

Primary administration of *Lactobacillus johnsonii* NCC533 in weaning period suppresses the elevation of proinflammatory cytokines and CD86 gene expressions in skin lesions in NC/Nga mice

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Abstract

The administration of probiotic lactic acid bacteria (LAB) has been studied for its potential to prevent atopic dermatitis (AD). The objective of this study was to assess the inhibitory mechanism of a skin lesion by LAB using an experimental model that we previously demonstrated in NC/Nga mice. *Lactobacillus johnsonii* NCC533 (La1) was administered orally to the La1 group from 20 to 22 days after birth, while phosphate-buffered saline was given to the control group. After the induction of skin lesions in 6-week-old mice, the expression of genes supposedly involved in AD was evaluated. Gene expression of the proinflammatory cytokines [interleukin-8 (IL-8), IL-12 and IL-23] was significantly enhanced in the lesional skin of the control group by the induction of the lesion, whereas gene expression of those in the La1 group was not elevated. Interestingly, expression of the costimulatory molecule CD86 showed a pattern similar to the expression of the cytokines in the lesional skin. Moreover, the La1 group showed a significantly lower gene expression of CD86 in Peyer's patches and mesenteric lymph nodes than the control group. The suppression of proinflammatory cytokines and CD86 by primary administration of La1 may significantly contribute to the inhibitory effect on the skin lesion.

Introduction

Atopic dermatitis (AD) is a highly pruritic chronic inflammatory skin disease with a wide variety of clinical manifestations. Regardless of a patient's age, mild to severe erythema, scaling, haemorrhage and excoriation with severe itch are generally present (Leung & Bieber, 2003). Interest in this disease has been sparked by reports of its increasing prevalence and significant adverse effects on quality of life.

At least two types of AD have been identified. The first, which affects 70–80% of patients, is extrinsic and is associated with immunoglobulin E (IgE)-mediated sensitization; the second, affecting 20–30% of patients, is intrinsic without IgE-mediated sensitization (Johansson *et al.*, 2001). Evidence suggests that various factors, including immunological abnormalities (Leung & Bieber, 2003), barrier dysfunction of the skin (Werner & Lindberg, 1985), exposure to allergens such as mites (Platts-Mills *et al.*, 1983) and environmental factors (Adinoff *et al.*, 1988), contribute to the pathogenesis and development of this disease.

It was first suggested by Isolauri *et al.* (2000) that the oral administration of lactic acid bacteria (LAB) could inhibit and/or prevent the development of AD in human infants. Several clinical studies supported this beneficial effect of probiotic bacteria on AD (Kalliomaki *et al.*, 2001, 2003; Rosenfeldt *et al.*, 2003; Viljanen *et al.*, 2005; Weston *et al.*, 2005). However, more studies will be required to ensure the potency of the oral administration of probiotics on AD, as Brouwer *et al.* (2006) reported that no beneficial effect was observed after probiotic administration to AD infants in their study.

The NC/Nga mouse was originally established as an inbred strain in 1957 (Kondo *et al.*, 1969; Festing, 1996) and is now used as a useful model for AD research. It develops human AD-like skin lesions mediated by elevated levels of serum IgE under conventional circumstances (Matsuda *et al.*, 1997) or as a result of the application of a mite antigen (Matsuoka *et al.*, 2003). In fact, this mouse model has been used for the evaluation of the preventive effects of functional foods, such as astragaloside, royal jelly and konjac

glucomannan, on AD (Kotani *et al.*, 2000; Taniguchi *et al.*, 2003; Onishi *et al.*, 2005).

This mouse model was previously used to demonstrate that the administration of *Lactobacillus johnsonii* strain NCC533 (La1) at the time of weaning can significantly inhibit the development of AD-like lesions (Inoue *et al.*, 2007). Our findings indicated that La1 definitely possessed the ability to inhibit the development of AD, although the timing of probiotic administration is a key to efficient immunomodulation.

The objective of this study was to assess the mechanism involved in the inhibitory effect on AD-like lesions by the primary administration of La1. The expression of the genes supposedly involved in the development of AD was analysed in skin and gut-associated lymph tissues using real-time RT-PCR to clarify the mechanism contributing to lesion inhibition.

