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ORIGINAL ARTICLE

μ-opioid Receptor-Mediated Alterations of Allergen-Induced Immune Responses of Bronchial Lymph Node Cells in a Murine Model of Stress Asthma

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ABSTRACT

Background: Psychological stress has a recognized association with asthma symptoms. Using a murine model of allergic asthma, we recently demonstrated the involvement of μ-opioid receptors (MORs) in the central nervous system in the stress-induced exacerbation of airway inflammation. However, the involvement of MORs on neurons and immunological alterations in the stress asthma model remain unclear.

Methods: MOR-knockout (MORKO) mice that express MORs only on noradrenergic and adrenergic neurons (MORKO/Tg mice) were produced and characterized for stress responses. Sensitized mice inhaled antigen and were then subjected to restraint stress. After a second antigen inhalation, bronchoalveolar lavage cells were counted. Before the second inhalation, bronchial lymph node (BLN) cells and splenocytes from stressed and non-stressed mice were cultured with antigen, and cytokine levels and the proportions of T cell subsets were measured.

Results: Stress-induced worsening of allergic airway inflammation was observed in wild-type and MORKO/Tg mice but not MORKO mice. In wild-type stressed mice, IFN-γ/IL-4 ratios in cell culture supernatants and the proportion of regulatory T cells in BLN cell populations were significantly lower than those in non-stressed mice. These differences in BLN cells were not observed between the stressed and non-stressed MORKO mice. Restraint stress had no effect on cytokine production or T cell subsets in splenocytes.

Conclusions: Restraint stress aggravated allergic airway inflammation in association with alterations in local immunity characterized by greater Th2-associated cytokine production and a reduced development of regulatory T cells, mediated by MORs.

KEY WORDS

adaptive immune response, allergic asthma, mu opioid receptor, murine model, psychological stress

INTRODUCTION

Bronchial asthma is a chronic airway inflammatory disease characterized by the accumulation and activation of inflammatory cells, such as eosinophils and mast cells, in the bronchial wall and airway lumen. Inflammatory mediators released from these cells upon stimulation, such as arachidonic acid metabolites and reactive oxygen species, induce the airway narrowing and airway hyper-responsiveness observed in asthmatics. Type 2 $CD4^+$ T (Th2) cells and Th2-biased cytokines, such as IL-4, IL-5 and IL-13, play a central

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role in regulating the behavior of inflammatory cells in asthmatic airways. It has been proposed that interferon-γ (IFN-γ)-producing type 1 $CD4^+$ T (Th1) cells inhibit the development of allergic diseases by counterbalancing the proliferation and development of Th2 cells, depending on the stage of inflammation.[1-4]

$CD4^+CD25^+$ regulatory T (Treg) cells play a critical role in the immune balance network by suppressing or limiting effector immune responses against inner and external insults, for example, preventing organ-specific autoimmunity and allograft rejection as well as maintaining self-tolerance.[5-8] Recent studies have demonstrated that the failure of Treg cells to suppress Th2-mediated immune responses to innocuous inhaled antigens leads to an inappropriate balance between allergen activation of Treg cells and effector Th2 responses, resulting in the development and maintenance of asthmatic airway inflammation characterized by uncontrolled Th2-biased immune responses.[9-11]

A number of clinical studies have demonstrated an association between psychological stress and asthma symptom aggravation.[12,13] For example, 20-35% of asthmatics experience an exacerbation of their symptoms during periods of stress.[14] In childhood asthma, high levels of chronic stress combined with an acutely negative life events have been shown to increase the risk of asthma attacks.[15] Furthermore, psychological stress has effects on the inflammatory and immune responses implicated in asthma, such as Th2 cytokines. For example, chronic stress caused by academic examinations has been shown to increase IL-5 production and eosinophil accumulation after antigen inhalation in college students with mild asthma.[16,17] However, the precise mechanisms by which psychological stress aggravates Th2 immune responses remain to be clarified.

Psychological stress upregulates the production of endogenous opioids, including endorphins, enkephalins and dynorphins. These endogenous opioids exert their effects, such as analgesia, neuroendocrine function and immunoregulation, by binding to opioid receptors consisting of three subtypes, μ, δ and κ.[18,19] We recently established a murine model of psychological stress-induced asthma exacerbation in which allergen-induced airway inflammation in restraint-stressed mice was more severe than in non-stressed mice. This increased airway inflammation in the restraint-stressed mice was associated with higher levels of Th2 cytokines. Using this model, we have demonstrated that μ-opioid receptors (MORs) in the central nervous system (CNS) are involved in psychological stress-induced asthma exacerbation.[20,21] However, MORs are expressed not only on neurons but also on several other cell types, including lymphocytes, and the function of MORs on these cells have been extensively studied.[22-25]

Therefore, we first aimed to confirm the involvement of neuron MORs in the aggravation of allergic airway inflammation evoked by the stress exposure, using mice that express MORs exclusively on neurons. Then we hypothesized that the aggravation of Th2 responses observed in psychological stress-induced asthma exacerbation may result from a failure in the development of Treg cells in airways and that MORs might be involved in these immune alterations. To address this hypothesis, we examined antigen-induced Th1, Th2 and regulatory cytokine production and T cell subset proportions in populations of bronchial lymph node (BLN) cells and splenocytes from wild-type and MOR-gene-knockout (MORKO) mice exposed to restraint stress.

METHODS

ANIMALS

Specific pathogen-free female C57BL/6 (B6) mice were purchased from Japan SLC (Hamamatsu, Japan). MORKO mice were generated and backcrossed with B6 mice as described previously.[26,27] MOR-transgenic MORKO (MORKO/Tg) mice were produced as discussed below. Animals were housed under a 12 h light/dark cycle with a constant temperature (22 ± 2℃). Sterilized food and water were available *ad libitum*. All experiments described below were approved by the Committee of Animal Experiments at Tohoku Pharmaceutical University and Tohoku University and conformed to the committee guidelines of each university.

PLASMID CONSTRUCT AND PRODUCTION OF MORKO/Tg MICE

The plasmid pDIL,[28] which contains the human dopamine β-hydroxylase (DBH) gene promoter connected to a cDNA fragment encoding the human interleukin 2 receptor α subunit (IL-2Rα), was donated by Dr. K. Kobayashi (Fukushima Medical University School of Medicine, Fukushima, Japan). We replaced the cDNA part of pDIL with a cDNA fragment encoding the human μ-opioid receptor gene, which we had previously cloned.[29] Figure 1 shows the transgene construct used in this study. The construct was microinjected into fertilized B6 mouse eggs, which were implanted into pseudopregnant female mice. Microinjections were conducted by Central Laboratories of Experimental Animals (Kawasaki, Japan) and YSNT Inst., Inc. (Shimotsuga, Japan). Successful integration of the transgene was confirmed by Southern blot analysis of tail DNA. The transgenic mice were then crossed with homozygous MORKO mice with a B6 genetic background[23] to obtain the Tg mouse (F1 offspring: heterozygous MORKO/Tg). Homozygous MORKO/Tg mice were produced by crossing the F1 offspring with homozygous MORKO mice.

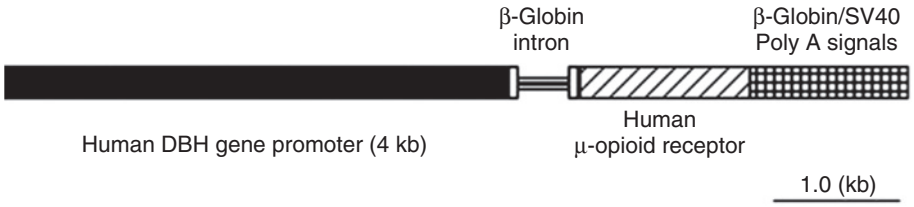

Fig. 1 Human DBH promoter—human MOR cDNA fusion gene in the plasmid. This construct includes the 4 kb DNA fragment of the human DBH promoter in addition to the rabbit β-globin second intron, human MOR cDNA, rabbit β-globin polyadenylation signal, and SV40 early gene polyadenylation signal.

