T_H1$ -cellular immunity axis and a shift toward  $T_H2$ -mediated humoral immunity, rather than generalized immunosuppression.<sup>33</sup> This may explain the present finding that

the  $T_H1/T_H2$  balance shifted to  $T_H1$  predominance when the effects of endogenous glucocorticoids were inhibited.

In the present study, no significant difference was found in  $T_H2$  cells between ovalbumin and metyrapone-RU486 mice after challenge, but a significant decrease was found in  $T_H2$  cytokines in the metyrapone-RU486 group. Also, no difference was found between control and ovalbumin mice for IL-4 and IL-5 production after ovalbumin stimulation. What are the causes of the negative results between these groups? The following discussion might bring some answers to the question. The general principle of an allergic airway murine model relies primarily on systemic intraperitoneal allergen injection and local challenge. However, repeated local challenge might cause tolerance in the animals.<sup>34-36</sup> In the present study, mice were exposed to antigens repeatedly for 10 days.

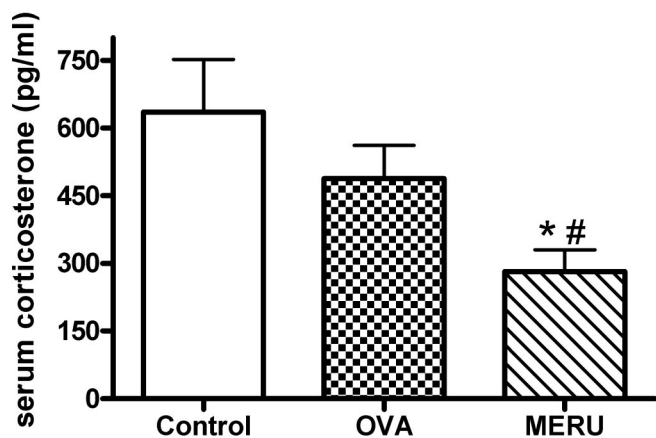


Figure 7. Serum levels of corticosterone. The blood samples in 3 different groups of mice were collected 24 hours after the final challenge, and the serum corticosterone levels were measured by enzyme-linked immunosorbent assay. \* $P < .05$  compared with those in the control group. # $P < .05$  compared with those in the ovalbumin (OVA) group. MERU indicates metyrapone-RU486.

Tolerance, at least in part, in the animals could not be ruled out and might lower the difference among the groups. Also, the *vitro* and the *vivo* analysis might partly contribute to the discrepancy between the  $T_H2$  cells and their cytokines in the present study. The cytokine levels in the cultured spleen cell supernatants reflected the cytokines secreted by the cells into the supernatants when the spleen cells were cultured with stimulator *in vitro*. However, the  $T_H2$  cells analyzed by flow cytometry might more directly reflect the *in vivo* state because the spleen cells were stained and analyzed for  $T_H1/T_H2$  cells immediately after they were isolated from the sensitized mice. When the isolated cells were cultured *in vitro*, the cytokine secretion may be influenced by many factors, such as culture time, types of stimulator, concentration of stimulators, sensitivity and reliability of the test method, and so on. Such factors contributed to the noise in the experiment, increased deviation, and reduced the gap between the non-sensitized and treatment groups.

Given the key role that  $T_H2$  cells and cytokines play in the pathophysiology of airway allergic disease, it was originally thought that inducing a  $T_H1$  response would be protective. However, in our study, we did not find that the inflammatory reaction decreased in the metyrapone-RU486 group mice, which showed a  $T_H1$ -predominant reaction. On the contrary, the eosinophils in the bone marrow and peripheral blood of the metyrapone-RU486 group mice increased significantly compared with those in the ovalbumin group mice. Studies using mouse models have provided conflicting results about the potential role of  $T_H1$  cells and IFN- $\gamma$  in airway inflammation. For example,  $T_H1$  cells and IFN- $\gamma$  were found to dampen allergic airway inflammation in some studies, whereas  $T_H1$  cells and IFN- $\gamma$  were found to have proinflammatory effects in other studies.<sup>37–39</sup> The potential role of

IFN- $\gamma$  is complex and depends on the stage of the disease. A line of evidence showed that total and free levels of serum cortisol and cortisone correlated with the severity and duration of asthma attacks and the low levels of adrenocortical hormones found in children were related to severe asthma attacks.<sup>40</sup> Shannon et al<sup>41</sup> found that IFN- $\gamma$  expression was increased and IL-4 expression was decreased in patients with severe asthma compared with moderate disease. Our study demonstrated that inhibition of endogenous glucocorticoids in sensitized mice led to a shift to  $T_H1$  predominate response and more severe systemic inflammation. Those findings implied that the levels of endogenous glucocorticoids might correlate with the severity of airway allergic disease and inducing a  $T_H1$  response would not always be protective in airway allergic disease.