Materials and methods

Mice and administration of La1

Four pregnant NC/Nga mice were obtained from a commercial supplier (Japan SLC, Shizuoka, Japan). Pups delivered from these mice were used in this experiment. They were divided into three groups and treated as follows.

La1 group ($n=9$): La1 was cultured for 20 h at 37 °C with 5 mL of anaerobic GAM broth (Nissui, Tokyo, Japan) supplemented with 1% glucose. The bacteria were collected by centrifugation (10 000 g, 4 °C) and suspended in phosphate-buffered saline (PBS). The suspension containing 10^{10} cells of La1 was orally administered from 20 to 22 days of age. The administration and La1 preparation were performed as described in Inoue *et al.* (2007).

Control group ($n=8$) and nontreated negative control (NT) group ($n=5$): PBS was orally administered during the same period as the La1 group. The mice were housed and reared as described in Inoue *et al.* (2007).

The experiment was conducted in accordance with the guidelines for studies with laboratory animals of the Kyoto Prefectural University Experimental Animal Committee.

Induction of human AD-like lesions

Human AD-like lesions were induced in 6-week-old mice. Mite antigen (Mite Extract *Dermatophagoides farinae*, LSL, Tokyo, Japan) diluted with PBS was applied three times weekly onto the back of the mice in the La1 and control groups, as described in Inoue *et al.* (2007). As a negative control, PBS was applied to the NT group. The severity of skin lesions was scored as described in Inoue *et al.* (2007).

Dissection and sample collection

The application of the mite antigen was carried out for 5 weeks (15 applications in total), as in the study by Inoue *et al.* (2007). At the time for the 16th application, the mice were dissected, and no mite antigen was applied.

Each mouse was anaesthetized with an intraperitoneal injection of 50 µL of pentobarbital sodium (Schering-Plough, Osaka, Japan). The antigen-applied region was photographed using a Nikon Coolpix 990 (Nikon, Tokyo, Japan). The skin from that region was then collected and immersed in RNAlater (Ambion, Tokyo, Japan) for RNA preservation.

All visible Peyer's patches (PPs) and mesenteric lymph nodes (MLNs) were collected and immersed in RNAlater. The samples in RNAlater were stored at -80 °C until use.

RNA extraction and cDNA synthesis

All samples were washed with diethylpyrocarbonate-treated water prior to RNA extraction to remove the remaining RNAlater.

The antigen-applied skin (1.5×1.5 cm) was ground in liquid nitrogen with a pestle and mortar and suspended with a Denaturation Solution of Totally RNA kit (Ambion, Tokyo, Japan). Total RNA was then extracted with a Totally RNA kit according to the manufacturer's instructions. The extracted total RNA was treated with DNase and further purified with the RNeasy Mini Kit and RNase-free DNase Set (Qiagen, Tokyo, Japan) according to the manufacturer's instructions.

MLNs were minced with a Biomasher (Hitec, Osaka, Japan) and RNA extraction and DNase treatment were performed with the RNeasy Mini Kit and RNase Free DNase Set according to the manufacturer's instructions.

Total RNA was extracted from PPs in the same manner as for MLNs, but PP tissue was minced by bead beating for 45 s.

From the total RNA (250 ng), cDNA was synthesized using ReverTraAce- α (TOYOBO, Osaka, Japan) according to the manufacturer's instructions using the Oligo (dT)20 primer.

Real-time PCR

Real-time PCR was performed using a light cycler system (Roche Applied Science, Tokyo, Japan). The expression of 12 genes (Table 1) including a housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), was evaluated in skin samples, and five of them [CD80, CD86, CD28, Cyto-toxic T-lymphocyte antigen 4 (CTLA-4) and GAPDH] were evaluated in the samples from PPs and MLNs. Amplification was carried out in a 20-µL reaction volume containing 2 µL of LightCycler FastStart DNA Master SYBR Green I (Roche Applied Science, Tokyo, Japan), 2.4 µL of 2.5 mM MgCl₂, 0.4 µL cDNA and 0.4 µM of each primer. The thermal cycling profile was 10 min at 95 °C followed by 45 cycles of 15 s at 95 °C, 5 s at appropriate annealing