In situ HYBRIDIZATION

Brains were removed quickly and frozen in powdered dry ice. Coronal sections (16 μm) were prepared in a cryostat, thaw-mounted onto gelatin-coated slides and stored at -80℃ until in situ hybridization was conducted. Because the coding regions of human and mouse MOR mRNA are highly homologous, 5'-noncoding regions of MOR cDNA were used as the templates to synthesize the RNA probes. ^{35}S-labeled antisense and sense RNA probes for the in situ hybridization were synthesized in the presence of [α^{35}S] UTP (30 TBq/mmol, Amersham, Buckingham, NY, USA), using a T3 RNA polymerase (Promega, Madison, WI, USA). For the in situ hybridization, sections were fixed in 4% formalin in 10 mM phosphate-buffered saline for 30 min, treated with proteinase K (0.5 μg/ml) for 10 min and immersed in 0.25% acetic anhydride in 0.1 M triethanolamine/saline for 10 min. After dehydration in an ethanol series (70, 85, 95, 100%), prehybridization was carried out at 55℃ for 1 hr in the following buffer: 50% formamide, 4x SSC (1x SSC = 150 mM NaCl, 15 mM tri-sodium citrate, pH 7.0), 5x Denhardt's solution, 10 mM EDTA, 250 μg/ml yeast tRNA, 20 mM dithiothreitol (DTT) and 500 μg/ml heat-denatured salmon sperm DNA, and the sections were then hybridized to the antisense or sense RNA probe at 55℃ for 17 h. For the hybridization, 10% dextran sulfate was added to the buffer. After the hybridization, sections were washed four times in 2x SSC/10 mM DTT for 10 min at 55℃, incubated with RNase A (50 μg/ml in 0.5 M NaCl/10 mM Tris/1 mM EDTA, pH 8.0) for 30 min at 37℃ and then washed twice in 50% formamide/2x SSC/10 mM DTT for 30 min at 55℃. The sections were dehydrated in the ethanol series and dried. The slides were exposed to Hyperfilm-β max (Amersham) and then dipped in autoradiographic emulsion NTB-3 (Kodak, Rochester, NY, USA) diluted 1 : 1 with water. After 3 weeks of exposure, the slides were developed with D-19 (Kodak) and counterstained with cresyl violet.

LIGAND BINDING AND AUTORADIOGRAPHY

Brains and adrenal glands were removed from wild-type, MORKO and MORKO/Tg mice and quickly frozen at -80℃. The brains were cryosectioned at 10 μm and thaw-mounted onto gelatin-coated slides. Tissue sections were preincubated with 50 mM Tris buffer (pH 7.4) containing 10 mM $MgCl_2$ and 1 mM EDTA for 10 min at 25℃. The sections were then incubated with 2 nM [^{3}H]DAMGO (50.5 Ci/mmol) (DuPont-New England Nuclear, Boston, MA, USA) for 60 min at 25℃. DAMGO [D-Ala^2, *N*-MePhe^4, Gly-ol^5] enkephalin is a MOR-selective agonist. After the incubation, sections were washed twice in fresh buffer for 120 min at 25℃ and in deionized water for 5 min at 4℃ and then dried for 60 min at 4℃. Sections were exposed to IP-film for 4 weeks and analyzed using the BAS3100II system (Fujix, Tokyo, Japan).

TAIL SUSPENSION TESTING

For tail suspension testing, mice were taped by their tail to a metal hook in a test chamber (20 × 20 × 25 cm) made of white plastic walls and floor. Each hook was connected to a computerized strain gauge that was adjusted to detect all movements of the animals (Tail suspension system; Neuroscience Inc., Osaka, Japan). The total duration of the immobility testing was 15 min.

STRESS PROCEDURES AND CORTICOSTERONE EIA

First, blood samples were obtained from naive wild-type, MORKO and MORKO/Tg mice tail veins. Next, after a 3-day interval, the 15-minute tail suspension procedure was performed on these mice. Blood samples were obtained again at 30 min after the tail suspension testing. Finally, after a 1-day interval, the physical restraint procedure was performed on these mice. For the restraint stress procedure, mice were placed in a 50 ml conical centrifuge tube with multiple ventilation holes. Mice were restrained vertically in the tube for 12 h, followed by 12 h of rest during which food and water were available *ad libitum*. This restraint allowed the mice to rotate from a supine to prone position but not to turn their heads toward their tails or take food and water. Restraint stress is generally considered to induce psychological stress in animals.[30] Mice were restrained for two cycles as described, and blood samples were then obtained. All

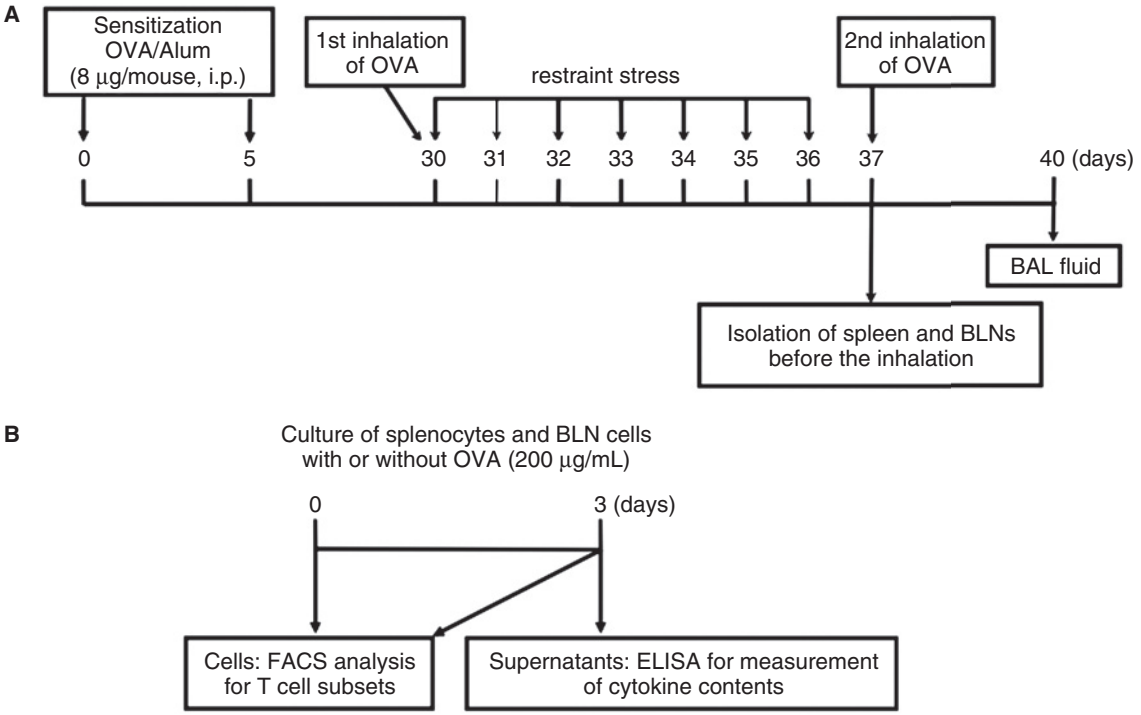

Fig. 2 Diagrammatic representation of the protocols. (**A**) Isolation of spleen, BLNs and BAL fluid. (**B**) Culture of splenocytes and BLN cells.

blood samples were immediately centrifuged for 20 min at 1,000 g, and obtained plasma samples were then stored at -80℃ until the analysis. Plasma corticosterone levels were determined using a Corticosterone Enzyme Immunoassay Kit (Assay Design Inc., Ann Arbor, MI, USA).

PROTOCOLS FOR SENSITIZATION, CHALLENGE AND STRESS EXPOSURE

Our previously published murine model of stress-induced exacerbation of allergic airway inflammation[21] was used in this study. Briefly, mice 6-8 weeks old were sensitized by intraperitoneal injections of chicken ovalbumin (OVA) (Grade V, Sigma, St. Louis, MO, USA) (8 μg/mouse) adsorbed with aluminum hydroxide (Wako Pure Chemical Industries, Osaka, Japan) (4 mg/mouse) on days 0 and 5. For restraint stress, each mouse was placed in a 50-ml conical centrifuge tube as described above for 6 h per day. On day 30, stressed mice in the 50-ml conical centrifuge tubes were left in plastic chambers for 6 h. For the first hour of this period, aerosolized OVA was pumped through the chambers to expose the mice in the tubes to OVA. Stressed mice were subjected to the restraint stress for 6 consecutive days, on days 31 to 36, at the same time each day. Non-stressed mice were only deprived of food and water for the same period as the stressed mice were exposed to stress. Food and water deprivation has been used as a non-stress condition in other rodent experiments investigating the effects of restraint stress.[31,32] On day 37, stressed and non-stressed mice were challenged with aerosolized OVA. On day 40, their lungs were lavaged twice with an injection of 0.25 ml of cold saline through the trachea, as described previously, and the recovered bronchoalveolar lavage (BAL) fluid was pooled. Approximately 0.4 ml of instilled fluid was consistently recovered from each mouse. BAL fluid was processed for counting total cells and cell differentials (Fig. 2A). In other experiments, stressed and non-stressed mice were sacrificed on day 37 to collect spleen and BLNs (Fig. 2A).

