Requirement for neuropeptide Y in the development of type-2 responses and allergen-induced airway hyperresponsiveness and inflammation

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Abbreviations

AHR:	Airway hyperresponsiveness
APC:	Antigen-presenting cell
BAL:	Bronchoalveolar lavage
DC:	Dendritic cell
HE:	Hematoxylin and eosin
HDM:	House dust mite
IFN:	Interferon
IL:	Interleukin
ILC2:	Group 2 innate lymphoid cell
Mch:	Methacholine
MLN:	Mediastinal lymph node
MNC:	Mononuclear cell
NIH:	National Institutes of Health
NPY:	Neuropeptide Y
OVA:	Ovalbumin
PAS:	Periodic acid-Schiff
RL:	Lung resistance
Th1:	T helper type 1
Th2:	T helper type 2
TSLP:	Thymic stromal lymphopoietin

ABSTRACT

Neuropeptide Y (NPY) is a neurotransmitter that is widely expressed in the brain and peripheral nervous system. Various immune cells express the NPY Y1 receptor. NPY modulates these cells via its Y1 receptor; however, involvement of NPY in the pathophysiology of bronchial asthma, particularly airway hyperresponsiveness (AHR), has not been defined. NPY-deficient and wild-type mice were intranasally sensitized and challenged to house dust mite (HDM) extract, and airway responses were monitored. After sensitization and challenge, NPY-deficient mice showed significantly lower AHR than wild-type mice, and numbers of eosinophils and levels of type-2 cytokines [interleukin (IL)-4, IL-5, and IL-13] in bronchoalveolar lavage fluid were significantly lower. Type-2 cytokine production from splenic mononuclear cells of HDM-sensitized mice was also significantly lower in NPY-deficient mice. Flow cytometry analysis showed that the numbers of CD4 T cells and CD11c+ antigen–presenting cells (APCs) were significantly lower in the lungs of NPY-deficient mice than in wild-type mice following sensitization and challenge. Significantly fewer CD11c+ APCs phagocytosed HDM in the mediastinal lymph nodes of NPY-deficient mice than in those of wild-type mice. Treatment with BIBO 3304, a NPY receptor antagonist, significantly suppressed development of HDM-induced AHR and inflammation in wild-type mice. These data identify an important contribution of NPY to allergen-induced AHR and inflammation through accumulation of dendritic cells in the airway and promotion of the type-2 immune response. Thus, manipulating NPY represents a novel therapeutic target to control allergic airway responses.

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INTRODUCTIION

Bronchial asthma is characterized by airway inflammation and airway hyperresponsiveness (AHR). Airway inflammation results from the accumulation of activated eosinophils and T cells at the site of inflammation. T cells, particularly T helper (Th) type-2 cells, which release interleukin (IL)-4, IL-5, and IL-13, play pivotal roles in the development of allergic airway inflammation and AHR (1, 2, 21). Current management based on inhaled corticosteroids and long-acting β2-adrenergic agonists is effective in controlling bronchial asthma in most patients. However, 5–10% of patients with asthma respond poorly to high doses of inhaled corticosteroid and/or systemic corticosteroid and develop prolonged inflammatory cell infiltration in the airways (15). In such patients with so-called "severe asthma" or "refractory asthma," uncontrolled and frequently exacerbated asthmatic symptoms greatly impair quality of life and have a considerable impact on healthcare costs (17). Therefore, development of an effective and novel pharmacotherapy is warranted.

Neuropeptide Y (NPY) is a 36-amino-acid peptide neurotransmitter that is widely expressed in the brain. NPY regulates a broad range of functions, such as feeding, anxiety, memory, and circadian rhythms. NPY is released from peripheral sympathetic nerves and is important in the regulation of blood pressure and energy homeostasis (23). Immunohistochemistry of lung biopsies has shown that lung tissue is highly innervated with NPY-positive nerve fibers entering the bronchus-associated lymphoid tissue, the branches of the pulmonary artery (13), and the respiratory tract (29). During sympathetic stimulation, NPY is co-released with norepinephrine in the lymph nodes close to immune cells (9, 38). The Y-1 receptor, an NPY receptor, is expressed on various immune cells, such as B cells, CD4 and CD8 T cells,

macrophages, dendritic cells (DCs), natural killer cells, and mast cells (36), and NPY acts on these cells via its Y1 receptor (36). NPY enhances IL-4 production, inhibits interferon (IFN)-gamma production by Th cells (18), and increases migration of immature DCs derived from human peripheral blood, which promotes Th2 differentiation (3). Thus, NPY is a potent immunomodulator that skews the immune profile toward type-2 immunity.

Serum NPY levels increase in asthmatic patients (6, 7), and NPY polymorphisms are associated with an increased risk for asthma in overweight subjects (16) and young adults (25). Ovalbumin (OVA)-induced eosinophilic airway inflammation was reported to be lower in NPY- or Y-1-deficient mice compared to wild-type mice (26). Thus, NPY might be involved in eosinophilic airway inflammation. However, the role of NPY in the pathophysiology of bronchial asthma has not been well defined. In particular, the AHR, the most important phenotype of asthma, airway responses induced by a protease allergen such as house dust mite (HDM) extract, and the mechanisms of how NPY contributes to allergic airway responses have not been elucidated.

In this study, we investigated the role of NPY in allergen-induced AHR and inflammation in HDM-sensitized and -challenged mice. We assessed NPY-deficient (NPY-/-) mice and the effects of treatment with an NPY receptor antagonist, and showed that both approaches attenuated development of AHR, airway inflammation, and the accumulation of CD11c+ antigen-presenting cells (APCs) in the airway. Thus, manipulating NPY may be beneficial for controlling asthmatic responses.

MATERIALS AND METHODS

Animals.

NPY-/- mice (129 background) were purchased from Charles River Laboratories (Yokohama, Japan). The NPY-/- mice were then backcrossed to C57BL/6J mice (Charles River) for nine generations. Eight-to-ten-week-old female NPY-/- mice and C57BL/6J (NPY+/+ mice) were used in all experiments. NPY-/- mice were viable and displayed normal reproductive fitness without a striking phenotype. No spontaneous disease was observed in the NPY-/- mice up to 6 months of age, when they were housed under specific pathogen-free conditions. All experiments were performed in accordance with the National Institutes of Health (NIH) guidelines. All procedures were conducted under a protocol approved by the institutional animal care and use committee of Okayama University (Okayama, Japan).

Experimental protocol (sensitization and airway challenge).

The HDM-induced airway-inflammation mouse model was prepared with reference to a previous report (12). NPY-/- and NPY+/+ mice were sensitized with 15 μg of HDM extract (Greer Laboratories, Lenoir, NC, USA) in 30 μL of PBS by intranasal instillation on days 0 to 2. The mice were subsequently challenged with 5 μg of HDM extract in 30 μL of PBS by intranasal instillation on days 14 to 17. AHR was measured as described below at 24 h after the last challenge, and samples were collected for further analyses.

Administration of the Y1 receptor antagonist.

The Y1 receptor antagonist BIBO3304 ((R)-N-[[4-(aminocarbonylaminomethyl)-phenyl] methyl]-N2-(diphenylacetyl)-argininamide trifluoroacetate) (Santa Cruz Biotechnology, Inc., Dallas, TX, USA) or vehicle was administered by intraperitoneal injection at a rate of 0.1 mg/kg or 1 mg/kg in 200 μL of PBS once per day, from day 13 to 17.

Determination of airway responsiveness.

Airway responsiveness was assessed by measuring changes in lung resistance in response to increasing doses of inhaled methacholine (10) using a FlexiVent^{TM} small-animal ventilator (SCIREQ, Montreal, PQ, Canada). Before testing, the mice were anesthetized by an intraperitoneal injection of ketamine (80 mg/kg) and xylazine (10 mg/kg), tracheostomized, and mechanically ventilated. No significant differences in baseline values were observed among the different groups.

Bronchoalveolar lavage.

Immediately after assessing airway function, the lungs were lavaged with Hanks' balanced salt solution (2 × 1 mL, 37°C) via a tracheal tube. The volume of bronchoalveolar lavage (BAL) fluid collected was measured in each sample, and the numbers of cells in the BAL fluid were counted. Cytospin slides were stained with May–Giemsa stain and differentiated in a blinded fashion by counting at least 200 cells under a light microscope (33).

Lung histology.

The lungs were fixed in 10% formalin, cut around the main bronchus, and embedded in paraffin blocks. The slides were stained with hematoxylin-eosin and periodic acid–Schiff (PAS) to identify mucus-containing cells under a light microscope. The numbers of mucus-containing cells (goblet cells) were counted in more than 10 bronchioles in 10 high-power fields per animal by measuring the length of the epithelium defined along the basement membrane and luminal area using the NIH Image Analysis system (14).

Lung homogenates.

Lung tissues were frozen at −80°C immediately after euthanasia. The lung tissues were mixed with a PBS-0.1% Triton-X100 solution containing proteinase inhibitors at a 1:2.5 ratio (w:v) (Sigma-Aldrich, St. Louis, MO, USA). The specimens were homogenized and then centrifuged at 14,000 rpm for 30 min. The supernatants were used to analyze cytokine levels by enzyme-linked immunosorbent assay (ELISA), as described below (20).

Culture of splenic mononuclear cells.

The spleens of HDM-sensitized mice were removed and placed in PBS. The cells were dispersed, and mononuclear cells (MNCs) were separated by density gradient cell centrifugation using Histopaque (Sigma-Aldrich). The cells were washed, counted, and resuspended to a fixed concentration in RPMI-1640 (Wako Pure Chemical Industries, Osaka, Japan) containing heat-inactivated 10% fetal calf serum (FCS) and penicillin/streptomycin. The cells (4×10^5) were plated in each well of a 96-well round-bottom plate and cultured at 37°C in a 5% CO_2 atmosphere in the presence or absence of

10 mg/mL HDM extract. The supernatants were removed at 48 h after the last challenge, and cytokine levels were analyzed by ELISA as described below (19).

Measurement of cytokines and chemokines.

Cytokine levels in the BAL fluid were measured using ELISA. All cytokine and chemokine ELISAs were performed according to the manufacturers' directions. The limits of detection were 2 pg/mL for IL-4, 7 pg/mL for IL-5, 1.5 pg/mL for IL-13, 2 pg/mL for IFN-gamma, 5 pg/mL for IL-17A, 2.8 pg/mL for IL-33, 0.71 pg/mL for thymic stromal lymphopoietin (TSLP), and 0.01 ng/mL for NPY. All kits, except that for NPY (EMD Millipore Corp., Billerica, MA, USA), were purchased from R&D Systems (Minneapolis, MN, USA). Lung homogenates were prepared as described previously (20).

Lung cell isolation.

Lungs of HDM-sensitized and -challenged mice were separated from the associated lymph nodes, removed, and placed in PBS containing 10% heat-inactivated FCS. The lung tissues were minced and incubated for 1 h at 37°C in 5 ml PBS containing 0.05% collagenase I (Sigma-Aldrich). The lung tissues were dispersed by passing through a 20-G needle several times, and the suspensions were strained through a cell strainer. The pulmonary MNCs were isolated by density-gradient cell centrifugation over Histopaque (Sigma-Aldrich) (11).

Flow cytometry.

The cells were incubated with antigen-presenting cell (APC)-conjugated anti-CD3, phycoerythrin (PE)-conjugated anti-CD8, FITC-conjugated anti-CD4, APC-conjugated anti-CD11b, and PE-conjugated anti-CD11c antibodies (BD Biosciences, San Diego, CA, USA), and then analyzed by flow cytometry using a MACSQuant Analyzer (Miltenyi Biotec, Bergisch Gladbach, Germany).

Analyses of group 2 innate lymphoid cells (ILC2s).