Table 1. Genes evaluated and their primer set in this study

Gene name	Primer 5'–3'	Annealing temperature (°C)	Reference
GAPDH	F- CCACCCAGAAGACTGTGGAT R- CACATTGGGGTAGGAACAC	62	Dieterich <i>et al.</i> (2000) Blavier <i>et al.</i> (2006)
INF- γ	F- ATCTGGAGGAACTGGCAAAA R- TGAGCTCATTGAATGCTTGG	58	This study Schroppel <i>et al.</i> (2004)
TGF- β	F- TGACGTCACTGGAGTTGTACGG R- GGTCATGTCATGGATGGTGC	62	Choi <i>et al.</i> (2003) This study
IL-8	F- ATGGCTGGGATTCACCTCAA R- AAGCCTCGCGACCATCTT	58	Heishi <i>et al.</i> (2003)
IL-10	F- TGCTATGCTGCCTGCTCTTA R- TCATTTCCGATAAGGCTTGG	58	This study
IL-12 p40	F- AGGTGCGTTCCTCGTAGAGA R- AAAGCCAACCAAGCAGAAGA	58	Nishihori <i>et al.</i> (2000)
IL-18	F- GAGGAAATGGATCCACCTGA R- ACAAACCTCCCCACCTAAC	60	This study Van Der Sluijs <i>et al.</i> (2005)
IL-23 p19	F- AATAATGTGCCCCGTATCCA R- CTGGAGGAGTTGGCTGAGTC	58	Leithauser <i>et al.</i> (2006) Becker <i>et al.</i> (2003)
CD80	F- ATGCTCACGTGCAGAGGA R- GACGGTCTGTTACGCTAATG	60	Serebrisky <i>et al.</i> (2000)
CD86	F- CAACTGGACTCTACGACTTC R- TGCTTAGACGTGCAGGTCAA	60	Serebrisky <i>et al.</i> (2000)
CD28	F- CTCTGGAATCTGCACGTCAA R- AACAGGACTCCAGCAACCAC	60	This study
CTLA-4 (CD152)	F- ACAGGTGACCCAACCTTCAG R- CAGTCCTGGATGGTGAGGT	62	This study

temperature (Table 1) and 15 s at 72 °C. Melting curves were generated for each sample to verify the specificity of the amplicons. For a standard curve preparation, a high-concentration mouse cDNA, previously obtained in our laboratory from a healthy BALB/c mouse, was serially diluted and included in every run. The arbitrary gene expression unit for each sample was calculated from the standard curve and normalized with the unit for GAPDH.

Statistical analysis

One-way ANOVA was used to analyse the differences among the means of experimental groups with regard to gene expression. The statistical analysis was performed with the arbitrary gene expression unit normalized by GAPDH. When a parameter of any of the experimental groups was significant, Scheffé's *posthoc* comparison was used. The difference among means was considered significant at $P < 0.05$. All data were analysed using Statcel (OMS, Saitama, Japan), which is an add-in application for Microsoft Excel (Seattle, WA USA).

Results

Skin lesion

Figure 1 shows the appearance of the antigen-applied region in three representative individuals from each experimental group. As in a previous study (Inoue *et al.*, 2007), the

administration of La1 at the age of 20–22 days remarkably inhibited the development of AD-like lesions. The skin scores in the control group were significantly higher than those in the La1 group from the 11th application to the end of the experimental period, except for the day of the 12th application (Fig. 2). The skin scores in the La1 and NT groups remained low or zero throughout the experimental period.

Gene expression in antigen-applied skin

The control group showed a significantly higher gene expression of interleukin-8 (IL-8), IL-10, IL-12 and IL-23 than the La1 and NT groups ($P < 0.05$; Fig. 3). The control group expressed approximately ninefold higher levels of IL-8 gene compared with the NT group while the La1 group showed comparable levels of IL-8 gene expression with the NT group. The La1 group showed about twofold higher expression of IL-12 and IL-23 than the NT group, but the control group showed a much higher expression of both genes. Gene expression levels of IL-12 and IL-23 were six and 4.3-fold higher in the control group than in the NT group, respectively. The IL-10 gene was expressed at a more than 10-fold higher level in the control group than in the NT group, whereas it was expressed at a 3.5-fold higher level in the La1 group than in the NT group.