PREPARATION AND CULTURE OF SPLENOCYTES AND BLN CELLS

Splenocytes and BLN cells were prepared and cultured as previously described.[33,34] For each experiment, BLN cells were prepared from BLNs collected from 6-8 wild-type mice and 5 MORKO mice, and splenocytes were prepared from the spleen of one mouse. Splenocytes and BLN cells were suspended at 5×10^6 cells/ml in complete media consisting of RPMI 1640 (Gibco BRL, Rockville, MD, USA) supplemented with 10% fetal bovine serum (ICN Biomedicals, Inc., Aurosa, OH, USA), 55 μM 2-mercaptoethanol (Wako Pure Chemical Industries), 2 mg/ml $NaHCO_3$, 50 U/ml penicillin G (Meiji Seika, Tokyo, Japan), 50 μg/ml streptomycin sulphate (Meiji Seika) and 2 mM L-glutamine (Wako Pure Chemical Industries). Cell viability of the suspension was more than

98%, as determined by trypan blue dye exclusion. The cells were cultured at 1×10^7 cells/well in 24-well culture plates (MICROPlate, Asahi Techno Glass, Chiba, Japan) in the presence or absence of OVA (200 μg/ml) for 3 days. After the culture, the number of viable cells was counted, and the samples were centrifuged. The supernatants were stored at -80℃ for cytokine measurements using an enzyme-linked immunosorbent assay (ELISA), and the cells were subjected to lymphocyte compartment analysis by flow cytometry before and after the culture (Fig. 2B).

CYTOKINE MEASUREMENTS

IL-4, IL-5, IL-10, IL-13, IFN-γ and TGF-β_1 levels in the supernatant were measured using specific ELISA kits (R&D systems, Minneapolis, MN, USA) according to the manufacturer's protocol. The sensitivity of detection was 2 pg/ml for IL-4, 7 pg/ml for IL-5, 4 pg/ml for IL-10, 1.5 pg/ml for IL-13, 2 pg/ml for IFN-γ and 2.9 pg/ml for TGF-β_1. Because IFN-γ and IL-4 are classical examples of antagonistic signature cytokines for Th1 and Th2 activity, respectively, the Th1/Th2 balance was monitored by IFN-γ/IL-4 ratio,[35] which was calculated with the values of these two cytokines obtained from the same experiment.

FLOW CYTOMETRIC ANALYSIS

$CD3^+CD4^+$, $CD3^+CD4^+T1/ST2^+$ and Treg compartments of splenocytes and BLN cells were analyzed as previously described.[34] Briefly, the cells were preincubated with anti-CD16/CD32 (FCγIII/II receptor; BD Biosciences PharMingen, San Diego, CA, USA) to reduce nonspecific binding of the subsequent antibodies. Anti-T1/ST2-fluorescein isothiocyanate (clone DJ8, 0.8 μg/10^6 cells) (Morwell Diagnostic, Zurich, Switzerland), anti-CD3e-allophycocyanin (clone 145-2C11, 0.8 μg/10^6 cells) (BD Biosciences PharMingen), anti-CD4-R-phycoerythrin (clone GK1.5, 0.5 μg/10^6 cells) (BD Biosciences PharMingen) or isotype control antibodies were added directly to the cells. T1/ST2 was employed as a surface marker for Th2 cells because it is preferentially expressed on the surface of murine Th2 cells and plays an important role in the activation of Th2 cells, including proliferation and Th2 cytokine production.[36,37] Treg cells were identified as $CD4^+CD25^+$ cells, using a mouse regulatory T cell staining kit (eBioscience, San Diego, CA, USA) according to the manufacturer's protocol. Cells were counted on a FACScan (BD Biosciences PharMingen), and the analyses were performed using Cell Quest (BD Biosciences PharMingen).

DATA ANALYSIS

In the behavior analyses, significant differences were statistically evaluated using an analysis of variance (ANOVA) followed by the Bonferroni *post hoc* test or a paired *t*-test. Significant differences between two groups were determined using the nonparametric Mann-Whitney *U*-test. These analyses were performed using Prism4 (GraphPad Software, San Diego, CA, USA). A p value of less than 0.05 was considered significant.

RESULTS

ANALYSIS OF TRANSGENE mRNA AND FUNCTIONAL MOR PROTEIN EXPRESSION

The expression of the human MOR was investigated in MORKO/Tg mice by *in situ* hybridization with selective probes for mouse and human MOR mRNA. In brainstem sections, the specific expression of human MOR mRNA was found in locus ceruleus (LC) regions in the MORKO/Tg mouse (Fig. 3A, B). Lower expression levels of the human MOR mRNA were found in other brain regions. The lack of detection of the expression signals in both wild-type mouse by the human selective probe and the MORKO/Tg mouse by the mouse selective probe confirmed the expression of human MOR mRNA by the transgene. Next, the functional expression of MOR protein was confirmed in MORKO/Tg mice by [^{3}H]DAMGO binding (Fig. 3C). In MORKO/Tg mice, the [^{3}H]DAMGO binding sites were specifically found in the cortex and thalamus, which were quite different from those in wild-type mice.

TAIL SUSPENSION TEST

Immobility times in the 15-min tail suspension assay were analyzed in wild-type, MORKO, and MORKO/Tg mice (Fig. 4A, B). Although all the studied genotypes showed a time-dependent increase in immobility time, there were significant differences in the manner of this increase among the genotypes (two-way repeated measures ANOVA: $p < 0.05$; $F = 5.13$; $df = 2.560$). MORKO mice showed significantly less immobility compared with wild-type mice from 5 to 10 min after initiation of the tail suspension ($p < 0.05$). Interestingly, this decreased immobility in MORKO mice was not found in MORKO/Tg mice. MORKO/Tg mice showed no difference from wild-type mice but did show a significant difference compared with MORKO mice ($p < 0.05$) in immobility time (Fig. 4B).

STRESS-INDUCED CORTICOSTERONE RELEASE

We analyzed both tail suspension and restrained stress-induced changes in plasma corticosterone concentration in wild-type, MORKO, and MORKO/Tg mice (Fig. 5). Both tail suspension and restraint stress significantly increased plasma corticosterone concentrations in wild-type (paired *t*-test: $p < 0.0005$, $t = -5.68$, $p < 0.0001$, $t = -7.56$, respectively), MORKO (paired *t*-test: $p < 0.0005$, $t = -4.54$, $p < 0.0001$, $t = -9.84$, respectively), and MORKO/Tg mice (paired *t*-test: $p < 0.0001$, $t = -7.39$, $p < 0.0001$, $t = -24.01$, respectively). In addition, the ANOVA showed significant differences among these genotypes in changes in

