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Anti-inflammatory potential of a heat-killed *Lactobacillus* strain isolated from Kimchi on house dust mite-induced atopic dermatitis in NC/Nga mice

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Running headline: The effect of *L. brevis* NS1401 on atopy

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Abbreviations: AD, Atopic dermatitis; Control, control group of mice; Df body, extract of *Dermatophagoides farinae*; H&E, hematoxylin and eosin; Non-induction, non-AD induced group of mice; PBST, 0.05% Tween[®] in PBS.

Abstract

Aims: Atopic dermatitis (AD) is an allergic skin disease driven by the Th2-prone immune response. Therefore, a fundamental approach to restoring the Th1/Th2 balance is needed to treat AD.

Methods and Results: Eighteen different *Lactobacillus* strains isolated from Kimchi were screened to identify those that stimulated immune cells to secrete Th1-type or Th2-type cytokines. *Lactobacillus brevis* NS1401 induced the greatest IFN- γ and IL-12 secretion and the least IL-4 production among the tested *Lactobacillus* strains. Furthermore, oral administration of heat-killed NS1401 ameliorated the symptoms of dust mite-induced AD in NC/Nga mice by decreasing the serum IgE level and reducing the number of mast cells and eosinophils in lesions. Also, the size and number of cells in the draining lymph nodes of NS1401-administered mice were significantly reduced. In agreement with these results, secretion of a Th1-type cytokine (IFN- γ) and allergen-specific IgG2a were increased, whereas secretion of Th2-type cytokines (IL-4, IL-5, and IL-10) and allergen-specific IgG1 were decreased upon administration of NS1401 in mice.

Conclusions: *Lactobacillus brevis* NS1401 alleviates the symptoms of AD by restoring the Th1/Th2 balance through enhancing Th1-prone immunity.

Significance and Impact of the Study: The immunomodulatory function of *Lactobacillus*

brevis NS1401 may provide effective new therapeutics against AD.

Keywords: atopic dermatitis, house dust mite, Kimchi, *Lactobacillus*, NC/Nga mouse

Introduction

Atopic dermatitis (AD), an inflammatory skin disorder, mainly affects young children, but 25% of individuals with AD continue to suffer from AD until adulthood owing to the disease's chronic and relapsing characteristics (Nutten 2015). AD is driven by a Th2-skewed immune response which induces an elevation in the level of IgE and infiltration of main AD effector cells such as mast cells and eosinophils (Galli and Tsai 2012).

Combination therapy with topical steroids and tacrolimus (FK506) is mainly used to treat AD patients (Liu *et al.* 1991; Auphan *et al.* 1995). However, these therapeutic agents only relieve clinical symptoms and cannot restore the Th1/Th2 balance. Moreover, patients treated with topical steroids suffer from cutaneous side effects such as skin atrophy and irreversible scar-like lesions (Furue *et al.* 2003). Also, systemic steroid therapy in patients

with chronic AD involves more severe adverse effects such as osteoporosis, growth retardation, and an increased infection ratio (Poetker and Reh 2010). Therefore, a new therapeutic approach for AD is needed to rebuild the Th1/Th2 balance by treatment with agents that induce strong Th1 immune responses to suppress Th2 immune responses.

Probiotics are beneficial microorganisms used as food supplements to improve health (Fuller 1991). Recent studies have suggested that probiotics may be an effective therapeutic tool to manage specific human diseases by modulating the overall immune system (Ruemmele *et al.* 2009). For example, the administration of some heat-killed probiotics to AD patients alleviates the symptoms of AD (Mori *et al.* 2011; Yamamoto *et al.* 2016).

In this study, we screened eighteen different *Lactobacillus* strains isolated from Kimchi based on their ability to induce immune cells to produce high amounts of Th1-type cytokines such as IFN- γ and IL-12 *in vitro*. *Lactobacillus brevis* NS1401 was selected as a strong Th1 inducer because NS1401 stimulated immune cells to secrete the greatest quantity of IFN- γ and IL-12 and the least amount of IL-4 among the tested *Lactobacillus*

strains. Furthermore, we demonstrated that oral administration of heat-killed NS1401 alleviated symptoms of house dust mite-induced AD in NC/Nga mice by restoring the Th1/Th2 balance via enhancing IFN- γ secretion.

Materials and methods

Isolation and species level identification of *Lactobacillus* strains

Eighteen different *Lactobacillus* strains were isolated from Kimchi, as previously described (Lee *et al.* 2011). See the Materials and Methods section of Supplementary Material for the details on isolation of *Lactobacillus* strains. To identify each *Lactobacillus* strain at the species level, 16S rDNA was sequenced and aligned using an established DNA database (<http://www.ncbi.nlm.nih.gov/BLAST>) (Lee *et al.* 2011). The identified species and strain names are summarized in Fig 1. Each *Lactobacillus* strain was subcultured more than three times prior to *in vitro* experimental analysis.