Gene expression of interferon- γ (IFN- γ), transforming growth factor- β (TGF- β and IL-18) was not significantly different among the experimental groups.

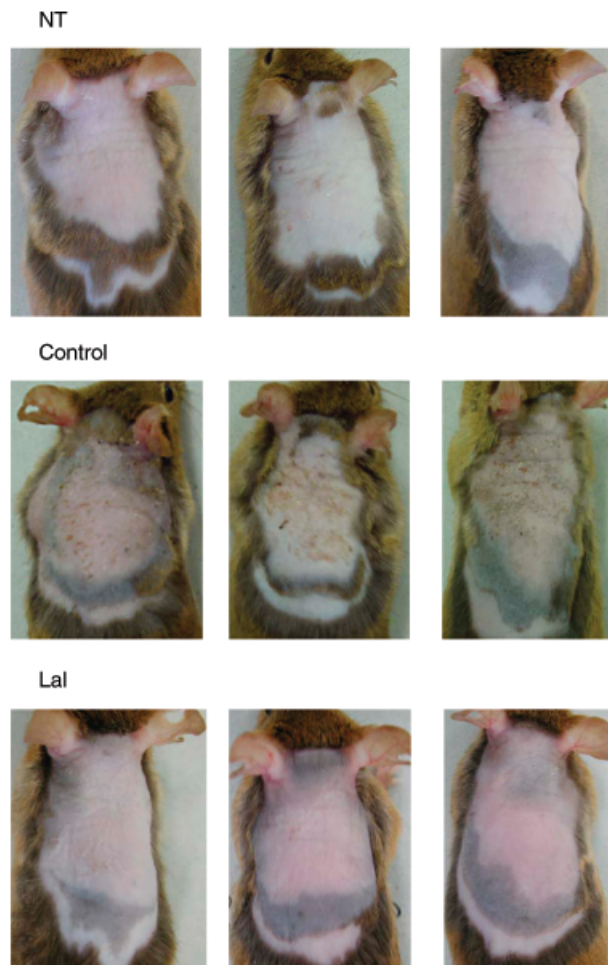


Fig. 1. Skin features of the antigen-applied region in NC/Nga mice after 15 antigen applications in each group. Control, mice were administered PBS from 20 to 22 days after birth; La1, mice were administered La1 at the same period as controls; NT, mice were administered PBS at the same period as controls without any subsequent mite antigen applications.

Gene expressions of CD86 and CTLA-4 in the control group were significantly higher than those in the La1 and NT groups ($P < 0.05$; Fig. 4). CD86 and CTLA-4 genes were expressed at three and 6.5-fold higher levels in the control group than the NT group. The La1 group showed a comparable level of CD86 and CTLA-4 gene expression with the NT group. The expression level of CD80 was significantly higher in the control group than in the La1 and NT groups; however, the difference in the expression level of this gene among the experimental groups was relatively small compared with that of CD86 and CTLA-4 (c. 1.8-fold higher in the control group than in the La1 and NT groups).

There was no significant difference in CD28 gene expression among the experimental groups.

