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Oral administration of *Lactobacillus plantarum* CJLP133 and CJLP243 alleviates birch pollen induced allergic rhinitis in mice

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Running headline: The effect of probiotics on airway allergy

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Abbreviations: AHR, airway hyperresponsiveness; BAL, bronchoalveolar lavage; BP, birch pollen; Penh, enhanced pause; PMN, polymorphonuclear leukocytes.

Abstract

Aims: In this study, we evaluated the therapeutic efficacy of selected probiotics in a mouse model of birch pollen (BP)-induced allergic rhinitis.

Methods and Results: Oral administration of *Lactobacillus plantarum* CJLP133 and

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CJLP243 ameliorated the symptoms of BP-induced allergic rhinitis by reducing airway hyperresponsiveness (AHR), and both the histological scores and the number of infiltrated cells in the nasal cavities and lungs. Compared with those from vehicle-treated mice, bronchoalveolar lavage (BAL) fluid and draining lymph node samples from CJLP133 and CJLP243-administrated mice showed diminished numbers of immune cells, increased secretion of a Th1-type cytokine (IFN- γ), and decreased production of Th2-type cytokines (IL-4, IL-5 and IL-13). Consistent with these results, levels of IL-4, IL-5, IL-13, serum IgE, and BP-specific serum IgG1 were decreased, whereas secretion of IFN- γ and BP-specific serum IgG2a were augmented upon administration of CJLP133 and CJLP243 in mice.

Conclusion: Oral administration of *Lactobacillus plantarum* CJLP133 and CJLP243 alleviates symptoms of BP-induced allergic rhinitis in mice by recovering Th1/Th2 balance via enhancement of the Th1-type immune response.

Significance and Impact of the Study: *Lactobacillus plantarum* CJLP133 and CJLP243 have therapeutic effects on BP-induced allergic rhinitis in an animal model.

Keywords: allergic rhinitis, birch pollen, CJLP133, CJLP243, *Lactobacillus plantarum*

Introduction

Birch pollen (BP)-induced allergic rhinitis is classified as a type I airway hypersensitivity, and is mediated by BP-specific IgE-primed inflammation in the nasal mucosa (Mastrorilli *et al.* 2016). Overall, a predominance of the Th2 response over the Th1 response plays a pivotal role in the development of allergic rhinitis (Cox *et al.* 2012). In particular, eotaxin, IL-4, IL-5, and IL-13 secreted by allergen-activated Th2 cells induce IgE overproduction by plasma cells, and mediate recruitment and activation of inflammatory cells in lesions (Cox *et al.* 2012).

Probiotics are live microorganisms that are beneficial to human health when consumed in the appropriate manner (Guarner and Schaafsma 1998). The beneficial effects of probiotics have been used for various purposes, including modulation of the systemic immune response (Macfarlane and Cummings 2002). *Lactobacillus plantarum* CJLP133 and CJLP243 were originally isolated from Kimchi, and immune cells stimulated with these probiotics produced high levels of a Th1-type cytokine, IFN- γ and TNF- α (Lee *et al.* 2011; Lee *et al.* 2012). Both CJLP133 and CJLP243 may exhibit good probiotics properties such as strong acid and bile tolerance, and high adhesive properties to intestinal epithelial cells (Lee *et al.* 2011). Previous

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study indicated that consumption of CJLP243 improved the growth performance of weaning pigs during acute infection of pathogenic enteric bacteria by enhancing Th1-type immune response (Lee *et al.* 2012). Furthermore, results from clinical trial showed that consumption of CJLP133 alleviates atopic dermatitis by restoring the Th1/Th2 balance (Han *et al.* 2012).

Atopic dermatitis and allergic rhinitis are classified as a type I hypersensitivity since the molecular pathogenesis of these diseases is so closely linked (Endo *et al.* 2014). In this study, we tested whether CJLP133 and CJLP243 could modulate the immune response in an animal model of BP-induced allergic rhinitis in mice. The experimental results demonstrated that oral administration of CJLP133 or CJLP243 can induce Th1 responses while suppressing Th2 responses, thereby ameliorating major symptoms of BP-induced allergic rhinitis in a mouse model.

Materials and methods

Preparation of BP allergen

Recombinant Bet v1 was purchased from Biomay (Linz, Austria), and BP was

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purchased from Allergon (Engelholm, Sweden). BP extract was prepared as previously described (Wiedermann *et al.* 1998). Briefly, 20 g of BP was extracted in 200 ml of PBS by stirring overnight at 4°C. Then, BP in PBS was centrifuged for 1 h at 4000 *g* at 4°C. The supernatant was filtered and dialyzed in PBS for 24 h. After dialysis, the concentration of protein in the BP extract was measured and the extract was stored at -20°C. No endotoxin activity was observed in BP extract (data not shown).