Measurement of cytokine secretions by immune cells *in vitro*

Splenocytes (1×10^6 cells well⁻¹) from C57BL/6 mice (OrientBio, Seongnam, Republic of Korea) or 5×10^4 cells well⁻¹ of J774 murine macrophages (ATCC, Manassas, VA, USA) mixed with heat-killed bacteria (1×10^5 CFU ml⁻¹) from each *Lactobacillus* strain were seeded at 96 well plates in 200 μ l of RPMI1640 medium supplemented with 10% (v/v) FBS and 1% penicillin/streptomycin. After three days of incubation, IFN- γ and IL-4 production from splenocytes and IL-12 secretion from macrophages were quantitated by sandwich ELISA kits according to the manufacturer's instructions (eBioscience, Inc., San Diego, CA, USA).

Oral administration of *Lactobacillus brevis* NS1401 after induction of house dust mite-induced AD in NC/Nga mice

Six-week old female NC/Nga mice were purchased from Central Lab Animal Incorporation (Seoul, 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. The induction of house dust mite-mediated AD in NC/Nga mice was performed using Biostir

AD cream containing extract of *Dermatophagoides farina* (Df body) (Central Lab Animal

Incorporation) according to a previously established method (Fig. 2a) (Matsuda *et al.* 2003).

Twenty four NC/Nga mice were then randomly separated into four groups. One group was orally administered 250 μ l of PBS containing heat-killed *Lactobacillus brevis* NS1401 (5×10^8 CFU per mouse) every day for 8 weeks after the induction of AD (Fig. 2a). The dexamethasone-treated group was orally administered 250 μ l of 12.5 μ g dexamethasone in PBS every day for 8 weeks after the induction of AD. The control group was orally administered only 250 μ l of PBS every day for 8 weeks after the induction of AD. We did not induce AD in the remaining group. Mice received proper care in accordance with a protocol approved by the institutional animal care and use committee of Korea University (protocol number: KUIACUC-2015-93).

Measurement of dermatitis score and histological examination of skin samples

The severity of dermatitis on the ears and dorsal skin was evaluated on days 0, 14, 28, 42, and 56 after the first oral administration of *Lactobacillus*. On day 56 after the first oral administration of *Lactobacillus*, dorsal skins between the head and tail of sacrificed mice

were obtained and prepared for histological examination. See the Materials and Methods

section of Supplementary Material for the details on dermatitis score and histological examination of skin samples.

Analysis of sizes and immune cells in draining lymph nodes

On day 56 after the first oral administration of *Lactobacillus*, we obtained four axillary lymph nodes including two proper axillary lymph nodes and two accessory axillary lymph nodes from the fore limbs of sacrificed mice. See the Materials and Methods section of Supplementary Material for the details on analysis of sizes and immune cells in draining lymph nodes

Measurement of cytokine secretions from draining lymph nodes and serum immunoglobulin levels

Single cell suspensions from each draining lymph node (5×10^6 cells well⁻¹) mixed with Df body ($10 \mu\text{g ml}^{-1}$) were seeded at 96 well plates in 200 μl of RPMI1640 medium supplemented with 10% (v/v) FBS and 1% penicillin/streptomycin. Two days after

incubation, IFN- γ , IL-4, IL-5 and IL-10 production were quantitated using sandwich ELISA kits according to the manufacturer's instructions (eBioscience, Inc.).

To measure serum immunoglobulin levels, Blood specimens from experimental mice were drawn from the retro-orbital sinus using a capillary tube on day 56 after the first oral administration of *Lactobacillus*. See the Materials and Methods section of Supplementary Material for the details on measurement of serum immunoglobulin levels.

Statistical analysis

Statistical significance of the experimental data was determined by Student's t-test. Significant differences at a confidence level of 95% and 99% are labeled with asterisks (*) and (**), respectively, on each graph.

Results

Effects of *Lactobacillus* strains isolated from Kimchi on cytokine secretions by immune cells

Eighteen different *Lactobacillus* strains were isolated from Kimchi and identified on the species level by 16S rDNA sequencing (Fig 1). Subsequently, the *Lactobacillus* strains were examined for their ability to stimulate immune cells to produce Th1-type cytokines (IFN- γ and IL-12) or a Th2-type cytokine (IL-4). The results showed that the treatment of all examined bacterial strains increased IFN- γ and IL-12 production in a strain-specific manner (Fig 1). In particular, the NS1401 and L67 strains induced the highest secretion of IFN- γ by splenocytes and the NS1401 and GP1B strains induced the greatest secretion of IL-12 by macrophages (Fig 1). However, most *Lactobacillus* strains induced a low level of IL-4 secretion, except MYA9, MYB5 and GP1B (Fig 1). Since NS1401 treatment induced the highest levels of IFN- γ and IL-12 and the lowest IL-4 production among the tested *Lactobacillus* strains, we selected NS1401 as the most effective Th1 inducer. Therefore, we evaluated the efficacy of NS1401 as a new therapeutic agent to treat AD.

