

Increased Susceptibility to Allergic Asthma with the Impairment of Respiratory Tolerance Caused by Psychological Stress

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Keywords

Asthma · Psychological stress · Immune tolerance · Regulatory T cell · Glucocorticoid

Abstract

Background: Bronchial asthma is characterized by type 2 T helper (Th2) cell inflammation, essentially due to a breakdown of immune tolerance to harmless environmental allergens. Etiologically, experiences of psychological stress can be associated with a heightened prevalence of asthma. However, the mechanisms underlying stress-related asthma development are unclear. In this study, we examined whether psychological stress increases susceptibility to allergic asthma by downregulating immune tolerance. **Methods:** Female BALB/c mice were sensitized with ovalbumin/alum, followed by ovalbumin inhalation. Ovalbumin inhalation induced immune tolerance before sensitization occurred. Some mice were exposed to restraint stress during tolerance induction or sensitization. Asthma development was evaluated by airway responsiveness, inflammation, cytokine expression, and IgE synthesis. Sensitization was evaluated by

measuring proliferation and cytokine production by splenocytes. The effects of stress exposure on the numbers and functions of dendritic cells and regulatory T (Treg) cells in bronchial lymph nodes and spleens were evaluated. To investigate the role of endogenous glucocorticoid in inhibiting immune tolerance after stress exposure, we examined the effects of (i) a glucocorticoid-receptor antagonist administered prior to stress exposure, and (ii) exogenous glucocorticoid (instead of stress exposure). **Results:** Asthmatic responses and Th2-biased sensitization, which were suppressed in tolerized mice, re-emerged in tolerized mice stressed during tolerance induction in association with decreased tolerogenic dendritic and Treg cell numbers. The effects of stress exposure on tolerized mice were abolished by administering a glucocorticoid-receptor antagonist and reproduced by administering exogenous glucocorticoid without stress. **Conclusions:** Our findings suggested that psychological stress can potentially increase allergic asthma susceptibility by inhibiting immune tolerance.

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Introduction

Bronchial asthma is a common cause of chronic illness that is estimated to affect more than 300 million people worldwide. Asthma is caused by various environmental factors, such as allergens, infection, and stress. Asthma manifests with airway inflammation, airway hyperresponsiveness (AHR), and tissue remodeling. Asthmatic inflammatory responses are caused by eosinophil infiltration, increased mucous production, and elevated serum IgE levels, which are orchestrated by type 2 T helper (Th2) cells and Th2 cytokines, such as interleukin (IL)-4, IL-5, and IL-13. Airway inflammation leads to AHR and mucosal remodeling [1–6].

Regulatory T (Treg) cells play a critical role in balancing the immune network by suppressing or limiting effector immune responses against internal and external insults, for example, by preventing organ-specific autoimmunity and allograft rejection, and maintaining self-tolerance [7–9]. Treg cells are generated by plasmacytoid dendritic cells (pDCs) characterized by CD11c^{mid} and PDCA-1 expression, or IL-10-producing myeloid DCs (mDCs) with CD11c^{high} and IL-10 expression [9–11]. Their generation appears to be dependent on microenvironmental factors such as IL-10 and TGF- β [12, 13]. Patients with severe asthma show reduced numbers of Treg cells in the blood and sputum, with impaired suppressive activities as compared with healthy subjects [14]. Moreover, the percentage of Treg cells in bronchoalveolar lavage fluid (BALF) of children [15] and adults [16] with asthma is lower than that in healthy subjects. The failure of Treg cells to suppress Th2-mediated immune responses to innocuous inhaled antigens may lead to an imbalance 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 [13, 17, 18]. Recent studies have investigated the mechanisms underlying the impairment of Treg cells in maintaining immune tolerance and the association with increased susceptibility to allergic asthma. Respiratory infections with viruses such as rhinovirus and respiratory syncytial virus (RSV) in early life are risk factors for asthma development in adult life. RSV infection impairs immune tolerance by inducing phenotypic changes in Foxp3⁺ Treg cells, including GATA-3 expression, Th2 cytokine production, and loss of suppressive functions, in an IL-4-dependent pathway [19]. However, how exactly ineffective tolerogenic immune responses develop in asthmatic patients remains unclear.

Several epidemiological studies have demonstrated an association between psychological stress and asthma development [20–23]. For example, perceived stress was strongly associated with the development of adult-onset asthma and hospitalization [24]. Experiences of abuse during childhood and adolescence were positively associated with adult-onset asthma in African American women [25]. Stressful life events, such as a broken life partnership, also contributed to asthma development [26]. However, the pathogenic mechanism of asthma development by psychological stress is unknown.

Various kinds of stressors have been reported to decrease Treg cell production. The number of Treg cells in the peripheral blood of patients with PTSD (posttraumatic stress disorder) was significantly lower than that in non-PTSD subjects [27, 28]. An acute laboratory stressor decreased the number of CD4⁺FOXP3⁺ Treg cells in the peripheral blood of human subjects [29].

Psychological stress upregulates the release of glucocorticoid (GC) by activating the hypothalamic-pituitary-adrenal cortex axis. GC evokes defensive responses to stress that help to maintain homeostatic balance, including regulatory effects on immune-system activities. By affecting the immune system, GC can potentially enhance Th2 immune responses through inhibiting the development of Treg cells essential for respiratory tolerance [30, 31].

Therefore, we hypothesized that exposure to psychological stress prevents the development of respiratory tolerance by inhibiting Treg cell generation via the enhanced release of GC, resulting in Th2-biased immune responses followed by increased susceptibility to allergic asthma. To test this hypothesis, we examined the inhibitory effects of psychological stress on respiratory tolerance induction with or without a GC-receptor antagonist in a murine model of allergic asthma.

Materials and Methods

Mice

Specific pathogen-free female BALB/c mice were purchased from CLEA Japan (Tokyo, Japan). OVA-specific TCR transgenic DO11.10 (Rag2^{-/-}) mice were kindly provided by Dr. Ryo Abe (Tokyo University of Science, Noda, Japan). Animals were housed under a 12-h light/dark cycle at a constant temperature (22 $\pm$ 2°C). Sterilized food and water were available ad libitum. All experiments described below were approved by the Committee of Animal Experiments at Tohoku Medical and Pharmaceutical University (approval numbers: 14002-cn, 15001-cn, and 16002-cn). We took the utmost care to alleviate any pain and suffering in the mice.

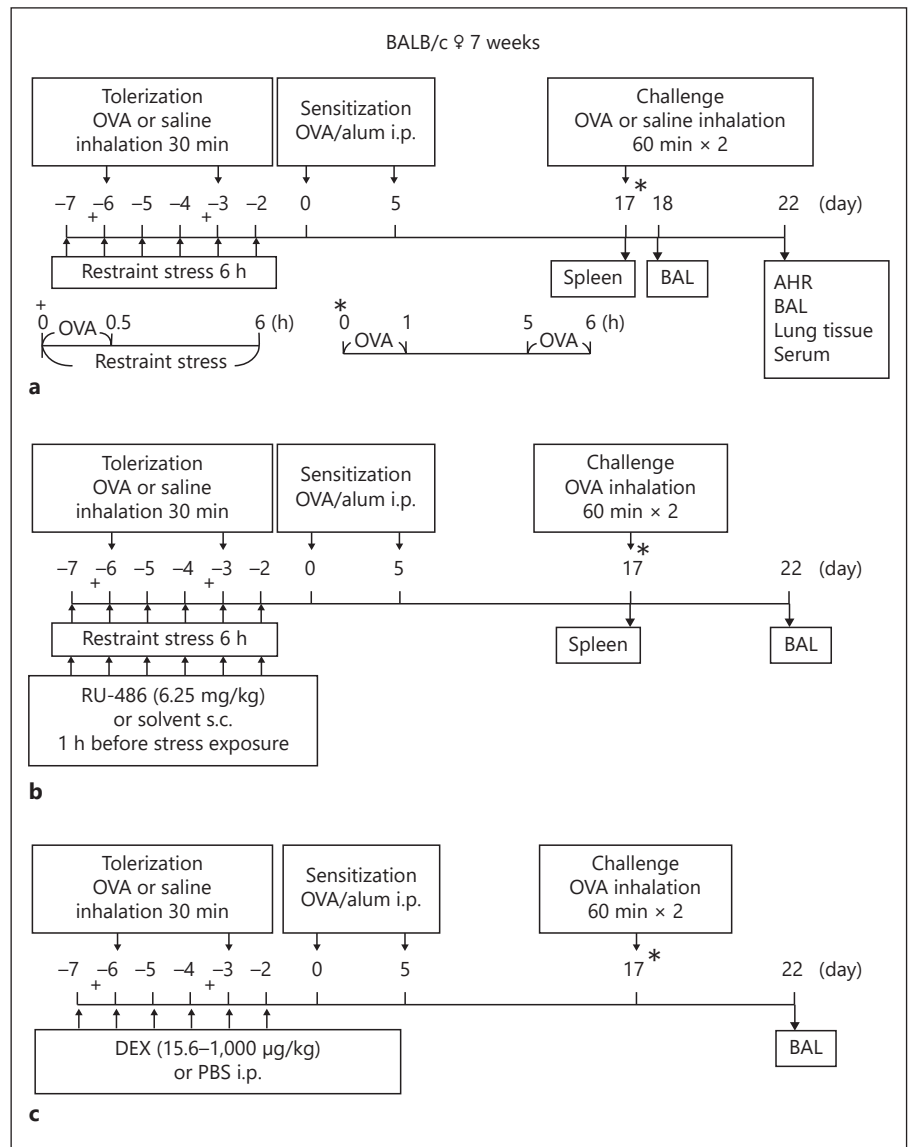


Fig. 1. Schematic representation of the protocols used for tolerization, stress exposure, sensitization, and antigen challenge.

Protocols for Tolerization, Sensitization, Antigen Challenge, and Stress Exposure

Seven-week-old mice were sensitized and made to inhale antigen, as described previously [32], with a minor modification (Fig. 1a). Briefly, mice were sensitized by intraperitoneal injections of chicken ovalbumin (OVA; grade V; Sigma-Aldrich, St. Louis, MO, USA; 8 µg/mouse) adsorbed onto aluminum hydroxide (Wako Pure Chemical Industries, Osaka, Japan; 4 mg/mouse) on days 0 and 5. Respiratory tolerance was induced by OVA inhalation without aluminum hydroxide on days -6 and -3, as described previously [33]. For the first 30 min of the period of stress exposure described below, aerosolized OVA (5 mg/mL in saline) flowed through the chambers for OVA exposure (tolerized mice). As a control for tolerance, saline alone was flowed through the chambers (asthmatic mice). For restraint stress, each mouse was placed in a 50-mL conical centrifuge tube

with multiple ventilation holes [32]. This restraint allowed mice to rotate from a supine to a prone position, but not to turn their heads toward their tails or consume food and water. Restraint stress is generally considered to induce psychological stress in animals [34]. During the tolerance phase, stressed/tolerized and stressed/asthmatic mice in 50-mL conical centrifuge tubes were left in plastic chambers for 6 h for 6 consecutive days (-7 to -2), at the same time each day. In another set of experiments, during the sensitization phase, restraint-stress exposure was performed on days 0–5 in each case. Nonstressed mice were only deprived of food and water during the period when stressed mice were exposed to stress. On day 17, all mice were challenged with aerosolized OVA (5 mg/mL) or saline. Before and after the challenge, bronchoalveolar lavage (BAL) was performed [32]. Briefly, the lungs were lavaged twice by injecting 0.25 mL of cold phosphate-buffered saline (PBS), and the recovered PBS was pooled. Ap-

proximately 0.4 mL of instilled fluid was consistently recovered from each mouse. BALFs were processed to measure cytokine contents and to count the total cell numbers and cell differentials. After centrifugation, BALF supernatants were stored at -80°C before performing cytokine measurements by an enzyme-linked immunosorbent assay (ELISA). Lung tissues were processed as described below.

Administration of a GC-Receptor Antagonist and Dexamethasone

In experiments using a GC-receptor antagonist, mifepristone (RU-486; Sigma-Aldrich) was dissolved in polyethylene glycol 400 (Nacalai Tesque, Kyoto, Japan) and subcutaneously administered (6.25 mg/kg body weight) 1 h before each stress exposure (Fig. 1b). In experiments involving GCs, dexamethasone (DEX; Sigma-Aldrich) in PBS was intraperitoneally injected at the indicated dose to substitute for restraint stress (Fig. 1c). The DEX dose administered was determined based on previous studies [35–38] to make the serum GC contents reach levels comparable to those after restraint-stress exposure. The solvents RU-486 and DEX were used as vehicle controls for these agents.