Oral administration of *Lactobacillus plantarum* CJLP133 and CJLP243 after induction of BP-induced allergic rhinitis in BALB/c mice

Six-week-old female BALB/c mice were purchased from Orient Bio Inc. (Seoungnam, Republic of Korea) and acclimated for one week prior to experiments. Mice were fed a standard rodent diet with purified water *ad libitum* and kept at 20-24°C and 40-60% humidity in the Korea University animal facility under a 12 h/12 h light-dark cycle. Allergic rhinitis was induced in BALB/c mice by sensitization to BP allergen, as previously described (Fig. 1a) (Tourdot *et al.* 2013). Briefly, mice were sensitized by intraperitoneal injection of 10 µg Bet v1 mixed with 2 mg Alum on days 0 and 7 (Fig. 1a). Fourteen days later, the mice were

challenged with 8 mg of BP extract in 30 ml of PBS via inhalation using an ultrasonic nebulizer (NE-U12) (Omron, Kyoto, Japan) for three consecutive days (Fig. 1a). All experimental mice were sacrificed one day after the final BP extract challenge (Fig. 1a).

Next, thirty-five BALB/c mice were randomly separated into five groups. Two groups were orally administered different strains of *Lactobacillus plantarum* (one strain per group). Each group received 400 µl of PBS containing *Lactobacillus plantarum* CJLP133 or CJLP243 (1×10^{10} CFU per mouse) daily starting one week before the sensitization until the mice were sacrificed (Won *et al.* 2012). As a positive control, the dexamethasone-treated group was orally administered 400 µl of PBS containing dexamethasone (12.5 µg) (Kim *et al.* 2004). As a negative control, the vehicle-treated group was orally administered only 400 µl of PBS. We did not induce allergic rhinitis in the remaining group (non-induction). Mice received proper care in accordance with the protocol, which was approved by the Institutional Animal Care and Use Committee of Korea University (protocol number: KUIACUC-2015-161).

Measurement of airway hyperresponsiveness (AHR)

AHR was measured using a plethysmograph (body volume change recorder) (All Medicus Co. Ltd, Seoul, Republic of Korea) one day after the final BP extract challenge. Prior to measuring AHR, each mouse was treated with a different concentration of aerosolized methacholine (0, 12.5, 25, 50, or 100 mg mL⁻¹) for 3 min. Enhanced pause (Penh) was measured after each nebulization.

Histological examination

Three different sections of nasal mucosa and lungs from each experimental mouse were fixed with 10% formalin (Sigma, St. Louis, MO, USA) and embedded in paraffin. Each 5 µm thick section was stained with hematoxylin and eosin. The degree of inflammation in the nasal mucosa was evaluated by counting the number of infiltrated cells in the lamina propria of the nasal mucosa in five randomly chosen regions (2500 µm²) in each sample under a microscope at ×200 magnification. The level of inflammation around the peribronchial and perivascular regions of the lungs in each sample was evaluated as described previously

(Kwak *et al.* 2003).

Analysis of bronchoalveolar lavage (BAL) fluid

Mice were anesthetized with diethyl ether via inhalation. After exposing the trachea, BAL was performed with 2 ml PBS. BAL fluids were centrifuged at 800 *g* for 5 min at 4°C. Cell pellets were resuspended in 200 ml RPMI and stained with a Diff-Quik staining kit (Sysmex Co., Kobe, Japan). Also, the levels of IFN- γ , IL-4, IL-5, IL-13, and eotaxin (CCL11) in BAL fluids were quantitated by sandwich ELISA kits according to the manufacturer's instructions (eBioscience, Inc., San Diego, CA, USA).

Analysis of draining lymph nodes

The size of the mediastinal lymph nodes was measured in experimental mice. Also, the total cell number in a single cell suspension from each lymph node was assessed using a hemocytometer under a microscope. Then, cells were cultured with 100 μ g/ml of BP extract at a density of 1×10^6 cells well⁻¹ in RPMI1640 medium containing 10% (v/v) fetal bovine

serum (FBS) and 1% penicillin/streptomycin. On day 2 after stimulation, the amount of IL-4, IL-5, IL-1,3 and IFN- γ in the supernatant was quantitated by sandwich ELISA.

Measurement of serum Ig levels

Blood samples from experimental mice were taken from the orbital venous plexus using a capillary tube. To obtain serum, samples were incubated on ice for 1 h and centrifuged at 8000 *g* for 10 min at 4 °C. The concentration of total IgE was measured by sandwich ELISA kits according to the manufacturer's instructions (eBioscience, Inc.). The levels of BP-specific IgG1 and IgG2a in each serum sample were measured as described previously (Tourdot *et al.* 2013).

Statistical analysis

Mean values were compared using Student's t-test for comparing independent variables. Significant differences were measured with p-values and are marked with asterisks (* $p < 0.05$, ** $p < 0.01$).