Oral administration of NS1401 reduces skin thickness and infiltration of mast cells and eosinophils in skin lesions of NC/Nga mice with AD-like symptoms

AD-like symptoms in NC/Nga mice were induced by Df body ointment application, as previously described (Fig. 2a) (Matsuda *et al.* 2003). NC/Nga mice were randomly divided into four groups. One group was orally administered heat-killed *Lactobacillus brevis* NS1401 (5×10^8 CFU per mouse) in PBS every day for 8 weeks after the induction of AD (Fig. 2a). An anti-inflammatory agent, dexamethasone, in PBS was given orally to another group of mice every day for 8 weeks after the induction of AD. The control group received PBS orally every day for 8 weeks after the induction of AD. AD was not induced in the remaining fourth group of mice. During the administration of PBS, NS1401, or dexamethasone in Df body-treated mice, dermatitis score was measured at one-week intervals until eight weeks after the first administration. All Df body-treated mice developed severe dermatitis on their back and ears before administration of NS1401 or dexamethasone (Fig. 2b). The dermatitis score of controls diminished slightly, but still maintained a certain level of severity until day 56 (Fig. 2b,c). Mice fed NS1401 or dexamethasone exhibited significant improvement until day 56 (Fig. 2b,c).

After eight weeks of oral administration, all experimental mice were sacrificed and dorsal skin was obtained in order to analyze histological aspects including skin thickness, and infiltration of mast cells and eosinophils. Control mice exhibited the thickest skin layer, which was over ten times thicker than that of the non-induction group (Fig. 3a). Ingestion of NS1401 as well as dexamethasone ameliorated the increase in skin thickness induced by Df body treatment (Fig. 3a). We also stained samples of all experimental mice with toluidine blue and Congo red to detect mast cells and eosinophils in skin lesions, respectively.

Consistent with results of the skin thickness assays, oral administration of NS1401 and dexamethasone to Df body-treated mice significantly reduced the number of mast cells and eosinophils in skin lesions compared with controls (Fig. 3b,c). Taken together, these data indicate a critical role of NS1401 in reducing skin thickness and effector cell infiltration in the inflamed skin of Df body-treated mice.

Oral administration of NS1401 diminishes the size and number of immune cells in draining lymph nodes of NC/Nga mice with AD-like symptoms

We then examined the size and number of cells of the axillary lymph nodes (draining lymph nodes), which are proximal to the dorsal skin and participate in the pathogenesis of

AD in NC/Nga mice. In control mice, we detected a pronounced enlargement of axillary lymph nodes, two times greater than in the non-induction group (Fig. 4a). NS1401 administration significantly ameliorated enlargement of the lymph nodes (Fig. 4a). In the dexamethasone-treated group, the size of the lymph nodes was similar to that in the non-induction group (Fig. 4a). The total number of cells of the axillary lymph nodes in controls also increased over two-fold compared with that in non-induced mice (Fig. 4b). Mice treated with NS1401 exhibited a statistically significant reduction in total cell number in the axillary lymph nodes (Fig. 4b). The total number of cells in the axillary lymph nodes in the dexamethasone-treated group was slightly decreased compared to that in the non-induction group owing to immune suppression (Fig. 4b). Absolute T cell and B cell numbers were also determined in experimental animals because those cells are the major immune cells which play important roles in AD pathogenesis. The NS1401-treated group as well as the dexamethasone-treated group had significantly diminished T cell and B cell numbers in axillary lymph nodes (Fig. 4c,d). Overall, these results indicate that oral administration of NS1401 significantly decreased the size and the number of immune cells in the draining lymph nodes of AD-induced mice.

Effect of NS1401 on cytokine production in draining lymph nodes of NC/Nga mice with AD-like symptoms

To examine allergen-specific T cell responses in NC/Nga mice with AD-like symptoms, we measured production of a Th1-type cytokine (IFN- γ) as well as Th2-type cytokines (IL-4, IL-5, and IL-10) after stimulation of draining lymph node cells with Df body. As shown in Fig. 5a, Th1-type cytokine (IFN- γ) production in draining lymph node cells in the NS1401-treated group was significantly increased compared with that in the control group. However, secretion of Th2-type cytokines (IL-4, IL-5, and IL-10) in the NS1401-treated group was significantly decreased compared with that in the control group (Fig. 5b,c,d). Both Th1-type and Th2-type cytokine production were severely decreased in the dexamethasone-treated group due to immune suppression (Fig. 5a,b,c,d). These results indicated that administration of NS1401 rebalances Th1/Th2 ratio by increasing the Th1-type immune response in Th2-prone AD-like syndrome.

Oral administration of NS1401 decreases total IgE and Df body-specific IgG1 levels, but increases Df body-specific IgG2a levels in sera of NC/Nga mice with AD-like symptoms

To systemically confirm the immunomodulatory effect of NS1401 administration, we measured serum levels of IgE from sacrificed mice. As shown in Fig. 5e, controls showed the greatest increase in production of IgE upon Df body ointment application. Oral administration of NS1401 as well as dexamethasone significantly decreased the production of IgE compared with that in controls (Fig. 5e). Next, we evaluated the effect of NS1401 administration on allergen-specific immunoglobulin responses in NC/Nga mice with AD-like symptoms. Compared with controls, the Df-specific IgG1 (Th2 type immunoglobulin) level was significantly reduced in NS1401-treated mice (Fig. 5f). However, the Df body-specific IgG2a (Th1-type immunoglobulin) level in NS1401-treated mice was significantly increased compared with that in controls (Fig. 5g). The dexamethasone-treated group showed decreased levels of both Df body-specific IgG1 and IgG2a compared with control mice, since this reagent has an immunosuppressive effect (Fig. 5f,g). The combined results strongly suggest that oral administration of NS1401 decreases the levels of IgE and IgG1, but increases the IgG2a level in sera of NC/Nga mice with AD-like symptoms by recovering the balance between Th1 and Th2 responses.