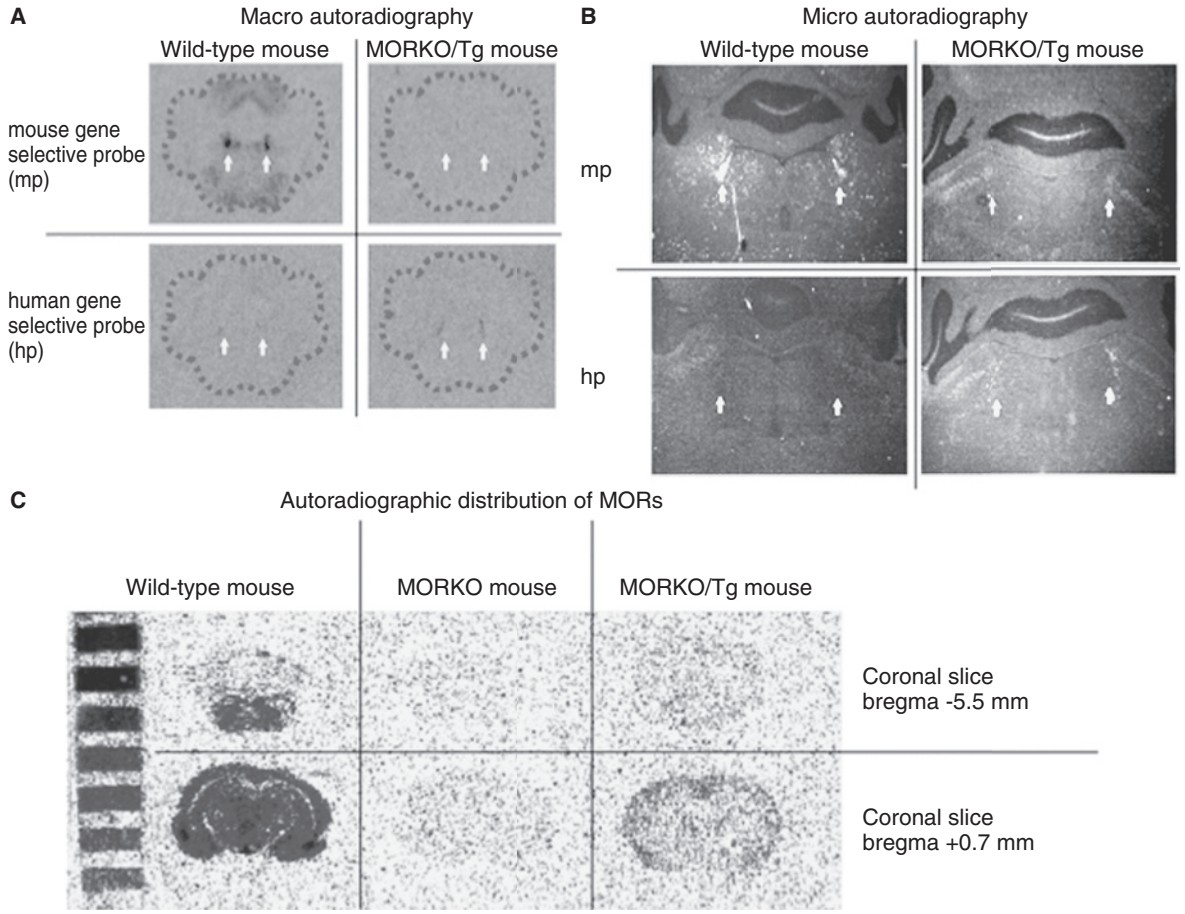

Fig. 3 Autoradiographs of MOR mRNA expression and [3H]DAMGO binding sites. Macro(**A**)- and micro(**B**)-autoradiographs of in situ hybridization for mouse and human MOR mRNA showed the LC region-specific expression of human MOR mRNA in coronal sections (bregma -5.5 mm) of the MORKO/Tg mouse. White arrows indicate the LC regions. (**C**) Autoradiographic distribution of [3H]DAMGO binding sites in coronal brain slices of wild-type, MORKO, and MORKO/Tg mice. Sections were taken through the brainstem: LC (bregma +5.5 mm: upper panel) and thalamus: cortex (bregma -0.7 mm: lower panel).

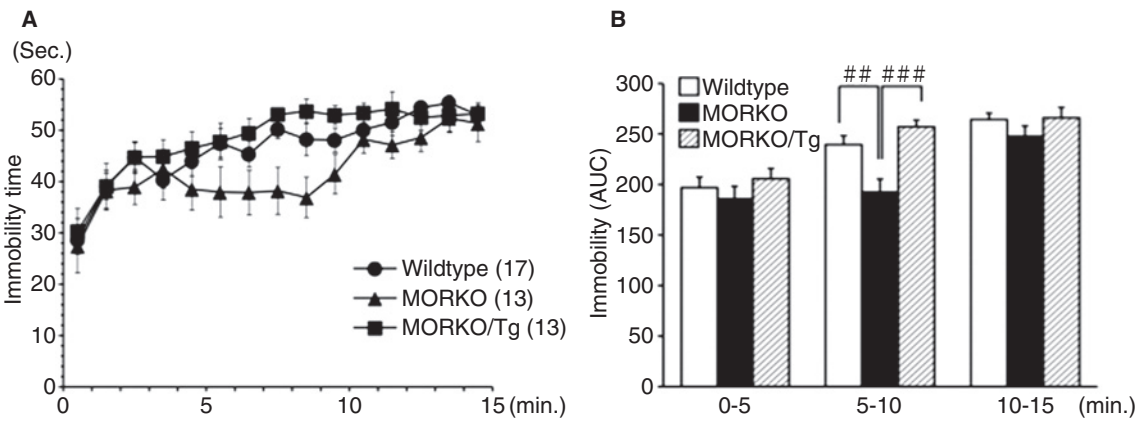

Fig. 4 Immobility in wild-type, MORKO, and MORKO/Tg mice in the 15-min tail suspension test. (**A**) Immobility times were measured in wild-type (n = 17), MORKO (n = 13), and MORKO/Tg (n = 13) mice. (**B**) AUC of the immobility times over 5 min in the tail suspension tests. ##, ### $p < 0.01$, 0.001, indicating a significant difference from the corresponding value in MORKO mice. Data are expressed as the mean ± SEM.

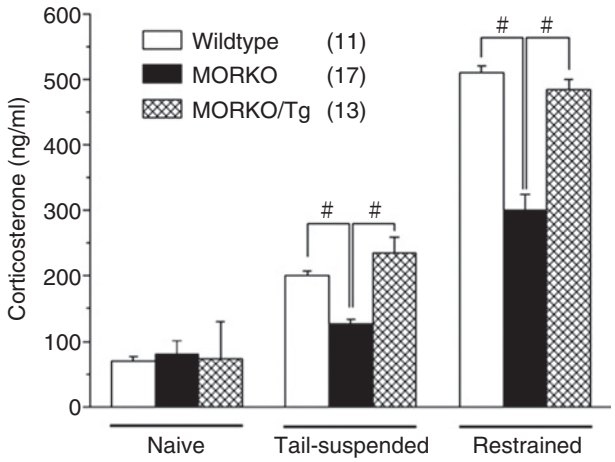

Fig. 5 Stress-induced increase in plasma corticosterone concentrations in wild-type, MORKO, and MORKO/Tg mice. Plasma corticosterone levels were analyzed in wild-type (n = 11), MORKO (n = 17), and MORKO/Tg (n = 13) mice before any test, after the tail suspension test, and after the restraint stress. #$p < 0.05$, showing a significant difference from the corresponding value in MORKO mice. Data are expressed as the mean ± SEM.

plasma corticosterone concentration (tail suspension stress: $p < 0.0005$; F = 11.14; df = 2.38; restrained stress: $p < 0.0001$; F = 14.04; df = 2.38). MORKO mice showed significantly lower plasma corticosterone concentrations compared with wild-type mice after both the tail suspension and restraint stress procedure ($p < 0.05$). Furthermore, MORKO/Tg mice showed the same level of increase in corticosterone concentration compared to wild-type mice, and this change was significantly different from MORKO mice ($p < 0.05$).

RESTRAINT STRESS-INDUCED AGGRAVATION OF ALLERGIC AIRWAY INFLAMMATION IN MORKO/Tg MICE

As previously demonstrated,[21] the stress-induced worsening of allergic airway inflammation observed in wild-type mice (non-stress vs. stress: total cells, 134.5 ± 57.1 vs. 340.6 ± 164.8 × 10^4 cells/ml, $p < 0.01$; eosinophils, 82.0 ± 35.8 vs. 262.4 ± 128.0 × 10^4 cells/ml, $p < 0.01$; lymphocytes, 16.7 ± 7.7 vs. 39.0 ± 30.1 × 10^4 cells/ml, $p < 0.01$) (Fig. 6A) was not observed in the MORKO mice (Fig. 6B). However, as shown in Figure 6C, the numbers of total cells and eosinophils in BAL fluid three days after the second antigen inhalation in stressed MORKO/Tg mice were significantly higher than those in non-stressed MORKO/Tg mice (non-stress vs. stress: total cells, 170.7 ± 67.8 vs. 320.6 ± 62.5 × 10^4 cells/ml, $p < 0.01$; eosinophils, 124.0 ± 43.7 vs. 251.5 ± 57.2 × 10^4 cells/ml, $p < 0.01$), although the difference in the number of lymphocytes did not reach significance (non-stress vs. stress:

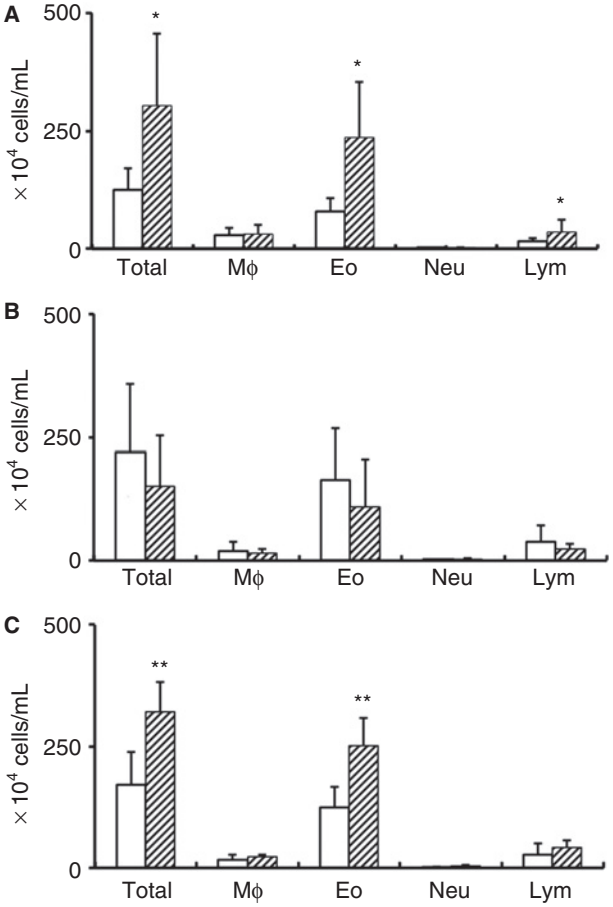

Fig. 6 Comparison of cell numbers in BAL fluids between stressed and non-stressed mice. The numbers of total cells (Total), macrophages (Mϕ), eosinophils (Eo), neutrophils (Neu) and lymphocytes (Lym) in BAL fluids were counted for non-stressed (open bars) and stressed (hatched bars) wild-type (5 and 6, respectively, in **A**), MORKO (7 and 8, respectively, in **B**) and MORKO/Tg (5 and 6, respectively, in **C**) mice three days after the 2nd OVA inhalation (day 40). Data are expressed as the mean ± S.D. *$p < 0.05$ and **$p < 0.01$ compared to non-stressed mice.