Results

Oral administration of CJLP133 and CJLP243 reduced AHR and leukocyte infiltration in lesions of mice with BP-induced allergic rhinitis

BP-sensitized and -challenged mice showed an increase in AHR upon methacholine exposure in a dose-dependent manner (Fig. 1b). Both the CJLP133- and CJLP243-treated groups displayed significantly lower levels of AHR compared with the vehicle-treated group (Fig. 1b). Also, the dexamethasone-treated group exhibited a significant reduction in AHR compared with the vehicle-treated group (Fig. 1b). Next, histological analyses of nasal mucosa and lung were performed to assess leukocyte infiltration in lesions of experimental mice. In the vehicle-treated group, a significant increase in leukocyte infiltration was found in the lamina propria of the nasal mucosa and the peribronchial and perivascular regions of the lungs, in comparison with that in both the CJLP133- and CJLP243-treated groups or the dexamethasone-treated group (Fig. 1c,d).

Effects of CJLP133 and CJLP243 administration on the secretion of inflammatory mediators in BAL fluid of mice with BP-induced allergic rhinitis

Previous observation showed that macrophages function as a protector against allergen-mediated AHR (Careau *et al.* 2010). Histological analyses revealed that the proportion of macrophage was markedly decreased in BAL fluid taken from the vehicle-treated group, whereas the proportion of polymorphonuclear leukocytes (PMNs) including eosinophils and neutrophils was markedly increased, compared with that from both the CJLP133- and CJLP243-treated groups or the dexamethasone-treated group (Fig. 2a,b). To estimate the degree of airway inflammation, the levels of Th1- or Th2-type cytokines were examined in BAL fluid from experimental mice. The level of a Th1-type cytokine (IFN- γ) significantly increased in both the CJLP133- and CJLP243-treated groups compared with that in the vehicle-treated group (Fig. 2c). In contrast, levels of the Th2-type cytokines IL-4, IL-5, and IL-13 in the BAL fluid were significantly decreased in both the CJLP133- and CJLP243-treated groups compared with levels in the vehicle-treated group (Fig. 2c). The level of eotaxin was also markedly decreased in both the CJLP133- and CJLP243-treated groups compared with the vehicle-treated group (Fig. 2c). The dexamethasone group showed the

lowest levels of both Th1- and Th2-type cytokines owing to immunosuppression (Fig. 2c).

Effects of CJLP133 and CJLP243 administration on the immune response in the draining lymph nodes of mice with BP-induced allergic rhinitis

To examine the effects of CJLP133 and CJLP243 administration on the immune response in BP-induced allergic rhinitis, the size and cell counts in the draining lymph nodes (mediastinal lymph nodes) were assessed in experimental mice. The size and total cell numbers in the draining lymph nodes of both the CJLP133- and CJLP243-treated groups decreased significantly in comparison with those in the vehicle-treated group (Fig.3 a,b). The size and cell numbers of the lymph nodes from the dexamethasone-treated group were similar to those in the non-induction group (Fig. 3a).

To explore BP-specific T cell responses in experimental mice, we monitored the secretion of several cytokines involved in the pathogenesis of BP-induced allergic rhinitis after stimulation of mediastinal lymph node cells with the allergen. Both the CJLP133- and CJLP243-treated groups displayed a marked increase in the level of the Th1-type cytokine (IFN- γ), whereas the levels of the Th2-type cytokines IL-4, IL-5, and IL-13 as well as eotaxin

in both the CJLP133- and CJLP243-treated groups were significantly decreased compared with the levels in the vehicle-treated group (Fig. 3c). The levels of cytokines secreted from the dexamethasone-treated group were comparable to those in the non-induction group (Fig. 3c).

Effects of CJLP133 and CJLP243 administration on Ig secretion in mice with BP-induced allergic rhinitis

To assess the systemic antibody responses resulting from oral administration of CJLP133 and CJLP243 in mice with BP-induced allergic rhinitis, serum levels of total IgE, BP-specific IgG1, and BP-specific IgG2a were determined by ELISA. In both the CJLP133- and CJLP243-treated groups, the levels of total IgE were significantly reduced compared with that seen in the vehicle-treated group (Fig. 4a). Also, the levels of BP-specific IgG1 were markedly decreased in both the CJLP133- and CJLP243-treated groups compared with that in the vehicle-treated group (Fig. 4b). However, the level of BP-specific IgG2a was increased in both the CJLP133- and CJLP243-treated groups compared with that in the vehicle-treated group (Fig. 4c). The levels of total IgE, BP-specific IgG1, and BP-specific

IgG2a in the dexamethasone-treated group were significantly reduced compared with that in the vehicle-treated group (Fig. 4).

Discussion

In the present study, the immunomodulatory roles of CJLP133 and CJLP243 administration were evaluated in a murine model of birch pollen allergy, a Th2 immune disorder. Previous studies demonstrated that splenocytes treated with CJLP133 and CJLP243 produced a high amount of a Th1-type cytokine, IFN- γ (Lee *et al.* 2011) and that administration of CJLP133 alleviates atopic dermatitis in clinical trials (Han *et al.*, 2012). Since the mechanisms of disease progression are quite similar in atopic dermatitis and allergic rhinitis (Endo *et al.* 2014), it is worthwhile to examine whether administration of CJLP133 or CJLP243 alleviates the symptoms of BP-induced allergic rhinitis.

