

# The Distinctive Effects of Acute and Chronic Psychological Stress on Airway Inflammation in a Murine Model of Allergic Asthma

Kaori Okuyama<sup>1</sup>, Keiko Ohwada<sup>1</sup>, Shinobu Sakurada<sup>2</sup>, Naoko Sato<sup>3</sup>, Ichiro Sora<sup>4</sup>, Gen Tamura<sup>5</sup>, Motoaki Takayanagi<sup>1</sup> and Isao Ohno<sup>1</sup>

## ABSTRACT

**Background:** Psychological stress has long been recognized to be associated with asthma symptoms. There appear to be individual differences in the susceptibility to even the same kind of stress, and furthermore, stress responses are different between the types of the stress, acute and chronic, even in the same person. However, the mechanisms linking stress to asthma are not well defined. Psychological stress upregulates the expression of endogenous opioids. The opioids stimulate the hypothalamus-pituitary-adrenal axis and sympathetic and adrenomedullary system, through the activation of  $\mu$ -opioid receptor (MOR) to release stress hormones, such as cortisol and catecholamines, respectively. These hormones can modulate immune responses via the induction of Th1 immunity.

**Methods:** Female BALB/c and C57BL/6, wild and MOR-deficient, mice sensitized with ovalbumin (OVA) were exposed to OVA with or without either acute or chronic restraint stress. Airway inflammation was evaluated by the measurement of the number of inflammatory cells and cytokine contents in bronchoalveolar lavage fluids.

**Results:** In BALB/c mice, but not in C57BL/6 mice, the number of total cells, eosinophils and lymphocytes in the acute stress group were significantly decreased compared with those in the non-acute stress group. In contrast, chronic stress significantly increased the cell numbers and the contents of IL-4 and IL-5 in both mouse strains. Furthermore, these exacerbations were abolished in MOR-deficient mice.

**Conclusions:** These results suggest that acute stress modifies the allergic airway responses distinctively depending on the genetic background, and MOR is involved in the chronic psychological stress-induced exacerbation of allergic airway inflammation.

## KEY WORDS

airway inflammation, bronchial asthma, opioid, psychological stress,  $\mu$ -opioid receptor

## INTRODUCTION

Bronchial asthma is a disease characterized by chronic airway inflammation associated with the accumulation and activation of inflammatory cells, such as eosinophils and mast cells, in the bronchial wall and airway lumen. The behavior of the inflammatory cells in asthmatic airways is tightly regulated by a

network of Th2 cytokines, such as IL-4, IL-5 and IL-13, which are produced mainly by Th2 cells. Inflammatory mediators released from these cells upon stimulation, including eosinophil granule protein, arachidonic acid metabolites and reactive oxygen species, evoke bronchoconstriction, bronchial mucosal oedema, epithelial shedding and airway hyper-responsiveness, which are the pathophysiological fea-

<sup>1</sup>Department of Pathophysiology, <sup>2</sup>Department of Physiology and Anatomy, <sup>3</sup>Department of Radiopharmacy, Tohoku Pharmaceutical University, <sup>4</sup>Division of Psychobiology, Department of Neuroscience and <sup>5</sup>Department of Respiratory and Infectious Diseases, Tohoku University Graduate School of Medicine, Miyagi, Japan.  
Correspondence: Isao Ohno, Department of Pathophysiology, To-

hoku Pharmaceutical University, 4-4-1 Komatsushima, Aoba-ku, Sendai, Miyagi 981-8558, Japan.

Email: iohno@tohoku-pharm.ac.jp

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tures observed in asthmatics.<sup>1-3</sup>

Bronchial asthma is one of the most common chronic diseases worldwide, and its prevalence has increased over the past 20 years. Although the underlying pathophysiology of the increased prevalence is not entirely understood, some reports suggest that increasing psychological stress is involved in the phenomenon.<sup>4</sup> Indeed, several recent reports indicated that between 20–35% of asthmatics experience exacerbations of symptoms during periods of stress.<sup>5</sup> For example, asthmatics who viewed highly emotional films showed increases in oscillatory resistance,<sup>6</sup> and chronic stress caused by academic examinations was shown to increase airway inflammation after antigen inhalation in college students with mild asthma.<sup>7</sup> However, in contrast, an improvement in airway constriction induced by antigen inhalation was observed during acute stress.<sup>8</sup> Furthermore, there appear to be individual differences in the susceptibility to even the same kind of stress, which might be attributable to genetic factors, utero and postnatal environmental factors and the timing of the exposure to psychological stress.<sup>9</sup>