Discussion

In this study, we conducted several experiments to elucidate whether *Lactobacillus brevis* NS1401 has potential therapeutic effects in AD. Since AD is a Th2-prone disease, we initially screened several *Lactobacillus* strains based on the ability to stimulate strong Th1-type immune responses that could rebalance the Th1/Th2 ratio by inducing Th1-type cytokines (Ashraf and Shah 2014). As a result, we selected NS1401 as the most effective Th1 inducer since NS1401 treatment caused the highest amount of Th1-type cytokine (IFN- γ and IL-12) secretion and the lowest Th2-type cytokine (IL-4) production among the tested *Lactobacillus* strains.

After initial screening, we tested whether NS1401 administration alleviates AD-like symptoms in NC/Nga mice induced by Df body ointment application. Prior to oral administration of NS1401, 21 days of Df body ointment application in NC/Nga mice induced AD-like symptoms in all experimental mice. After feeding of NS1401, mice showed significant relief of AD-like symptoms. In fact, the dermatitis score and skin thickness were significantly reduced in NS1401-treated mice, compared with control mice.

To mimic AD-like symptoms in this animal model, dorsal skin inflammation was induced by Df body ointment application. Axillary lymph nodes acted as draining lymph nodes, which may modulate dorsal skin inflammation. Oral administration of NS1401 reduced the size of the axillary lymph nodes and the number of effector T and B cells in the axillary lymph nodes. These results may indicate that Th1 immune responses induced by NS1401 administration can inhibit Th2-prone effector cells. In support of this idea, oral administration of NS1401 produced a significant reduction in the number of mast cells and eosinophils in skin lesions compared with those in controls. However, keratinocytes also play an important role in developing inflammatory response in atopic dermatitis patients (Brunner *et al.* 2017). Therefore, measurement of cytokine secretions in skin tissue has to be evaluated for more precise examination of skin inflammation.

The infiltrated mast cells and eosinophils in skin regions of mice with AD are the major effector cells responsible for severe inflammation (Galli and Tsai 2012). When allergens interact with IgE bound to mast cells, the mast cells are activated and release inflammatory mediators, such as histamine, serine proteinases, and eicosanoid mediators (Liu *et al.* 2011). IL-4, a Th2-type cytokine, amplifies IgE-induced mast cell degranulation by inducing FcεRI

expression on the surface of mast cells (Ozdemir *et al.* 2010). Moreover, IL-4 in inflamed tissue stimulates recruitment of eosinophils, which worsens skin lesions by releasing cytotoxic proteins (Ozdemir *et al.* 2010). NS1401 administration in mice resulted in a reduction in mast cell infiltration along with decreased IgE and IL-4 production. IL-5, another Th2-type cytokine, also contributes to an increased number of eosinophils in skin lesions by promoting eosinophil production in bone marrow (Modena *et al.* 2016; Tan *et al.* 2016). The NS1401-treated group exhibited less IL-5 production, followed by decreased infiltration of eosinophils. IL-10, also a Th2-type cytokine, stimulates mast cell growth and inhibits Th1-type immune responses (Thompson-Snipes *et al.* 1991; Ohmen *et al.* 1995). Th1-prone responses induced by NS1401 treatment decreased IL-10 production. Therefore, NS1401 administration may reduce the infiltration of AD-inducing immune cells by enhancing the Th1-prone immune response.

Th1 cells typically produce IFN- γ , which enhances cell-mediated immunity. However, Th1-type cells can cause an immunoglobulin switch to IgG2a by modulating B cell activity (Garraud *et al.* 2003). The typical cytokine produced by Th2 cells is IL-4, which promotes B cell-mediated immunity and induces a switch to IgG1/IgE production (Garraud *et al.* 2003).

IL-10 is another Th2-type cytokine which induces IgG1 (Garraud *et al.* 2003). Oral administration of NS1401 diminished the production of total IgE and allergen (Df body)-specific IgG1, whereas it led to increased production of allergen (Df body)-specific IgG2a. These lines of evidence further support the idea that heat-killed NS1401 administration enhances the Th1 immune response and alleviates the symptoms of AD by rebalancing the Th1/Th2 immune response. In agreement with our observation, previous clinical trials revealed that oral feeding some heat-killed probiotics to AD patients improved the symptoms of AD (Mori *et al.* 2011; Yamamoto *et al.* 2016). In terms of safety and stability, heat-killed probiotics has an advantage over live probiotics. Therefore, food application of heat-killed probiotics could have broader than that of live probiotics. Results from this study may provide useful information to develop dried food additive which alleviates the symptoms of AD. Clinical studies will be required to further exam the efficacy of NS1401 in AD.

Acknowledgement

This work was supported by the Nong Shim Corporation, Republic of Korea.