The cells isolated from digested lungs were stained with biotin-conjugated antibody mixtures for lineage markers (CD4, CD5, CD8, CD11c, CD11b, CD19, NK1.1, Gr-1, TER119, FcεRI, and B220), Pacific blue-conjugated anti-Sca-1, PECy7-conjugated c-Kit (CD117), APC-conjugated anti-IL-7R (CD127), FITC-conjugated anti-T1/ST2, APC-Cy7-conjugated anti-CD25, and PE-conjugated anti-streptavidin, and analyzed using the MACSQuant Analyzer. Lin^{-}Scac-KitIL-7RCD25$ST2^{dim}$ cells were identified as lung ILC2s (31). The data were analyzed using FlowJo software (TreeStar, Ashland, OR, USA). APC-Cy7-conjugated anti-CD25; Pacific blue-conjugated anti-Sca-1; biotin-conjugated anti-CD4, anti-CD5, anti-CD8, anti-CD11b, anti-NK1.1, anti-Gr-1, anti-TER119, and anti-B220; and PE-conjugated anti-streptavidin were obtained from BD Biosciences. FITC-conjugated anti-T1/ST2 was obtained from MD Bioscience (St Paul, MN, USA). APC-conjugated anti-IL-7R and biotin-conjugated anti-FcεRI were obtained from BioLegend (San Diego, CA, USA). PECy7-conjugated c-Kit was purchased from eBioscience (La Jolla, CA, USA). Biotin-conjugated anti-CD11c and anti-CD19 were obtained from TONBO Biosciences (San Diego, CA, USA).

DC migration assay.

DC migration from the lungs to the mediastinal lymph nodes (MLNs) was analyzed. The HDM extract was labeled with a DyLight 405 Microscale Protein Labeling kit (Thermo Scientific, Waltham, MA, USA). NPY-/- mice and NPY+/+ mice were sensitized with unlabeled HDM extract on days 0–2 and were challenged with labeled HDM extract on days 14–17. The cells were harvested from the MLNs at 24 h after the challenge and analyzed by flow cytometry.

Immunohistochemistry for NPY

Immunohistochemistry was performed on paraffin sections using an automated Bond Max stainer (Leica Biosystems, Melbourne, Australia) with mouse monoclonal anti-NPY antibody (ab112373, dilution 1:1,000; Abcam, Cambridge, UK) as the primary antibody. NPY-positive cells were examined under light microscopy (final magnification: ×400). Evaluation of immunostaining was performed by an expert pathologist (Y.G.).

Statistical analysis.

All results are expressed as mean ± standard error. Analysis of variance was used to determine differences between the groups. Pairs of samples distributed parametrically were compared using the unpaired two-tailed Student's *t*-test, and samples distributed nonparametrically were compared using the Mann–Whitney *U*-test. *P*-values < 0.05 were considered significant.

RESULTS

AHR and allergic airway inflammation decrease in NPY-/- mice

AHR was monitored at 24 h after the last HDM challenge in NPY+/+ and NPY-/- mice. Sensitization and challenge by intranasal administration of HDM extract increased AHR in NPY+/+ mice, as shown by a significant increase in lung resistance compared to that in non-sensitized and non-challenged mice (Fig. 1A). By contrast, sensitized and challenged NPY-/- mice developed less of an increase in lung resistance compared to sensitized and challenged NPY+/+ mice, but, nonetheless, the changes were significantly greater than in NPY-/- mice that were non-sensitized and non-challenged.

We assessed BAL fluid following sensitization and HDM challenge. Eosinophils increased significantly in sensitized and challenged mice, compared to non-sensitized and non-challenged mice. However, the numbers of eosinophils were significantly lower in the BAL fluid of NPY-/- mice than in that of NPY+/+ mice (Fig. 1B). The numbers of PAS-positive goblet cells were also significantly lower in NPY-/- mice compared to numbers in NPY+/+ mice following sensitization and HDM challenge (Fig. 1C, D).

Airway cytokine levels

Sensitization and challenge with HDM extract resulted in significant increases in IL-4, IL-5, and IL-13 levels in NPY+/+ mice. By contrast, NPY-/- mice had significantly lower levels of IL-4, IL-5, and IL-13 following sensitization and challenge

(Fig. 2A–C). Levels of IFN-gamma and IL-17A did not differ between NPY-/- mice and NPY+/+ mice (Fig. 2D, E).

Splenic MNCs from NPY-/- mice release lower levels of Th2 cytokines

To determine whether the attenuated Th2 cytokine secretion observed in NPY-/- mice was due to impaired Th2 cytokine production, we assessed cytokine production in splenic MNCs *in vitro*. The levels of IL-5, IL-13, and IFN-gamma from HDM re-stimulated splenic MNCs in NPY-/- mice were significantly lower than in those of NPY+/+ mice (Fig. 2G–I). No significant differences were observed in the IL-4 levels of the two strains of mice, although a lower trend was observed in NPY-/- mice (Fig. 2F). These data imply that NPY contributes to systemic sensitization of Th2 cells.

The numbers of CD4 T cells and CD11c+ APCs in the lungs decrease in NPY-/- mice

To determine whether the accumulation of T cells and CD11c+ APCs in the airways of sensitized and challenged mice was affected by NPY expression, we assessed the numbers of T cells and CD11c+ cells in the lungs. Numbers of CD4 T cells and CD11c+ APCs were significantly lower in NPY-/- mice than in NPY+/+ mice following sensitization and challenge (Fig. 3A, C); however, numbers of CD8+ T cells in the two strains of mice did not differ (Fig. 3B).

Migration of CD11c+ APCs from lungs to the MLNs is attenuated in NPY-/- mice

NPY induces migration of human DCs (3); therefore, we investigated migration of DCs in this model. The numbers of violet+ and CD11c+ APCs that phagocyted

labeled-HDM in the MLNs of NPY-/- mice were significantly lower in sensitized and labeled-HDM challenged mice than in NPY+/+ mice (Fig. 4B).

These data indicate that NPY plays a crucial role in the migration of DCs to regional lymph nodes, mediating type-2 immune responses, and eliciting allergic airway responses.

Numbers of ILC2s and IL-33 levels in the lungs

Numbers of ILC2s and IL-33 levels increased significantly in sensitized and challenged mice compared to non-sensitized and non-challenged mice. However, there were no differences between NPY+/+ and NPY-/- mice (Fig. 5A–C).

NPY expression in lung tissue

NPY expression in NPY+/+ mice was evaluated by immunohistochemistry 24 hours after the last challenge (Fig.5D). NPY expression was found mainly in alveolar walls, vascular endothelial cells, and some of the inflammatory cells including mononuclear cells and granulocytes around the bronchus of HDM-sensitized and -challenged NPY+/+ mice (Fig. 5D c-e), whereas relatively few NPY+ cells were detected in non-sensitized and non-challenged NPY+/+ mice (Fig. 5D a, b).

The Y1 receptor antagonist suppresses AHR, allergic airway inflammation, and cytokine levels in the lungs

We assessed the AHR of HDM-sensitized and -challenged mice treated with vehicle, low-dose Y1 receptor antagonist (0.1 mg/kg/day), and high-dose Y1 receptor antagonist (1 mg/kg/day) at 24 h after the last HDM challenge. The mice treated with

vehicle developed AHR more frequently than did the non-sensitized and non-challenged mice. Administering the high-dose Y1 receptor antagonist significantly attenuated the increase in AHR compared to the vehicle-treated mice following sensitization and challenge (Fig. 6A).

The numbers of inflammatory cells in BAL fluid were assessed in vehicle and Y1 receptor-antagonist–treated mice. The numbers of total cells, lymphocytes, and eosinophils were significantly lower in the BAL fluid of high-dose Y1 receptor-antagonist–treated mice that were sensitized and challenged compared to numbers in vehicle-treated mice (Fig. 6B). The numbers of PAS-positive goblet cells were significantly lower in high-dose Y1 receptor antagonist–treated mice than in the vehicle-treated mice following sensitization and challenge with HDM extract (Fig. 6C, D).

We then measured cytokine levels in BAL fluid using ELISA. Sensitization and challenge with HDM extract resulted in significant increases in IL-5 and IL-13 levels in the vehicle-treated mice. By contrast, mice treated with the high-dose Y1 receptor antagonist showed significantly lower levels of IL-5 and IL-13 following sensitization and challenge (Fig. 6E–G).

The Y1 receptor antagonist suppresses CD11c+ APCs in the lungs

To determine whether accumulation of immune cells in the airways of sensitized and challenged mice was affected by the Y1 receptor antagonist treatment, we assessed the numbers of T cells and CD11c+ APCs in the lungs. The numbers of CD11c+ APCs were significantly lower in the mice treated with the high-dose Y1 receptor antagonist than in the mice treated with vehicle following sensitization and challenge, although the numbers of CD4 T cells and CD8 T cells did not differ among

the groups (Fig. 7A–C). These data imply that the NPY-Y-1 axis contributes to migration of DCs to the airway and induces allergic airway responses.

DISCUSSION

In this study, we demonstrated that NPY contributes to both systemic sensitization and local activation of Th2 cells, as well as to the accumulation of CD11c+ APCs in the airways and migration of CD11c+ APCs to MLNs following sensitization and challenge with HDM extract. These data identify the important contribution of NPY to allergen-induced AHR and airway inflammation through migration of DCs to regional lymph nodes and promotion of the type-2 immune response. We also demonstrated for the first time that a Y1 receptor antagonist suppressed allergen-induced AHR and airway inflammation, which are important bronchial asthma phenotypes. Thus, manipulating NPY represents a novel therapeutic target to control allergic airway responses.

T cells, particularly Th2 cells that release IL-4, IL-5, and IL-13, play pivotal roles in the development of AHR and eosinophilic inflammation (1, 2, 21). Furthermore, DCs, representative of lung APCs, are critical for activating lung immune responses (30). In our study, the numbers of CD4 T cells in the lungs and the levels of Th2 cytokines in BAL fluid were significantly lower in NPY-/- mice than in NPY+/+ mice. The numbers of CD11c+ APCs, which are recognized as DCs in the lung, were also significantly lower in NPY-/- mice. Furthermore, the numbers of CD11c+ APCs, which phagocytosed fluorescently labeled HDM in the MLNs of NPY-/- mice, were significantly lower than those of NPY+/+ mice following sensitization and challenge. Buttari et al. reported that NPY induces dose-dependent migration of human monocyte-derived immature DCs by activating extracellular regulated kinase and p38 mitogen-activated protein kinases, and that this phenomenon was suppressed by a Y1 receptor antagonist (BIBP3226) (3). Wheway et al. (36) reported that activation of bone

marrow–derived Y1-/- DCs with lipopolysaccharide led to normal expression of activation markers; however, uptake of an antigen, such as OVA-FITC or FITC-dextran, by immature Y1-/- DCs decreased compared to that of Y1+/+ immature DCs as determined by a flow cytometry analysis. In our study, the numbers of CD11c+ APCs also decreased significantly in Y1 receptor-antagonist–treated mice compared to vehicle-treated mice. Thus, the NPY-Y1 axis plays a critical role in the function of DCs in the process of acquired immunity of HDM-induced airway inflammation.

T cells express the Y1 receptor; therefore, NPY can directly act on T cells. It has been reported that NPY enhances IL-4 production and inhibits IFN-gamma production by Th cells (18), and in the absence of any additional factors, directly induces marked secretion of cytokines (IL-2, IFN-gamma, IL-4, and IL-10) from T cells (22). According to these *in vitro* findings, NPY directly induces the cytokine secretion ability of Th cells, particularly Th2 cytokines. *Ex vivo* re-stimulation of MLN MNCs with OVA resulted in reduced levels of IL-5 and unchanged levels of IFN-gamma in NPY-/- mice after sensitization and challenge with OVA, compared to NPY+/+ mice (26). In their model, sensitization and challenge with OVA did not induce IFN-gamma production by T cells. By contrast, in our model, HDM sensitization induced the Th2 and Th1 subtypes. In our study, *ex vivo* re-stimulation of splenic MNCs with HDM extract resulted in lower production of IL-5, IL-13, and IFN-gamma in NPY-/- mice compared to that in NPY+/+ mice. *In vivo* sensitization and a deficit of NPY may reduce migration of DCs and differentiation of effector Th cells. DCs have been reported to affect the release of NPY and the activation of Y1 receptors (35, 36); therefore, NPY-/- DCs were considered to impair its function during *ex vivo* re-stimulation of splenic MNCs with HDM extract in our study.