In the present study, the eosinophil counts in the bone marrow, blood, and lungs were increased but the IL-5 levels were down-regulated in the metyrapone-RU486 group. Concurrently, IL-5 has been recognized as the major maturation and differentiation factor for eosinophils. However, dissociations between IL-5 and eosinophils have also been reported. For example, anti-IL-5 treatment has failed to completely abolish eosinophils.<sup>42</sup> It was reported that peripheral blood mononuclear cells from children with asthma and healthy children produced similar amounts of IL-5 when stimulated *in vitro*. However, children with asthma had higher eosinophil counts and circulating eosinophil cationic protein levels than healthy children.<sup>43</sup> These findings suggest that there may be IL-5-independent pathways that are able to, at least in part, regulate eosinophil inflammation. IL-3 and granulocyte-monocyte colony-stimulating factor, which have been shown to be expressed at increased concentrations in asthmatic airways, are the only known candidates capable of inducing eosinophil differentiation, migration, and survival.<sup>44,45</sup> In our study, we found that eosinophil inflammation in the metyrapone-RU486 group was independent of IL-5. It is well known that inhibition of cytokine-stimulated eosinophil survival is 1 of the most important actions of glucocorticoids; however, the precise mechanism by which glucocorticoids reduce tissue eosinophils has not been established. Our findings indicate that the levels of the endogenous glucocorticoids might contribute to the IL-5-independent pathway to regulate eosinophils, the precise mechanisms by which are worthy of further investigation.

In conclusion, the endogenous glucocorticoids, the end effectors of the HPA axis, could protect against systemic inflammatory reaction and modulate the  $T_H1/T_H2$  balance of immune system in an airway allergic murine model. Our study implied that endogenous glucocorticoids might play an important role in the pathophysiologic processes of airway allergic disease, thus regulating endogenous glucocorticoid secretion, which might be another treatment target of airway allergic disease. By inhibition of endogenous glucocorticoids, the  $T_H2$ -dominant immune responses switched to the  $T_H1$ -dominant immune responses in sensitized mice, but that did not attenuate the severity of the allergic inflammatory reac-

tion. In contrast, it resulted in more severe systemic inflammation.

## ACKNOWLEDGMENTS

We are grateful to Weihua Chen, Jian Ying, Jianhua Huang, and Bin Wu for their technical support throughout the study.