Although the precise mechanisms linking psychological stress to asthmatic responses, especially exacerbations, are not well understood, one contributing factor could be the effects of stress on inflammatory and immune responses. This interpretation is supported by findings in asthmatics<sup>7</sup> and animal models.<sup>10-12</sup> Psychological stress is associated with the activation of the hypothalamic-pituitary-adrenal (HPA) axis and sympathetic and adrenomedullary (SAM) system, resulting in the enhanced secretion of cortisol and catecholamines, respectively.<sup>13</sup> These stress hormones have been reported to shift the immune response from Th1 to Th2 by the downregulation of Th1 cytokines expression.<sup>14</sup>

Psychological stress also upregulates the mRNA expression and production of endogenous opioids<sup>15-17</sup> including endorphins, enkephalins and dynorphins. The endogenous opioids are involved in multiple stress-induced behavioral responses by affecting many physiological processes such as analgesia, neuroendocrine function and control of respiration.<sup>18</sup> These opioids exert their effects through the binding to opioid receptors consisting of three subtypes,  $\mu$ ,  $\delta$  and  $\kappa$ . The functions of the HPA axis and SAM system are modulated by opioids in stressful conditions through the binding of the ligands to  $\mu$ -opioid receptors (MOR).<sup>19,20</sup> MOR is also expressed on inflammatory cells including T cells, B cells, monocytes/macrophages, polymorphonuclear leukocytes and dendritic cells.<sup>21,22</sup> The activation of MOR on lymphocytes was reported to induce the differentiation of Th2 cells and a shift from Th1 to Th2 responses.<sup>23-26</sup>

Therefore, we hypothesized that the distinctive effects of acute and chronic stress on allergic airway responses are different depending on the genetic back-

ground, and that asthmatic exacerbations caused by psychological stress are mediated by the activation of MOR. To address this hypothesis, we examined the effects of acute and chronic stress on allergic airway inflammation in a murine model of asthma, using two different strains, C57BL/6 and BALB/c mice, and also the effect of MOR gene knockout on stress-induced exacerbations of allergic airway inflammation.