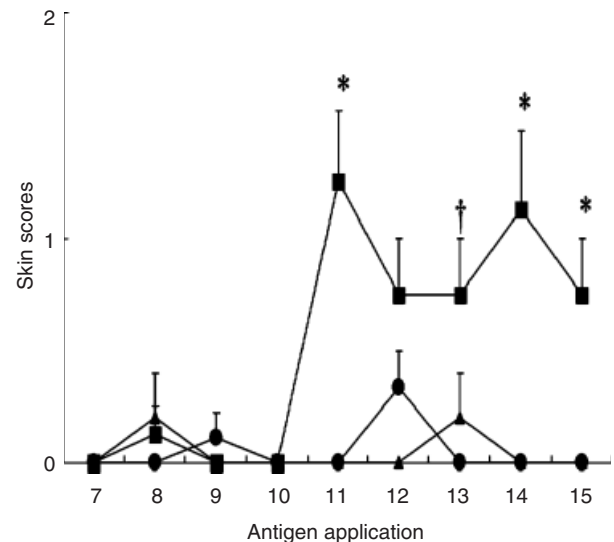


Fig. 2. Skin scores of NC/Nga mice from the seventh to the 15th mite antigen application. Control, mice were administered PBS from 20 to 22 days after birth (■); La1, mice were administered La1 at the same period as controls (●); NT, mice were administered PBS at the same period as controls without any subsequent mite antigen applications (▲). Data are shown as means, and bars represent the SE. * $P < 0.05$, control vs. La1 and NT; † $P < 0.05$, control vs. La1.

Gene expression of CD80, CD86, CD28 and CTLA-4 in PPs and MLNs

PPs and MLNs showed similar expression patterns with the genes evaluated in this study (Fig. 5). In both organs, the control and NT groups showed significantly higher gene expression of CD86 than the La1 group ($P < 0.05$). The La1 group showed lower gene expression of CD80 and CTLA-4 than the other experimental groups in PPs, but the difference between the control and La1 groups was not significant. In MLN, CTLA-4 gene expression was significantly lower in the La1 group than the other two experimental groups ($P < 0.05$). No significant difference was found in the CD80 gene expression level in MLN among the experimental groups. There were no significant differences in the gene expression of CD28 among the experimental groups in either organ.

Discussion

Since the first report by Isolauri *et al.* (2000), the oral administration of probiotic bacterium has received attention as a nondrug-dependent therapy for AD. This is evidenced by the large number of studies, both in agreement and in disagreement, that have been published on the topic (Murch, 2001; Kalliomaki *et al.*, 2001, 2003; Niers *et al.*, 2003; Rosenfeldt *et al.*, 2003; Weston *et al.*, 2005; Viljanen *et al.*, 2005; Hoekstra & Niers, 2006; Shaw, 2006). However,

Fig. 3. Gene expression of cytokines in antigen-applied skin. The cytokine genes evaluated in this study are detailed in Table 1. The filled bar represents the NT group. The shaded and blank bars represent the control and La1 groups, respectively. The arbitrary unit for each gene was normalized by that for GAPDH and was used for the statistical analysis. Data are presented as fold-change compared with the NT group (mean $\pm$ SE). * $P < 0.05$.

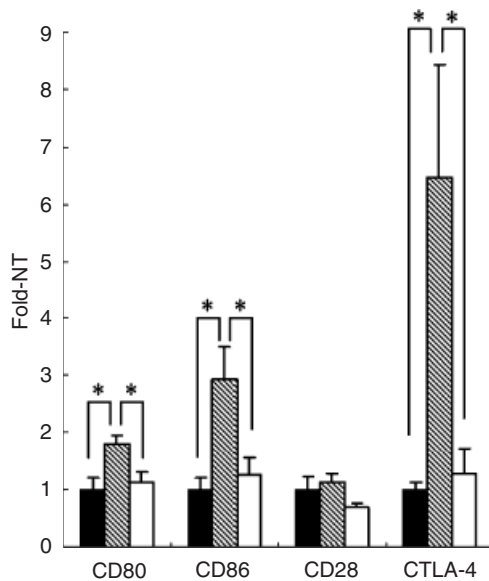
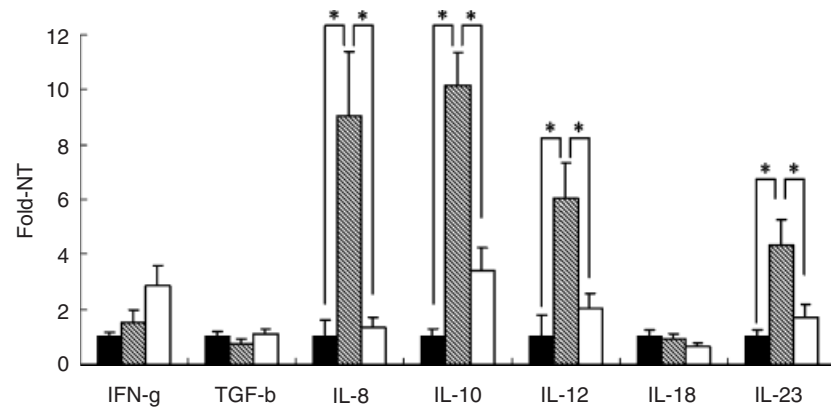