Conflict of Interest

H. J. Lee, J. H. Kim, S. H. Park and H. J. Kim are currently employed by Nong Shim Corporation, Republic of Korea. None of the other authors had any conflict of interest.

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Figure legends

Figure 1 Screening of *Lactobacillus* strains isolated from Kimchi based on the ability to induce cytokine secretions by immune cells. Splenocytes (1×10^6 cells well⁻¹) from C57BL/6 mice or J774 murine macrophages (5×10^4 cells well⁻¹) were co-cultured with each strain of heat-killed bacteria (1×10^5 CFU ml⁻¹). ELISAs were performed to detect IFN- γ and IL-4 secretions from splenocytes and IL-12 secretion from macrophages. Data shown are the mean $\pm$ SEM from three independent experiments.

Figure 2 Experimental schedule and effects of *Lactobacillus* NS1401 on dermatitis lesions of Df body induced AD in NC/Nga mice. (a) Experimental schedule of Df body-induced AD in NC/Nga mice. ▼ Df body ointment application; Day 0, first administration of NS1401 or

dexamethasone. (b) Dermatitis scores were evaluated after administration of NS1401 or

dexamethasone. Mouse groups: (□) Non-induction, (●) Control, (○) NS1401, (■)

Dexamethasone. The data shown are means $\pm$ SEM ($n = 6$). Significant differences compared

with the control group are represented as * $P < 0.05$ or ** $P < 0.01$. (c) Severity of dermatitis

was assessed on dorsal skin samples of NC/Nga mice on day 56 after administration. The

results shown are representative of each group of mice ($n = 6$). Non-induction, non-AD

induced group of mice; Control, control group of mice that orally received PBS; NS1401,

mice that orally received NS1401 in PBS; Dexamethasone, mice that orally received

dexamethasone in PBS.

Figure 3 Oral administration of NS1401 ameliorates skin swelling and infiltration of mast

cells and eosinophils into inflamed skin. (a) After H&E staining, skin thickness was measured

in samples (0.025 mm^2) under a microscope at $\times 200$ magnification. (b) After Toluidine blue

staining, the number of mast cells in samples (0.025 mm^2) was counted under a microscope

at $\times 200$ magnification. (c) After Congo red staining, the number of eosinophils in the

samples (0.025 mm^2) was quantified under a microscope at $\times 400$ magnification. The results

shown in the *left* panels are representative of each group of mice ($n = 6$). Data shown in the

right panels are means $\pm$ SEM ($n = 6$). Non-induction, non-AD induced group of mice;

Control, control group of mice that orally received PBS; NS1401, mice that orally received

NS1401 in PBS; Dexamethasone, mice that orally received dexamethasone in PBS.

Significant differences compared with the control group are represented as * $P < 0.05$ or **

$P < 0.01$.

Figure 4 Oral administration of NS1401 decreases the size and absolute cell number of

draining lymph nodes after AD induction by Df body treatment in NC/Nga mice. (a) The size

of the proper axillary lymph nodes and accessory axillary lymph nodes was assessed in each

group of experimental mice. Results shown in the *left* panel are representative of each

group of mice ($n = 6$). Data shown in the *right* panel are means $\pm$ SEM ($n = 6$). (b) The

absolute cell numbers of the axillary lymph nodes from experimental mice. Data shown are

means $\pm$ SEM ($n = 6$). (c) The absolute T cell numbers of axillary lymph nodes from

experimental mice. Single cell suspensions from each lymph node were stained with FITC-

conjugated anti-TCR β antibody. After staining, cells were analyzed by flow cytometry. Data

shown are means $\pm$ SEM ($n = 6$). (d) Absolute B cell numbers of axillary lymph nodes from

experimental mice. Single cell suspensions from each lymph node were stained with PE-

conjugated anti-B220 antibody. After staining, cells were analyzed by flow cytometry. Data shown are means $\pm$ SEM ($n = 6$). Non-induction, non-AD induced group of mice; Control, control group of mice that orally received PBS; NS1401, mice that orally received NS1401 in PBS; Dexamethasone, mice that orally received dexamethasone in PBS. Significant differences compared with controls are represented as * $P < 0.05$ or ** $P < 0.01$.

Figure 5 Oral administration of NS1401 regulates the production of Th1/Th2 cytokines in draining lymph nodes and modulates the levels of different immunoglobulin isotypes in sera on NC/Nga mice after AD induction. Experimental mice were sacrificed on day 56 after administration. Single cell suspensions from each draining lymph node (5×10^6 cells well^{-1}) were stimulated with Df body ($10 \mu\text{g ml}^{-1}$). Two days after stimulation, production of IFN- γ (a), IL-4 (b), IL-5 (c), and IL-10 (d) were measured by ELISA. Also, ELISAs were performed to detect serum levels of total IgE (e), Df body-specific IgG1 (f), and Df body-specific IgG2a (g) from each group of experimental mice. Data shown are means $\pm$ SEM ($n = 6$). Non-induction, non-AD induced group of mice; Control, control group of mice that orally received PBS; NS1401, mice that orally received NS1401 in PBS; Dexamethasone, mice that orally received dexamethasone in PBS. Significant differences compared to controls are represented as * $P < 0.05$ or ** $P < 0.01$.