Plasma Corticosterone Measurements

Before and immediately after restraint-stress exposure on day -7 and immediately after restraint-stress exposure on days -5 and -2 , blood was collected from stressed and nonstressed mice as described [32]. Plasma samples separated from blood were stored at -80°C for corticosterone concentration measurements using an enzyme immunoassay kit (Enzo Life Sciences, Farmingdale, NY, USA) according to the manufacturer's protocol. The sensitivity of detection was 32 pg/mL.

AHR Measurements

Measurements of airway resistance (R_L) were conducted using the Buxco FinePointe Resistance and Compliance system (DSI, Minneapolis, MN, USA), as described [32] (Fig. 1a). Mice were sequentially challenged with aerosolized PBS (baseline) followed by increasing doses of methacholine (Sigma-Aldrich), ranging from 0.625 to 5 mg/mL. Maximum-resistance values were recorded during a 3-min period following each challenge. The concentration of methacholine that provoked a 200% increase in R_L (PC200) was calculated by linear interpolation of the dose-response curves.

Histological Analysis of the Lungs

Sections of lung tissues collected on day 22 were stained with periodic acid-Schiff (PAS) to evaluate mucus-containing goblet cells in the bronchial epithelium, or with hematoxylin and eosin to study eosinophil infiltration in peribronchial areas. The number of PAS-positive cells and eosinophils were determined as described previously [32].

OVA-Specific IgE Measurement

The concentrations of OVA-specific IgE in serum collected on day 22 were measured by ELISA, as described previously [32]. The sensitivity of detection was 1.9×10^{-2} EU/mL for IgE.

Preparation of Bronchial Lymph Node Cells

Bronchial lymph nodes (BLNs) obtained on day -2 from 10 nontolerized mice and from 3 tolerized mice with or without the stress exposure were pooled and weighed. BLN cells were prepared

as described previously [39], processed for flow cytometric analysis, and used for the transfer and coculture experiments described below.

Adoptive Transfer of BLN DCs

DCs as CD11c-positive cells were isolated from BLN cells from tolerized and stressed/tolerized mice on day -2 using an autoMACS Pro Separator (Miltenyi Biotec, Bergisch Gladbach, Germany) with anti-mouse CD11c (N418) MicroBeads (Miltenyi Biotec) and adoptively transferred (day -2) into naïve mice as described previously [39]. The mice were subsequently immunized (days 0–5) with OVA/alum and challenged with OVA (day 17), and BAL was performed (day 22) as described above.

Coculture of BLN DCs with Naïve DO11.10 T Cells

CD11c-positive DCs were isolated from BLN cells of tolerized and stressed/tolerized mice as mentioned above. CD4⁺ T cells were isolated from splenocytes of naïve DO11.10 (Rag2^{-/-}) female mice using an autoMACS Pro Separator (Miltenyi Biotec) with anti-mouse CD4 (L3T4) MicroBeads (Miltenyi Biotec). DCs (1×10^4 cells/well) were cocultured with CD4⁺ T cells (2×10^5 cells/well) in the presence of OVA (100 $\mu\text{g/mL}$). After culture for 3 days, cells were processed for flow-cytometric analysis.

Adoptive Transfer of CD4⁺ T Splenocytes

Splenocytes were prepared from the 4 mouse groups 1 day after the last stress exposure. CD4⁺ T cells were purified from the splenocytes, as described above. CD4⁺ T cells (2×10^7 cells/0.2 mL of saline for each mouse) were transferred into naïve BALB/c mice via the tail vein (day -1). The mice were subsequently immunized (days 0–5) with OVA/alum and challenged (day 17) with OVA, and BAL was performed (day 22) as mentioned above.

Culture of Splenocytes

Splenocytes were prepared from mice of all 4 experimental groups on day 17. The cells were cultured at 1×10^6 cells in 200 μL /well in 96-well culture plates (MICROPlate; Asahi Techno Glass, Chiba, Japan) in the presence or absence of OVA (200 $\mu\text{g/mL}$) for 72 h. The samples were then centrifuged, and the supernatants were stored at -80°C for subsequent cytokine measurements. Cell proliferation was measured by performing WST-8 assays with cell-count reagent SF (Nacalai Tesque) according to the manufacturer's protocol.

Culture of Bone Marrow-Derived DCs

Bone marrow-derived DCs (BM-DCs) were prepared from BM cell cultures from female BALB/c mice (6–10 weeks old) incubated with recombinant mouse GM-CSF for 8 days in RPMI 1640 [40]. The cultured cells were more than 95% CD11c⁺. BM-DCs (2×10^4 cells in 200 μL /well in 96-well culture plates; MICROPlate, Asahi Techno Glass) were stimulated with OVA (100 $\mu\text{g/mL}$) in the absence or presence of DEX at the indicated concentration for 24 h. Cell proliferation was measured by WST-8 assay as mentioned above.

Flow Cytometric Analysis

Cells were preincubated with anti-CD16/CD32 (F γ gamma III/II receptor; BD Biosciences, Franklin Lakes, NJ, USA) to reduce nonspecific binding of the subsequent antibodies. Dead cells were excluded using a LIVE/DEAD Fixable Blue Dead Cell Stain Kit

(Life Technologies/Thermo Fisher Scientific Corporation, Grand Island, NY, USA). The cells were stained for surface antigens with anti-CD11c-PE (clone N418; BioLegend, San Diego, CA, USA), anti-PDCA1-APC (clone 927; BioLegend), anti-CD3ε-FITC (clone 145-2C11; Miltenyi Biotec), anti-CD4-AF700 (clone RM4-5; BD Biosciences), anti-DO11.10-TCR-APC (clone KJ1-26; eBioscience), or isotype-control antibodies. For intracellular staining, cells were stimulated with phorbol 12-myristate 13-acetate (5 ng/mL), ionomycin (500 ng/mL), and monensin (2 mM) for 4 h before surface-antigen staining. After fixation and permeabilization, the cells were incubated with an anti-IFN-γ-PE (clone XMG1.2; eBioscience), anti-IL-4⁺-PE-CF594 (clone 11B11; BD Biosciences), anti-IL-10-FITC (clone JES5-16E3; BioLegend), anti-Foxp3-PE or PE-Cy7 (clone FJK-16s; eBioscience), or isotype-control antibody. IL-10-producing mDCs were identified as CD11c^{high} MHC II^{high} IL-10⁺ cells, pDCs as CD11c^{mid} PDCA-1⁺ cells, DO11.10 Th1 cells as KJ1-26⁺IFN-γ⁺ cells, Th2 cells as KJ1-26⁺IL-4⁺ cells, and Treg cells as CD3⁺CD4⁺Foxp3⁺ cells. The cells were counted on a FACSAria II flow cytometer (BD Biosciences), and the analyses were performed using FACSDiva software (BD Biosciences). The flow cytometric gating strategies used to evaluate cell subsets are shown in online supplementary Figure 1 (for all online suppl. material, see www.karger.com/doi/10.1159/000488289).

Measurements of Cytokine Contents

The concentrations of IL-4, IL-5, and IL-13 in BALFs, and the concentrations of IL-4 and IFN-γ in cell culture supernatants were measured using 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, 1.5 pg/mL for IL-13, and 8 pg/mL for IFN-γ.

Statistical Analysis

Data are expressed as the mean ± standard deviation (SD) from multiple independent experiments (as indicated by *n* values). Significant differences between 2 groups were determined by the non-parametric Mann-Whitney U test. For comparisons between multiple groups, analysis of variance and Bonferroni post hoc tests were performed. These analyses were performed using Prism5 (GraphPad Software, San Diego, CA, USA). *p* < 0.05 was considered statistically significant.

Results

Effects of Stress on Plasma Corticosterone Concentrations

Corticosterone concentrations (in ng/mL) in stressed mice were significantly higher than those in nonstressed mice after the 1st, 3rd, and 6th stress exposures on days -7, -5, and -2, respectively (1st: 327.2 ± 250.3 [*n* = 9] vs. 88.9 ± 29.8 [*n* = 8], *p* < 0.05; 3rd: 547.4 ± 256.0 [*n* = 7] vs. 90.0 ± 95.3 [*n* = 5], *p* < 0.05; 6th: 365.5 ± 205.7 [*n* = 11] vs. 85.1 ± 63.7 [*n* = 8], *p* < 0.01). Before stress exposure, no significant differences in corticosterone concentrations occurred between stressed and nonstressed mice. Corti-

costerone concentrations after stress exposure were significantly higher at all time points than before stress exposure, while no such increases were observed in non-stressed mice (online suppl. Fig. 2).

Effects of Stress on Asthmatic Responses in Tolerized Mice

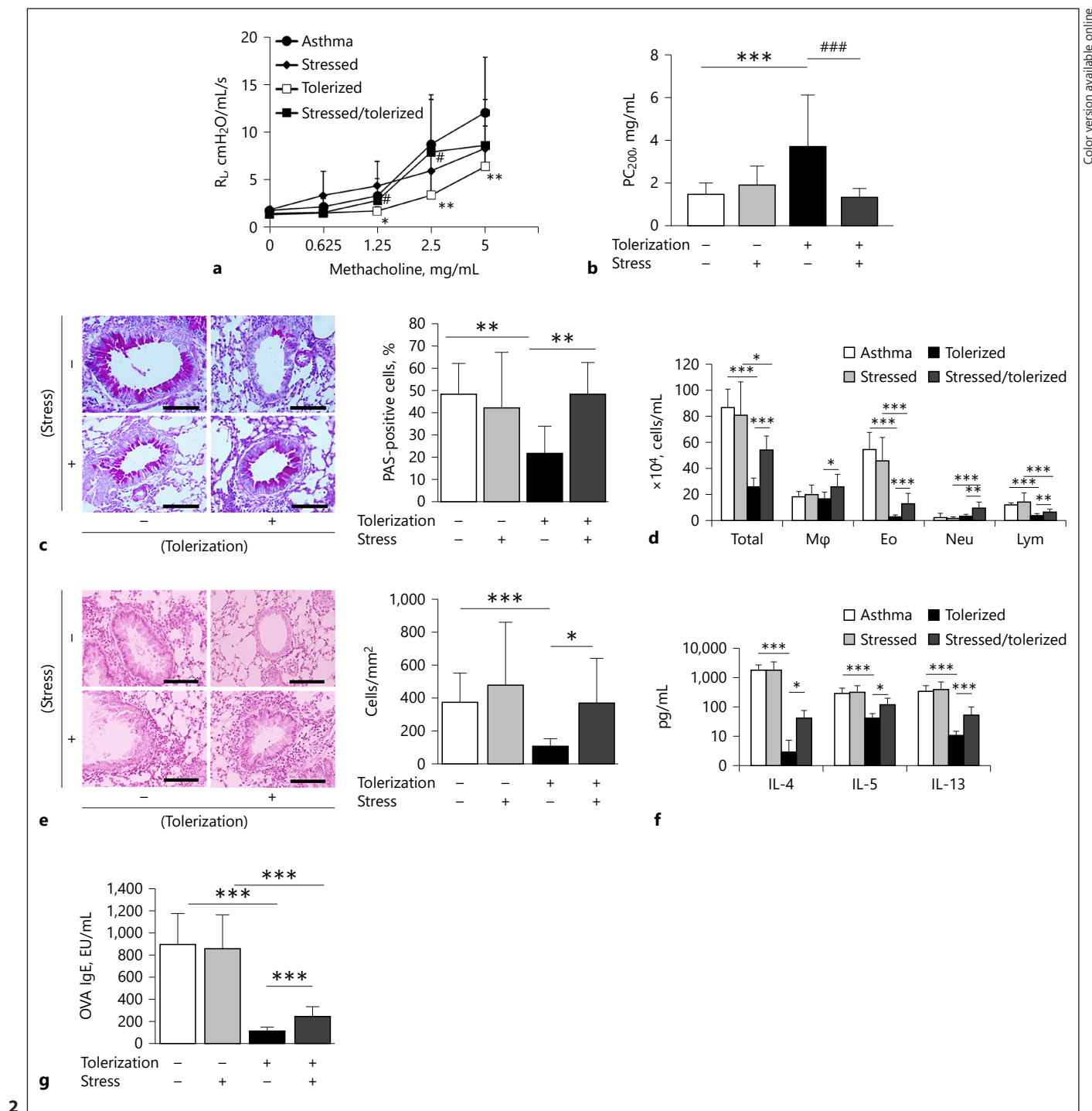
To investigate the effects of restraint stress on asthmatic responses in tolerized mice, we compared airway responsiveness, inflammation, cytokine expression, and IgE synthesis after antigen challenge in the 4 mouse groups (Fig. 2). As expected, tolerized mice showed significantly lower AHR, mucus production, numbers of total cells, eosinophils, and lymphocytes in BALFs, peribronchial eosinophil accumulation, Th2 cytokine content in BALF, and OVA-specific IgE in serum than asthmatic mice. In contrast, those airway responses and IgE levels were significantly higher in stressed/tolerized mice than in tolerized mice. However, no significant differences occurred in airway responses or IgE levels between asthmatic and stressed/asthmatic mice. The IFN-γ content in BALFs did not significantly differ between the 4 groups. Significant differences were not observed in total cell and cell differential counts in BALFs before OVA challenge (day 17) or after saline challenge (day 22) between the 4 groups (data not shown). These data indicated that stress indirectly enhanced allergic responses by inhibiting the development of respiratory tolerance. However, tolerized mice with stress exposure during sensitization (after immune tolerance establishment) showed low numbers of inflammatory cells in the BALF, similar to tolerized mice without stress exposure. No significant differences in the numbers of inflammatory cells were observed between asthmatic mice (with or without stress exposure) during sensitization (online suppl. Fig. 3).