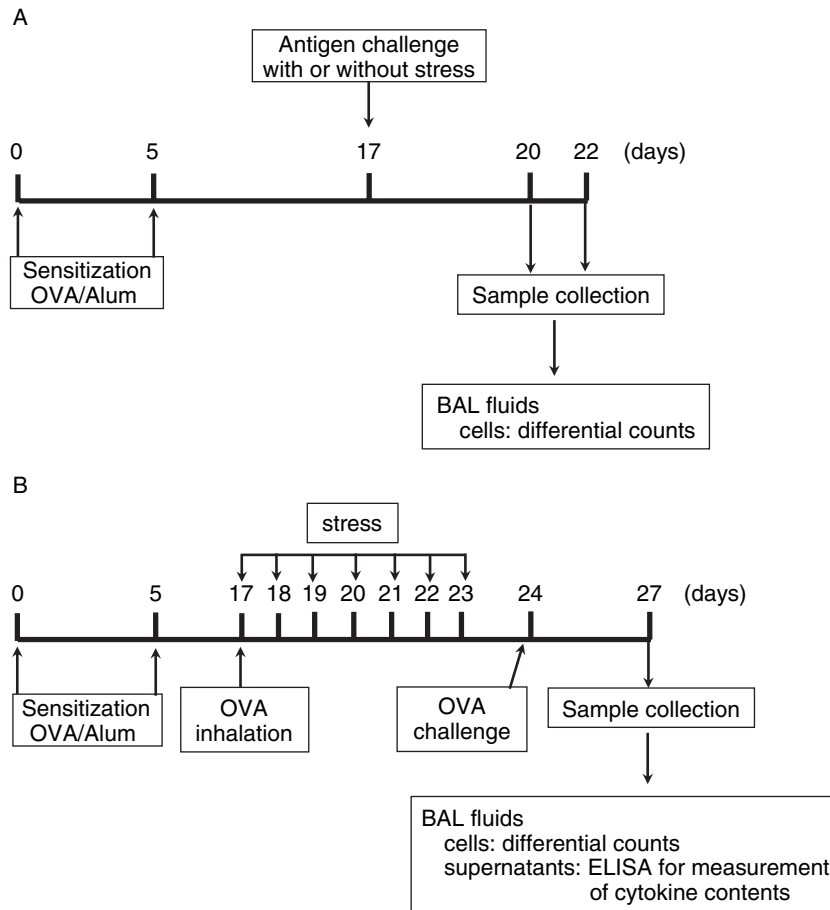
## METHODS

### ANIMALS

Specific pathogen-free (SPF) female C57BL/6 (B6) and BALB/c mice were purchased from Japan SLC (Hamamatsu, Japan). MORKO mice were generated as described previously.<sup>27</sup> Congenic MORKO mice backcrossed with B6 mice<sup>28</sup> and wild type B6 mice (Japan SLC) as control were used in this study. Animals were housed under a 12 hour light/dark cycle with constant temperature ( $22 \pm 2^\circ\text{C}$ ). Sterilized food and water were available ad libitum. All experiments described below were approved by and conformed to the guidelines of the Committee of Animal Experiments at Tohoku Pharmaceutical University.

### PROTOCOLS FOR ACUTE STRESS

B6 and BALB/c mice, 6–8 weeks of age, were sensitized and made to inhale antigen as previously described.<sup>29</sup> Briefly, mice were sensitized by intraperitoneal (i.p.) injections of chicken ovalbumin (OVA) (Grade V, Sigma, St. Louis, MO, USA) (8  $\mu\text{g}/\text{mouse}$ ) adsorbed with aluminum hydroxide (Wako Pure Chemical Industries, Osaka, Japan) on day 0 and 5. On day 17, mice were challenged with aerosolized OVA (5% in saline) or saline as a control for 1 hour two times at a 4 hour interval, with (acute-stressed mice) or without (non acute-stressed mice) acute stress. Acute-stressed mice were exposed to restraint stress for 6 hours during the two periods of OVA aerosol administration and during the interval. Restraint stress is generally regarded as inducing psychological stress in animals.<sup>23</sup> Each mouse was placed in a 50-mL conical centrifuge tube with multiple ventilation holes. Mice were not physically squeezed and felt no pain. This restraint allowed the mice to rotate from a supine to prone position, but not to turn their heads toward tails, not to take food and water. Non-acute-stressed mice were not exposed to the restraint but were deprived of food and water for the same duration that the acute-stressed mice were exposed to stress (Fig. 1A). Three days for BALB/c and five days for B6 after the antigen challenge, bronchoalveolar lavage (BAL) fluids were collected as previously described.<sup>29</sup> Total cell numbers in the BAL fluids were counted with a haemocytometer. Smears of BAL cells prepared with a Cytospin IV (Shandon, Runcorn, UK) were stained with Diff-Quik solution (International Reagents Corp., Kobe, Japan) for dif-



**Fig. 1** Diagrammatic representation of the protocols for sensitization, challenge and stress exposure.

ferential cell counting. The percentages of cell differentials were determined by counting at least 200 cells under light microscopy. Counts of total cells and cell differentials in BAL fluids were performed by a person unaware of the experiment.

### PROTOCOLS FOR CHRONIC STRESS

BALB/c, B6 and MORKO mice, 6–8 weeks of age, were sensitized as mentioned above. On day 17, mice were challenged with aerosolized OVA or saline for 1 hour with (chronic-stressed mice) or without (non-chronic-stressed mice) the restraint stress. Chronic-stressed mice were further exposed to the restraint stress for 6 consecutive days, day 18 to 23, at the same time everyday (Fig. 1B). Non-chronic-stressed mice were only deprived of food and water for the same period that the chronic-stressed mice were exposed to stress. On day 24, the mice were challenged with aerosolized OVA or saline for 1 hour. Three days after the last challenge, on day 27, BAL fluids were collected, and total cells and cell differentials were counted, as described above. After centrifugation of BAL fluid, supernatants were stored at  $-80^{\circ}\text{C}$  for cy-