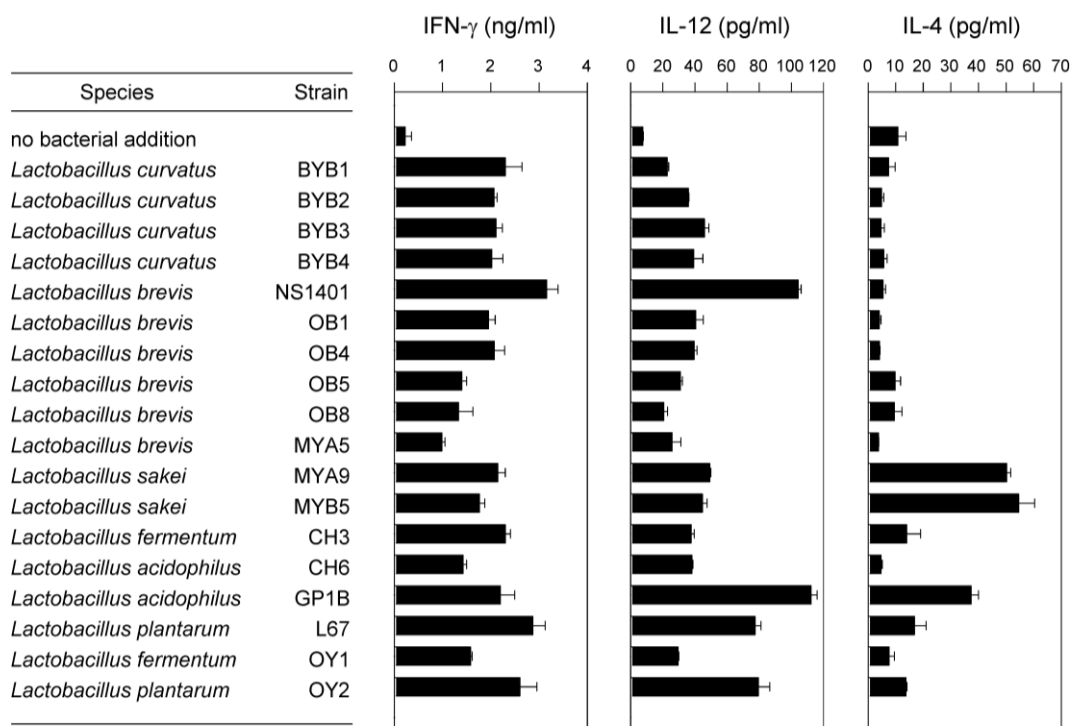
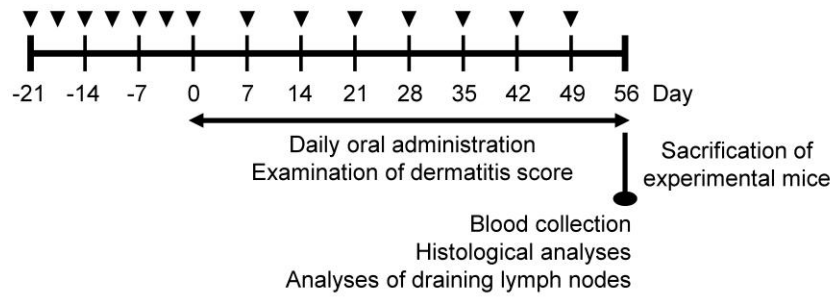
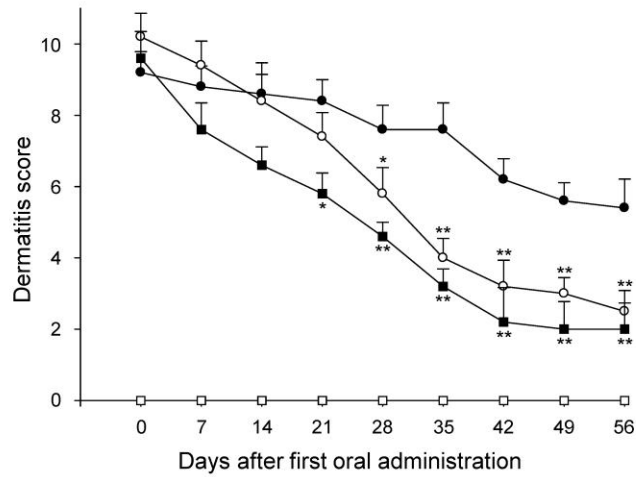


Figure 1

(a)



(b)



(c)