Fig. 4. Gene expression of CD80, CD86, CD28 and CTLA-4 in antigen-applied skin. The filled bar represents the NT group. The shaded and blank bars represent the control and La1 groups, respectively. The arbitrary unit for each gene was normalized by that for GAPDH and was used for the statistical analysis. Data are presented as fold-change compared with the NT group (mean $\pm$ SE). * $P < 0.05$.

the potency of probiotic therapy on AD still needs to be elucidated because the inhibitory mechanism has not been fully established. This is attributed primarily to the lack of appropriate animal models for understanding the positive effects of probiotics.

The experimental model previously presented (Inoue *et al.*, 2007) seems to be useful in clarifying the inhibitory mechanisms on AD by LAB administration. It was confirmed in that study that the primary administration of LAB significantly decreases the number of infiltrated mast cells in antigen-applied skin, the IgE concentration and IgG1/G2a ratio in the serum, and the scratching scores in NC/Nga mice.

The induction of AD-like lesions and its inhibition by the primary administration of La1 were successfully reproduced in this study, as in a previous experiment (Figs 1 and 2; Inoue *et al.*, 2007).

Gene expression of IL-8, IL-12 and IL-23 was significantly higher in the mite antigen-applied skin of the control group than in that of the NT group (Fig. 3). In agreement with these results, the upregulation of these cytokines has been reported in humans with chronic AD (Hamid *et al.*, 1996; Mullings *et al.*, 2001; Kopp *et al.*, 2003). IL-8, the C-X-C chemokine, clearly plays a key role in neutrophil emigration (Kunkel *et al.*, 1991) and IL-12 is considered to be involved in chronic AD (Hamid *et al.*, 1996; Yawalkar *et al.*, 2000). Moreover, a repeated subcutaneous injection of IL-23 seems to result in skin inflammation (Kopp *et al.*, 2003). With regard to the functions of those cytokines, it is quite likely that they participated as highly causative agents of skin lesions.

In contrast, the level of gene expression of these cytokines in the La1 group was comparable with that in the NT group and was significantly lower than that in the control group (Fig. 3). This suggested that the administration of La1 in early life could impede the overexpression of these proinflammatory cytokines and that this impediment was presumably involved in the inhibitory process on skin lesions by La1 administration.

The higher gene expression of IL-10 in the antigen-applied region of the control group than that in the La1 and NT groups (Fig. 3) did not seem to agree with the theory that IL-10 has the potential to inhibit inflammation, the so-called immune regulatory function (Asseman *et al.*, 1999). However, IL-10 was significantly overexpressed in AD patients relative to healthy controls according to Aleksza *et al.* (2002) and Ohmen *et al.* (1995). Thus, the impediment of IL-10 overexpression may also have partly contributed to the inhibitory effect on skin lesions by La1.

Considering the fact that IL-12 and IL-23 are involved in innate immunity (Langrish *et al.*, 2004) and IL-8 is the product of active dendritic cells (DCs) (Ramoner *et al.*,