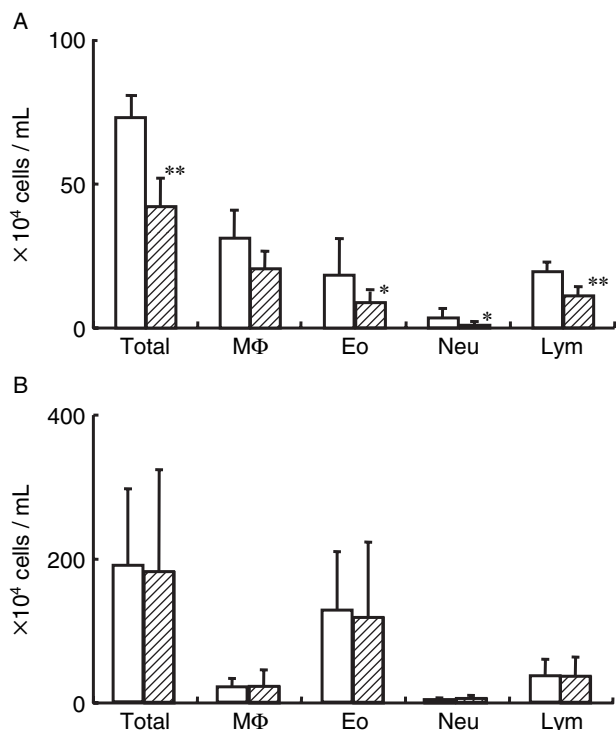
tokine measurements by enzyme-linked immunosorbent assay (ELISA). Since, in rodent model, stress loading for relatively short duration (hours or repeated for a few days) has been deemed “acute stress”, and for relatively long duration (weeks) “chronic stress”,<sup>10-12,30</sup> we termed a single exposure to restraint stress “acute” and repeated (for 7 days) exposure to the stress “chronic” in this study.

### CYTOKINE ASSAYS

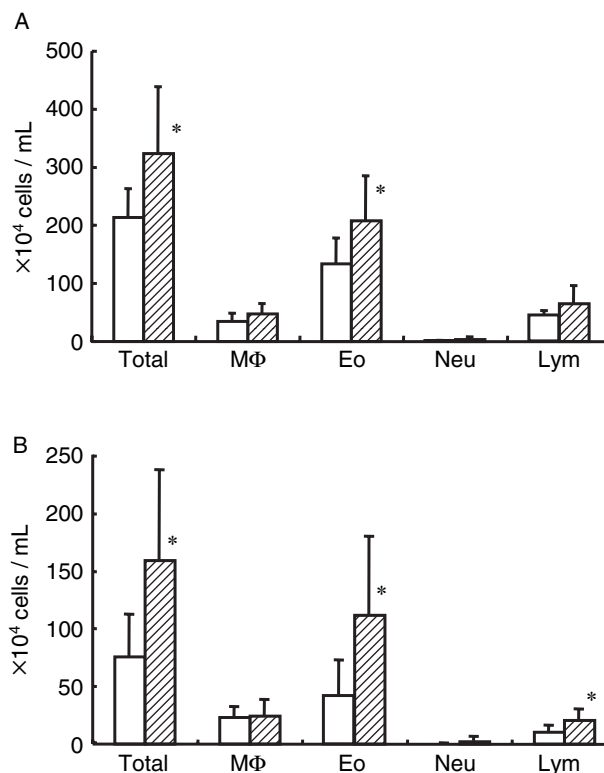
IL-4 and IL-5 contents in BAL fluids were measured using an ELISA kit (R&D systems, Minneapolis, MN, USA) according to the manufacturer’s protocol. The sensitivity of detection was 2 pg/mL for IL-4 and 7 pg/mL for IL-5.

### DATA ANALYSIS

Data were presented as mean  $\pm$  SD. Significant differences between two groups were determined using the nonparametric Mann-Whitney *U*-test. These analysis were performed using Prism4 (GraphPad Software, San Diego, CA, USA). A *p* value of less than 0.05 was taken as significant.