Effects of Stress on Sensitization in Tolerized Mice

Because restraint stress caused a re-emergence of antigen-induced asthmatic responses in tolerized mice, we next investigated the effects of psychological stress on immune tolerance. As expected, pretreating mice with aerosolized OVA inhibited splenocyte proliferation upon OVA stimulation in vitro (vs. pretreatment with aerosolized saline). However, restraint stress exposure during the induction phase of tolerance significantly increased splenocyte proliferation in vitro compared with findings in tolerized mice (Fig. 3a). Moreover, we compared the OVA-induced cytokine expression (IFN-γ and IL-4) by the splenocyte in the 4 groups (Fig. 3b, c). With regard to IFN-γ (Fig. 3b), tolerance had no effects on the expression

of IFN- γ either with or without stress exposure, whereas the stress exposure decreased the cytokine expression in tolerized mice but not in nontolerized mice. On the other hand, tolerance significantly decreased IL-4 expression either with or without stress exposure, while the stress exposure did not significantly alter the cytokine expres-

sion in either nontolerized or tolerized mice (Fig. 3c). Therefore, the deviation of the Th1/Th2 balance, as seen by the IFN- γ /IL-4 ratios, toward Th2-dominant responses caused by the stress exposure in tolerized mice (Fig. 3d) could be mainly due to the decreased expression of IFN- γ .



(For legend see next page.)

Effects of Stress on the Development of Tolerogenic DCs and Tregs

Because restraint stress inhibited the induction of respiratory tolerance, we analyzed Treg cells, pDCs, and IL-10-producing mDCs as Treg-generating DCs in BLNs. The numbers and percentages of pDCs (Fig. 4a, b) and Treg cells (Fig. 4c, d) were significantly increased in toler-

ized mice compared with nontolerized mice under the nonstress condition. The stress exposure significantly decreased the numbers (Fig. 4a), but not the percentages (Fig. 4b), of pDCs and both the numbers (Fig. 4c) and percentages (Fig. 4d) of Treg cells in stressed/tolerized mice compared with tolerized mice. Few IL-10-producing mDCs were detected in BLN cells.

Fig. 3. Effects of stress on sensitization. On day 17, splenocytes were prepared from mice of each group of sensitized mice and stimulated in vitro with OVA. **a** After a 72-h culture, cell proliferation was measured in WST-8 assays. The content of IFN- γ (**b**) and IL-4 (**c**), and the content ratios of IL-4/IFN- γ (**d**) in culture supernatants were determined by ELISA. Data are expressed as the mean $\pm$ SD of triplicate cultures from 3 independent experiments. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

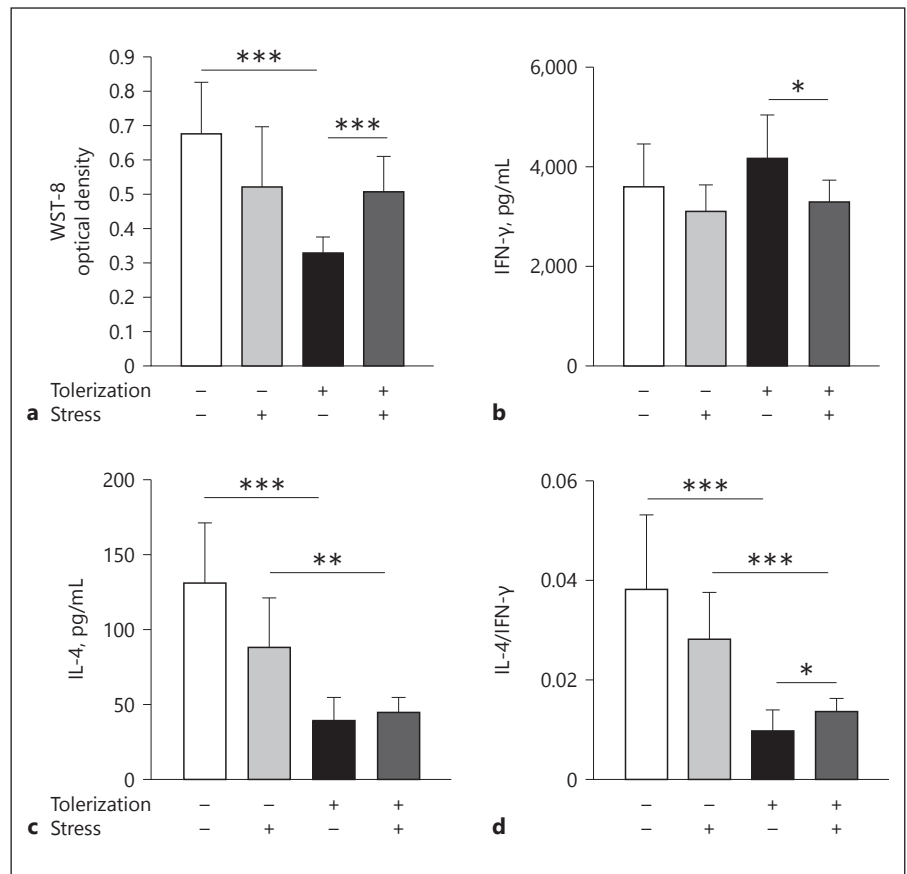
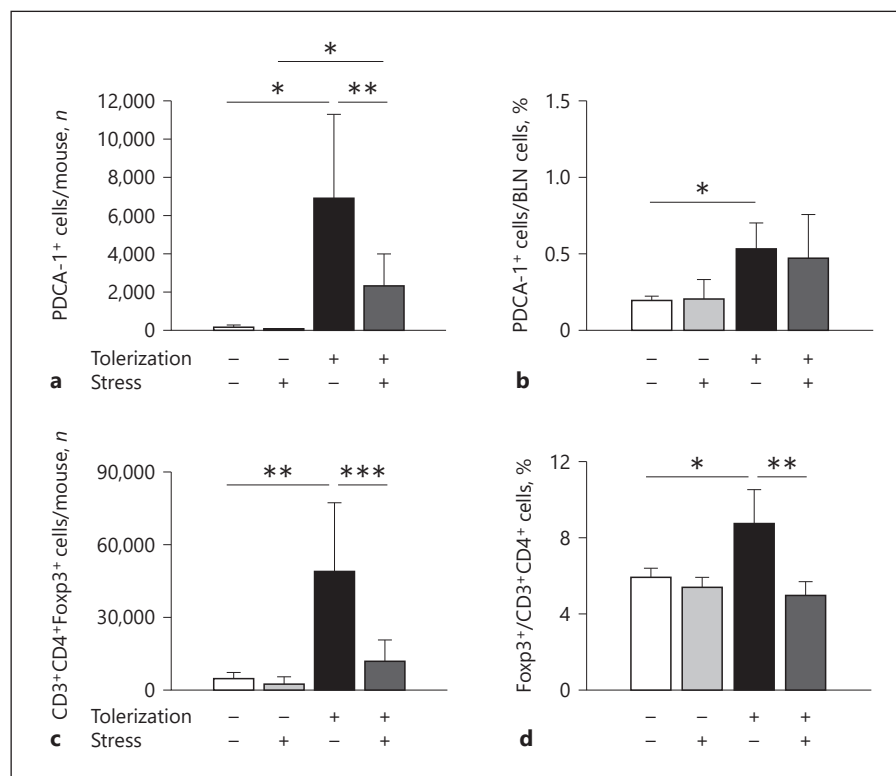


Fig. 2. Effects of stress during the tolerance induction phase on allergic airway responses. Airway reactivity (R_L ; **a**) and airway sensitivity (PC200; **b**) were measured 5 days after the last OVA challenge (day 22). Data are expressed as the mean $\pm$ SD ($n = 9-13$ from 4 independent experiments). * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$, compared with asthmatic mice; # $p < 0.01$ and ### $p < 0.001$, compared with tolerized mice. **c** Lungs were isolated from mice of each group 5 days after the last OVA challenge (day 22). Lung sections were prepared and stained with PAS. Representative images of lung sections (left; scale bar, 100 μ m) and the percentage of PAS-positive cells (right) from the 4 mouse groups are shown. Data are expressed as the mean $\pm$ SD ($n = 5-7$ mice from 3 independent experiments). **d** BALFs were obtained from mice of each group 5 days after the last OVA challenge (day 22). Total cells (Total), macrophages (M Φ), eosinophils (Eo), neutrophils (Neu), and lymphocytes (Lym) were counted. Data are expressed as the mean $\pm$ SD

($n = 7-9$ from 3 independent experiments). **e** Lung sections were stained with hematoxylin and eosin. Representative images of lung sections (left; scale bar, 100 μ m) and the eosinophil counts in the peribronchial area (right) are shown. Data are expressed as the mean $\pm$ SD ($n = 5-8$ mice from 3 independent experiments). **f** Effects of stress on BALF cytokine contents. BALFs were obtained from mice of each group 1 day after the last OVA challenge (day 18). BALF IL-4, IL-5, and IL-13 levels were measured by ELISA. Data are expressed as the mean $\pm$ SD ($n = 3-9$ from 3 independent experiments). **g** Effects of stress on OVA-specific IgE in serum. Sera were obtained from mice of each treatment group 5 days after the last OVA challenge (day 22). OVA-specific IgE in serum was measured by ELISA. Data are expressed as the mean $\pm$ SD ($n = 5-7$ from 3 independent experiments). * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

Fig. 4. Effects of stress on tolerogenic DCs and Treg cells in BLN. BLNs were collected after the final stress exposure (day -2). The numbers and percentages of PDCA-1⁺ cells (a, b), and CD3⁺CD4⁺Foxp3⁺ cells (c, d) were determined. Data from 3 independent experiments for nontolerized groups and 4 independent experiments for tolerized groups are expressed as the mean $\pm$ SD. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.



An adoptive transfer experiment was performed to examine the effects of stress exposure on DC functions that promote the development of respiratory tolerance in our stress/tolerance model. As expected, airway inflammation was suppressed in mice transferred with CD11c⁺ DCs from tolerized mice as compared with mice without transfer, although mice transferred with CD11c⁺ DCs from stressed/tolerized mice showed no significant difference compared to mice transferred with CD11c⁺ DCs from tolerized mice (online suppl. Fig. 4A). Furthermore, we investigated the effects of stress on the function of DCs in Treg development. Treg, Th2, and Th1 cell development promoted by DCs from the tolerized and stressed/tolerized mouse groups were not different (online suppl. Fig. 4B).