## REFERENCES

1. Quraishi SA, Davies MJ, Craig TJ. Inflammatory responses in allergic rhinitis: traditional approaches and novel treatment strategies. *J Am Osteopath Assoc*. 2004;104(5 suppl 5):S7–S15.
2. Bochner BS, Busse WW. Allergy and asthma. *J Allergy Clin Immunol*. 2005;115:953–959.
3. Kroegel C, Costabel U. Immune functions of constitutive pulmonary cells: the salt in the soup. *Eur Respir J*. 1994;7:2106–2107.
4. Krouse JH, Brown RW, Fineman SM, et al. Asthma and the unified airway. *Otolaryngol Head Neck Surg*. 2007;136(5 suppl):S75–S106.
5. Ciprandi G, Cirillo I. The lower airway pathology of rhinitis. *J Allergy Clin Immunol*. 2006;118:1105–1109.
6. Busse WW, Coffman RL, Gelfand EW, Kay AB, Rosenwasser LJ. Mechanisms of persistent airway inflammation in asthma: a role for T-cells and T-cell products. *Am J Respir Crit Care Med*. 1995;152:388–393.
7. Cher DJ, Mosmann TR. Two types of murine helper T-cell clone: delayed-type hypersensitivity is mediated by Th1 clones. *J Immunol*. 1987;138:3688–3694.
8. Mosmann TR, Coffman RL. Th1-cell and Th2-cell: different patterns of lymphokine secretion lead to different functional-properties. *Ann Rev Immunol*. 1989;7:145–173.
9. Finkelman FD, Katona IM, Urban JF Jr, et al. IL-4 is required to generate and sustain in vivo IgE responses. *J Immunol*. 1988;141:2335–2341.
10. Lopez AF, Sanderson CJ, Gamble JR, Campbell HD, Young IG, Vadas MA. Recombinant human interleukin-5 is a selective activator of human eosinophil function. *J Exp Med*. 1988;167:219–224.
11. Gajewski TF, Joyce J, Fitch FW. Antiproliferative effect of IFN- $\gamma$  in immune regulation: differential selection of Th1 and Th2 murine helper lymphocyte-T clones using recombinant IL-2 and recombinant IFN- $\gamma$ . *J Immunol*. 1989;143:15–22.
12. Webster JI, Tonelli L, Sternberg EM. Neuroendocrine regulation of immunity. *Ann Rev Immunol*. 2002;20:125–163.
13. Adcock IM. Molecular mechanisms of glucocorticosteroid actions. *Pulm Pharmacol Ther*. 2000;13:115–126.
14. Barnes PJ, Adcock IM. Transcription factors and asthma. *Eur Respir J*. 1998;12:221–234.
15. Chrousos GP. Seminars in Medicine of the Beth-Israel-Hospital, Boston: the hypothalamic-pituitary-adrenal axis and immune-mediated inflammation. *N Engl J Med*. 1995;332:1351–1362.
16. Sasaki H, Yanai M, Shimura S, et al. Late asthmatic response to ascaris antigen challenge in dogs treated with metyrapone. *Am Rev Respir Dis*. 1987;136:1459–1465.
17. Herrscher RF, Kasper C, Sullivan TJ. Endogenous cortisol regulates immunoglobulin E-dependent late phase reactions. *J Clin Invest*. 1992;90:596–603.
18. Peebles RS Jr, Togias A, Bickel CA, Diemer FB, Hubbard WC, Schleimer RP. Endogenous glucocorticoids and antigen-induced acute and late phase pulmonary responses. *Clin Exp Allergy*. 2000;30:1257–1265.
19. Donley MP, Schulkin J, Rosen JB. Glucocorticoid receptor antagonism in the basolateral amygdala and ventral hippocampus interferes with long-term memory of contextual fear. *Behav Brain Res*. 2005;164:197–205.
20. Heikinheimo O, Kekkonen R. Dose-response relationships of Ru-486. *Ann Med*. 1993;25:71–76.
21. Lahteenmaki P, Heikinheimo O, Croxatto H, et al. Pharmacokinetics and metabolism of Ru-486. *J Steroid Biochem*. 1987;27:859–863.
22. Kawai S, Nieman LK, Brandon DD, et al. Pharmacokinetic properties of the antiglucocorticoid and antiprogesterone steroid Ru 486 in man. *J Pharmacol Exp Ther*. 1987;241:401–406.
23. Georen S, Ahnblad P, Stjarne P, Wikstrom AC, Stierna P. Significance of endogenous glucocorticoid sensitivity for airway eosinophilia in a murine model of allergy. *Acta Otolaryngol*. 2005;125:378–385.
24. De Bie JJ, Hessel EM, Van Ark I, et al. Effect of dexamethasone and endogenous corticosterone on airway hyperresponsiveness and eosinophilia in the mouse. *Br J Pharmacol*. 1996;119:1484–1490.
25. Sternberg EM, Hill JM, Chrousos GP, et al. Inflammatory mediator-induced hypothalamic pituitary-adrenal axis activation is defective in streptococcal cell-wall arthritis-susceptible Lewis rats. *Proc Natl Acad Sci U S A*. 1989;86:2374–2378.