**Fig. 2** The effect of acute restraint stress on the accumulation of inflammatory cells in BAL fluids after antigen challenge. BAL fluids were obtained from non-acute-stressed and acute-stressed BALB/c (A) and C57BL/6 (B) mice 3 days after antigen challenge. The numbers of total cells (Total), macrophages (MΦ), eosinophils (Eo), neutrophils (Neu) and lymphocytes (Lym) were counted. Open and striped columns represent non-acute-stressed and acute-stressed mice, respectively. Data are expressed as mean  $\pm$  S.D. ( $n = 8-9$ ). \*  $p < 0.05$ , \*\*  $p < 0.01$



**Fig. 3** The effect of chronic restraint stress on the accumulation of inflammatory cells in BAL fluids after antigen challenge. BAL fluids were obtained from non-chronic-stressed and chronic-stressed BALB/c (A) and C57BL/6 (B) mice 3 days after the second antigen challenge. The numbers of total cells (Total), macrophages (MΦ), eosinophils (Eo), neutrophils (Neu) and lymphocytes (Lym) were counted. Open and striped columns represent non-chronic-stressed and chronic-stressed mice, respectively. Data are expressed as mean  $\pm$  S.D. ( $n = 8-9$ ). \*  $p < 0.05$

## RESULTS

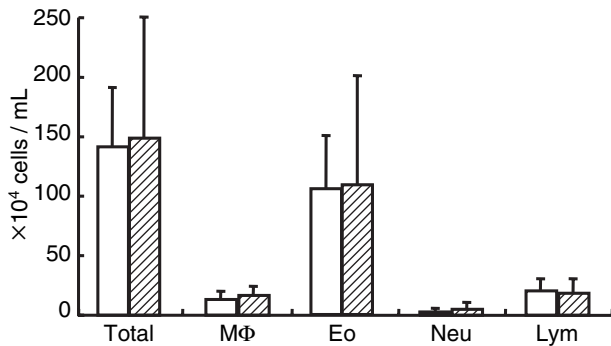
### EFFECT OF ACUTE STRESS ON THE AIRWAY INFLAMMATION

The numbers of total cells, eosinophils, neutrophils and lymphocytes in the BAL fluids of the acute-stressed BALB/c mice ( $n = 7$ ) were significantly decreased compared with those of the non-acute-stressed BALB/c mice ( $n = 7$ ) 3 days after the OVA challenge ( $42.4 \pm 8.7$  vs  $73.0 \pm 7.9 \times 10^4$  /mL,  $p < 0.01$ ;  $9.2 \pm 4.0$  vs  $18.6 \pm 12.2 \times 10^4$  /mL,  $p < 0.05$ ;  $1.0 \pm 1.1$  vs  $3.7 \pm 2.8 \times 10^4$  /mL,  $p < 0.05$  and  $11.2 \pm 3.4$  vs  $19.5 \pm 3.6 \times 10^4$  /mL,  $p < 0.01$ , respectively) (Fig. 2A). In contrast, in B6 mice, there was no significant difference in the numbers of BAL cells between acute-stressed and non-acute-stressed mice (Fig. 2B).

### EFFECT OF CHRONIC STRESS ON THE AIRWAY INFLAMMATION

The numbers of total cells and eosinophils in BAL flu-

ids of chronic-stressed BALB/c mice ( $n = 9$ ) were significantly increased compared with those of non-chronic-stressed BALB/c mice ( $n = 8$ ) 3 days after the second OVA challenge ( $323.5 \pm 114.1$  vs  $213.3 \pm 49.0 \times 10^4$  /mL,  $p < 0.05$  and  $208.0 \pm 76.4$  vs  $133.7 \pm 43.6 \times 10^4$  /mL,  $p < 0.05$ , respectively) (Fig. 3A). In B6 mice, while acute stress did not change the numbers of inflammatory cells, chronic stress produced a significant increment in the numbers of total cells, eosinophils and lymphocytes in BAL fluids ( $n = 8$ ) compared with non-chronic-stressed mice ( $n = 7$ ) 3 days after the second challenge ( $159.2 \pm 78.2$  vs  $75.8 \pm 36.0 \times 10^4$  /mL,  $p < 0.05$ ;  $111.7 \pm 68.0$  vs  $42.2 \pm 30.1 \times 10^4$  /mL,  $p < 0.05$  and  $20.6 \pm 9.3$  vs  $10.3 \pm 5.6 \times 10^4$  /mL,  $p < 0.05$ , respectively) (Fig. 3B). However, in MORKO mice, no significant alterations in the numbers of the BAL cells were observed in chronic-stressed mice compared with those in non-chronic-stressed mice (Fig. 4).