The data clearly showed that oral administration of CJLP133 or CJLP243 can alleviate the severity of BP-induced allergic rhinitis in a mouse model by rebalancing the Th1 and Th2 immune responses via enhancing the Th1 response. First, AHR was significantly reduced in both the CJLP133- and CJLP243-treated groups compared with the vehicle-treated group.

Also, oral administration of both CJLP133 and CJLP243 significantly lowered the number of infiltrated PMNs such as eosinophils and neutrophils, and increased the number of macrophages in upper respiratory tissues and BAL fluids. These results can be explained by the observed increase in the amount of a Th1-type cytokine (IFN- γ) and the decrease in the amounts of the Th2-type cytokines IL-4, IL-5, and IL-13 in BAL fluids and draining lymph nodes (mediastinal lymph nodes) in both the CJLP133- and CJLP243-treated groups compared with the vehicle-treated group.

Eotaxin is an important chemokine for the recruitment of eosinophils, major contributors to allergic reactions (Rothenberg *et al.* 1995). A previous study indicated that IFN- γ is a potent inhibitor of eotaxin synthesis (Miyamasu *et al.* 1999). The level of eotaxin was also significantly reduced in BAL fluids from CJLP133- and CJLP243-treated mice compared with vehicle-treated mice. Therefore, this evidence also supports that administration of CJLP133 and CJLP243 restore Th1 and Th2 immune balance via an increase in the Th1 immune response.

Our results also demonstrated that oral administration of both CJLP133 and CJLP243 significantly reduced serum levels of IgE and BP-specific IgG1 and increased BP-specific IgG2a levels. Since IFN- γ augments IgG2a production (Garraud *et al.* 2003), whereas IL-4

promotes IgE and IgG1 production (Garraud *et al.* 2003), these results are another line of evidence indicating that administration of CJLP133 and CJLP243 can enhance the Th1-type immune response in mice.

Similar to our findings, some strains of probiotics are powerful inducers of the Th1 immune response, and, so, can be used as therapeutic options for BP-induced allergic rhinitis (Yang *et al.* 2013). Clinical studies will be necessary to further understand the efficacy of CJLP133 and CJLP243 in BP-induced allergic rhinitis.

Acknowledgement

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Conflict of Interest

H. Ahn, H. S. Yun, Y. M. Chung, B. Kim are currently employed by the CJ CheilJedang Corporation, Republic of Korea. None of the other authors had any conflict of interests.

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Figure legends

Figure 1 Experimental design and effects of *Lactobacillus plantarum* CJLP133 and CJLP243

on AHR and lesions associated with BP-induced allergic rhinitis in mice. (a) Experimental

design used for induction of BP-induced allergic rhinitis in mice. ▼, allergen (BP)

application; day 0, first administration of allergen (BP). (b) AHR was measured one day after

the final BP extract challenge using a whole-body plethysmograph. Prior to measuring AHR,

each mouse inhaled a different concentration of aerosolized methacholine. Mouse groups:

(●) Non-induction, (○) Vehicle, (▼) CJLP133, (△) CJLP243, (■) Dexamethasone. (c,d)

Histological analysis of nasal mucosa sections (c) and lungs (d) of experimental mice. Tissues

were fixed and stained with Hematoxylin and eosin (H&E). (c) After H&E staining, the

number of infiltrated cells in the lamina propria of the nasal mucosa was counted in the

samples (2500 μm^2) under a microscope at $\times 200$ magnification. The images shown in the left

panels are representative of each group of mice. Data shown in the *right* panels are the mean numbers of infiltrated cells. (d) Histological evaluation of lung tissues was performed under a microscope at $\times 100$ magnification. The results shown in the *left* panels are representative of each group of mice. Data shown in the *right* panels are the average of the peribronchial and perivascular inflammation scores. Non-induction, non-BP-treated mice; vehicle, PBS (vehicle)-treated mice; CJLP133, mice orally administered CJLP133 in PBS; CJLP243, mice orally administered CJLP243 in PBS; dexamethasone, mice orally administered dexamethasone in PBS. The data shown are means $\pm$ SEM ($n = 7$). Significant differences compared with the vehicle are represented as * $P < 0.05$ or ** $P < 0.01$.

Figure 2 Oral administration of *Lactobacillus plantarum* CJLP133 and CJLP243 reduces infiltration of inflammatory cells and modulates cytokine production in the BAL fluid of mice after induction of BP-induced allergic rhinitis. (a) Infiltration of inflammatory cells in the BAL fluid from each group of experimental mice. Red arrows indicate PMNs. (b) The proportion of cells in the BAL fluid from each group of experimental mice was assessed using Diff Quik staining. Mouse groups: (□) Non-induction, (▨) Vehicle, (▩) CJLP133, (⊠) CJLP243, (■) Dexamethasone. (c) ELISAs were performed to detect IFN- γ , IL-4, IL-5,

IL-13, and eotaxin in the BAL fluid. Non-induction, non-BP-treated mice; vehicle, PBS

(vehicle)-treated mice; CJLP133, mice orally administered CJLP133 in PBS; CJLP243, mice

orally administered CJLP243 in PBS; dexamethasone, mice orally administered

dexamethasone in PBS. The data shown are means $\pm$ SEM ($n = 7$). Significant differences

compared with the vehicle are represented as * $P < 0.05$ or ** $P < 0.01$.