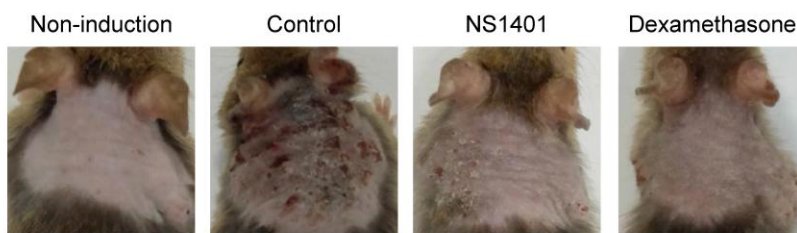
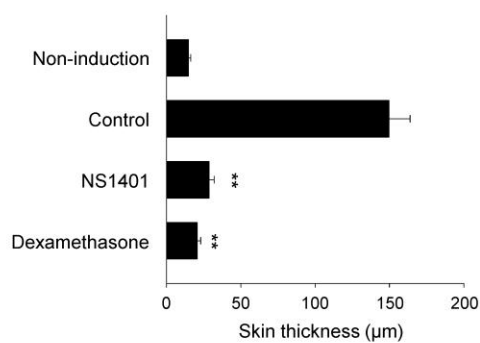
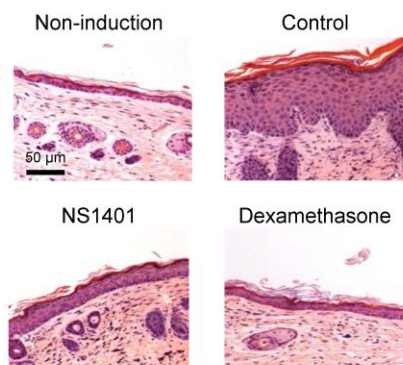
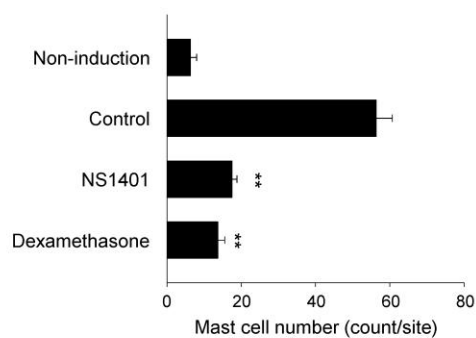
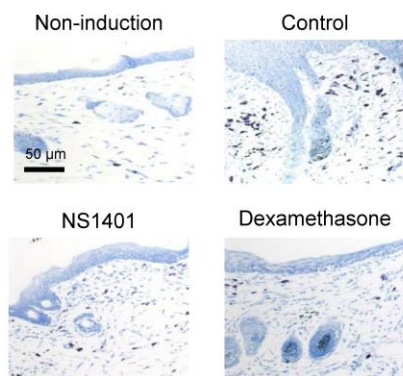


Figure 2

(a)



(b)



(c)

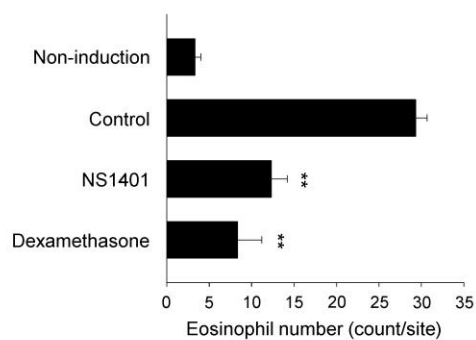
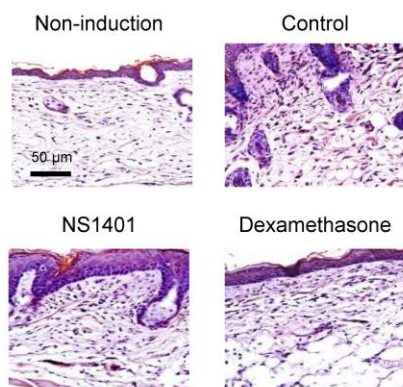
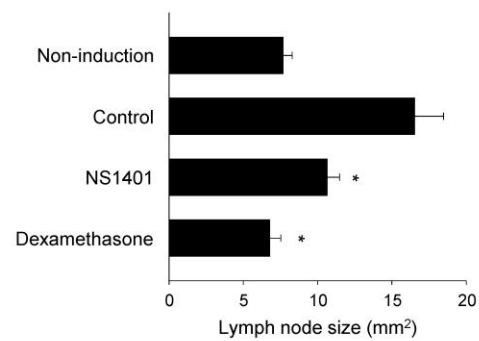
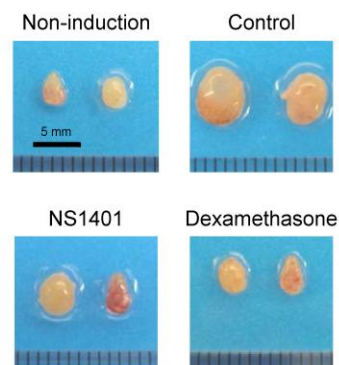
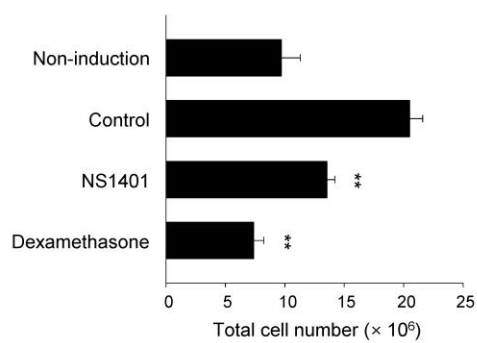