ILC2s, a newly identified innate immune cell with the capacity for Th2 cytokine production in response to airway epithelial cell–derived IL-25, IL-33, and TSLP, have been reported to induce the innate immune response and enhance Th2 allergic inflammation (8, 28). ILC2s are associated with corticosteroid-resistant pathophysiology in patients with severe asthma (17). In our study, the levels of IL-33 and the numbers of ILC2s in the lung were analyzed; however, they were not lower in NPY-/- mice or Y1 receptor-antagonist–treated mice than in NPY+/+ mice or vehicle-treated mice. Thus, although Y1 and Y5 receptors are expressed on airway epithelial cells (27), the NPY-Y1 axis may not play a critical role in the secretion of innate cytokines by airway epithelial cells. By contrast, Wallrapp et al. (34) reported that ILC2s express the neuropeptide receptor Nmur1 in steady and activated states. Neuromedin U, which is a ligand of Nmur1, activates ILC2s *in vitro*, and *in vivo* co-administration of NMU with IL-25 strongly amplifies allergic inflammation (34). In our study, numbers of ILC2s did not differ between NPY-/- and NPY+/+ mice; however, the effector function of ILC2s was not fully investigated. Therefore, neuro-immune crosstalk in ILCs needs to be further investigated.

NPY receptors are G-protein-coupled receptors, and consist of at least five subtypes (Y1, Y2, Y4, Y5, and Y6) (23). NPY modulates the immune system, particularly via its Y1 receptor, and the Y1 receptor is expressed on various immune cells (36). BIBO3304 is an antagonist of the Y1 receptor, which suppresses NPY-induced food intake after an intraventricular injection (37). In our study, mice treated with a Y1 receptor antagonist did not show the side effect of reduced body weight. The migration rate of BIBO3304 to the central nervous system was low; therefore, food intake might not have been affected. Treatment with the Y1 receptor antagonist only

during the challenge phase suppressed AHR and airway inflammation in our study. In the *ex vivo* re-stimulation of splenic MNCs with HDM extract experiment, we showed the critical role of NPY in HDM sensitization. DCs present antigens to effector Th cells, and Th cells secrete type-2 cytokines in the airways during the challenge phase. Our data imply that the Y1 receptor antagonist inhibited those processes and was sufficient to suppress the asthmatic phenotype, indicating the possibility of a treatment for asthmatic patients.

Li et al. showed that increased expression of NPY in airway epithelium of forkhead box p1/p4–deficient mice induced an AHR phenotype in a paracrine manner with airway smooth muscle but without airway inflammation, and that NPY amplified methacholine-induced bronchoconstriction in vitro (24). Wu et al. showed that early postnatal exposure of mice to side-stream tobacco smoke increased the density of NPY nerve fibers in trachea smooth muscle and AHR (39). Thus, the bronchoconstricting action of NPY may worsen the asthmatic phenotype. In contrast, although NPY was reported to cause a contraction in isolated airways of guinea pigs, its bronchoconstricting action was very small, where less than 6% of responses were elicited by standard spasmogens (4, 32). In our study, methacholine-induced contractions in naive NPY-/- mice did not differ from that in naive NPY+/+ mice, and the systemic Y1 receptor antagonist treatment did not suppress methacholine-induced contractions in naive mice. Although NPY is released with norepinephrine by sympathetic nerve stimulation (9, 38), our study indicates that the role of NPY in cholinergic airway contraction is small under normal conditions. It has also been reported that in most mammalian species, including mice, there is little innervation of

airway smooth muscle by sympathetic fibers (5). Thus, the direct bronchoconstricting action of NPY itself on airway smooth muscle in vivo is controversial.

Repeated allergen challenges have been reported to increase the levels of NPY in BAL fluid (27), and we showed that NPY could increase AHR through activation of the immune response in airway inflammation. Interestingly, NPY was expressed in several cell types in the lung tissue following sensitization and challenge in our study. However, it remains unclear which cell type mainly secretes NPY for eliciting AHR. Further investigations are warranted.

In summary, we identified a critical role for NPY in the development of AHR, airway inflammation, accumulation of CD11c+ APCs in the airways, migration of CD11c+ APCs in MLNs, and activation of Th2 cells. Furthermore, we demonstrated that a Y1 receptor antagonist attenuated AHR and airway inflammation. Our data imply that controlling the NPY-Y1 axis will provide a novel interventional strategy for treating asthma.

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DISCLOSURES

No conflicts of interest, financial or otherwise are declared by the authors.

AUTHOR CONTRIBUTIONS

Conceived and designed research: NO, NM; Performed experiments: NO, AT, DM, SS, UF, JI, YG; Analyzed data: NO, NM, AT, JI, YG; Interpreted results of experiments: NO, NM, AT, DM, SS, UF, KK, AK, YM; Prepared figures: NO, NM, AT, YG; Drafted manuscript: NO, NM, AT; Approved final version of manuscript: NO, NM, AT, DM, SS, UF, JI, YG, KK, AK, YM.

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614

615 **FIGURE LEGENDS**

616 Figure 1. Neuropeptide Y (NPY)-/- mice develop reduced airway hyper-responsiveness
617 (AHR) and airway inflammation following sensitization and challenge. A: AHR in
618 NPY+/+ and NPY-/- mice after sensitization and challenge with HDM. 24 hours after
619 the last challenge, lung resistance was monitored in response to increasing
620 concentrations of inhaled methacholine, as described in MATERIALS AND
621 METHODS. Values are means±SE (n= 8–12 in each group). *P<0.05. B: cellular
622 composition in bronchoalveolar lavage fluid. Values are means±SE (n=8–12 in each
623 group). Significant differences (*P <0.05) vs NPY+/+ PBS and NPY-/- PBS mice.
624 #P<0.05 vs NPY+/+ HDM mice. C: development of goblet cell metaplasia in the
625 airways of NPY+/+ and NPY-/- mice. D: goblet cell metaplasia was quantified in
626 periodic acid Schiff (PAS)-stained sections, as described in MATERIALS AND
627 METHODS. Values are means±SE (n=5 in each group). Significant differences (*P
628 <0.05) vs NPY+/+ PBS and NPY-/- PBS mice. #P<0.05 vs NPY+/+ HDM mice. NPY,
629 neuropeptide Y; HDM, house dust mite; Mac, macrophage; Lym, lymphocyte; Neu,
630 neutrophil; Eos, eosinophil.

631

632 Figure 2. Cytokine levels in bronchoalveolar lavage (BAL) fluid and the lung and
633 cytokine production from splenic mononuclear cells. A-E: T helper type 2 (Th2)
634 cytokine levels [interleukin (IL)-4, IL-5 and IL-13] in BAL fluid, interferon (IFN)-
635 gamma and IL-17 levels in the lung were measured by ELISA, as described in
636 MATERIALS AND METHODS. Values are means±SE (n=8–12 in each group).

Significant differences (*P <0.05) vs NPY+/+ PBS and NPY-/- PBS mice. #P<0.05 vs NPY+/+ HDM mice. F-I: Th2 cytokine levels (IL-4, IL-5 and IL-13) and IFN-gamma levels in supernatants from spleen cultured in the presence or absence of HDM (10 mg/ml) determined by ELISA. Values are means±SE (n=3–4 in each group). *P<0.05. NPY, neuropeptide Y; HDM, house dust mite.

Figure 3. The numbers of T cells and CD11c+ antigen-presenting cells (APCs) in the lung following sensitization and challenge. A-B: the numbers of CD4 and CD8 T cells in the lung of NPY+/+ and NPY-/- mice after sensitization and challenge. Numbers of cells in the lung were determined as described in MATERIALS AND METHODS. Values are means±SE (n=6–7 in each group). Significant differences (*P <0.05) vs NPY+/+ PBS and NPY-/- PBS mice. #P<0.05 vs NPY+/+ HDM mice. C: the numbers of CD11c+ cells in the lungs of NPY+/+ and NPY-/- mice following sensitization and challenge. Values are means±SE (n=4–5 in each group). Significant differences (*P <0.05) vs NPY+/+ PBS and NPY-/- PBS mice. #P<0.05 vs NPY+/+ HDM mice. NPY, neuropeptide Y; HDM, house dust mite.

Figure 4. The numbers of CD11c+ violet+ cells in mediastinal lymph nodes (MLNs) in NPY+/+ and NPY-/- mice. The numbers of CD11c+violet+cells which phagocyted labeled-HDM in the lungs were counted by flowcytometry in MLN, as described in MATERIAL AND METHODS. Values are means±SE (n=5 in each group). *P <0.05. NPY, neuropeptide Y; HDM, house dust mite.

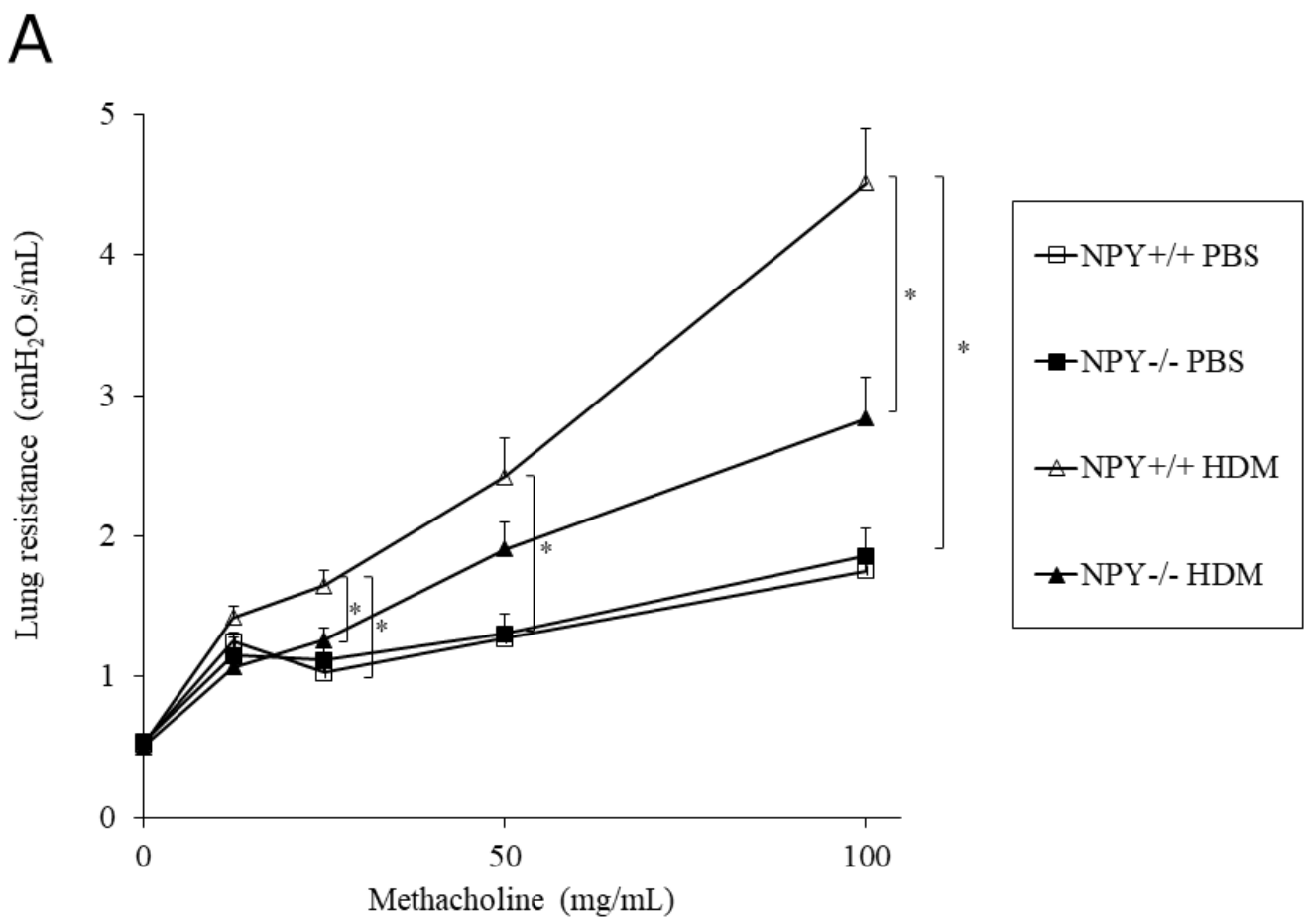
Figure 5. The numbers of group 2 innate lymphoid cells (ILC2s) and interleukin (IL)-33 and thymic stromal lymphopoietin (TSLP) levels in the lung following sensitization and challenge, and NPY expression in lung tissue. A: the numbers of ILC2s in the lung of NPY+/+ and NPY-/- mice after sensitization and challenge. Numbers of cells in the lung were determined as described in MATERIALS AND METHODS. Values are means±SE (n=4–5 in each group). Significant differences (*P <0.05) vs NPY+/+ PBS and NPY-/- PBS mice. There were no differences between NPY+/+ HDM and NPY-/- HDM mice. B-C: IL-33 and TSLP levels in the lung were measured by ELISA, as described in MATERIALS AND METHODS. Values are means±SE (n=8–12 in each group). Significant differences (*P <0.05) vs NPY+/+ PBS and NPY-/- PBS mice. There were no differences between NPY+/+ HDM and NPY-/- HDM mice. D: Immunohistochemical staining of NPY in non-sensitized and non-challenged NPY+/+ mice (a, b) and HDM-sensitized and -challenged NPY+/+ mice (c, d) with different magnifications (a and c: ×100, b and d: ×400). (e) NPY staining in HDM-sensitized and -challenged NPY+/+ mice (×400). Arrowheads: vascular endothelial cells; bold arrows: mononuclear cells; thin arrows: granulocytes. NPY expression was evaluated by immunohistochemistry 24 hours after the last challenge as described in Materials and Methods. NPY+ cells are indicated by brown staining. NPY, neuropeptide Y; HDM, house dust mite.