**Fig. 4** The effect of chronic restraint stress on the accumulation of inflammatory cells in BAL fluids after antigen challenge in MORKO mice. BAL fluids were obtained from non-chronic-stressed and chronic-stressed MORKO mice 3 days after the second antigen challenge. The numbers of total cells (Total), macrophages (MΦ), eosinophils (Eo), neutrophils (Neu) and lymphocytes (Lym) were counted. Open and striped columns represent non-chronic-stressed and chronic-stressed mice, respectively. Data are expressed as mean  $\pm$  S.D. ( $n = 6-7$ ).

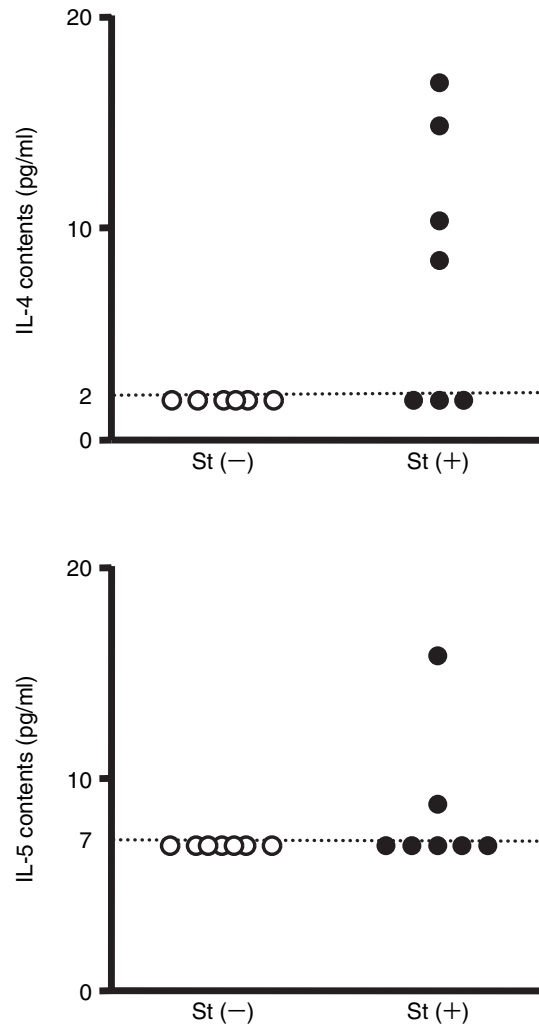
**EFFECT OF CHRONIC STRESS ON CYTOKINE CONTENTS IN BAL FLUIDS**

In the wild type of B6 mice, the contents of IL-4 and IL-5 in the BAL fluids 3 days after the second OVA challenge were relatively increased in chronic-stressed mice to in non-chronic-stressed mice in which the contents in any sample were under the limitation of detection (Fig. 5). In contrast, in MORKO mice ( $n = 8$ ), the contents of the cytokines in any sample were under the limitation of detection even in chronic-stressed mice, as well in non-chronic-stressed mice.

**DISCUSSION**

We demonstrated for the first time in the current study the following. Firstly, the effects of acute psychological stress on allergic airway inflammation were different between two mouse strains. Acute restraint stress decreased the cell numbers in BAL fluids after OVA inhalation in BALB/c mice, but not in B6 mice. Secondly, chronic psychological stress exacerbated the allergic airway inflammation independently of the mouse strain. Chronic restraint stress increased the cell numbers in BAL fluids after OVA inhalation in both mouse strains. Thirdly, MOR is involved in the chronic psychological stress-induced exacerbation of allergic airway inflammation.

In BALB/c mice, we observed bipolar effects of stress; acute restraint stress improved and chronic restraint stress enhanced the airway inflammation. Our findings in BALB/c mice were consistent with the findings by Forsythe *et al.*<sup>10</sup> We examined the effects



**Fig. 5** Cytokine contents in BAL fluids from wild type mice. BAL fluids were obtained from non-chronic-stressed (St (-); open circles) and chronic-stressed (St (+); closed circles) wild type mice 3 days after the second antigen challenge. The contents of IL-4 and IL-5 in BAL fluids were measured by ELISA. Dotted lines indicate the lower limitation of the detection by ELISA.