Figure 3 Oral administration of *Lactobacillus plantarum* CJLP133 and CJLP243 decreases

the total number of immune cells and modulates cytokine production in the draining lymph

nodes of mice after induction of BP-induced allergic rhinitis. (a) The size of the mediastinal

lymph nodes in each group of experimental mice were compared. (b) Total cell numbers in

the mediastinal lymph nodes from each group of experimental mice. (c) Single cell

suspensions from each draining lymph node (1×10^6 cells well⁻¹) were stimulated with BP

extract (100 mg ml⁻¹). Two days after stimulation, production of IFN- γ , IL-4, IL-5, and IL-13

in the culture supernatants was measured by ELISA. Non-induction, non-BP treated mice;

vehicle, PBS (vehicle)-treated mice; CJLP133, mice orally administered CJLP133 in PBS;

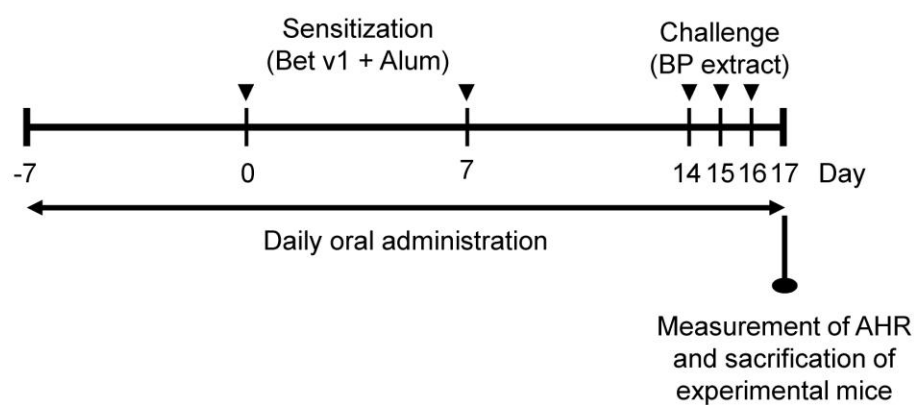
CJLP243, mice orally administered CJLP243 in PBS; dexamethasone, mice orally

administered dexamethasone in PBS. The data shown are means $\pm$ SEM ($n=7$). Significant

differences compared with the vehicle are represented as * $P < 0.05$ or ** $P < 0.01$.

Figure 4. Oral administration of *Lactobacillus plantarum* CJLP133 and CJLP243 modulates the levels of different Ig isotypes in sera. Sera were isolated one day after the final BP extract challenge from each experimental mouse. ELISAs were performed to detect the serum levels of total IgE (a), BP-specific IgG1 (b), and BP-specific IgG2a (c) in each group of experimental mice. Non-induction, non-BP-treated mice; vehicle, PBS (vehicle)-treated mice; CJLP133, mice orally administered CJLP133 in PBS; CJLP243, mice orally administered CJLP243 in PBS; dexamethasone, mice orally administered dexamethasone in PBS. The data shown are means $\pm$ SEM ($n = 7$). Significant differences compared with the vehicle are represented as * $P < 0.05$ or ** $P < 0.01$.

(a)



(b)