Figure 6. Treatment with the Y1 receptor antagonist (Y1Ri) suppresses airway hyper-responsiveness (AHR), airway inflammation, and T helper type-2 (Th2) cytokine levels in bronchoalveolar lavage (BAL) fluid following sensitization and challenge. A: AHR after sensitization and challenge with HDM. 24 hours after the last challenge, lung

684 resistance was monitored in response to increasing concentrations of inhaled
685 methacholine, as described in MATERIALS AND METHODS. Values are means±SE
686 (n=8–18 in each group). Significant differences (*P<0.05) vs PBS/vehicle and
687 PBS/Y1Ri 1mg/kg mice. #P<0.05 vs HDM/Y1Ri 0.1mg/kg and HDM/Y1Ri 1mg/kg
688 mice. B: cellular composition in bronchoalveolar lavage fluid. Values are means±SE
689 (n=8–18 in each group). Significant differences (*P <0.05) vs PBS/vehicle and
690 PBS/Y1Ri 1mg/kg mice. #P<0.05 vs HDM/vehicle mice. C: development of goblet cell
691 metaplasia in the airways. D: goblet cell metaplasia was quantified in periodic acid
692 Schiff (PAS)-stained sections, as described in MATERIALS AND METHODS. Values
693 are means±SE (n=6–7 in each group). Significant differences (*P <0.05) vs
694 PBS/vehicle and PBS/Y1Ri 1mg/kg mice. #P<0.05 vs HDM/vehicle mice. E-G: Th2
695 cytokine levels [interleukin (IL)-4, IL-5 and IL-13] in BAL fluid were measured by
696 ELISA, as described in MATERIALS AND METHODS. Values are means±SE (n=8–
697 18 in each group). Significant differences (*P <0.05) vs PBS/vehicle and PBS/Y1Ri
698 1mg/kg mice. #P<0.05 vs HDM/vehicle mice. HDM, house dust mite; Mac,
699 macrophage; Lym, lymphocyte; Neu, neutrophil; Eos, eosinophil.
700
701 Figure 7. The numbers of T cells and CD11c+ APCs in the lung following treatment
702 with the Y1 receptor antagonist (Y1Ri). A-B: the numbers of CD4 and CD8 T cells in
703 the lung after sensitization and challenge. Numbers of cells in the lung were determined
704 as described in MATERIALS AND METHODS. Values are means±SE (n=5–9 in each
705 group). Significant differences (*P <0.05) vs PBS/vehicle mice. There were no
706 differences between HDM/vehicle and HDM/Y1Ri 1mg/kg mice. C: the numbers of

CD11c+ cells in the lungs of NPY+/+ and NPY-/- mice following sensitization and challenge. Values are means±SE (n=9 in each group). Significant differences (*P <0.05) vs PBS/vehicle mice. #P<0.05 vs HDM/vehicle mice. NPY, neuropeptide Y; HDM, house dust mite.

Figure 1



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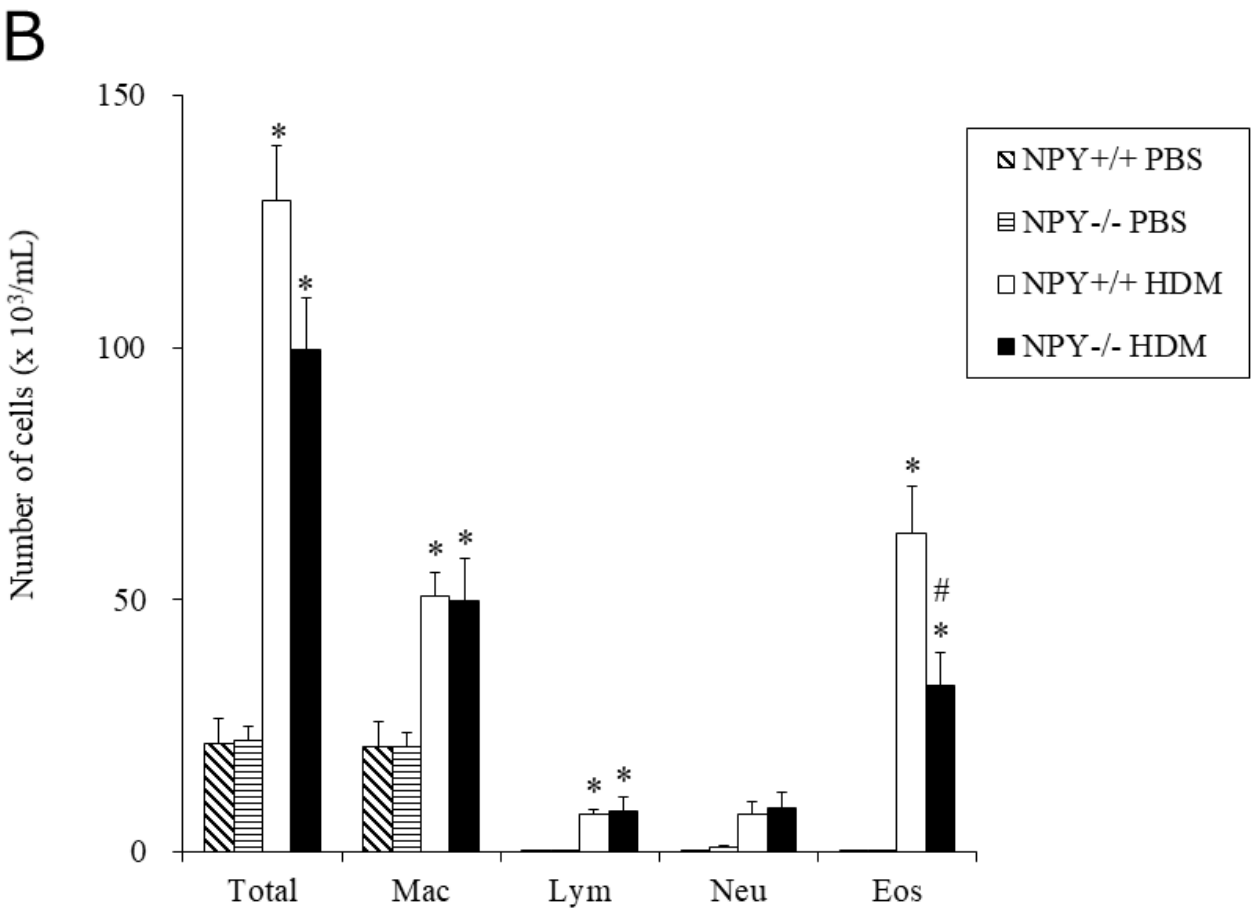

Figure 1

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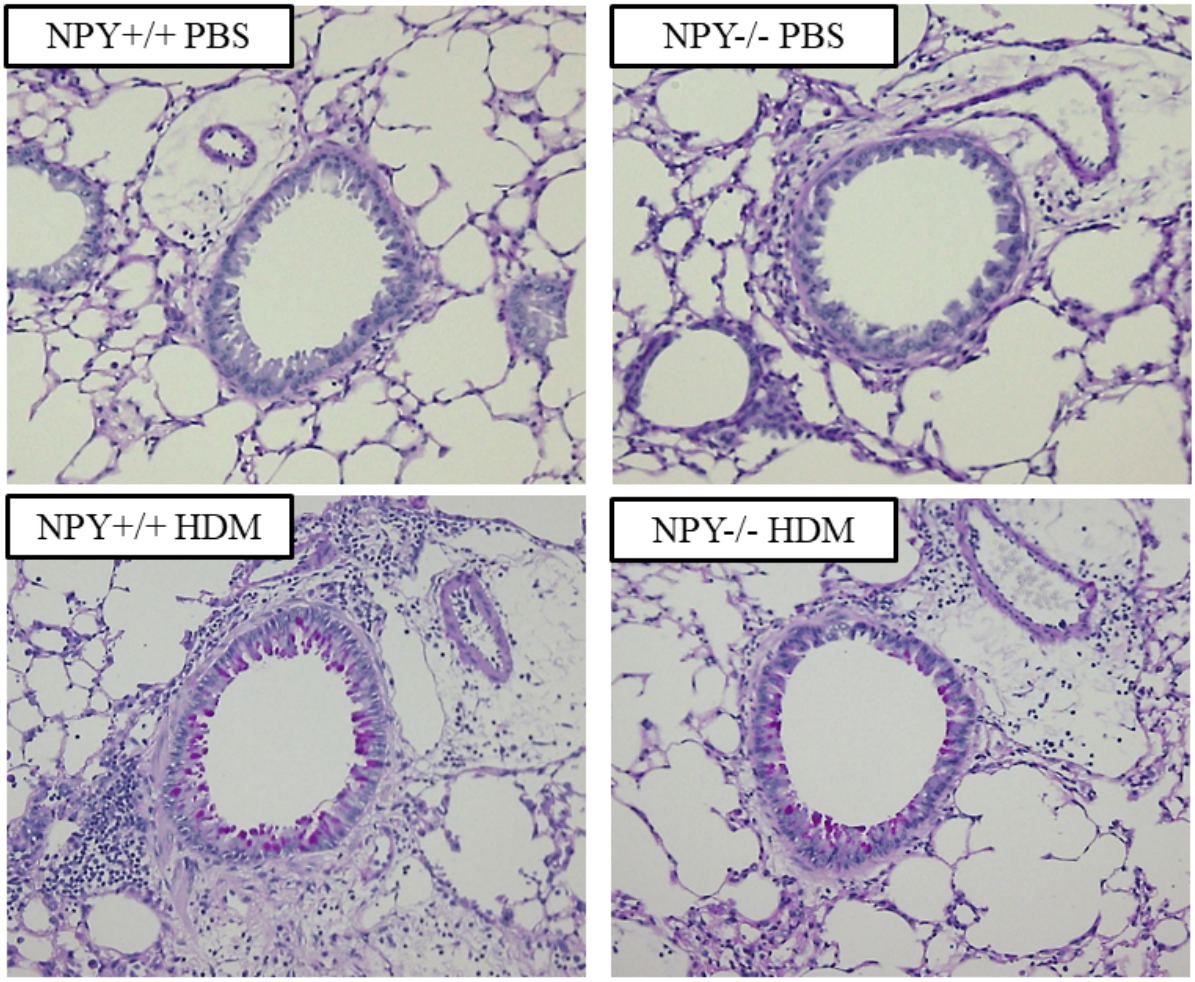