Transfer of the Inhibitory Effects of Stress on the Acquisition of Respiratory Tolerance by CD4⁺ T Cells

Analyses of BAL cells after sensitization followed by OVA inhalation in mice adoptively transferred with CD4⁺ T cells showed similar numbers of total cells and eosinophils between the 4 groups (Fig. 5a) compared to the variable levels observed between groups using the conventional mouse model (Fig. 2d). Adoptively trans-

ferring CD4⁺ T cells from tolerized mice resulted in significant decreases in the numbers of total cells and eosinophils in BALFs, relative to those numbers observed after transferring CD4⁺ T cells from asthmatic mice or mice injected with saline (vehicle control). The cell numbers in BALFs measured after adoptively transferring CD4⁺ T cells from stressed/tolerized mice were significantly higher than those after transferring CD4⁺ T cells from tolerized mice. However, no significant differences were found in the total numbers of cells or eosinophils between mice transferred with CD4⁺ T cells from asthmatic and stressed/asthmatic mice. The number of lymphocytes in BALFs showed a tendency similar to that of the numbers of total cells and eosinophils, although the differences between groups were not significant. In addition, the percentage of Treg cells in splenocytes from tolerized mice was significantly higher than that in asthmatic mice. In contrast, the percentage of Treg cells in splenocytes was significantly lower in stressed/tolerized mice than in tolerized mice (Fig. 5b). The percentage of CD3⁺CD4⁺ cells in splenocytes was not significantly different between the 4 groups (data not shown).

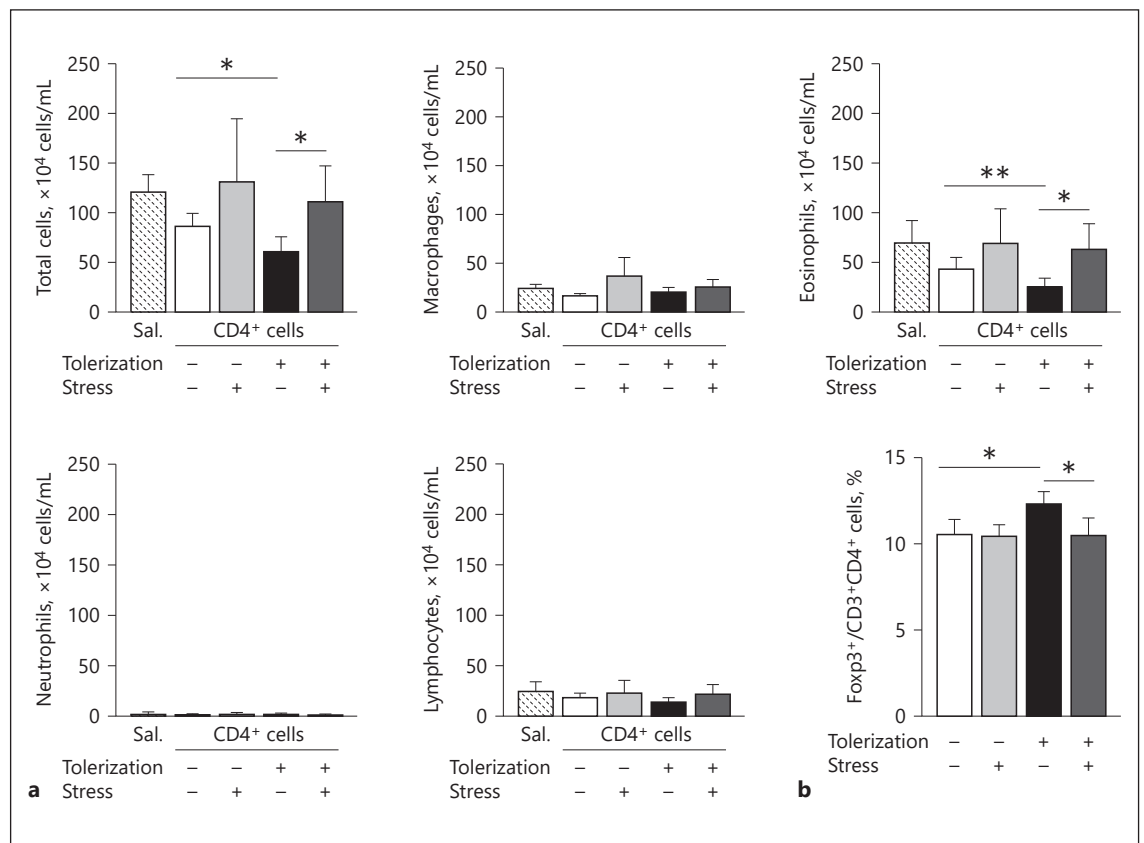


Fig. 5. Adoptive transfer of spleen CD4⁺ T cells. CD4⁺ T cells were isolated from spleens of mice of each mouse 1 day after the final stress exposure (day -1). **b** Spleen CD3⁺CD4⁺Foxp3⁺ cells were analyzed by flow cytometry. CD4⁺ T cells were intravenously injected into naïve BALB/c mice. Saline (Sal.) was injected as a control. One day postinjection, mice of all groups were sensitized with

OVA/alum and challenged with OVA. BALFs were obtained from mice of each group 5 days after the last OVA challenge. **a** Total cells, macrophages, eosinophils, neutrophils, and lymphocytes were counted. Data are expressed as the mean $\pm$ SD ($n = 3-9$ from 2 independent experiments). * $p < 0.05$ and ** $p < 0.01$.

Effects of a GC-Receptor Antagonist on Stress-Induced Inhibition of Respiratory Tolerance

The sizes and weights of BLNs and number of BLN cells in stressed/tolerized mice were lower than those in nonstressed/tolerized mice (Fig. 6a-c in solvent-treated groups). The atrophy of BLNs caused by exposure to restraint stress was abolished by treatment with RU-486 (Fig. 6a, b). Furthermore, the numbers of BLN cells, Treg cells, and tolerogenic DCs in stressed/tolerized mice recovered to the levels in nonstressed/tolerized mice after RU-486 treatment (Fig. 6c, d, f). Stress exposure appeared to affect the numbers of total cells, eosinophils, neutrophils, and lymphocytes in BALFs observed in tolerized and stressed/tolerized mice treated with solvent, but not in those treated with RU-486 (Fig. 7).

Effects of GC as a Substitute for the Stress on Respiratory Tolerance

Because the effects of stress exposure were diminished by administering the GC-receptor antagonist just before stress exposure, we examined whether exogenous GC could reproduce the effects of stress exposure. DEX administration significantly increased the numbers of total cells (250, 1,000 μ g/kg), eosinophils (250, 1,000 μ g/kg), and lymphocytes (250 μ g/kg) in BALFs as compared to PBS administration in tolerized mice, but not in nontolerized mice (Fig. 8).

Effects of GC on BM-DC Proliferation

The proliferation of BM-DCs stimulated with OVA was reduced by DEX in a dose-dependent manner (Fig. 9).

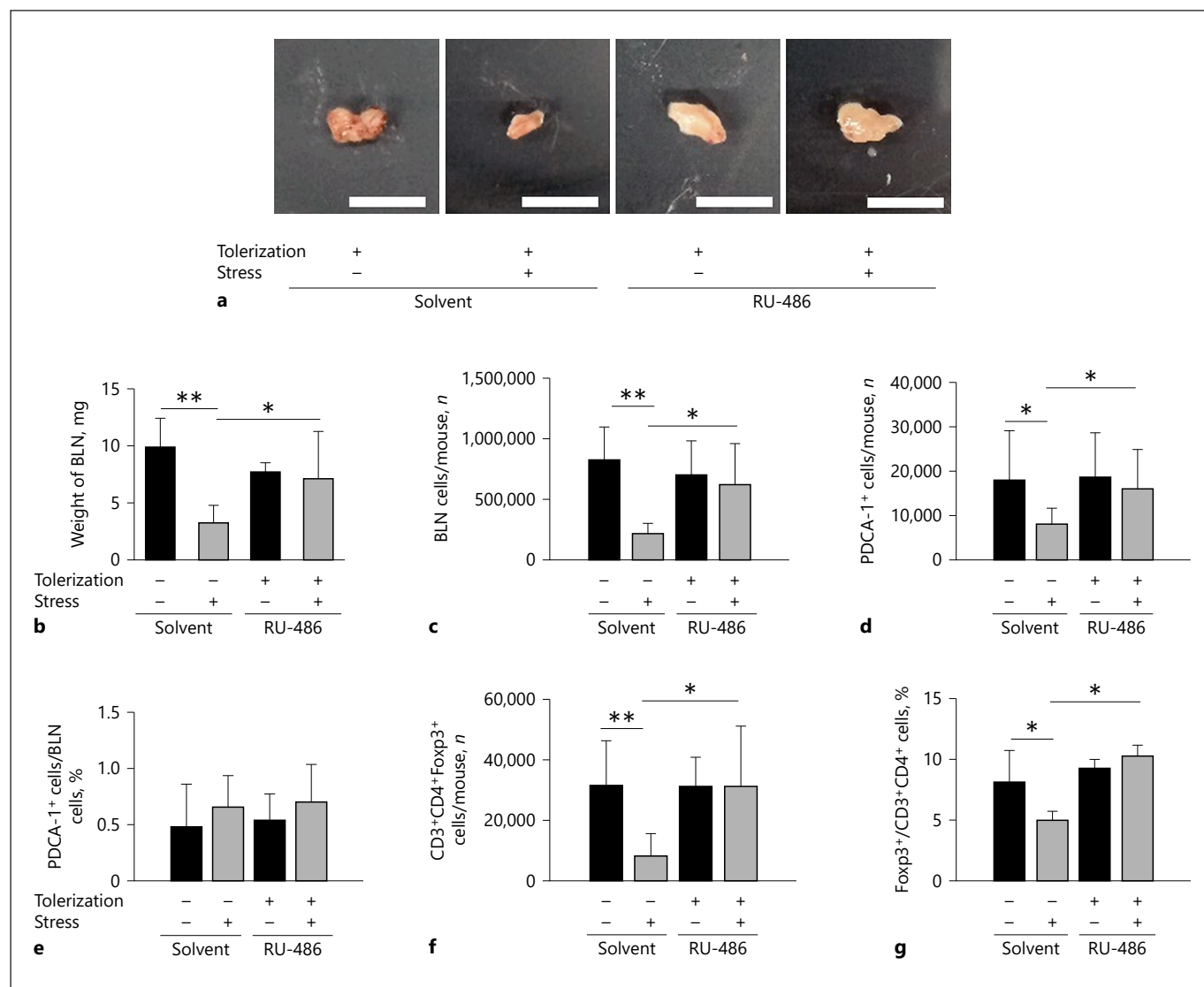


Fig. 6. Effects of a GC-receptor antagonist on BLN enlargement and cellular changes induced by stress. Tolerized mice with or without stress exposure were administered a GC-receptor antagonist, RU-486, or the solvent as a control before each stress exposure. BLNs were collected after the final stress exposure (day -2) and weighed. Representative images (**a**; scale bar, 5 mm) and weights (**b**) of BLNs in each mouse group are shown. Data are expressed as the mean $\pm$ SD ($n = 7-8$ from 3 independent experi-

ments). Cell populations of BLN cells were analyzed by flow cytometry. The numbers of total BLN cells (**c**), PDCA-1⁺ cells (**d**), and CD3⁺CD4⁺Foxp3⁺ cells (**f**) were determined and are expressed as percentages of PDCA-1⁺ cells (**e**) and CD3⁺CD4⁺Foxp3⁺ cells (**g**) in BLN cells. Data are expressed as the mean $\pm$ SD ($n = 6$ from 2 independent experiments). * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

Discussion

In this study, we demonstrated for the first time that exposure to psychological stress during the induction phase of respiratory tolerance, but not during the sensitization phase, promoted the development of Th2-biased sensitization and, thereby, asthmatic airway responses to

inhaled allergen, while restraint-stress exposure had no effect on sensitization and allergic airway inflammation (Fig. 2; online suppl. Fig. 3). Preventing the development of immune tolerance by stress exposure was confirmed by the re-emergence of Ag-specific splenocyte proliferation and Th2-predominant cytokine secretion in stressed/tolerized mice, both of which were suppressed in tolerized

Fig. 7. Effects of a GC-receptor antagonist on the stress-induced inhibition of respiratory tolerance. A GC-receptor antagonist, RU-486, or solvent was administered before each stress exposure. BALFs were obtained from mice of each treatment group 5 days after the last OVA challenge (day 22). Total cells (Total), macrophages (MΦ), eosinophils (Eo), neutrophils (Neu), and lymphocytes (Lym) were counted. Data are expressed as the mean ± SD (n = 5–8 from 4 independent experiments). * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$; # $p < 0.05$, ## $p < 0.01$, and ### $p < 0.001$, compared with solvent-treated control mice.