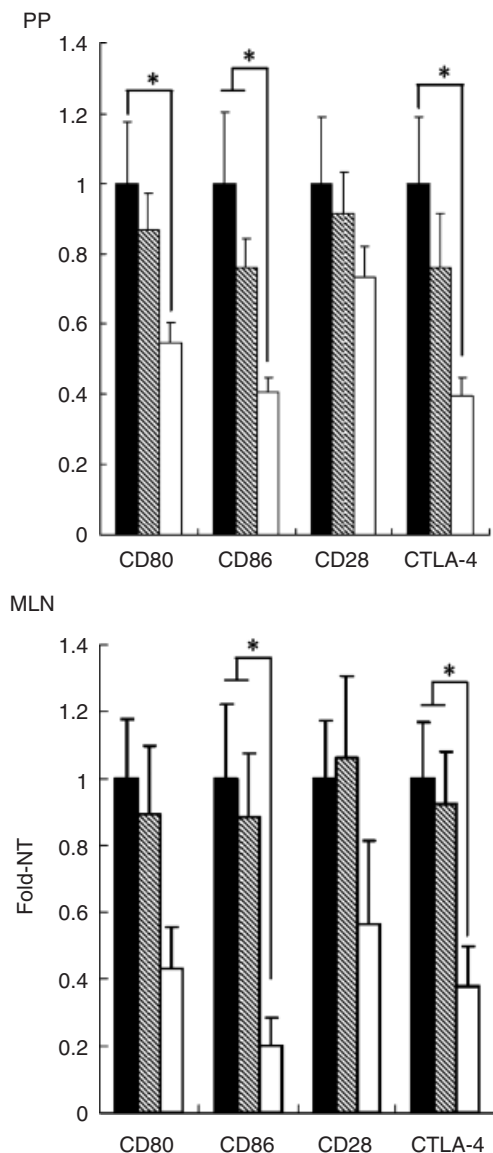


Fig. 5. Gene expression of CD80, CD86, CD28 and CTLA-4 in PPs and MLNs. The filled bar represents the NT group. The shaded and blank bars represent the control and La1 groups, respectively. The arbitrary unit for each gene was normalized by that for GAPDH and was used for the statistical analysis. Data are presented as fold-change compared with the NT group (mean $\pm$ SE). * $P < 0.05$.

1998; Verhasselt *et al.*, 1998; Caron *et al.*, 2001), we hypothesized that the primary administration of La1 may have affected the activity of the cells involved in innate immunity such as macrophages (M ϕ) and DCs after maturation. Thus, the costimulatory molecules, CD80 and CD86, were further examined in this study because their expression could be affected if the above hypothesis was plausible.

CD80 and CD86 are generally expressed in antigen presenting cells (APCs), such as M ϕ and DCs (Bhatia *et al.*,

2006), and are occasionally upregulated with proinflammatory cytokines (Windhagen *et al.*, 1995; Willmann & Dunne, 2000; Caron *et al.*, 2001). Moreover, overexpression of CD80 and especially of CD86 has been observed in humans with AD and atopic rhinitis (Ohki *et al.*, 1997; Hattori *et al.*, 2001; Schuller *et al.*, 2001), although their expression has not yet been investigated in this mouse model. It is noteworthy that CD80 and CD86 are in fact the positional candidate genes for AD in humans, according to the genome screens conducted by Lee *et al.* (2000).

Indeed, the gene expression of costimulatory molecules, especially CD86, in the antigen-applied skin of the control group was significantly higher than that of NT, while the La1 group showed a comparable level of expression of these genes in antigen-applied skin (Fig. 4). These results indicated that the overexpression of costimulatory molecules might contribute to the development of skin lesions in this mouse model as well as human AD. This result agrees with the observation that IL-10 is preferentially produced by T cells as a result of the costimulation through CD86 (Nakajima *et al.*, 1997).

The attenuation of CD86 by the primary La1 administration was presumably a part of the inhibitory mechanism on the skin lesion. It has been demonstrated that the blockade of CD86 by monoclonal antibodies significantly improved the allergic pulmonary inflammation and allergic airway hyper-responsiveness in model mice (Mark *et al.*, 1998; Haczku *et al.*, 1999).

Interestingly, the expression of CD86 was significantly attenuated not only in antigen-applied skin but also in gut-associated lymph tissues (PPs and MLNs) of the La1 group, whereas this was not the case in the control group (Fig. 5). This strongly suggests that the immunomodulatory effect of La1 may have been achieved first in the gut and was subsequently expressed in a systemic immune site, including the cutaneous immune system, through the common mucosal immune system, the immunological relationship of the mucosal immune system with the systemic immune system (Iijima *et al.*, 2001; Mowat, 2003).