of acute and chronic stress further in another strain, B6 mice. In B6 mice, acute restraint stress did not alter the airway inflammation while chronic restraint stress made the inflammation worse. Differences in immune reactivity between BALB/c and B6 mice have been observed under the condition of acute stress,<sup>31-33</sup> as well as without stress.<sup>33,34</sup> Shanks *et al.*<sup>31</sup> showed that BALB/c mice are more sensitive to acute stress than B6 mice in terms of the enhancement of the B cell antibody response upon exposure to acute stress. Plasma cortisol and catecholamine levels increase more intensely in BALB/c than in B6 mice in response to acute stress.<sup>32,33</sup> Furthermore, Forsythe *et al.*<sup>10</sup> showed that administration of a corti-

costeroid receptor antagonist prevented the acute stress-induced improvement of airway inflammation. These findings could explain the difference in the effect of acute stress on allergic responses between the two strains observed in the present study. Our results suggest that acute stress modifies allergic airway responses distinctively depending on the genetic background.

We observed that chronic psychological stress induced the exacerbation of allergic airway inflammation accompanied by the increased expression of Th2 cytokines, and that these exacerbations were abolished in MORKO mice. Since we hypothesized that MOR was involved in asthmatic exacerbations induced by psychological stress, the examination of the role of MOR was focused in the current study on the chronic stress model associated with the exacerbations. Chronic psychological stress was reported to lead to a shift of the immune responses from Th1 to Th2 in splenocytes stimulated non-specifically with concanavalin A (ConA).<sup>23</sup> In addition, chronic psychological stress exacerbates asthma symptoms in asthmatics<sup>7</sup> and animal models<sup>10-12</sup> in association with the upregulation of IL-4 and IL-5 expression. However, the mechanisms linking chronic stress with an enhancement of the Th2 response were not determined in those studies. It was reported that psychological stress upregulates the mRNA expression and production of endogenous opioids,<sup>15-17</sup> which exert multiple stress-induced behavioral responses through binding to opioid receptors. Especially, MOR plays a role in the immune alterations induced by psychological stress. With regard to the Th1/Th2 response, Wang *et al.*<sup>23</sup> reported that the production of Th1 cytokines after ConA stimulation was decreased in splenocytes from mice exposed to chronic restraint stress, and that the effect of chronic stress was significantly abolished in MORKO mice. Sacerddote *et al.*<sup>25</sup> demonstrated that chronic administration of naloxone, a opioid receptors antagonist, during sensitization with keyhole-limpet hemocyanin induced a shift from Th2 to Th1 responses in splenocytes upon stimulation *in vitro* with the specific antigen. Taken together with these findings, the chronic stress-induced exacerbation of allergic airway inflammation observed here might be attributable to a further immunodeviation to Th2 immunity through the activation of MOR during the exposure to restraint stress. However, the mechanisms by which MOR contributes to the increment of the Th2 response during the stress period were not determined in the present study. Psychological stress stimulates the HPA axis and SAM system via the activation of MOR to release stress hormones, cortisol and catecholamine, respectively.<sup>35</sup> These hormones have been reported to shift the immune response from Th1 to Th2 through the reduced production of Th1 cytokines such as IL-12.<sup>14</sup> It has also been proposed that prolonged activation of the HPA axis and

SAM system may result in a counterregulatory response in stimulated lymphocytes and consequent downregulation of the expression and/or function of glucocorticoid receptors.<sup>36</sup> The downregulation would reduce the sensitivity to endogenous cortisol, resulting in the exacerbation of inflammatory responses, such as asthmatic airway ones. MOR on immune cells is likely involved in the chronic stress-induced exacerbation. Roy *et al.*,<sup>26</sup> using murine splenocytes, reported that *in vitro* activation of MOR by chronic morphine treatment can differentiate naïve Th cells to Th2 cells. At present, there has been only one report that suggested the mechanisms by which chronic stress induced asthmatic exacerbations, in which a neurokinin (NK)-1 receptor antagonist was shown to improve the exacerbation in a murine model of asthma.<sup>37</sup> Although the relationship between the NK-1 receptor and MOR in exacerbation is unclear, it would be important to investigate candidates other than MOR in chronic stress-induced asthma exacerbations.

In conclusion, this study demonstrated that psychological stress affects allergic airway inflammation distinctively depending on not only the types of the stress but also the genetic background of hosts, and that the MOR is a key molecule in the exacerbation of allergic airway inflammation induced by chronic psychological stress. Our findings could suggest that the regulation of MOR activation under stressful conditions might have the potential to be a useful strategy for the treatment of stress-induced asthma.

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