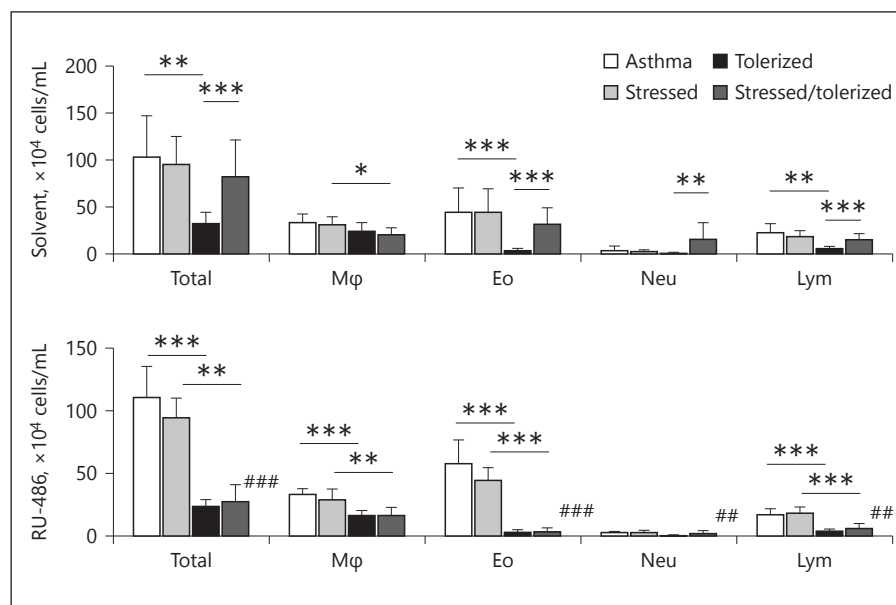
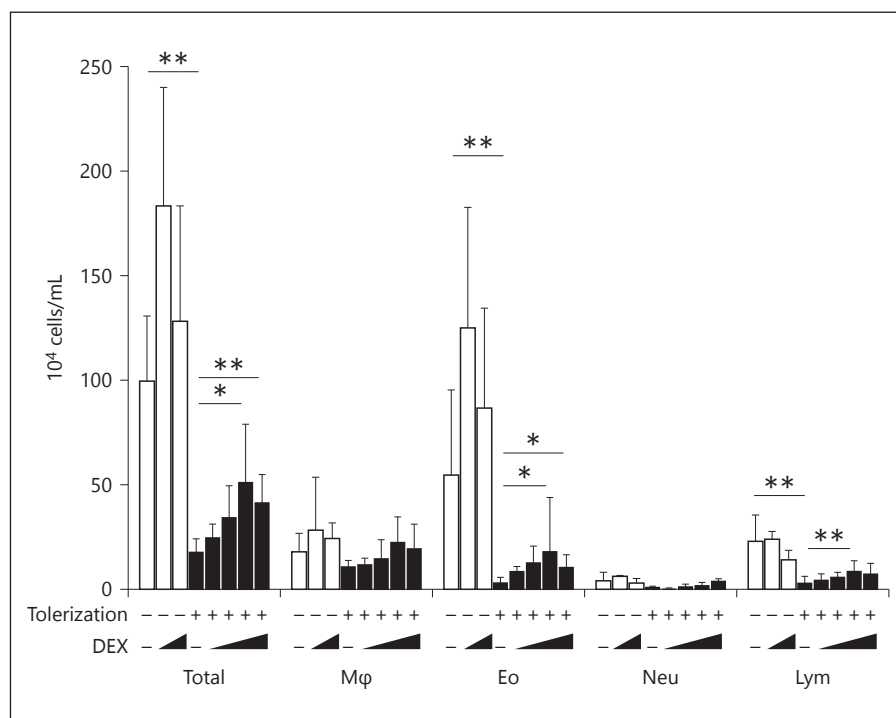


Fig. 8. Effects of DEX on the numbers of inflammatory cells. Solvent or 15.6, 62.5, 250, or 1,000 µg DEX/kg body weight was administered instead of stress exposure to nontolerized (open columns) and tolerized (closed columns) mice. BALFs were obtained from mice of each treatment group 5 days after the last OVA challenge (day 22). Total cells (Total), macrophages (MΦ), eosinophils (Eo), neutrophils (Neu), and lymphocytes (Lym) were counted. Data are expressed as the mean ± SD (n = 5–7 from 2 independent experiments). * $p < 0.05$ and ** $p < 0.01$.



mice as compared with asthmatic mice (Fig. 3). Stress exposure might inhibit the development of respiratory tolerance by dampening the tolerogenic DC-Treg cell axis (Fig. 4, 5), which could result from GC release upon stress exposure (Fig. 5–9).

In this study, the induction of immune tolerance by OVA inhalation correlated with BLN swelling, which was inhibited by stress exposure (Fig. 6). BLN atrophy following stress exposure has been shown in recent reports, revealing that stress leads to adverse physiological effects, such as atrophy of the thymus and lymph nodes [41, 42].

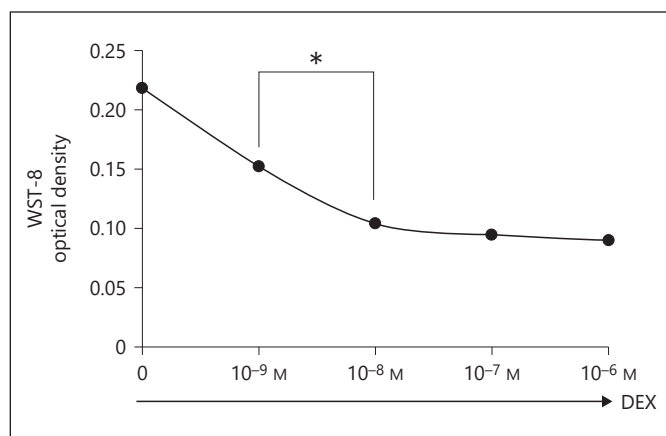


Fig. 9. Effects of DEX on BM-DC proliferation. BM-DCs were generated from female BALB/c mice. BM-DCs ($2/10^4$ cells in 200 μ L/well) in 96-well culture plates were stimulated with OVA (100 μ g/mL) in the absence or presence of DEX at the indicated concentrations for 24 h. Cell proliferation was measured in WST-8 assays.

Stress exposure caused the reduction of the absolute number of pDCs in BLNs (Fig. 4a), whereas the percentage of pDCs (Fig. 4b) and the ability of DCs to generate Treg cells (online suppl. Fig. 4B) were not altered and, furthermore, the effect of stress was not transferred by DCs (online suppl. Fig. 4A). Therefore, it is assumed that the potential roles for tolerogenic DCs in this model might not be strong. However, the transfer of CD4⁺ T cells from the spleen of stressed/tolerized mice caused the re-emergence of allergic airway inflammation, which was suppressed by the transfer of CD4⁺ T cells from tolerized mice (Fig. 5a), and the percentage of Treg cells in transferred splenocytes was significantly lower in stressed/tolerized mice than in tolerized mice (Fig. 5b). Thus, stress exposure during the tolerance-induction phase may have disturbed the induction, but not the function, of tolerogenic DCs, leading to insufficient differentiation of Treg cells, resulting in the failure to prevent Th2-biased immune sensitization. The inhibitory effects of stress exposure on Treg cell development are supported by studies showing that traumatic stress and a mental stressor reduced Treg cell numbers in peripheral blood in humans [27–29].

In airway immune responses, the stress exposure significantly increased IL-4 expression (Fig. 2f), although the stress had no effects on IFN- γ expression (data not shown), in BALF in tolerized mice. Furthermore, the number (Fig. 4c) and percentage (Fig. 4d) of Treg cells in BLNs were decreased by the stress exposure. On the other

hand, in the spleen, the deviation of the Th1/Th2 balance toward Th2-dominant responses caused by the stress exposure in tolerized mice (Fig. 3d) could be mainly due to the decreased expression of IFN- γ , while the percentage of Treg cells was decreased by the stress exposure (data not shown). Together with these findings, although the stress exposure has a potential to inhibit the development of tolerance leading to Th2-dominant immune responses, the main mechanisms underlying the deviation towards Th2-dominant immune responses might be different depending on the site of immune responses, for example systemic immunity in spleen and local immunity in airways.

To investigate the mechanisms underlying the inhibitory activity of stress on immune tolerance, we assessed the contribution of GC released upon stress exposure (online suppl. Fig. 2). The anti-tolerogenic effect of stress exposure was abolished by administering RU-486 (a GC-receptor antagonist) before each stress exposure during the tolerance-induction phase. RU-486 administration caused a re-emergence of BLN swelling (Fig. 6a) and retrieved the numbers of BLN cells, pDCs, and Treg cells in BLNs (Fig. 6) and inflammatory cells in BALFs after antigen challenge (Fig. 7) to the levels in tolerized but not stressed mice. Furthermore, the administration of DEX as a substitute for restraint stress during the tolerance-induction phase showed a similar effect as stress exposure, as demonstrated by the significant increase in the numbers of total cells, eosinophils, and lymphocytes in BALFs, compared with those in tolerized mice administered PBS (Fig. 8). These results indicated that GC signaling played a critical role in the stress exposure-induced perturbation of immune tolerance. GC can induce apoptosis of several types of immune cells [43], including mDCs [44] and pDCs [45]. Because RU-486 caused a re-emergence of BLN swelling and restored the number of pDCs, we investigated the effect of GC on DC proliferation in vitro. As expected, DEX reduced the proliferation of BM-DCs in a dose-dependent fashion (Fig. 9). Therefore, it is likely that inhibiting the acquisition of immune tolerance by stress exposure was mediated by GC, which decreased the number of pDCs and impaired Treg differentiation.

GC is recognized worldwide as a gold standard for treating and preventing asthma by its anti-allergic activities, including the reduction of airway inflammation and improvement of AHR through limiting cytokine production by T cells and epithelial cells, and impairing the recruitment, survival, and activation of eosinophils and other inflammatory cells [2, 46]. However, GC has

been shown to amplify Th2 immune responses via several mechanisms. GC treatment can potentiate Th2 differentiation, by either reducing IL-12 secretion from antigen-presenting cells [47] or directly suppressing Th1 cell polarization [48]. GC administration during sensitization enhanced Th2 responses in an allergic mouse model [31]. In addition, GC treatment during antigen inhalation (to promote the development of respiratory tolerance) eliminated IL-10-producing DCs required for the development of IL-10-producing Treg cells, leading to Th2-biased sensitization and allergic airway responses upon antigen exposure [30]. Interestingly, the development of respiratory tolerance was prevented by systemic, but not local, GC administration [49], in accordance with our finding that the GC level in the circulation had increased. Systemic administration of GC to immunocompetent humans and naïve mice significantly reduced the number, but not the suppressive function, of Foxp3⁺ Treg cells in blood [50]. More importantly, either systemic or local GC administration during the sensitization and allergen-induced inflammation phases decreased the number of Foxp3⁺ Treg cells in mouse lungs [51]. In contrast, both GC inhalation and systemic GC treatment increased the number and function of CD4⁺CD25^{high} Treg cells in blood and BALF in pediatric asthma [15], and Foxp3⁺ Treg cells in blood in adult asthma [52]. Whether the effect of GC on human Treg cells is inhibitory or stimulatory might depend on the immunocompetence status, such as healthy (immunocompetent) or asthmatic. The studies mentioned above and this study involved the use of wild-type, naïve mice so that the effect of GC treatment on Treg cells would be inhibitory, as observed with healthy subjects. The reason for the dependency was not determined and is beyond the aims of this study.

To our knowledge, it has not yet been reported that psychological stress prevents the development of respiratory tolerance. However, some studies have investigated the mechanisms underlying the impairment of immune tolerance associated with increased susceptibility to allergic asthma. Respiratory infections with viruses, such as rhinovirus and RSV, in early life are risk factors for asthma development in adult life. Simultaneous rhinovirus infection and tolerance induction in mice inhibited the acquisition of immune tolerance induced by intranasal OVA, attenuating the development of Foxp3⁺ Treg cells through OX40 ligand expression on lung DCs and thymic stromal lymphopoietin and IL-33 expression by airway epithelial cells, which increased the susceptibility to allergic airway responses [53]. Krishnamoorthy et al.