27.5 ± 23.4 vs. 41.8 ± 15.5 × 10^4 cells/ml).

THE EFFECT OF RESTRAINT STRESS ON CYTOKINE PRODUCTION BY SPLENOCYTES AND BLN CELLS

The levels of the Th2 cytokines IL-4, IL-5 and IL-13 in splenocyte culture supernatants stimulated with OVA were not significantly different between stressed and non-stressed wild-type mice (n = 8 for IL-4, n = 7 for IL-5 and n = 8 for IL-13). Similarly, the production of the Th1 cytokine IFN-γ and the regulatory cytokines IL-10 and TGF-β_1 by OVA-stimulated splenocytes was not significantly influenced by stress exposure (n = 10 for IFN-γ, n = 10 for IL-10 and n = 9 for TGF-β_1) (Table 1). IFN-γ/IL-4 ratio, as a measure of the Th1/

Table 1 Cytokine production by splenocytes and BLN cells

	splenocytes		BLN cells	
	OVA (-)	OVA (+)	OVA (-)	OVA (+)
IL-4	9.4 ± 2.6†	49.4 ± 23.8	8.3 ± 1.0	74.0 ± 24.6
	7.9 ± 6.1	41.7 ± 35.5	8.6 ± 3.2	118.2 ± 60.2
IL-5	0.0 ± 0.0	1504.4 ± 1094.0	244.9 ± 108.4	3110.4 ± 529.9
	2.9 ± 4.6	1430.8 ± 1075.6	184.1 ± 93.6	3645.0 ± 998.6
IL-13	67.0 ± 46.8	6110.4 ± 3570.7	440.1 ± 230.6	5512.2 ± 1079.3
	102.0 ± 73.5	5244.8 ± 2477.4	584.3 ± 286.1	6885.0 ± 2554.8
IFN-γ	100.0 ± 104.7	1681.2 ± 629.3	8.0 ± 4.7	125.3 ± 39.0
	338.0 ± 303.8	1116.0 ± 606.9	8.8 ± 5.6	102.1 ± 47.3
IL-10	11.4 ± 4.9	934.0 ± 540.0	44.8 ± 27.1	1065.0 ± 106.9
	15.2 ± 7.1	730.6 ± 458.5	46.9 ± 17.6	1160.0 ± 255.7
TGF-β_1	370.8 ± 169.8	453.8 ± 222.5	54.0 ± 59.3	193.1 ± 121.0
	391.3 ± 239.9	411.1 ± 271.8	0.0 ± 0.0	148.2 ± 58.1

† mean ± SD (pg/ml) in non-stressed mice / mean ± SD (pg/ml) in stressed mice

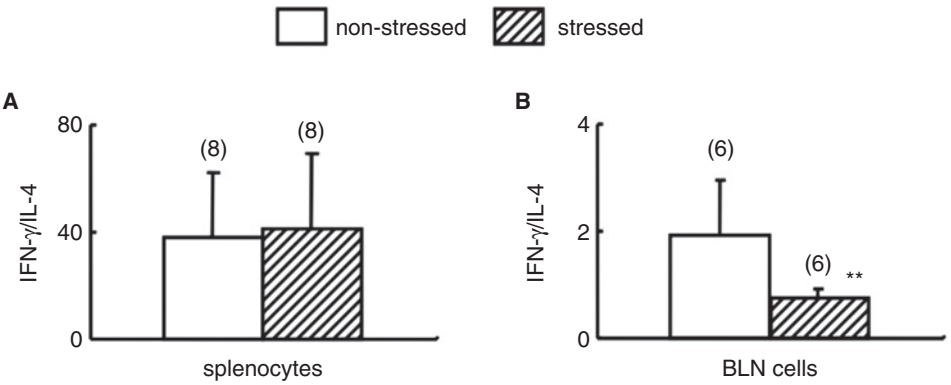

Fig. 7 IFN-γ/IL-4 ratio in splenocytes and BLN cells. The IFN-γ/IL-4 ratio was calculated as described in "Methods" in splenocytes (**A**) and BLN cells (**B**) from non-stressed (open bars) and stressed (hatched bars) wild-type mice. The numbers of samples for each condition are shown in parentheses. Data are expressed as the mean ± S.D. $^{**}p < 0.01$.

Th2 balance, in the culture supernatants was not significantly different between stressed (n = 8) and non-stressed (n = 8) wild-type mice (Fig. 7A). There were no significant differences between non-stressed and stressed wild-type mice in cytokine levels in the culture supernatants without OVA (n = 6 for IL-4, IL-5, IL-13, IFN-γ, IL-10 and TGF-β_1) (Table 1).

Similar to the results for cytokine production by splenocytes, stress exposure did not significantly affect cytokine production by BLN cells cultured with (n = 6 for IL-4, n = 5 for IL-5, n = 6 for IL-13, n = 6 for IFN-γ, IL-10 and TGF-β_1) or without (n = 6 for IL-4, n = 5 for IL-5, n = 6 for IL-13, n = 6 for IFN-γ, n = 4 for IL-10 and n = 6 for TGF-β_1) OVA (Table 1). However, the IFN-γ/IL-4 ratio in the culture supernatants of BLN cells stimulated with OVA from stressed wild-type mice was significantly lower than that from non-stressed wild-type mice (0.7 ± 0.2: n = 6 vs. 1.9 ± 1.0: n = 6, $p < 0.01$) (Fig. 7B).

THE EFFECT OF RESTRAINT STRESS ON T CELL SUBSET PROPORTIONS IN SPLENOCYTES AND BLN CELLS

In splenocytes, there was no significant difference in the numbers of viable cells after culture with OVA between non-stressed and stressed wild-type mice (data not shown). In both non-stressed and stressed wild-type mice, there were significant increases in the proportion of $CD3^+CD4^+$ and $CD3^+CD4^+T1/ST2^+$ cells after culture with OVA. The proportion of these cells in stressed wild-type mice was not significantly different from those in non-stressed wild-type mice, either before (n = 4) or after (n = 5) culture. The proportion of $CD4^+CD25^+$ cells after culture with OVA was not significantly increased compared with the percentage before culture in either non-stressed or stressed wild-type mice, and there were no significant differences in the proportion of these cells between stressed and

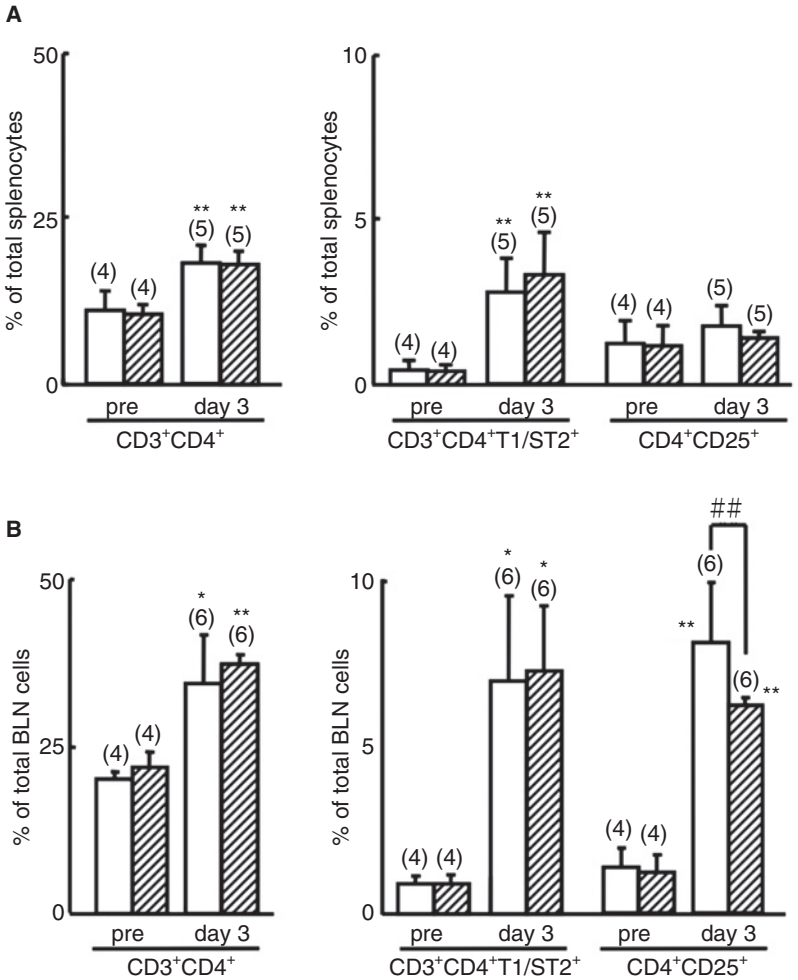

Fig. 8 The proportion of T cell subsets in splenocytes and BLN cells. Splenocytes (**A**) and BLN cells (**B**) from non-stressed (open bars) and stressed (hatched bars) wild-type mice were analyzed for the percentages of $CD3^+CD4^+$, $CD3^+CD4^+T1/ST2^+$ and $CD4^+CD25^+$ cells before (pre) and 3 days after the culture with OVA. The numbers of $CD3^+CD4^+$, $CD3^+CD4^+T1/ST2^+$ and $CD4^+CD25^+$ cells are shown as percentages of the total number of splenocytes and BLN cells. The numbers of mice examined and the experiment numbers are shown in parentheses in **A** and **B**, respectively. Data are expressed as the mean ± S.D. $^*p < 0.05$ and $^{**}p < 0.01$ compared with pre, $\#\#p < 0.01$.

non-stressed wild type mice, either before (n = 4) or after (n = 5) culture (Fig. 8A).