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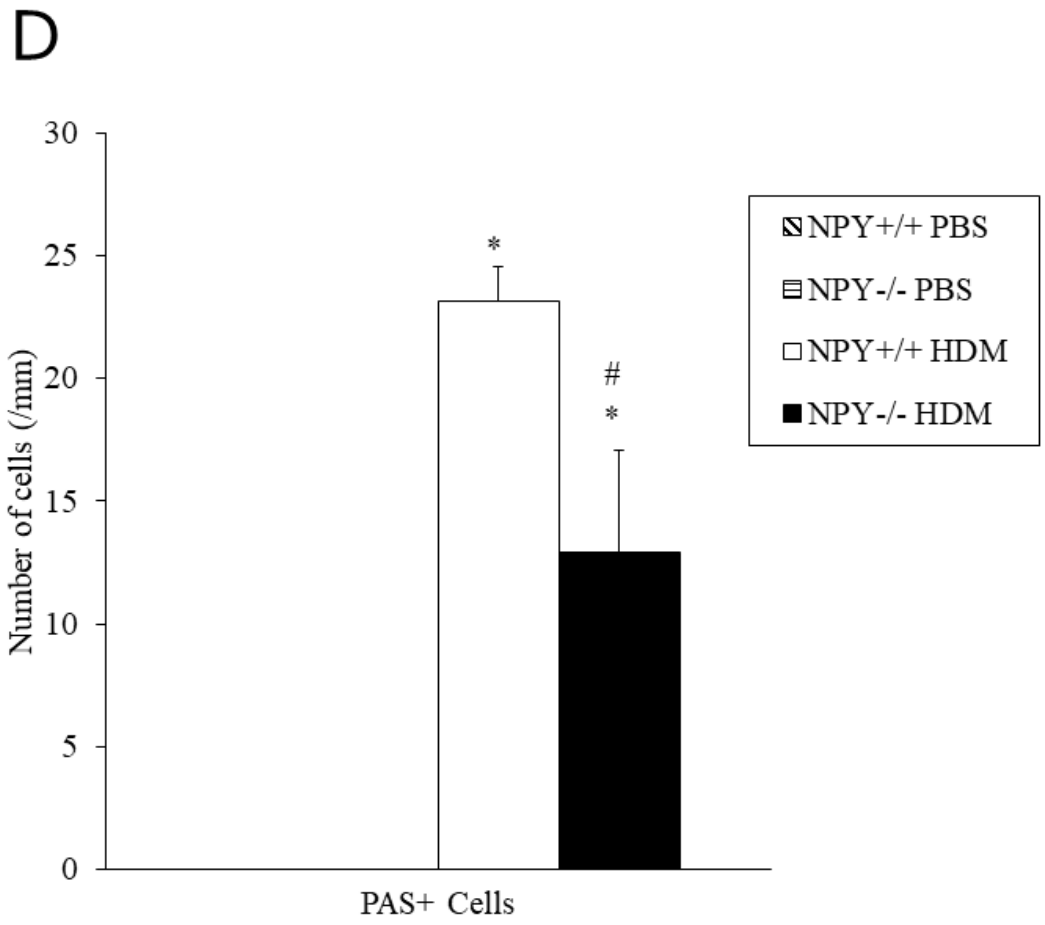

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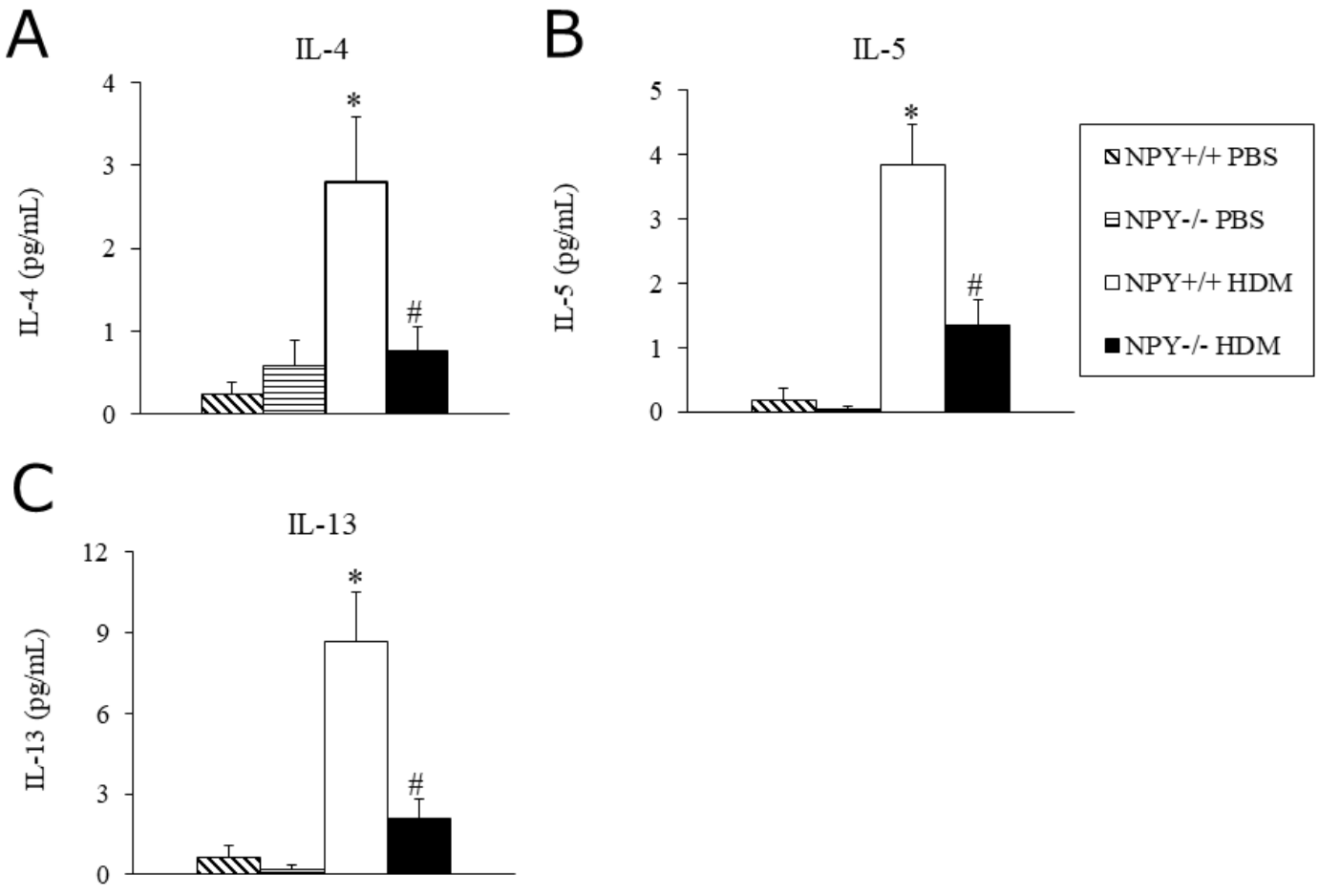

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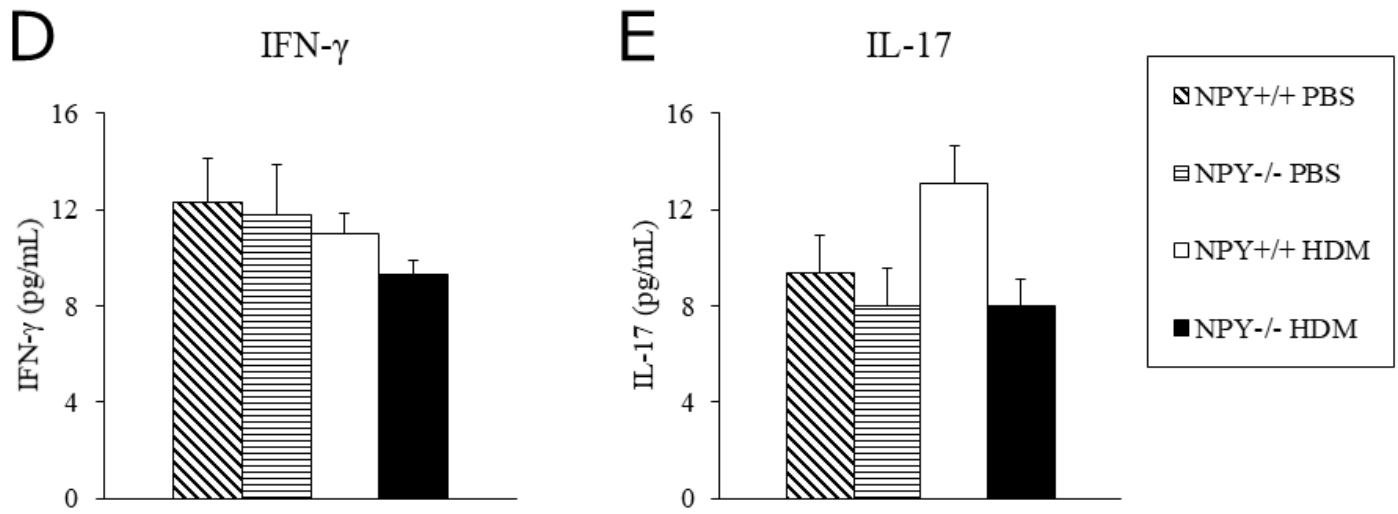