[19] demonstrated that after OVA tolerization by breast milk from maternal mice exposed to OVA, recurrent RSV infection in pups induced allergic responses, while the allergic responses were not observed in uninfected pups, in adult life (6 weeks old). RSV infection impaired immune tolerance by inducing phenotypic changes in Foxp3⁺ Treg cells, including GATA-3 expression, Th2 cytokine production, and loss of suppressive functions, in an IL-4-dependent pathway [19]. The potential of IL-33 in overcoming acquired immune tolerance was confirmed recently [54]. It was revealed that Foxp3⁺ Treg cells express the IL-33 receptor ST2, the expression of which was enhanced by the ligand, that IL-33 evoked Th2-like properties in those cells by inducing GATA-3 expression and the synthesis of Th2 cytokines associated with the loss of suppressive function, and that intranasal IL-33 administration to tolerized mice (during or after sensitization) caused allergen-induced airway inflammation.

In conclusion, psychological stress exposure inhibited the development of respiratory tolerance by GC-dependent suppression of tolerogenic DC and Treg cell induction, resulting in increased susceptibility to allergic asthma through Th2-biased immune sensitization. It is necessary to further explore the neuroendocrine pathway linking stress exposure to GC release, including neuropeptides such as opioids involved in stress-related asthma exacerbations [55, 56], as well as the endocrine-immune pathway, especially the GC-airway epithelium interaction leading to Th2-dominant immune responses.

Acknowledgements

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Statement of Ethics

Studies on animal subjects were approved by the Committee of Animal Experiments at Tohoku Medical and Pharmaceutical University (approval numbers: 14002-cn, 15001-cn, and 16002-cn). We took the utmost care to alleviate any pain and suffering in the mice.

Disclosure Statement

The authors have no conflicts of interest to declare.

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Author Contributions

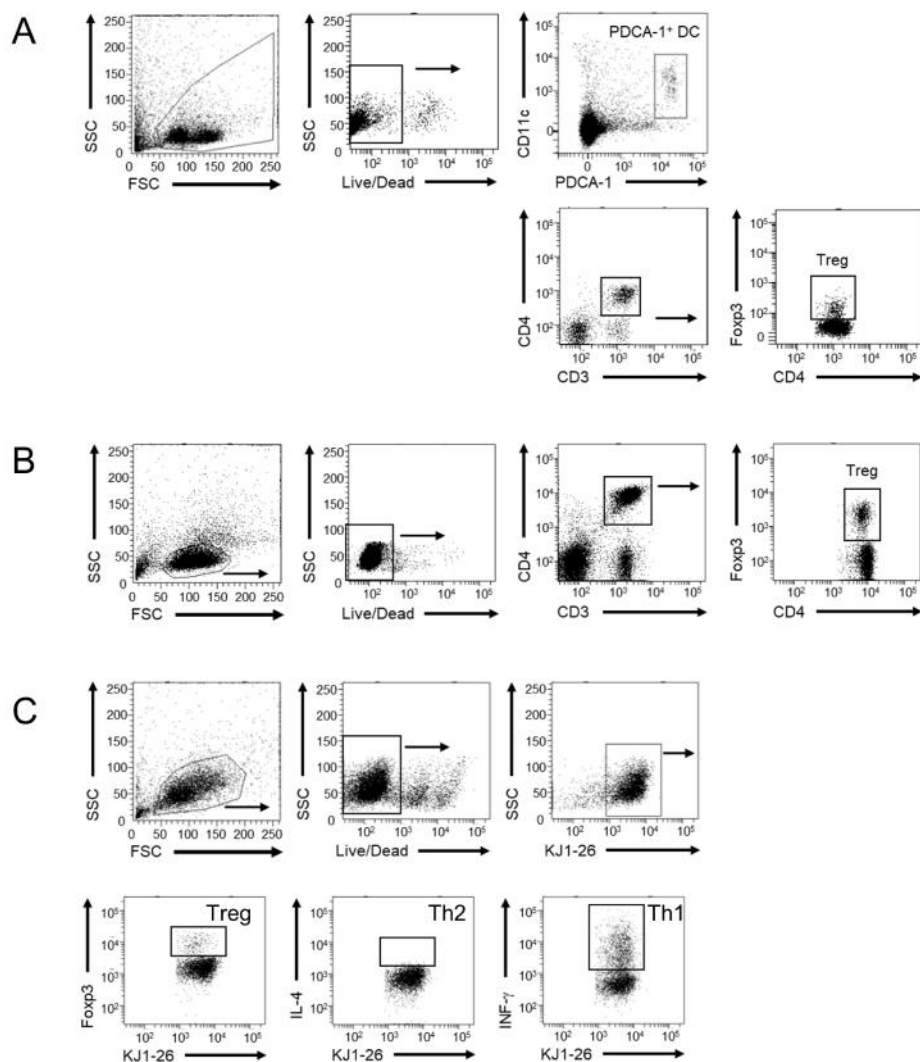
T.K. and I.O. designed the study. T.K., R.O., and T.I. performed the majority of experiments. C.M. contributed to the flow cytometric analysis of lung cells and lymph node cells. T.M. contributed to the analysis of histology. Y.O. contributed to the analysis of AHR. N.O., T.T., and M.T. suggested key experiments and edited the manuscript. T.K. and I.O. wrote the manuscript.

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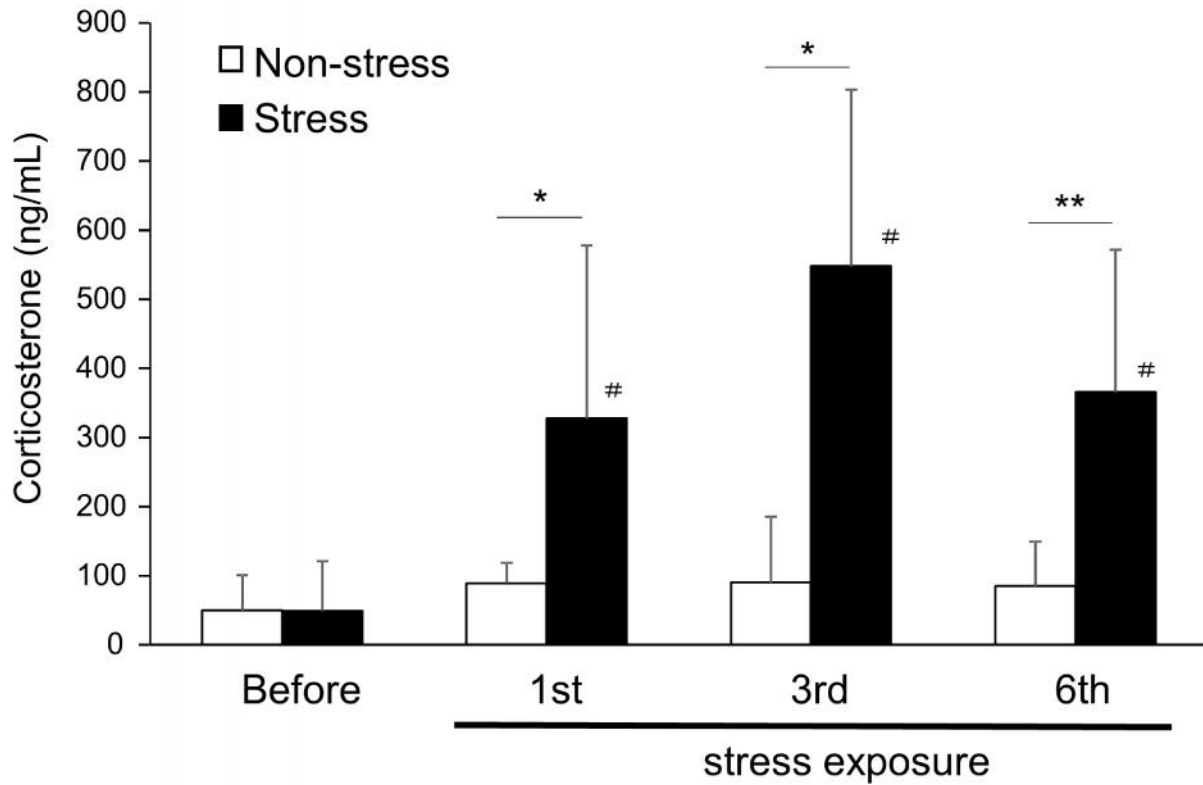
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Supplemental Figure 1



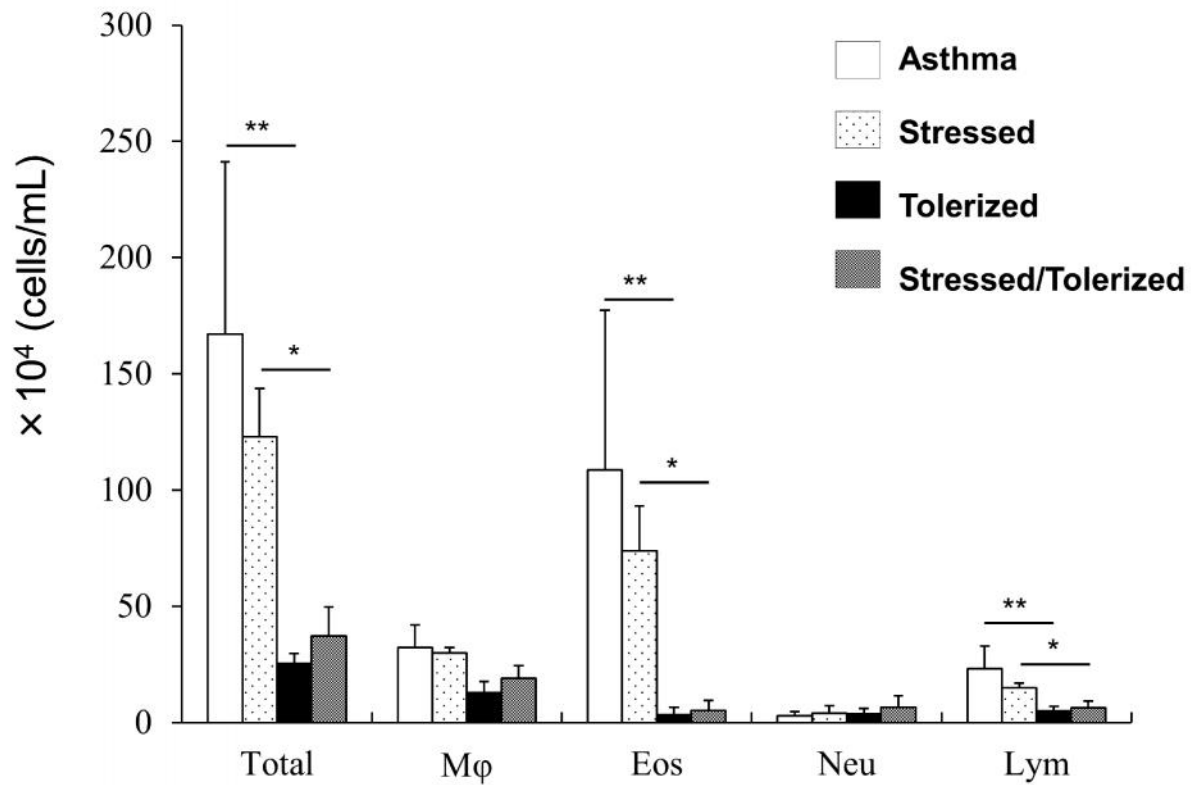
Supplemental Figure. 1. Gating strategy for DCs and T cells shown in **Figure 4 and 5 (A)**, **Figure 6 (B)** and Supplemental Figure 4 (C).