In BLN cells, there was no significant difference in the numbers of viable cells after the culture with OVA between non-stressed and stressed wild-type mice (data not shown). Similar to splenocytes, culturing the cells with OVA significantly increased the proportion of $CD3^+CD4^+$ and $CD3^+CD4^+T1/ST2^+$ cells from both non-stressed and stressed wild-type mice. There were no significant differences in the proportion of these cells between stressed and non-stressed wild-type mice, either before (n = 4) or after (n = 6) culture. Regarding the $CD4^+CD25^+$ population in BLN cells, in both non-stressed and stressed wild-type mice, the proportion of these cells was significantly increased after the culture with OVA (non-stressed: 7.3 ± 1.7: n = 6 vs. 1.2 ± 0.5: n = 4 %, $p < 0.01$, stressed: 5.6 ± 0.2: n = 6 vs. 1.1 ± 0.4: n = 4 %, $p < 0.01$). Although no significant difference was observed between the mice in the proportion of $CD4^+CD25^+$ cells before the culture, this proportion in stressed mice was significantly lower than that from non-stressed mice after the culture (5.6 ± 0.2: n = 6 vs. 7.3 ± 1.7: n = 6 %, $p < 0.01$) (Fig. 8B).

ABOLISHMENT OF THE EFFECT OF RESTRAINT STRESS ON BLN CELLS IN MORKO MICE

The stress-induced aggravation of allergen-mediated

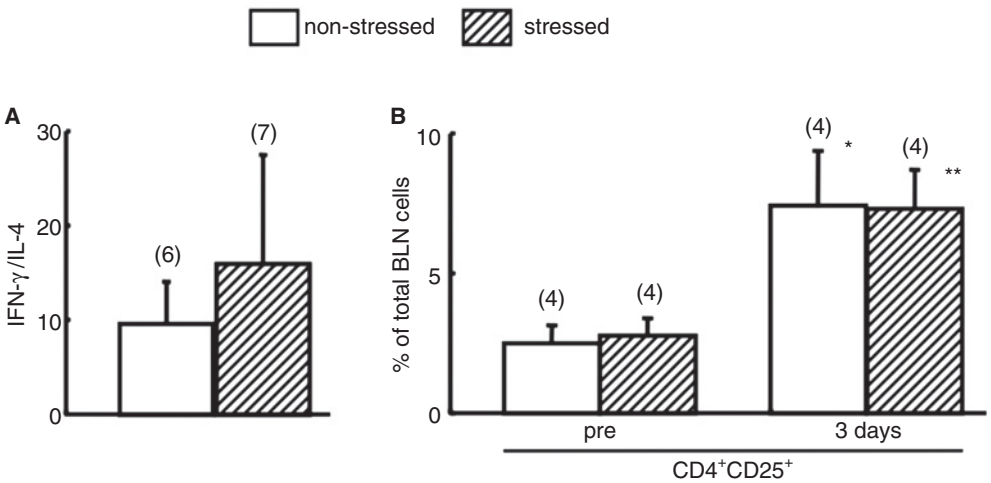

Fig. 9 IFN-γ/IL-4 ratio and the proportion of $CD4^+CD25^+$ cells in BLN cells of MORKO mice. BLN cells from non-stressed (open bars) and stressed (hatched bars) MORKO mice were cultured with OVA for 3 days. The IFN-γ/IL-4 ratio (**A**) was calculated as in Figure 7, and the proportion of $CD4^+CD25^+$ cells was analyzed as in Figure 8. The numbers of experiments for each condition are shown in parentheses. Data are expressed as the mean ± S.D. $^*p < 0.05$ and $^{**}p < 0.01$ compared with pre.

airway inflammation was abolished in MORKO mice, as previously reported.[21] Therefore, we investigated whether MOR was involved in the immunological alterations observed in BLN cells from stressed wild-type mice. In contrast to the case of wild-type mice, the IFN-γ/IL-4 ratio in the culture supernatants of OVA-stimulated BLN cells from stressed MORKO mice ($n = 7$) was not significantly different from that of the non-stressed MORKO mice ($n = 6$) (Fig. 9A). Similar to wild-type mice, the proportion of $CD4^+CD25^+$ cells from stressed and non-stressed MORKO mice showed a similar increase after culture with OVA. However, no significant difference was observed in the proportion of $CD4^+CD25^+$ cells between non-stressed ($n = 4$) and stressed ($n = 4$) MORKO mice (Fig. 9B).

DISCUSSION

In the present study, we produced Tg mice, in which the expression of MORs is under the control of the human DBH gene promoter. Furthermore, by mating these Tg mice with MORKO mice, we produced MORKO/Tg mice that express MORs only on noradrenergic and adrenergic neurons. This human DBH gene promoter has been previously used for producing transgenic mice, successfully expressing phenylethanolamine-N-methyltransferase[38] or IL-2Rα[28] on noradrenergic and adrenergic neurons. The expression of human MOR mRNA in coronal brainstem sections of MORKO/Tg mice was mostly limited to the LC, in which the cell bodies of noradrenergic neurons are congregated. Furthermore, we confirmed the functional expression of MOR protein on cortex regions, into which many noradrenergic neurons project, in MORKO/Tg mice. Despite these alterations, no abnormalities in growth, fertility, or general activity were detected in MORKO/Tg mice.

MORKO/Tg mice also showed the same level of immobility in the tail suspension test and stress-induced plasma corticosterone concentrations as wild-type mice, and these levels were significantly different from MORKO mice. MORKO mice showed a significantly decreased immobility time in the tail suspension test and a reduced stress-induced change in plasma corticosterone concentration compared with wild-type mice, which is consistent with our previous report.[39] These results indicate that the MORs on noradrenergic and adrenergic neurons has functional roles in stress sensibility or responses.

Next, we compared the effect of restraint stress on allergic airway inflammation between wild-type, MORKO and MORKO/Tg mice. We found that the stress-induced exacerbation of airway inflammation observed in wild-type mice, which was abrogated in MORKO mice, was recovered in MORKO/Tg mice. These results indicate that MORs on neurons in the CNS and periphery, in this case noradrenergic and adrenergic neurons, are involved in the stress-induced aggravation of allergic airway inflammation. MORs are expressed not only on neurons but also on several other types of cells, including lymphocytes, and the functions of these receptors on lymphocytes have been extensively studied, including cell differentiation and the production of pro-inflammatory and Th2 cytokines.[22-25] Previously, we demonstrated that the effect of stress in wild-type mice was completely

abolished by the intracerebroventricular injection of an MOR antagonist, β-funaltrexamine, that selectively inhibits the activation of MORs in the CNS, but this abolishment was not seen by the peritoneal injection of naloxone methiodide, a nonselective opioid receptor antagonist that cannot pass through the blood-brain barrier.[21] The peritoneal injection of naloxone methiodide would inhibit the activation of MORs on immune cells and neurons in the periphery. Therefore, together with our previous findings, the current results strongly suggest that the effect of restraint stress on allergic airway inflammation is mediated by MORs on cells in the CNS, including noradrenergic and adrenergic neurons, although the involvement of MORs on immune cells present in the CNS during the stress exposure cannot be completely excluded.

We have demonstrated for the first time the effects of restraint stress as psychological stress on allergen-specific immune responses at the site of inflammation in a murine model of psychological stress-induced asthma exacerbations and the involvement of MORs in these immunological alterations associated with restraint stress.