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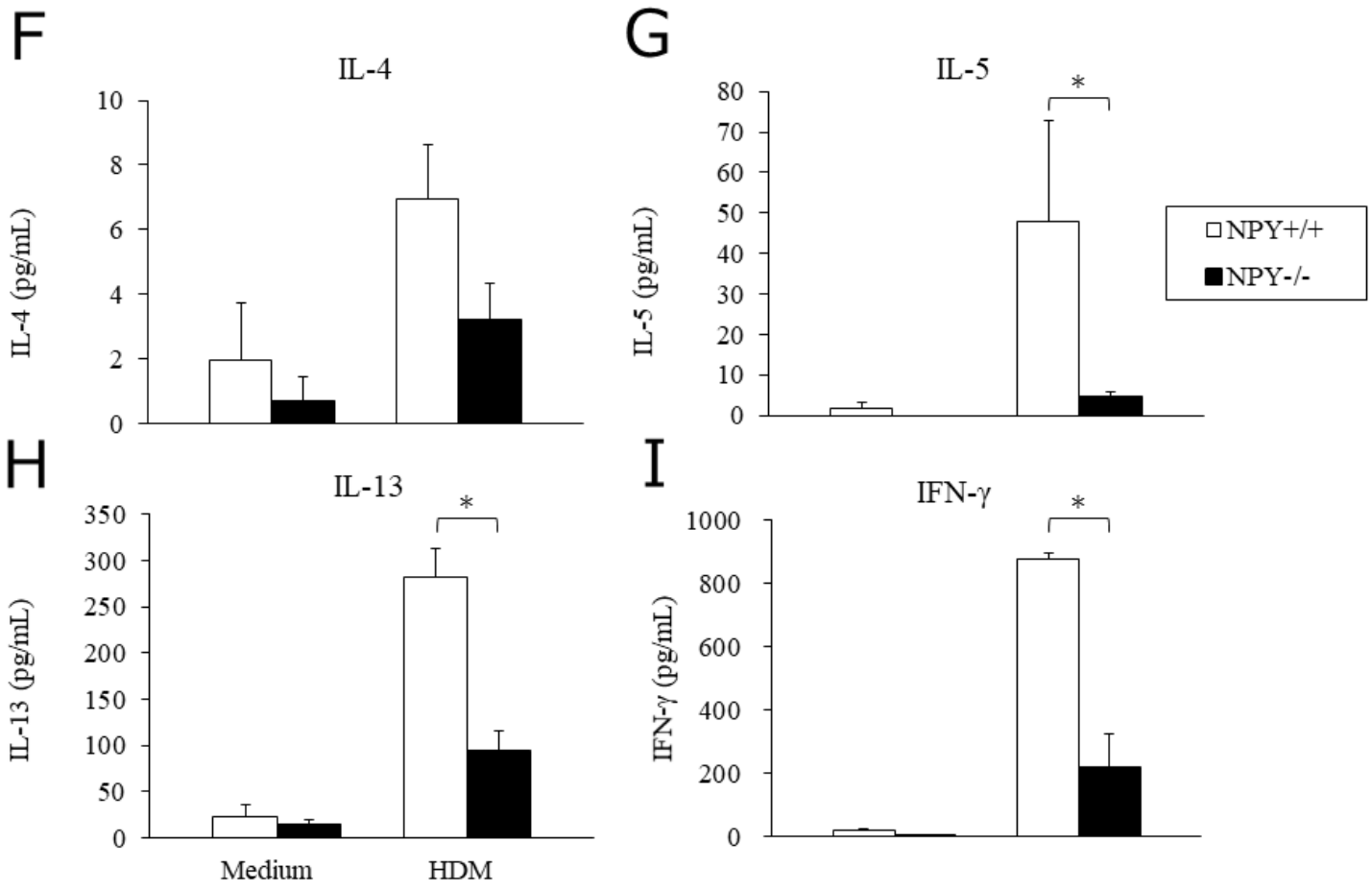

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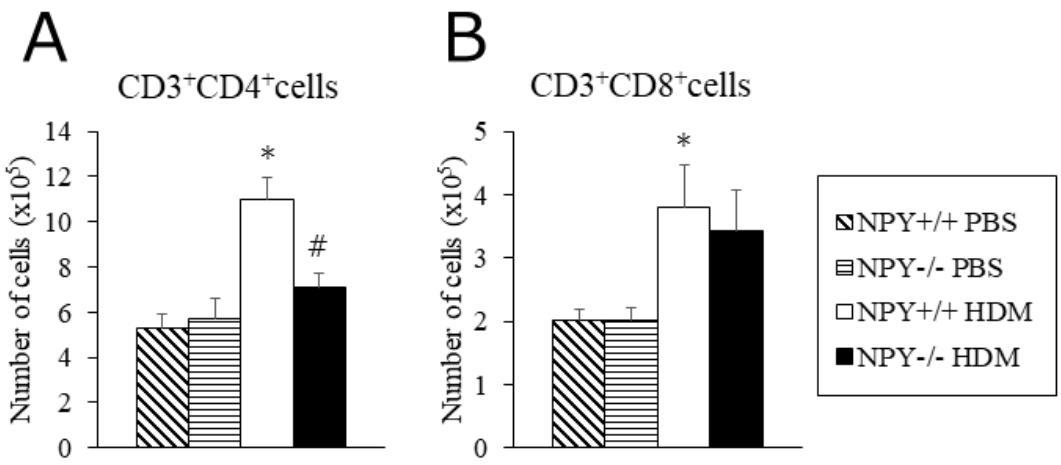

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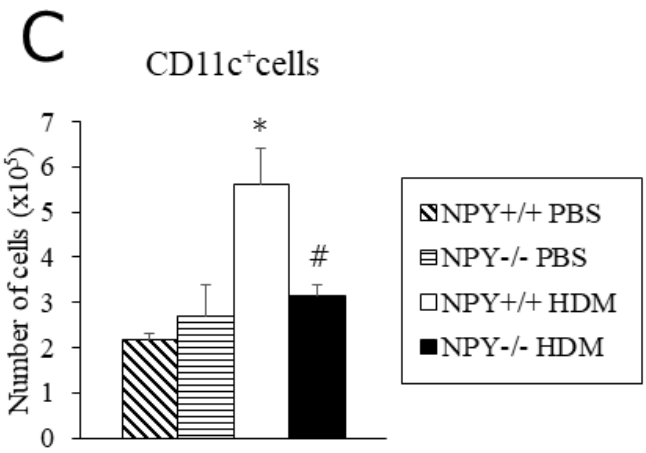

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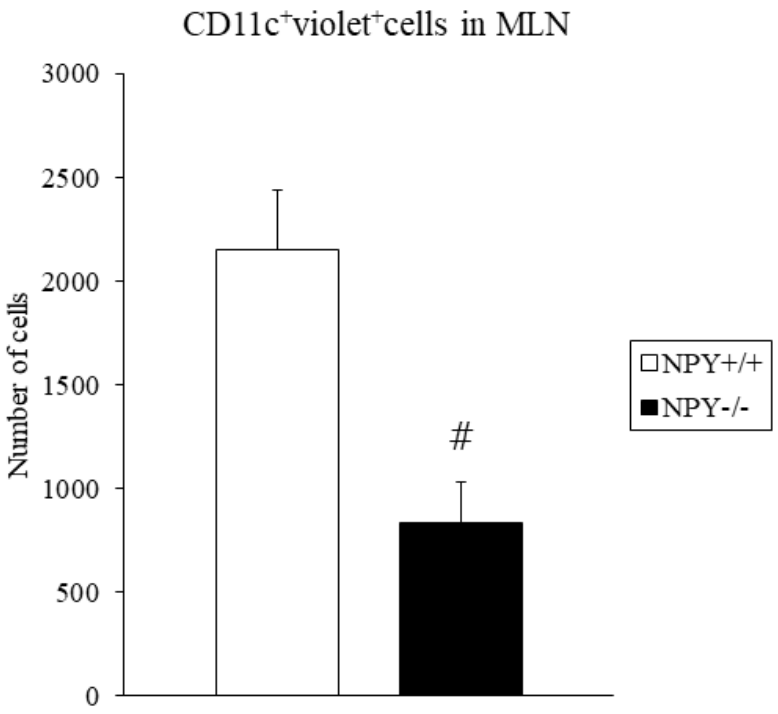

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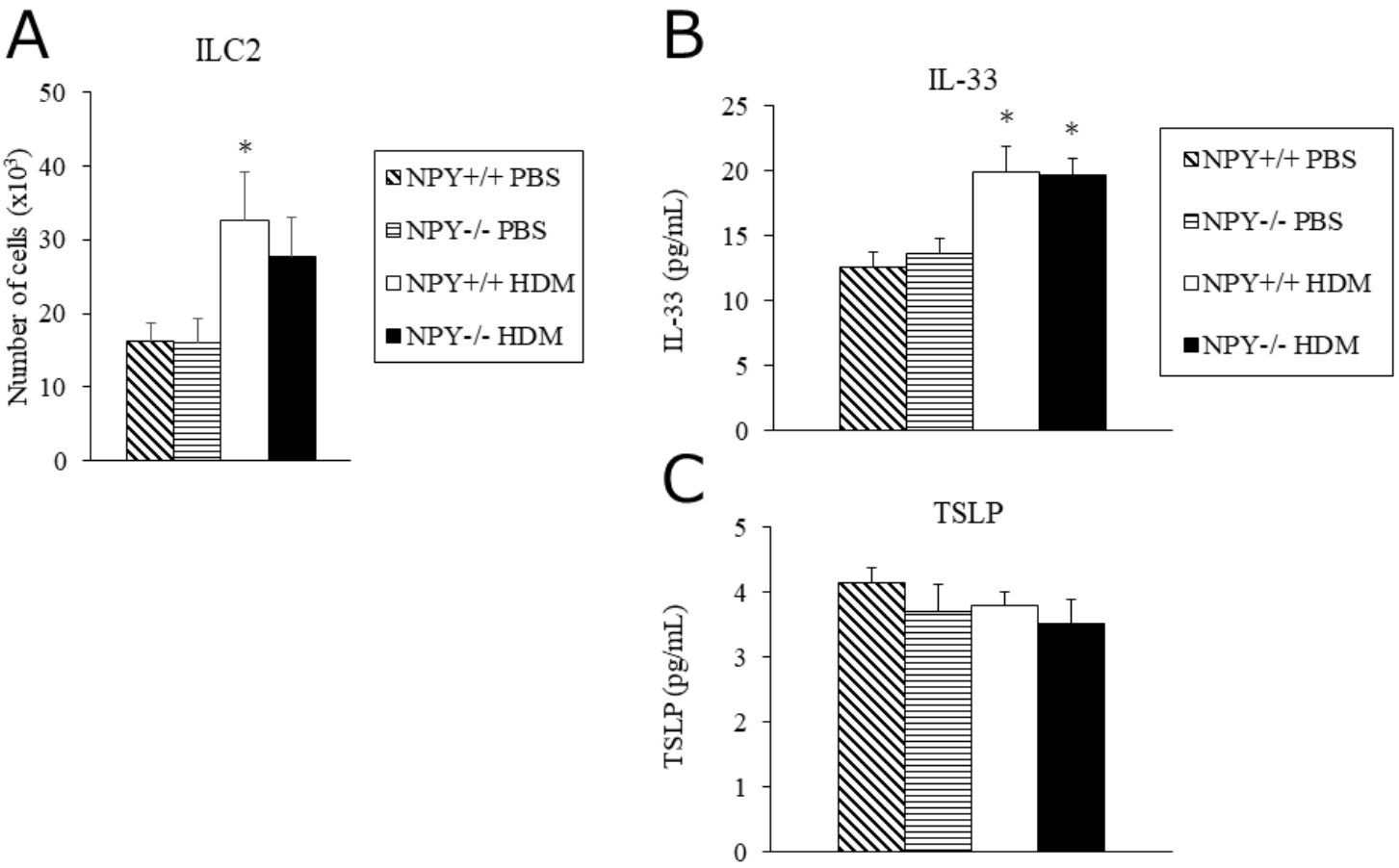