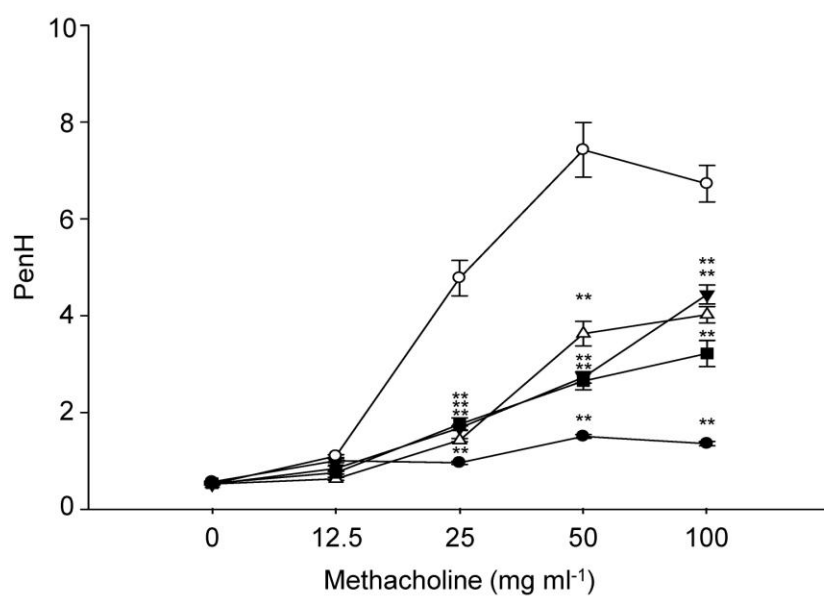
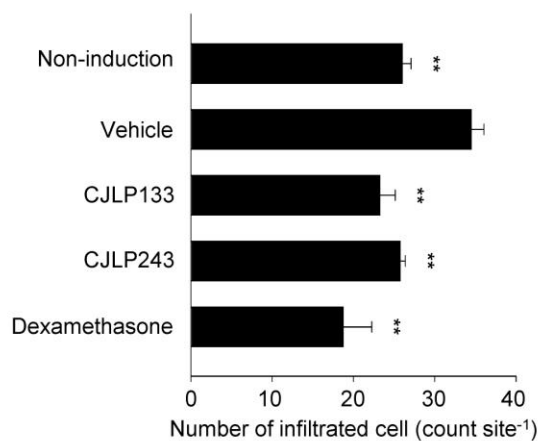
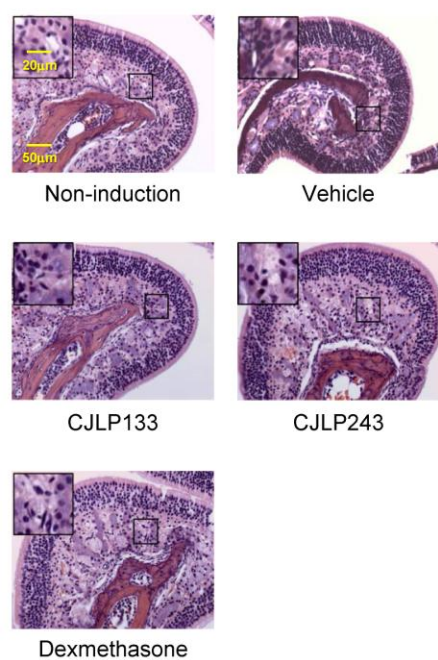


Figure 1

(c)



(d)

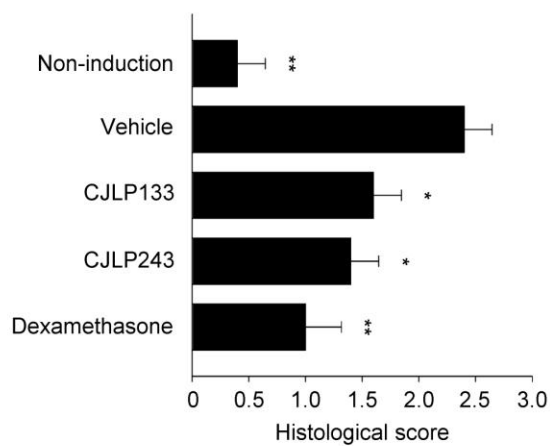
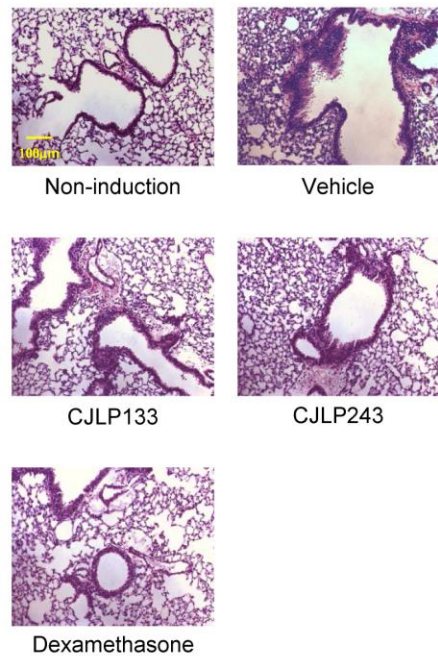
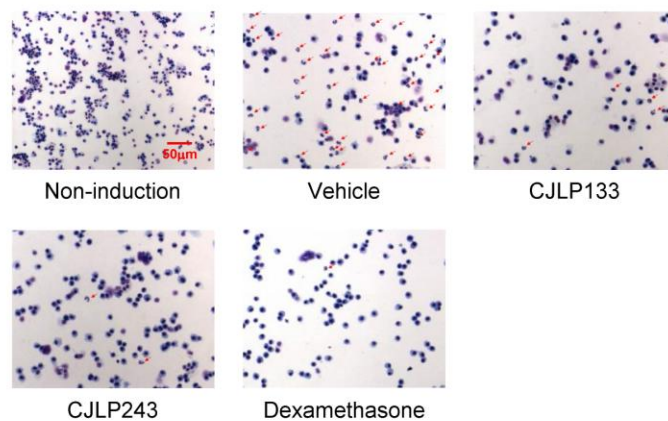


Figure 1^{cont.}

(a)



(b)