To investigate stress-induced allergen-specific immune responses, we compared cytokine production profiles and the proportions of T cell subsets in BLN cell populations from stressed and non-stressed mice. Levels of Th1, Th2 and regulatory cytokines produced by BLN cells upon stimulation with the allergen were not significantly different between stressed and non-stressed mice. However, IFN-γ/IL-4 ratios in stressed mice were significantly lower than those in non-stressed mice, suggesting that restraint stress shifted the allergic immune response towards a Th2 character. Regarding the T cell subset proportions, culturing the cells with OVA increased the proportion of Th2 cells in both groups. However, there was no significant difference in the proportion of Th2 cells either before or after the culture with the allergen between the stressed and non-stressed mice. However, whereas the proportion of Treg cells increased after the culture in both groups, the final Treg percentage in stressed mice was significantly lower than that in non-stressed mice after the culture. The increase in the Treg cell population after the exposure to allergen is consistent with the finding by Thunberg S *et al*. that allergen provocation increased the number of $CD4^+CD25^{bright}$ Treg cells in asthmatic lungs.[40] In the current study, CD4 and CD25 were used as markers to identify the Treg population, as in other murine models of allergic asthma.[41-43] The entire population of $CD4^+CD25^+$ T cells, expressing both low and high CD25 levels, exhibits a regulatory function in mice.[44-46] However, the expression of CD25 after the culture with antigen could also represent activated $CD4^+$ cells. Further characterization of more specific markers, including Foxp3, and inhibitory functions in $CD4^+CD25^+$ cells would be needed to distinguish Tregs from activated $CD4^+$ cells.

Mental stress has been reported to reduce the number of Treg cells in peripheral blood in human subjects. A brief laboratory stressor was shown to cause a decrease of $CD4^+Foxp3^+$ Treg cells in healthy young males.[47] A significant reduction in the proportion of Treg cells was observed in patients with post-traumatic stress disorder (PTSD) compared to individuals without PTSD.[48] Regarding changes in immune regulation in response to stressors in allergic subjects, Höglund *et al*. reported systemic immunological responses to examination stress in healthy and atopic individuals.[49] The ratio of Th1 and Th2 cytokines produced by peripheral blood mononuclear cells stimulated with PHA/PMA was decreased in response to stress in atopic individuals but not in controls, similar to our findings. In contrast to our findings, the proportion of Treg ($CD4^+CD45RO^+CD25^{bright}$) cells in peripheral blood increased in response to stress in atopic subjects. However, this increase of Treg cells was also observed in control subjects. The discrepancy in the change in Treg cell proportion may be attributable to sites investigated (systemic or local) or stimulation (with or without allergen), as well to differences in the subjects examined. The importance of the site and allergen exposure in the effect of stress on immune regulation is discussed below.

Deficiency and dysfunction of Treg cells are observed in asthmatics and are thought to be responsible for Th2-predominant immune responses in asthma.[9-11] The recovery of Treg cell numbers has been shown to ameliorate allergen-induced airway inflammation and hyper-responsiveness in murine models of allergic asthma,[42,43,50] and successful allergen-specific immunotherapy has been correlated with increased numbers and the activation of allergen-specific Treg cells.[51,52] Furthermore, the presence of Treg cells in the lungs has been inversely correlated with eosinophilic airway inflammation in murine models of allergic asthma.[53] Therefore, we assume that the lower number of Treg cells induced by stressors might allow more pronounced Th2 responses in allergen-challenged conditions, resulting in the aggravation of allergic airway inflammation observed in our model. This interpretation is supported by a report showing that the reconstitution of a T cell repertoire with a limited number of Treg cells resulted in severe Th2-associated inflammatory disease upon the subsequent transfer of effector T cells.[41]

Using our stress model, we previously demonstrated that MORs in the central nervous system were involved in the stress-induced aggravation of allergic airway inflammation.[21] In the current study, the effect of stress on the IFN-γ/IL-4 ratio and the number of Treg cells in BLN cell populations cultured with allergen was not observed in MORKO mice. Considering these results, it might be assumed that

restraint stress evokes an alteration of the immune responses to allergen through the activation of MORs, resulting in the exacerbation of allergen-induced airway inflammation. Although the hypothalamic-pituitary-adrenal axis and the sympathetic and adrenomedullary system have been proposed as relevant pathways through which stress modifies inflammatory/immune responses to induce asthma exacerbation,[54] the psycho-neuro-immunological cascade involved in the worsening of asthma symptoms due to psychological stress is not fully understood. In this study, we have reported for the first time potential components, MORs and Treg cells, in neurological and immunological pathways linking stress to asthma exacerbation.

Stress is perceived in the brain, which subsequently activates physiologic systems in the body. Accordingly, immunological alterations would be expected to occur not specifically in BLNs, but also in the spleen. However, a stress effect on immune responses to specific antigen was not observed in splenocytes. Sensitized mice were exposed to allergen by inhalation at the beginning of the stress exposure on day 30; consequently, BLN cells but not splenocytes were in contact with the allergen during the period of stress exposure. In this stress model, when mice were treated with saline by inhalation on day 30, no significant difference was observed between the stressed and non-stressed mice in airway inflammation elicited by the second allergen challenge on day 37 (data not shown). These findings suggest that the simultaneous presence of allergen with immune cells during the stress exposure was required for the development of stress-induced altered immune responses to the subsequent allergen stimulation. Immature dendritic cells, having captured allergens, migrate to regional lymph nodes,[55] where they induce different subsets of T cells, including Treg cells.[56,57] Stress might exert some effects on this induction of Treg cells[7,10,11] such that the proportion of Treg cells in the stressed mice is reduced after the culture with allergen, whereas it is not different in the non-stressed mice.

In conclusion, we produced mice that express MORs only on noradrenergic and adrenergic neurons, and we confirmed the contribution of MORs in the CNS to restraint stress-induced worsening of allergic airway inflammation. We also demonstrated that upon allergen stimulation, the profile of cytokine production was more Th2 dominated and the development of Treg cells was reduced in BLN cells from allergic mice exposed to restraint stress, and that MORs were involved in these immunological alterations induced by stress exposure. The mechanisms by which the activation of MORs induced the alterations of immune responses to allergen were not determined in the current study. Further investigations, including functional analysis and adoptive transfer of BLN cells from wild-type and MORKO mice with or without stress, are needed to clarify the pathways linking psychological stress to asthma exacerbation in terms of a neuro-immunological cascade.

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REFERENCES

1. Bousquet J, Jeffery PK, Busse WW, Johnson M, Vignola AM. Asthma: From bronchoconstriction to airways inflammation and remodeling. *Am J Respir Crit Care Med* 2000;**161**:1720-45.
2. Kay AB. The role of T lymphocytes in asthma. *Chem Immunol Allergy* 2006;**91**:59-75.
3. Barnes PJ. Immunology of asthma and chronic obstructive pulmonary disease. *Nat Rev Immunol* 2008;**8**:183-92.
4. Holgate ST. Pathogenesis of asthma. *Clin Exp Allergy* 2008;**38**:872-97.
5. Bacchetta R, Gambineri E, Roncarolo MG. Role of regulatory T cells and Foxp3 in human diseases. *J Allergy Clin Immunol* 2007;**120**:227-35.
6. Tang Q, Bluestone JA. The Foxp3+ regulatory T cell: a jack of all trades, master of regulation. *Nat Immunol* 2008; **9**:239-44.
7. Workman CJ, Szymczak-Workman AL, Collison LW, Pillai MR, Vignali DA. The development and function of regulatory T cells. *Cell Mol Life Sci* 2009;**66**:2603-22.
8. Wing K, Sakaguchi S. Regulatory T cells exert checks and balances on self tolerance and autoimmunity. *Nat Immunol* 2010;**11**:7-13.
9. Robinson DS. Regulatory T cells and asthma. *Clin Exp Allergy* 2009;**39**:1314-23.
10. Lloyd CM, Hawrylowicz CM. Regulatory T cells in asthma. *Immunity* 2009;**31**:438-49.
11. Akdis CA, Akdis M. Mechanisms and treatment of allergic disease in the big picture of regulatory T cells. *J Allergy Clin Immunol* 2009;**123**:735-46.
12. Rietveld S, Everaerd W, Creer TL. Stress-induced asthma: a review of research and potential mechanisms. *Clin Exp Allergy* 2000;**30**:1058-66.
13. Chetta A, Foresi A, Marangio E, Olivieri D. Psychological implications of respiratory health and disease. *Respiration* 2005;**72**:210-5.
14. Isenberg SA, Lehrer PM, Hochron S. The effects of suggestion and emotional arousal on pulmonary function in asthma: a review and a hypothesis regarding vagal mediation. *Psychosom Med* 1992;**54**:192-216.
15. Sandberg S, Paton JY, Ahola S *et al*. The role of acute and chronic stress in asthma attacks in children. *Lancet* 2000; **356**:982-7.
16. Kang DH, Coe CL, McCarthy DO *et al*. Cytokine profiles of stimulated blood lymphocytes in asthmatic and healthy adolescents across the school year. *J Interferon Cytokine*