Figure 5

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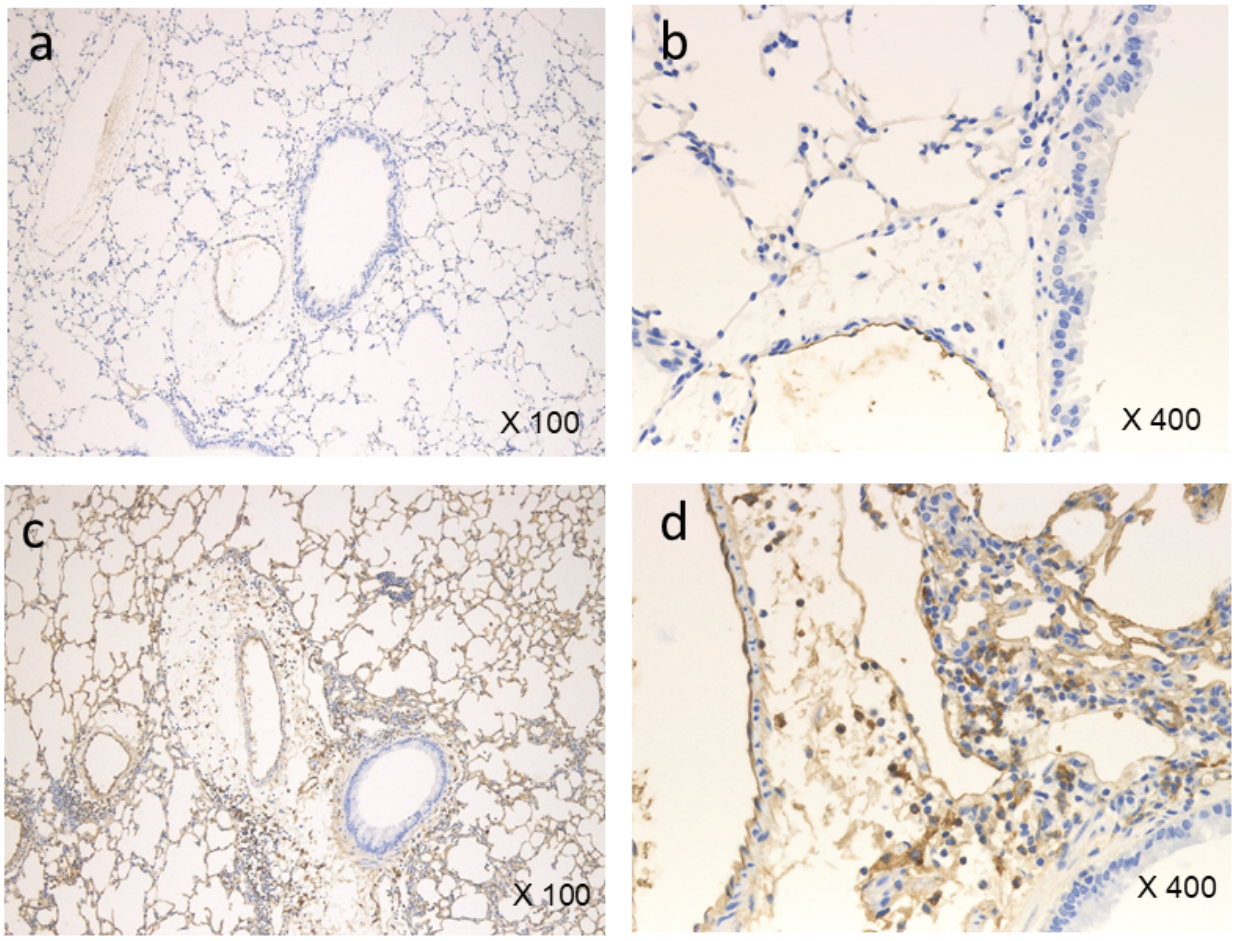

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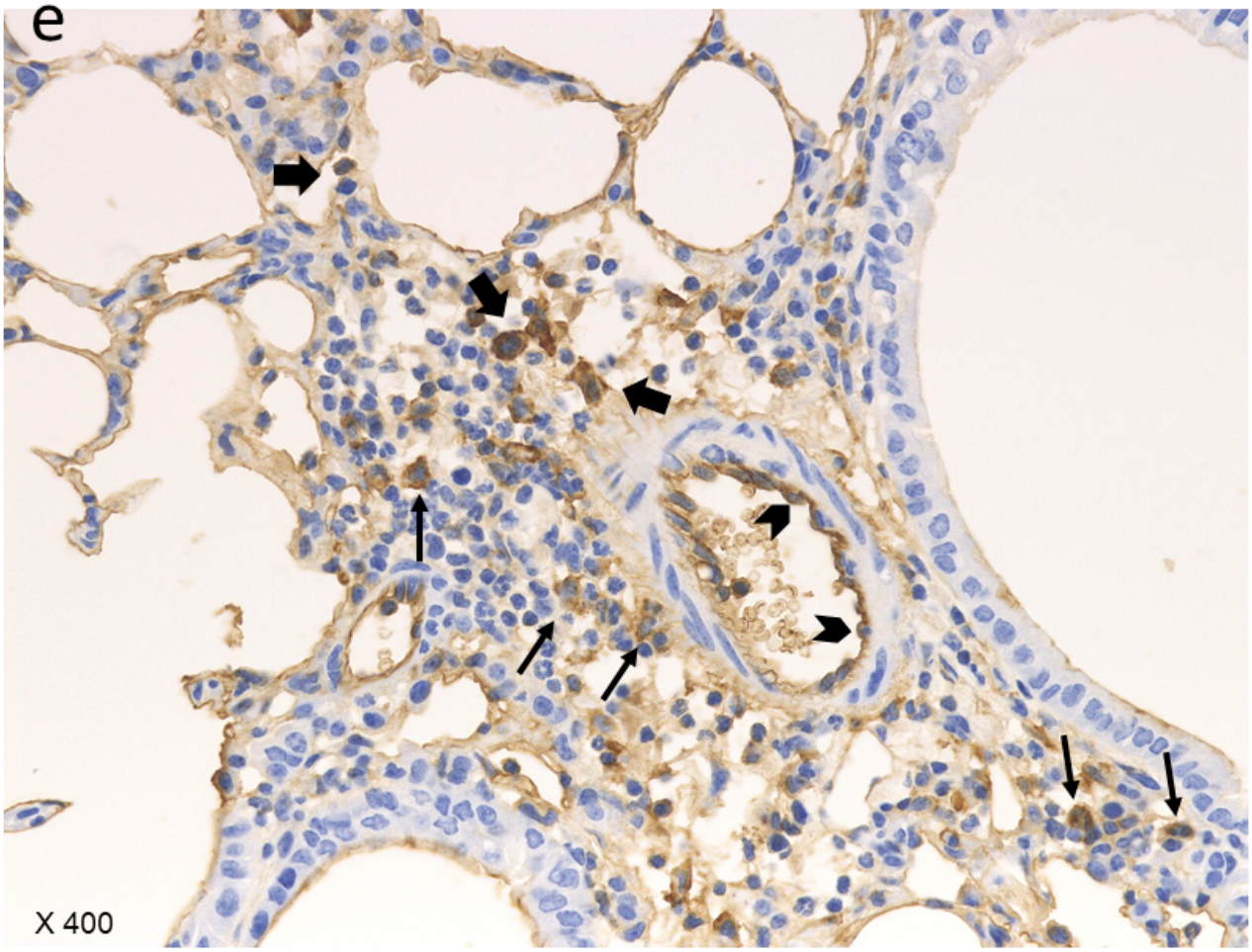

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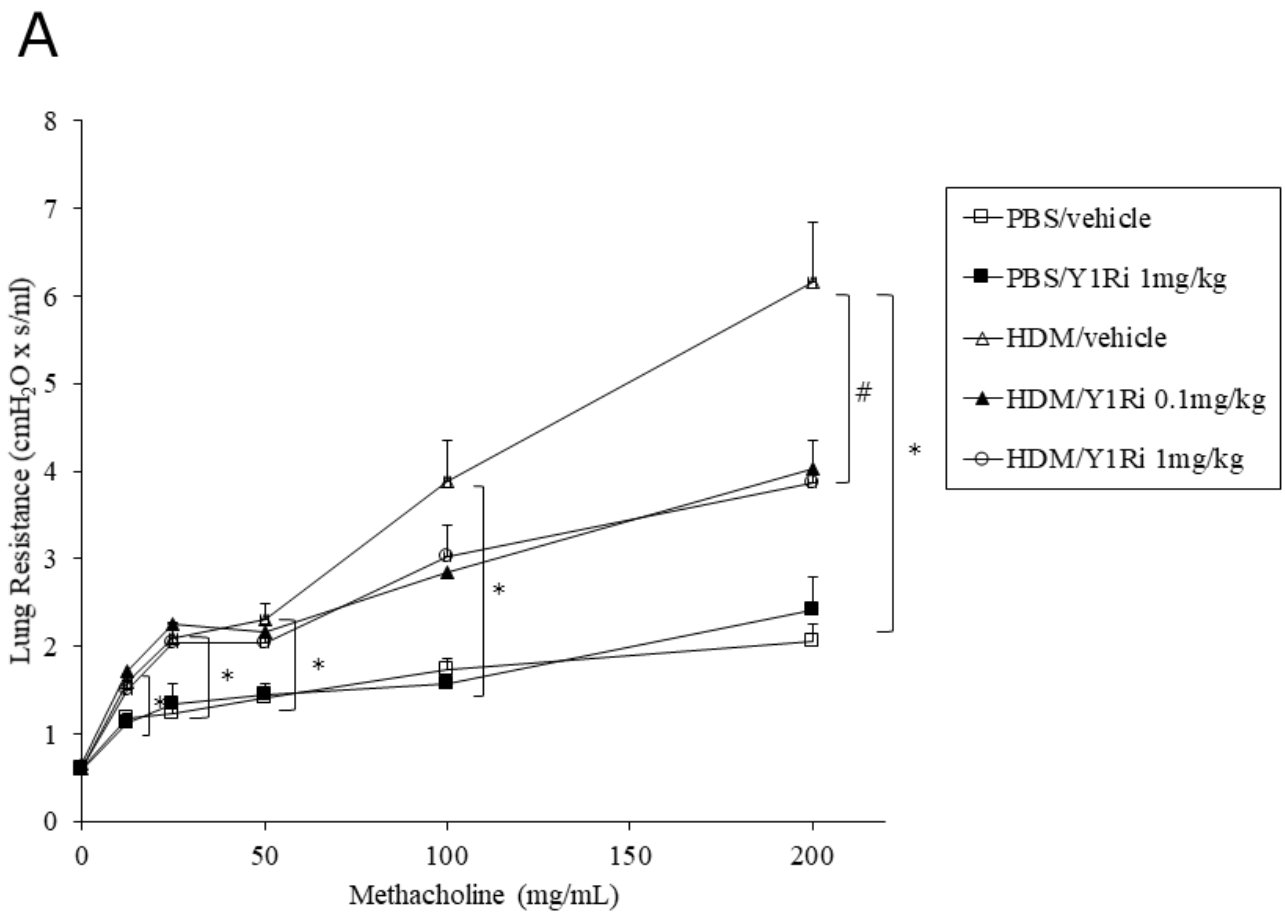

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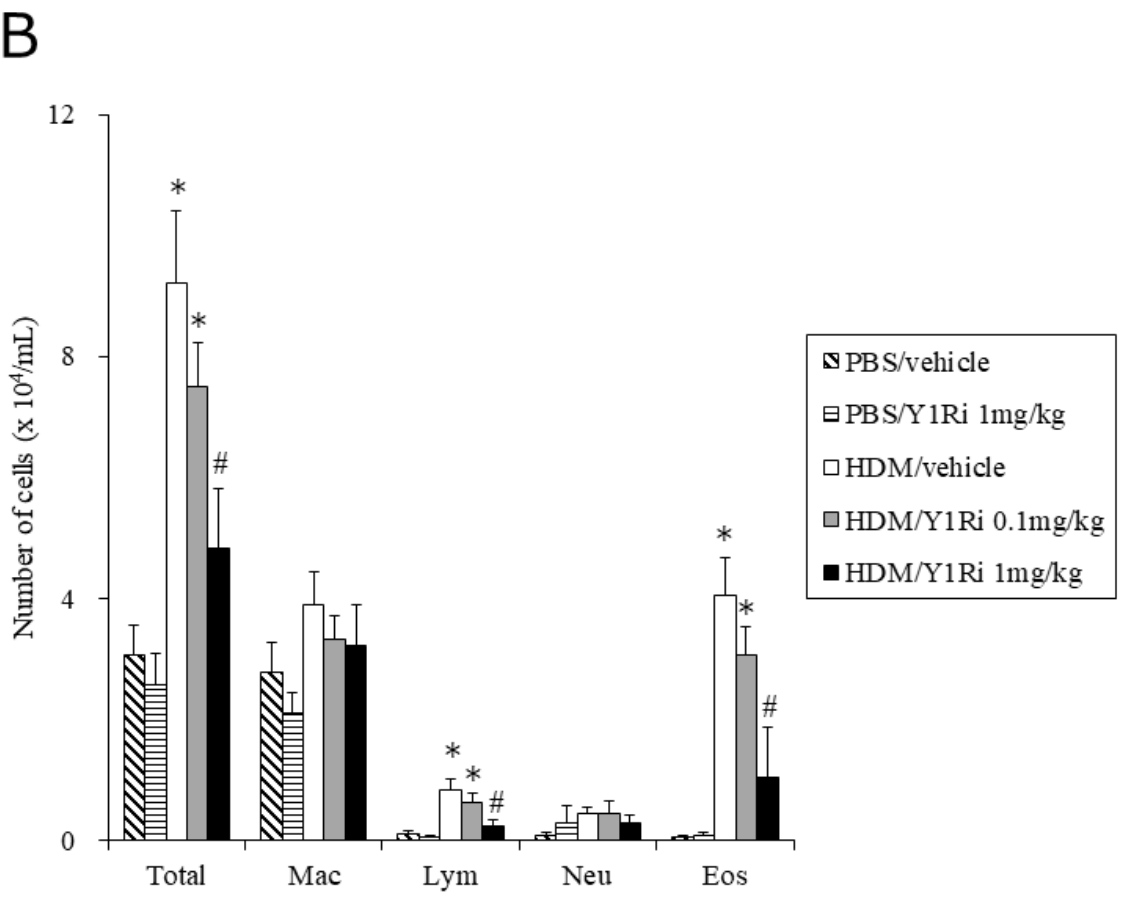