Supplemental Figure 2



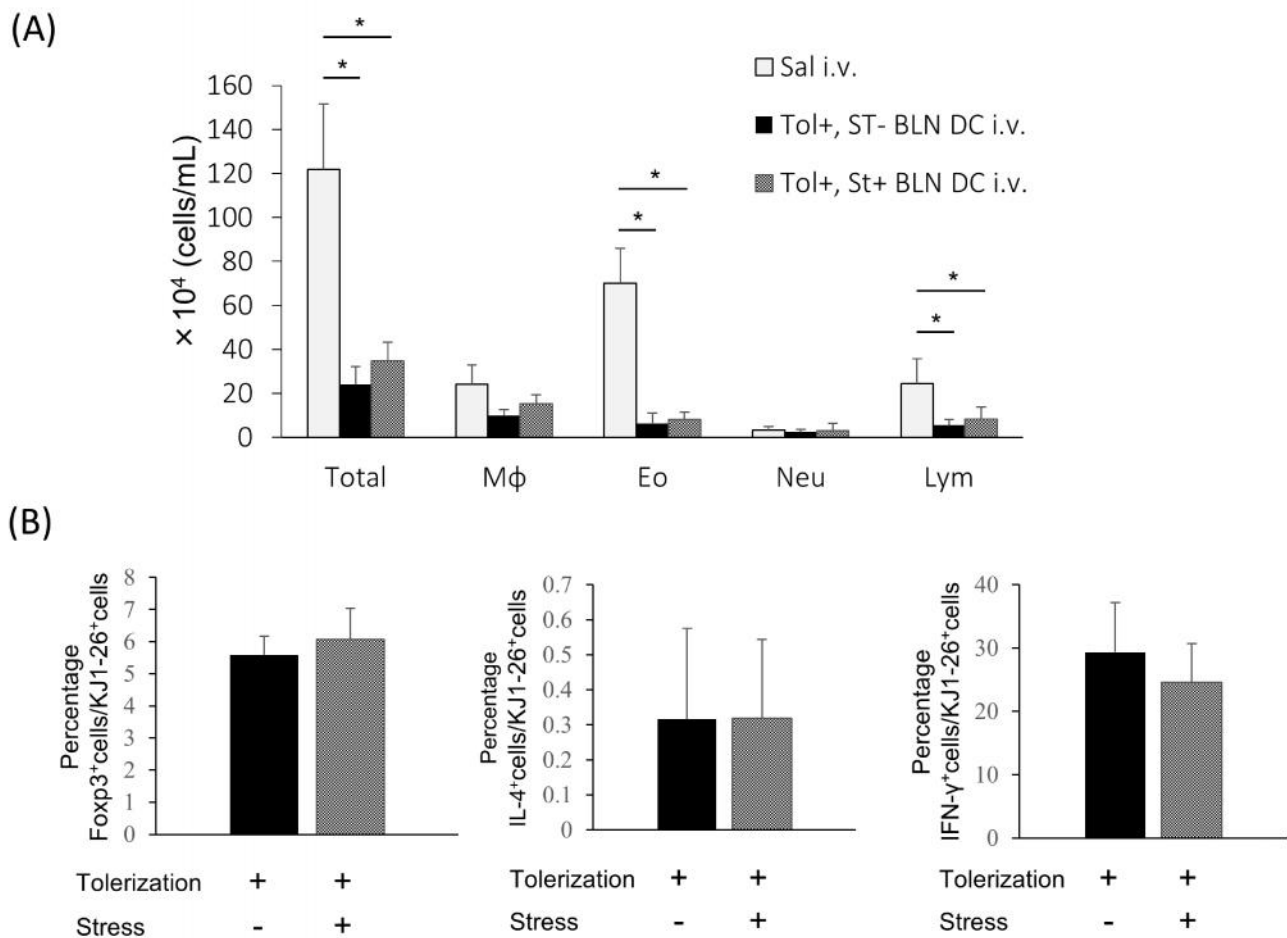
Supplemental Figure. 2. Effects of stress on the plasma corticosterone concentration. Blood was collected from stressed and non-stressed mice before the 1st and after the 1st, 3rd, and 6th stress exposures, and plasma corticosterone levels were measured in enzyme immunoassays. Data are expressed as the mean $\pm$ SD ($n = 3\text{--}11$ from 4 independent experiments). [#] $P < 0.05$ compared to values before stress exposure. $*P < 0.05$ and $**P < 0.01$, compared to non-stressed mice on the same day.

Supplemental Figure 3



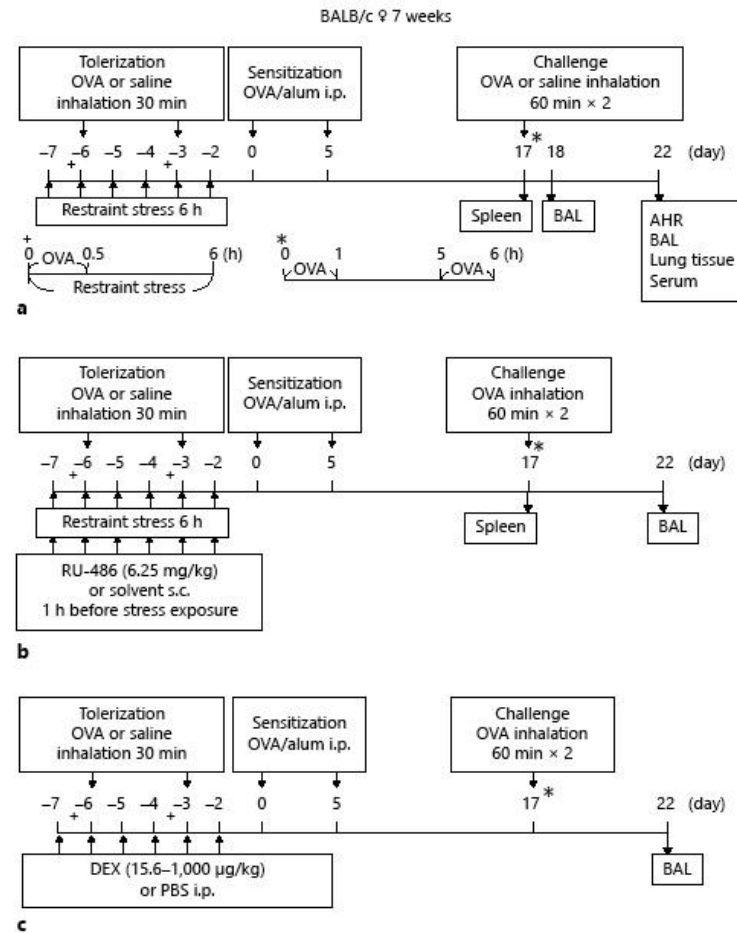
Supplemental Figure. 3. Effects of stress during the sensitization phase on allergic airway inflammation. BALF was obtained from mice of each treatment group 5 days after the last OVA challenge (day 22). Mice were exposed to stress for 6 consecutive days (days 0–5) during sensitization. Total cells (Total), macrophages (MΦ), eosinophils (Eo), neutrophils (Neu) and lymphocytes (Lym) were counted. Data are expressed as the mean $\pm$ SD ($n = 4\text{--}5$ from 2 independent experiments). * $P < 0.05$ and ** $P < 0.01$.

Supplemental Figure 4

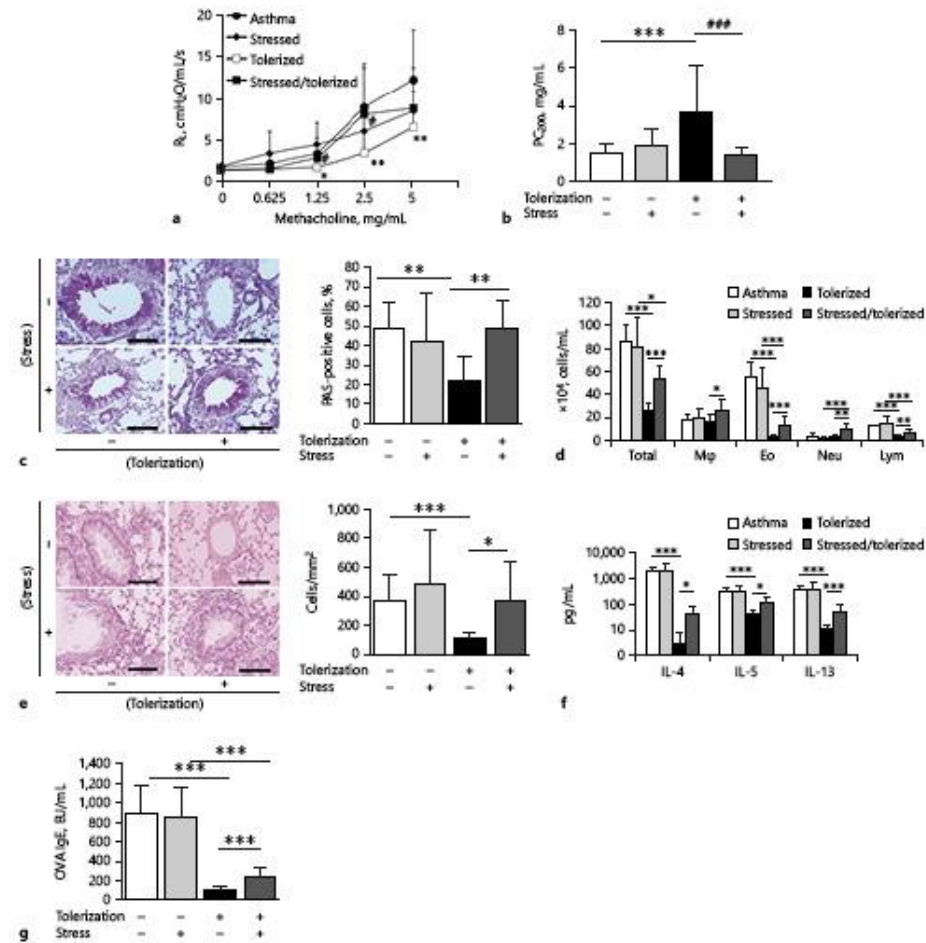


Supplemental Figure. 4. Adoptive transfer of CD11c⁺ DCs from BLN (A). CD11c⁺ DCs were isolated from BLNs of tolerized mice with or without stress exposure (day -2). CD11c⁺ DCs were intravenously injected into naïve BALB/c mice. Saline (Sal) was injected as a control. After injection, all mice were sensitized with OVA/alum and challenged with OVA. BALFs were obtained from mice of each treatment group 5 days after the last OVA challenge (day 22). Total cells (Total), macrophages (Mφ), eosinophils (Eo), neutrophils (Neu), and lymphocytes (Lym) were counted. Data are expressed as the mean ± SD (n = 4–5 from 2 independent experiments). Coculture of DCs from BLN and DO11.10 CD4⁺ T cells (B).

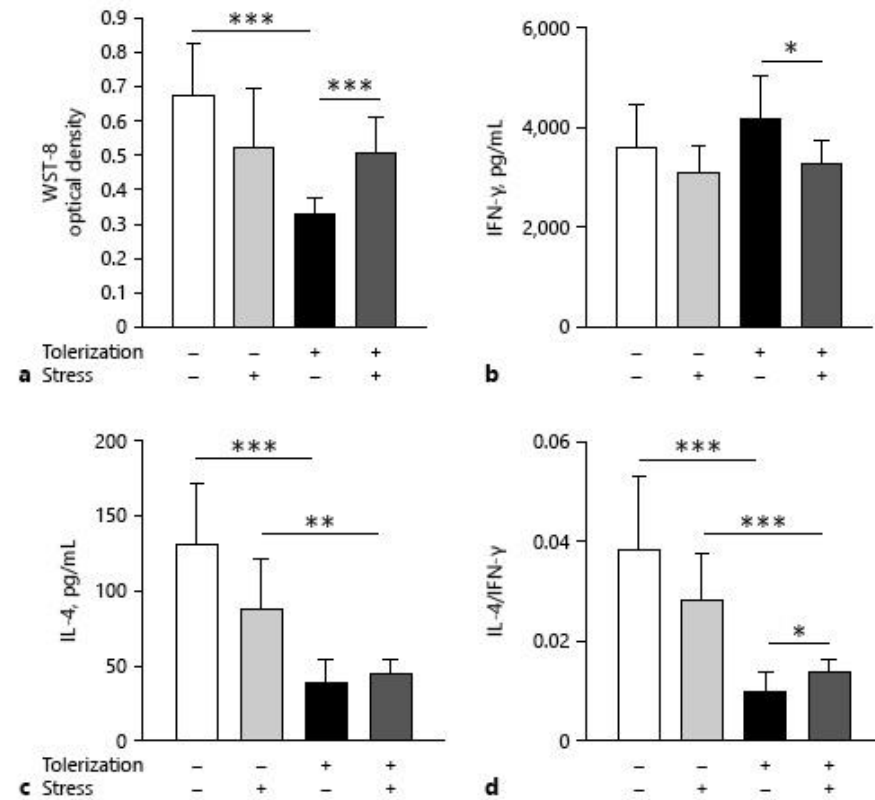
CD11c⁺ DCs were isolated from BLNs of tolerized mice, with or without stress exposure (day -2). CD4⁺ T cells were purified from naive DO11.10 (Rag2^{-/-}) mice and cultured with DCs in the presence or absence of OVA (100 µg/ml) for 72 h. In the last 4 h of the 72-h incubation, the cocultured DCs and CD4⁺ T cells in all treatment groups were stimulated with PMA (5 ng/ml), ionomycin (500 ng/ml), and monensin (2 µM). Subsequently, cells were collected and analyzed by flow cytometry. The percentage of gated KJ1-26⁺ cells was determined. Data are expressed as the mean ± SD (n = 4 from 2 independent experiments).