Res 1997;**17**:481-7.
17. Liu LY, Coe CL, Swenson CA, Kelly EA, Kita H, Busse WW. School examinations enhance airway inflammation to antigen challenge. *Am J Respir Crit Care Med* 2002; **165**:1062-7.
18. Lopez-Carderon A, Ariznavarreta C, Chen CL. Influence of chronic restraint stress on pro-opiomelanocortin mRNA and β-endorphin in the rat hypothalamus. *J Mol Endocrinol* 1991;**7**:197-204.
19. Drolet G, Dumont EC, Gosselin I, Kinkead R, Laforest S, Trottier L-F. Role of endogenous opioid system in the regulation of the stress response. *Prog Neuro-Psychopharmacol Biol Psychiatry* 2001;**25**:729-41.
20. Okuyama K, Ohwada K, Sakurada S *et al*. The distinctive effects of acute and chronic psychological stress on airway inflammation in a murine model of allergic asthma. *Allergol Int* 2007;**56**:29-35.
21. Okuyama K, Wada K, Sakurada S *et al*. The Involvement of μ-opioid receptors in the central nervous system in the worsening of allergic airway inflammation by psychological stress in mice. *Int Arch Allergy Immunol* 2010;**152**: 342-52.
22. Roy S, Cain KJ, Chapin RB, Charboneau RG, Barke RA. Morphine modulates NF kappa B activation in macrophages. *Biochem Biophys Res Commun* 1998;**245**:392-6.
23. Wetzel MA, Steele AD, Eisenstein TK, Adler MW, Henderson EE, Rogers TJ. Mu-opioid induction of monocyte chemoattractant protein-1, RANTES, and IFN-gamma-inducible protein-10 expression in human peripheral blood mononuclear cells. *J Immunol* 2000;**165**:6519-24.
24. Greeneltch KM, Kelly-Welch AE, Shi Y, Keegan AD. Chronic morphine treatment promotes specific Th2 cytokine production by murine T cells in vitro via a Fas/Fas ligand-dependent mechanism. *J Immunol* 2005;**175**:4999-5005.
25. Roy S, Wang J, Charboneau R, Loh HH, Barke RA. Morphine induces CD4+ T cell IL-4 expression through an adenylyl cyclase mechanism independent of the protein kinase A pathway. *J Immunol* 2005;**175**:6361-7.
26. Sora I, Takahashi N, Funada M *et al*. Opiate receptor knockout mice define μ receptor roles in endogenous nociceptive responses and morphine-induced analgesia. *Proc Natl Acad Sci U S A* 1997;**94**:1544-9.
27. Hall FS, Li XF, Goeb M *et al*. Congenic C57BL/6 μ-opiate receptor (MOR) knockout mice: baseline and opiate effects. *Genes Brain Behav* 2003;**2**:114-21.
28. Kobayashi K, Morita S, Sawada H *et al*. Immunotoxin-mediated conditional disruption of specific neurons in transgenic mice. *Proc Natl Acad Sci U S A* 1995;**92**:1132-6.
29. Ide S, Minami M, Satoh M, Uhl GR, Sora I, Ikeda K. Buprenorphine antinociception is abolished, but naloxone-sensitive reward is retained, in μ-opioid receptor knockout mice. *Neuropsychopharmacology* 2004;**29**:1656-63.
30. Posevitz V, Vizler C, Benyhe S, Duda E, Borsodi A. Restraint stress and anti-tumor immune response in mice. *Acta Biologica Hungarica* 2003;**54**:167-76.
31. Mercado AM, Padgett DA, Sheridan JF, Marucha PT. Altered kinetics of IL-1α, IL-1β, and KGF-1 gene expression in early wounds of restrained mice. *Brain Behav Immun* 2002;**16**:150-62.
32. Ashcraft KA, Hunzeker J, Bonneau RH. Psychological stress impairs the local $CD8^+$ T cells response to mucosal HSV-1 infection and allows for increased pathogenicity via a glucocorticoid receptor-mediated mechanism. *Psychoneuroendocrinology* 2008;**33**:951-63.
33. Okuyama K, Wada K, Chihara J, Takayanagi M, Ohno I. Sex-related splenocyte function in a murine model of allergic asthma. *Clin Exp Allergy* 2008;**38**:1212-9.
34. Wada K, Okuyama K, Ohkawara Y, Takayanagi M, Ohno I. Gender differences in transcriptional regulation of IL-5 expression by bronchial lymph node cells in a mouse model of asthma. *Respirology* 2010;**15**:629-35.
35. Barthold SW. Specificity of infection-induced immunity among *Borrelia burgdorferi* sensu lato species. *Infect Immun* 1999;**67**:36-42.
36. Loehning M, Stroehmann A, Coyle AJ *et al*. T1/ST2 is preferentially expressed on murine Th2 cells, independent of interleukin 4, interleukin 5, and interleukin 10, and important for Th2 effector function. *Proc Natl Acad Sci U S A* 1998;**95**:6930-5.
37. Meisel C, Bonhagen K, Loehning M *et al*. Regulation and function of T1/ST2 expression on $CD4^+$ T cells: Induction of type 2 cytokine production by T1/ST2 cross-linking. *J Immunol* 2001;**166**:3143-50.
38. Kobayashi K, Sasaoka T, Morita S *et al*. Genetic alteration of catecholamine specificity in transgenic mice. *Proc Natl Acad Sci U S A* 1992;**89**:1631-5.
39. Ide S, Sora I, Ikeda K *et al*. Reduced emotional and corticosterone responses to stress in mu-opioid receptor knockout mice. *Neuropharmacology* 2010;**58**:241-7.
40. Thunberg S, Gafvelin G, Nord M *et al*. Allergen provocation increases TH2-cytokines and FOXP3 expression in the asthmatic lung. *Allergy* 2010;**65**:311-8.
41. Milner JD, Ward JM, Keane-Myers A, Paul WE. Lymphopenic mice reconstituted with limited repertoire T cells develop severe, multiorgan, Th2-associated inflammatory disease. *Proc Natl Acad Sci U S A* 2007;**104**:576-81.
42. Kearley J, Robinson DS, Lloyd CM. $CD4^+CD25^+$ regulatory T cells reverse established allergic airway inflammation and prevent airway remodeling. *J Allergy Clin Immunol* 2008;**122**:617-24.
43. McGee HS, Agrawal DK. Naturally occurring and inducible T-reguratory cells modulating immune responses in allergic asthma. *Am J Respir Crit Care Med* 2009;**180**:211-25.
44. Sakaguchi S, Sakaguchi N, Asano M, Itoh M, Toda M. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *J Immunol* 1995;**115**:1151-64.
45. Thornton AM, Shevach EM. $CD4^+CD25^+$ immunoregulatory T cells suppress polyclonal T cell activation in vitro by inhibiting interleukin 2 production. *J Exp Med* 1998; **188**:287-96.
46. Itoh M, Takahashi T, Sakaguchi N *et al*. Thymus and autoimmunity: production of $CD25^+CD4^+$ naturally anergic and suppressive T cells as a key function of the thymus in maintaining immunologic self-tolerance. *J Immunol* 1999;**162**:5317-26.
47. Freier E, Weber CS, Nowottne U *et al*. Decrease of $CD4^+$ $FOXP3^+$ T regulatory cells in the peripheral blood of human subjects undergoing a mental stressor. *Psychoneuroendocrinology* 2010;**35**:663-73.
48. Sommershof A, Aichinger H, Engler H *et al*. Substantial reduction of naïve and regulatory T cells following traumatic stress. *Brain Behav Immun* 2009;**23**:1117-24.
49. Hoelund CO, Axen J, Kemi C *et al*. Changes in immune regulation in response to examination stress in atopic and healthy individuals. *Clin Exp Allergy* 2006;**36**:982-92.
50. Kearley J, Barker JE, Robinson DS, Lloyd CM. Resolution

of airway inflammation and hyperreactivity after in vivo transfer of $CD4^+CD25^+$ regulatory T cells is interleukin 10 dependent. *J Exp Med* 2005;**202**:1539-47.
51. Scadding G, Durham S. Mechanisms of sublingual immunotherapy. *J Asthma* 2009;**46**:322-34.
52. Akdis M. Immune tolerance in allergy. *Curr Opin Immunol* 2009;**21**:700-7.
53. Matsumoto K, Inoue H, Fukuyama S *et al*. Frequency of $Foxp3^+CD4^+CD25^+$ T cells is associated with the phenotypes of allergic asthma. *Respirology* 2009;**14**:187-94.
54. Chen E, Miller GE. Stress and inflammation in exacerbations of asthma. *Brain Behav Immun* 2007;**21**:993-9.
55. Xia W, Pinto CE, Kradin RL. The antigen-presenting activities of Ia^+ dendritic cells shift dynamically from lung to lymph node after an airway challenge with soluble antigen. *J Exp Med* 1995;**181**:1275-83.
56. Leech MD, Benson RA, De Vries A, Fitch PM, Howie SE. Resolution of Der p1-induced allergic airway inflammation is dependent on $CD4^+CD25^+Foxp3^+$ regulatory cells. *J Immunol* 2007;**179**:7050-8.
57. Strickland DH, Stumbles PA, Zosky GR *et al*. Reversal of airway hyperresponsiveness by induction of airway mucosal $CD4^+CD25^+$ regulatory T cells. *J Exp Med* 2006;**203**:2649-60.