Figure 3

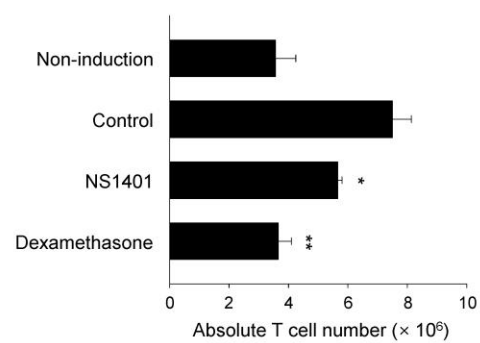
(a)



(b)



(c)



(d)

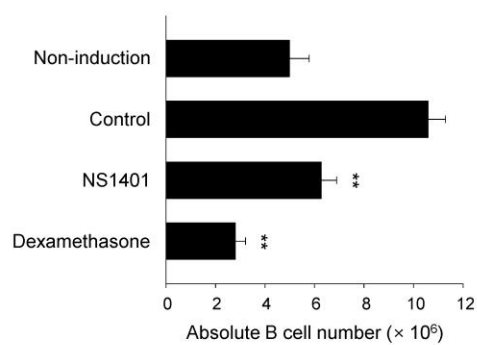


Figure 4

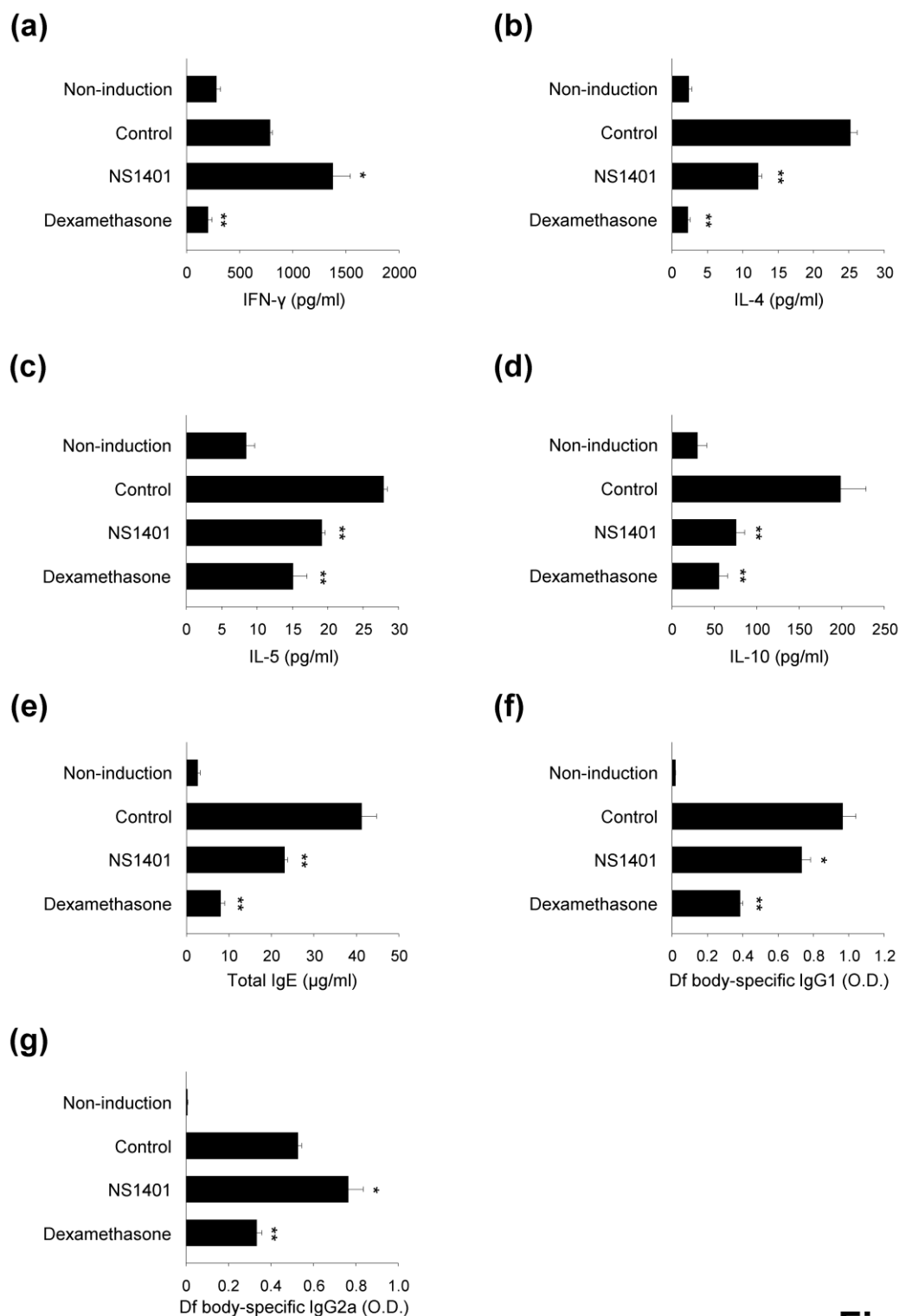


Figure 5