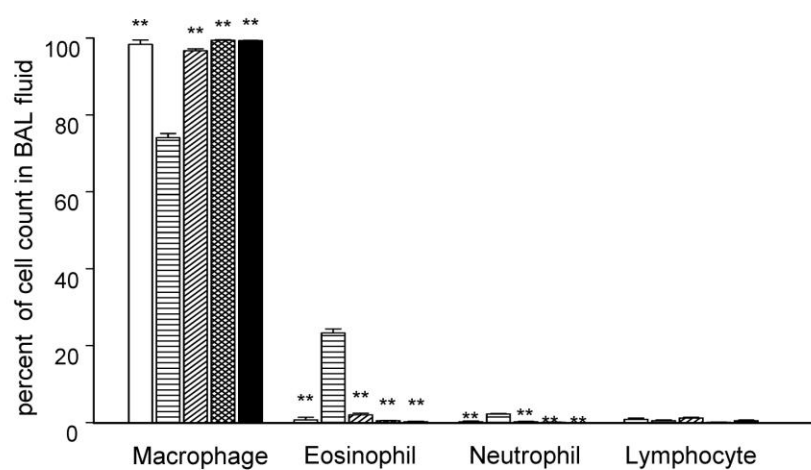


Figure 2

(c)

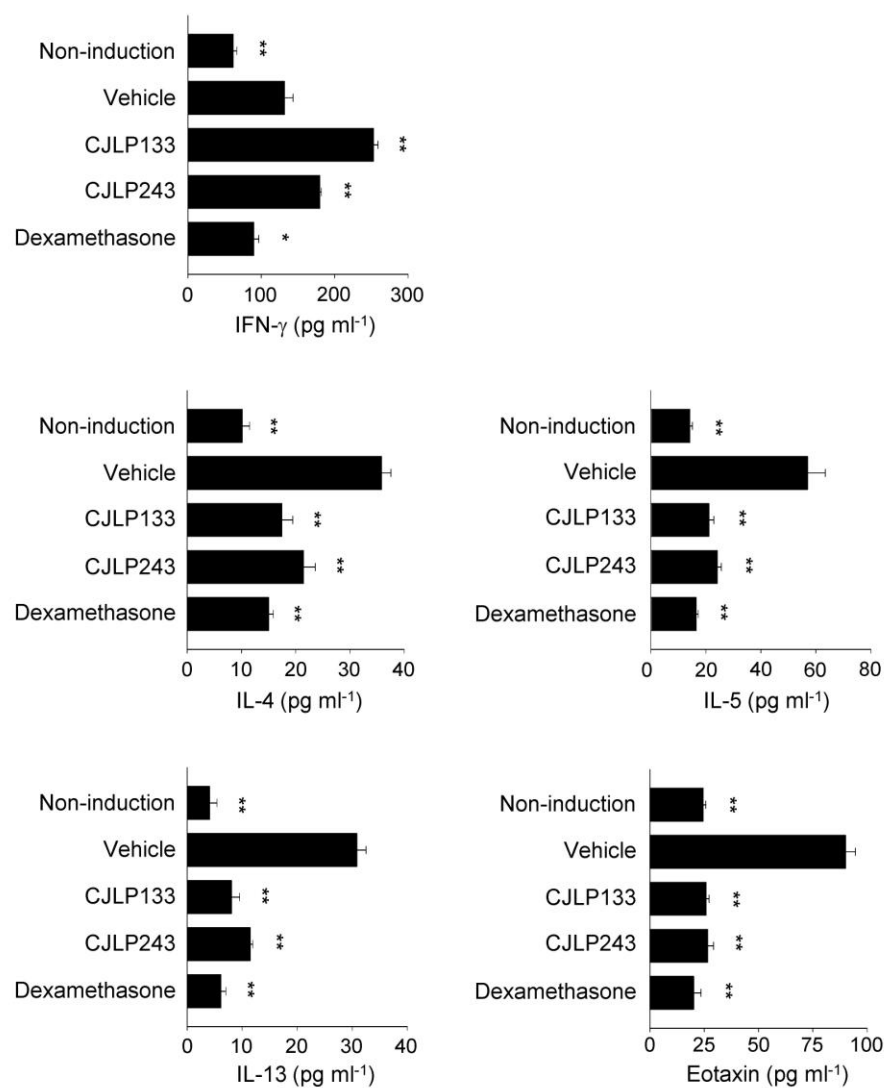


Figure 2^{cont.}

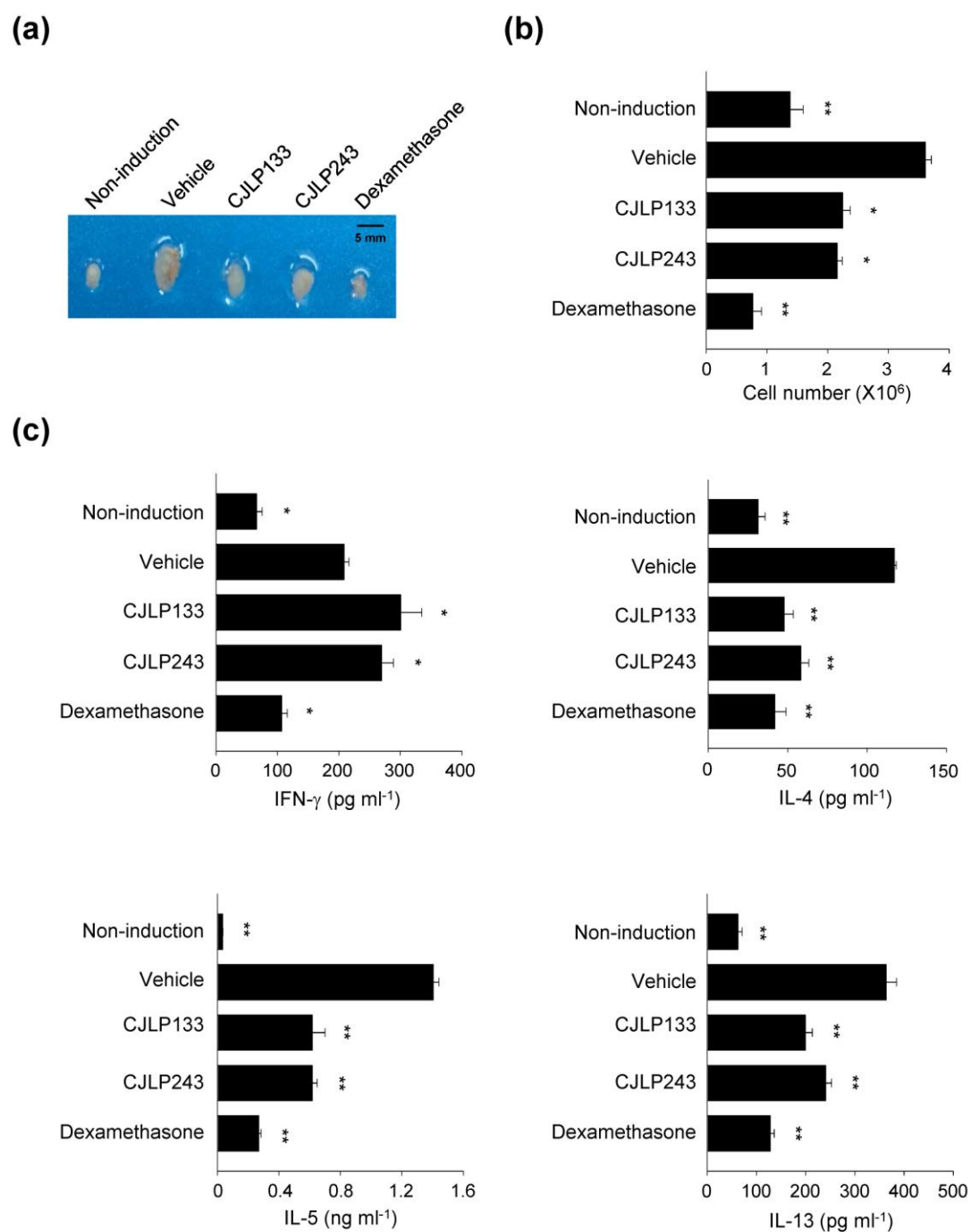