Gene expressions of CD80 and CD86 were unexpectedly higher in PPs and MLNs of the NT group than those of the La1 group (Fig. 5). However, it is worth mentioning that, in our evaluation, CD80 and CD86 gene expression levels in PPs and MLNs of healthy Balb/c mice were comparable with those of the La1 group, and not the NT group (mean expression levels of CD80 and CD86 in PPs and MLNs of healthy Balb/c mice were 0.75 and 0.16, respectively, and 0.26 and 0.34 for the NT group). These data imply genuine dysfunction of the intestinal immune system in this mouse model. Consistent with this implication, it has been demonstrated that oral tolerance could not be developed in this mouse model, while this was not the case in Balb/c mice (Sakai *et al.*, 2006). Moreover, this mouse model is used as a

model for food allergy, which is associated with an elevated level of serum IgE (Sakamoto *et al.*, 1999). Interestingly, abnormality of the intestinal immune system in patients with AD was also reported by Majamaa *et al.* (1996). According to the latter study, levels of intestinal inflammation markers were significantly higher in patients with AD than in healthy controls, while upregulation of CD86 was also reported in inflamed intestine (Vuckovic *et al.*, 2001). Although the gut immune system of this mouse model and that of the AD patients merit further investigation, it seems likely that dysfunction of the gut immune system is enhanced upon oral administration of La1.

The gene for CTLA-4 showed the most surprising expression pattern in this experiment (Fig. 3). CTLA-4 is a known counter receptor of CD80 and CD86, as is CD28. However, in contrast to CD28, CTLA-4 is reported as a negative receptor for T-cell activation. Briefly, the binding of CD80 and CD86 to CTLA-4 is considered to result in the suppression of the differentiation of the naïve T cell into Th1- and/or Th2-type cells (Thompson & Allison, 1997). Thus, in theory, the immune response in the control group should have been suppressed. However, this was clearly not the case, which suggested another role of CTLA-4 in AD and/or drastically increased numbers of T cells. In accordance, Choi *et al.* (2005) demonstrated the overexpression of CTLA-4 in AD infants but not in healthy controls. Jones *et al.* (2006) suggested that there were some polymorphisms on CTLA-4 genes and that at least some of them seemed to contribute to the development of AD in human infants. Furthermore, Wu *et al.* (1997) proposed that the interaction of CD80/86–CTLA-4 is sufficient to activate T-cell differentiation. Given these results, it is suggested that the overexpression of CTLA-4 in the control group might be related to the development of skin lesions. In the La1 group, gene expression was probably attenuated in response to the decrease in costimulation provided by CD80/86. Further studies are required to classify its exact role in AD.

This study demonstrated that the gene expression of proinflammatory cytokines (IL-8, IL-12 and IL-23) and CD86 was enhanced by the repeated application of mite antigens in NC/Nga mice and that the elevation in these gene expressions was suppressed by the administration of La1 in the weaning period. Although it is still unclear from this study which exactly type of cells are expressing CD86 and producing proinflammatory cytokines, and where these cells are localized, the suppression of these genes by La1 may participate to a large degree in the inhibitory effect on skin lesions in this experimental model. The oral administration of La1 in early life may have affected cell number and/or cell activity of CD86- and perhaps CD80-bearing cells in mucosal and cutaneous immune sites. The administered La1 may have achieved this immunomodulatory effect first in the mucosal and subsequently in the systemic immune system.

This immunomodulatory process by La1 may involve cells such as Mφ and DCs because these cells are the most plausible CD86-expressing cells. However, it has been reported that B and/or T cells also express CD86 during atopic disease (Jirapongsananuruk *et al.*, 1998; Nakada *et al.*, 1999; Hattori *et al.*, 2001) and the cytokines that were upregulated in the antigen-applied skin are theoretically produced by various kinds of cells.

Further immunohistochemical or flow cytometry studies focused on the costimulatory molecules will provide useful information regarding the localization and population of cells affected by La1 and such information would certainly help to reveal more precisely the inhibitory mechanism on skin lesions by La1.

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