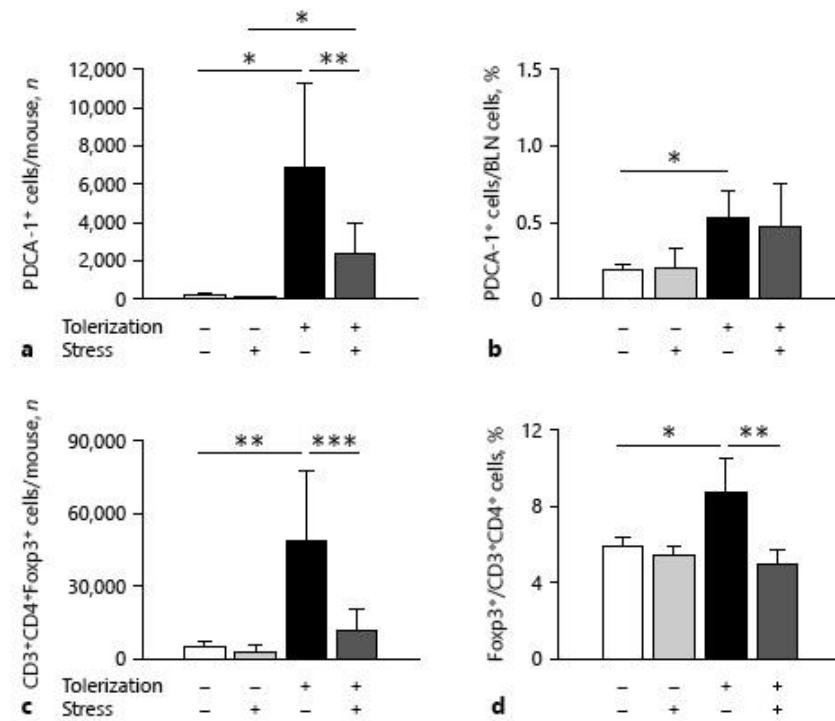
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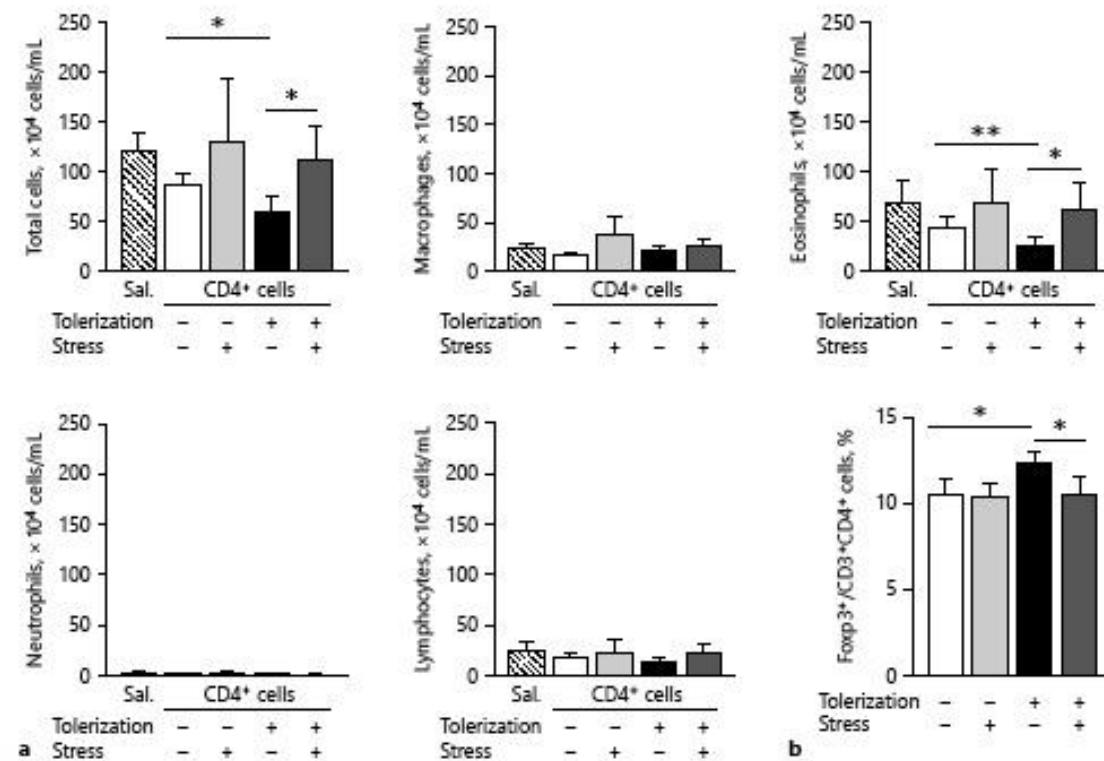
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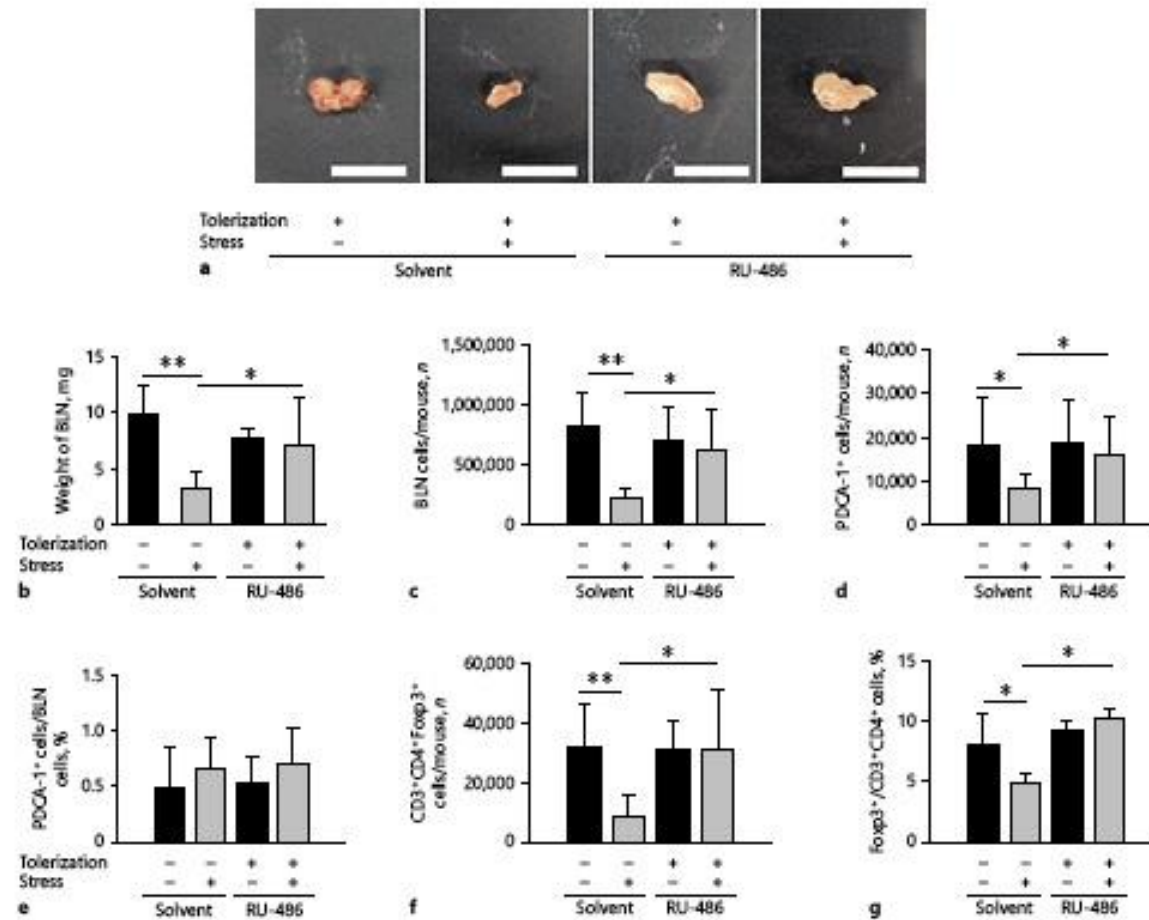
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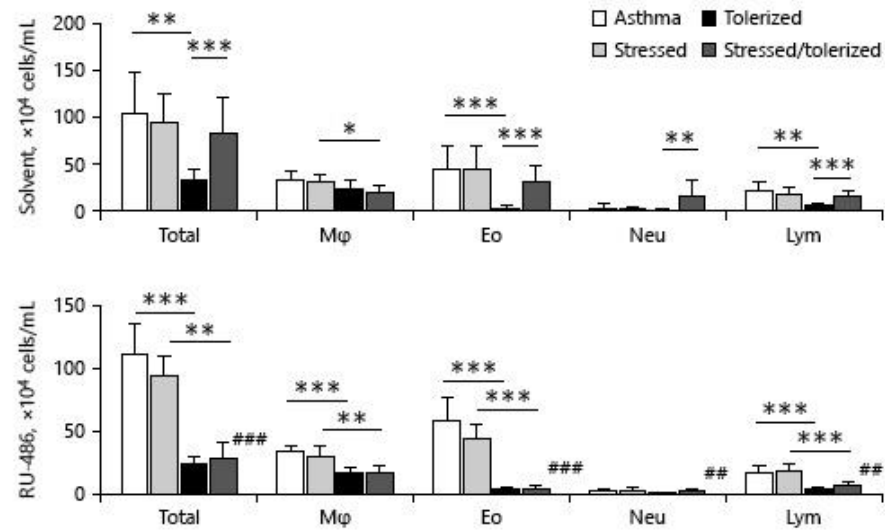
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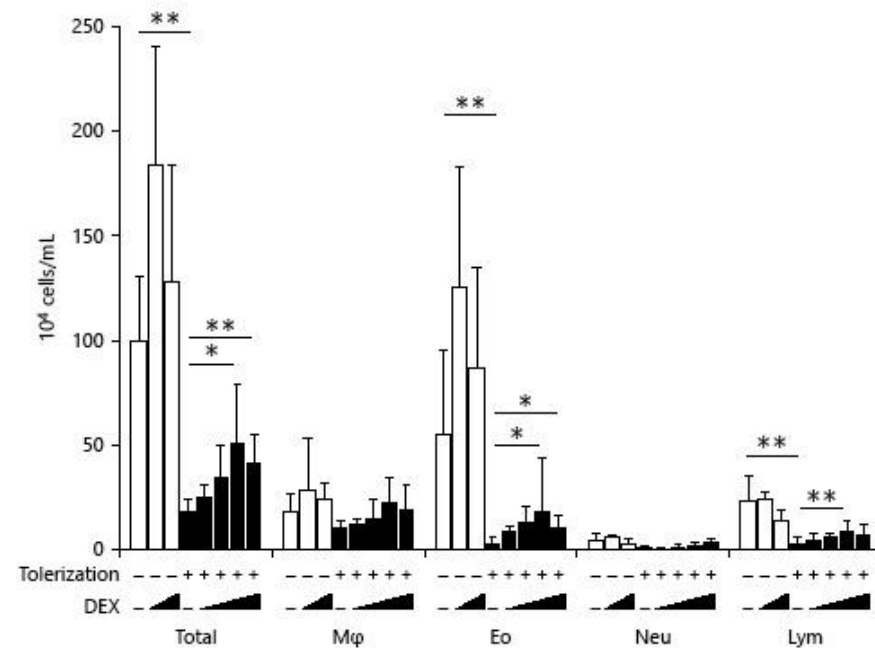
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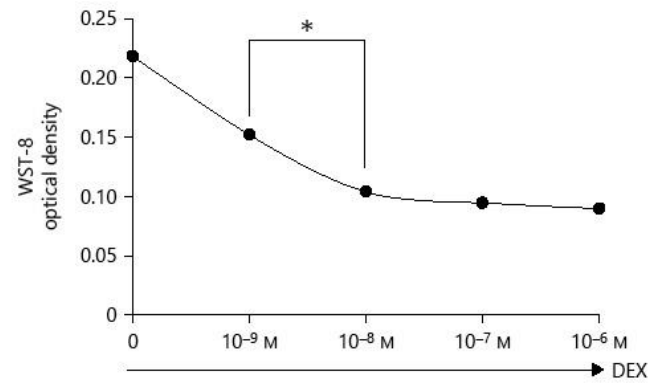
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