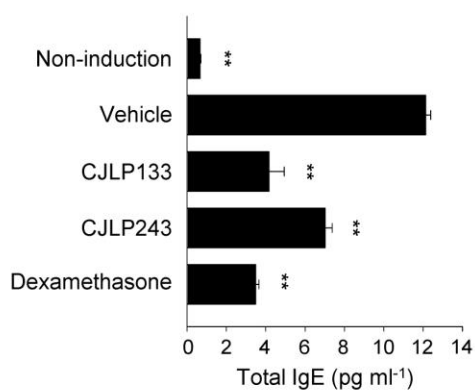
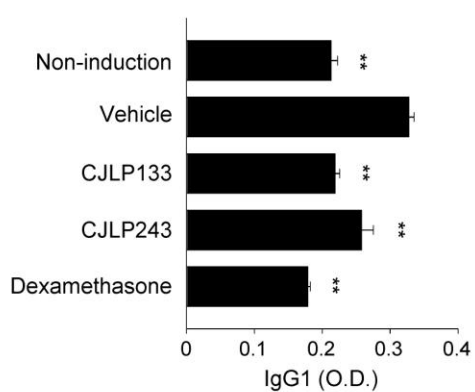


Figure 3

(a)



(b)



(c)

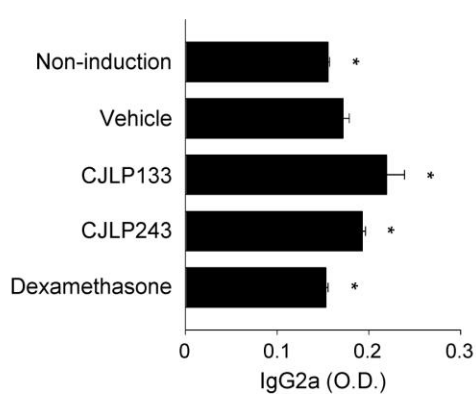


Figure 4