Figure 6

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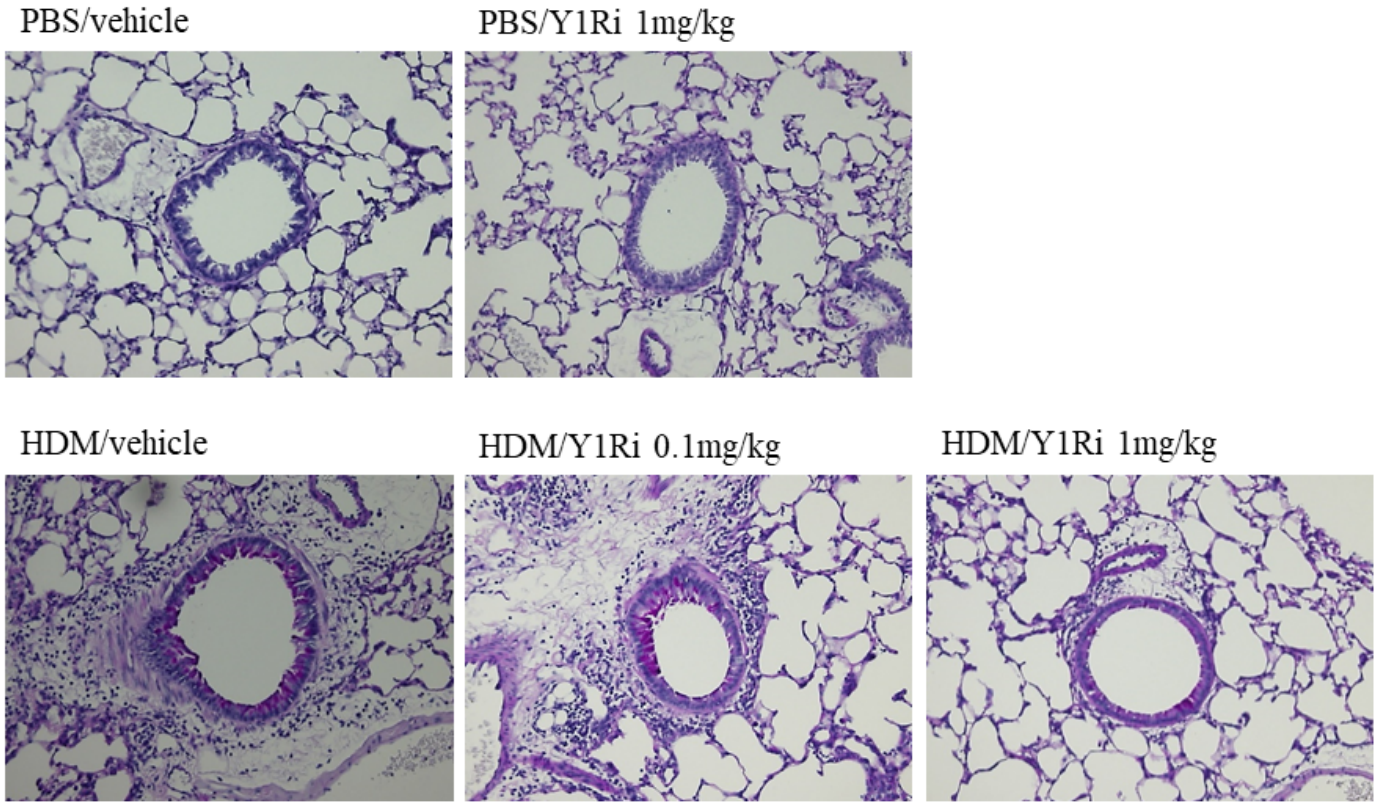

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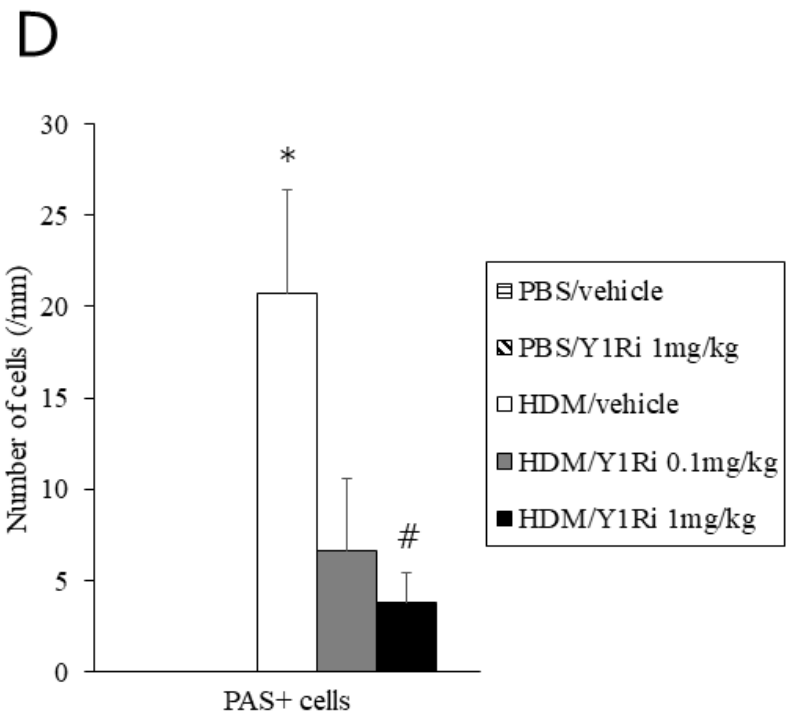

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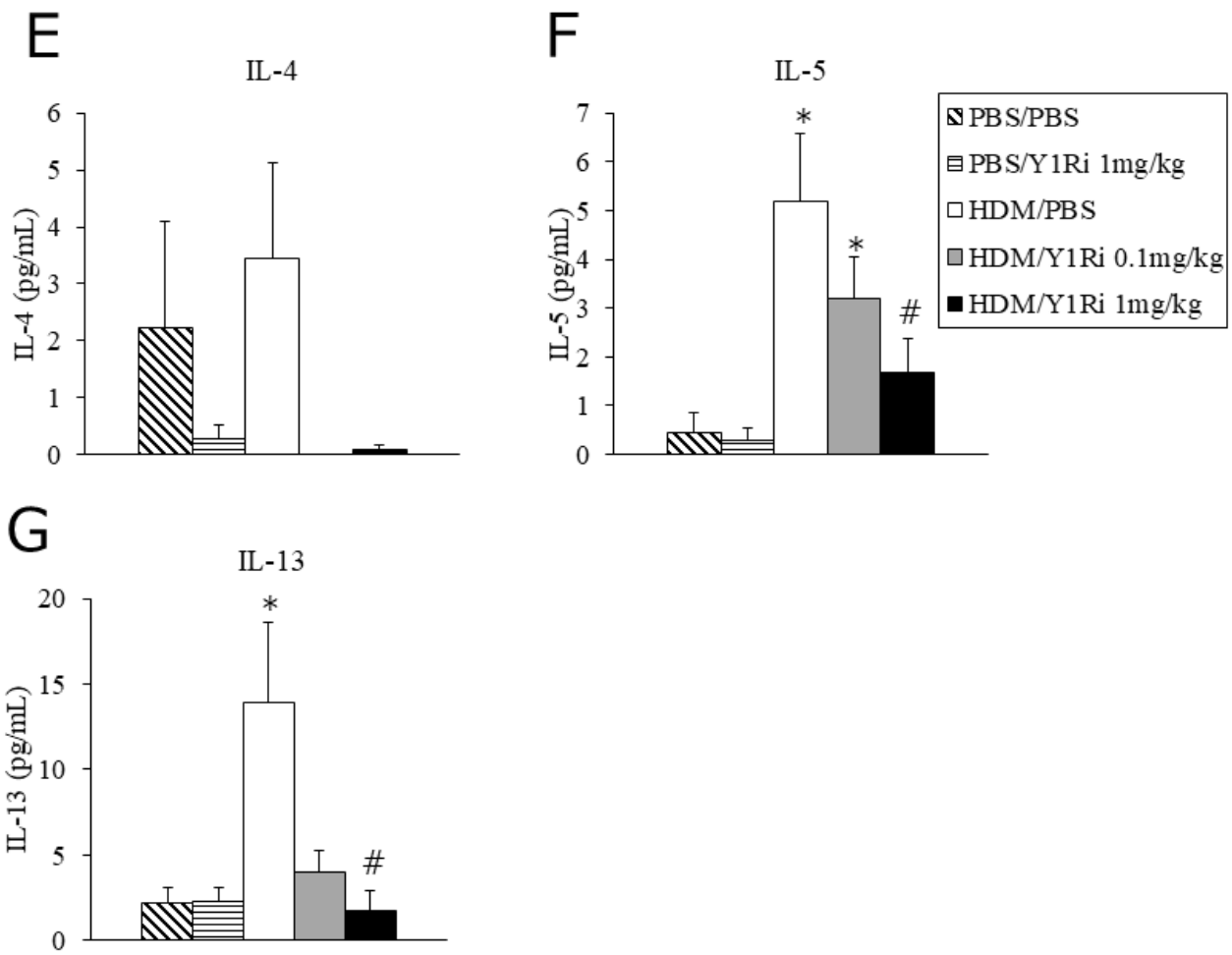

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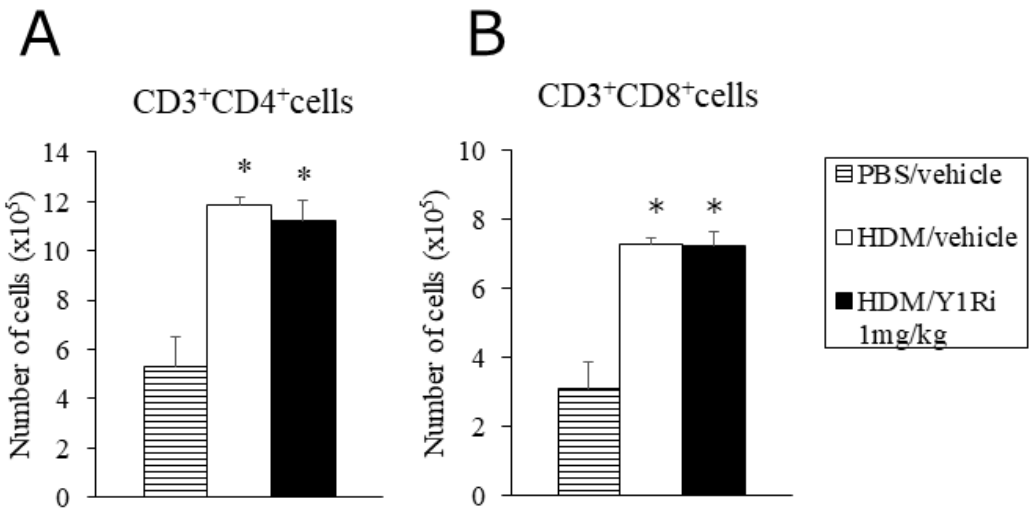

Figure 7