26. Wei RT, Listwak SJ, Sternberg EM. Lewis hypothalamic cells constitutively and upon stimulation express higher levels of mRNA for pro-inflammatory cytokines and related molecules: comparison with inflammatory resistant Fischer rat hypothalamic cells. *J Neuroimmunol*. 2003;135:10–28.
27. Eum SY, Creminon C, Haile S, Lefort J, Vargaftig BB. Inhibition of airways inflammation by dexamethasone is followed by reduced bronchial hyperreactivity in BP2 mice. *Clin Exp Allergy*. 1996;26:971–979.
28. Kauffmann F, Guiochon-Mantel A, Neukirch F. Is low endogenous cortisol a risk factor for asthma? *Am J Respir Crit Care Med*. 1999;160:1428–1428.
29. Landstra AM, Postma DS, Boezen HM, van Aalderen WM. Role of serum cortisol levels in children with asthma. *Am J Respir Crit Care Med*. 2002;165:708–712.
30. Buske-Kirschbaum A, von Auer K, Krieger S, Weis S, Rauh W, Hellhammer D. Blunted cortisol responses to psychosocial stress in asthmatic children: a general feature of atopic disease? *Psychosom Med*. 2003;65:806–810.
31. Wamboldt MZ, Laudenslager M, Wamboldt FS, Kelsay K, Hewitt J. Adolescents with atopic disorders have an attenuated cortisol response to laboratory stress. *J Allergy Clin Immunol*. 2003;111:509–514.
32. Silverman ES, Breault DT, Vallone J, et al. Corticotropin-releasing hormone deficiency increases allergen-induced airway inflammation in a mouse model of asthma. *J Allergy Clin Immunol*. 2004;114:747–754.
33. Elenkov IJ. Glucocorticoids and the Th1/Th2 balance. *Ann N Y Acad Sci*. 2004;1024:138–146.
34. Tsitoura DC, DeKruyff RH, Lamb JR, Umetsu DT. Intranasal exposure to protein antigen induces immunological tolerance mediated by functionally disabled CD4(+) T cells. *J Immunol*. 1999;163:2592–2600.
35. Tsitoura DC, Blumenthal RL, Berry G, DeKruyff RH, Umetsu DT. Mechanisms preventing allergen-induced airways hyperreactivity: role of tolerance and immune deviation. *J Allergy Clin Immunol*. 2000;106:239–246.
36. McCusker C, Chicoine M, Hamid Q, Mazer B. Site-specific sensitization in a murine model of allergic rhinitis: role of the upper airway in lower airways disease. *J Allergy Clin Immunol*. 2002;110:891–898.
37. Randolph DA, Stephens R, Carruthers CJ, Chaplin DD. Cooperation between Th1 and Th2 cells in a murine model of eosinophilic airway inflammation. *J Clin Invest*. 1999;104:1021–1029.
38. Ford JG, Rennick D, Donaldson DD, et al. IL-13 and IFN- $\gamma$ : interactions in lung inflammation. *J Immunol*. 2001;167:1769–1777.
39. Cui J, Pazdzioro S, Miyashiro JS, et al. TH1-mediated airway hyperresponsiveness independent of neutrophilic inflammation. *J Allergy Clin Immunol*. 2005;115:309–315.
40. Nomura S, Fujitaka M, Sakura N, Ueda K. Adrenocortical function in asthmatic children: low levels of adrenocortical hormones in children with persistent attacks. *Eur J Pediatr*. 1997;156:323–328.
41. Shannon J, Ernst P, Yamauchi Y, et al. Differences in airway cytokine profile in severe asthma compared to moderate asthma. *Chest*. 2008;133:420–426.
42. Flood-Page PT, Menzies-Gow AN, Kay AB, Robinson DS. Eosinophil's

- 
- role remains uncertain as anti-interleukin-5 only partially depletes numbers in asthmatic airway. *Am J Respir Crit Care Med.* 2003;167:199–204.
43. Miller GE, Gaudin A, Zysk E, Chen E. Parental support and cytokine activity in childhood asthma: the role of glucocorticoid sensitivity. *J Allergy Clin Immunol.* 2009;123:824–830.
44. Robinson DS, Hamid Q, Ying S, et al. Predominant Th2-Like bronchoalveolar lymphocyte-T population in atopic asthma. *N Engl J Med.* 1992;326:298–304.
45. Asquith KL, Ramshaw HS, Hansbro PM, Beagley KW, Lopez AF, Foster PS. The IL-3/IL-5/GM-CSF common beta receptor plays a pivotal

role in the regulation of Th2 immunity and allergic airway inflammation. *J Immunol.* 2008;180:1199–1206.

*Requests for reprints should be addressed to:*

Xinmin Zhang, PhD  
Huashan Hospital  
Fudan University  
12 Wulumuqi Middle Rd  
Shanghai, China 200040  
E-mail: xmzhang54@yahoo.com

---