Lactic acid bacteria have been reported to have various immune-regulating activities. We also found in the previous study that the oral administration of heat-killed Lactobacillus plantarum NRIC0380 induced CD4⁺CD25⁺Foxp3⁺ cells (Treg cells). We examine in this present study the influence of NRIC0380 on the function of intestinal dendritic cells (DCs) in vitro and in vivo. The aldehyde dehydrogenase (ALDH) activity was significantly induced in DCs obtained from the mesenteric lymph node (MLN) by culturing with NRIC0380. The oral administration of NRIC0380 also significantly increased ALDH-positive cells in MLN. NRIC0380 significantly enhanced the production of TGF-β from MLN cells in vitro. These effects were not apparent in cells from the Peyer’s patch (PP) and spleen (SPL). NRIC0380 also significantly enhanced the expression of B7-H1 on DCs of all organs in vitro. The effects of NRIC0380 on DCs, especially those located in MLN, might be involved in its function to induce Treg cells.

Key words: aldehyde dehydrogenase; dendritic cell; lactic acid bacterium; mesenteric lymph node; regulatory T cell

Lactic acid bacteria (LAB) are well known as probiotics. LAB have been reported to have such immune-regulating functions as anti-allergic, anti-inflammation, anti-infection and anti-tumor activities.1–3 The anti-allergic activity of LAB had been anticipated, because the content of LAB living in the intestines of children with atopic dermatitis was much lower than that of healthy children.4 Many studies have then demonstrated the anti-allergic activity of LAB in murine experimental models as well as in humans.5–7 It has also been reported that LAB could inhibit some inflammatory diseases in such murine models as autoimmune diabetes and inflammatory bowel disease.8–10 Some of these reports have suggested that T regulatory cells (Treg cells) might be involved in the anti-allergic and anti-inflammatory activity of LAB.1,4 Treg cells had been found as a type of T cell with immune suppressive functions.11 We have reported that the ratio of CD4⁺CD25⁺Foxp3⁺ cells to whole CD4⁺ cells in the spleen (SPL) and Peyer’s patch (PP) was significantly increased by an oral administration of the Lactobacillus plantarum NRIC0380 strain of LAB when using a murine model.12 A full understanding of the mechanism for inducing Treg cells by LAB is required for their safe and effective application.

The immune-regulating mechanism of LAB, especially their Th1-inducing ability, has been reported in previous studies.13–15 Several studies have revealed that LAB were recognized by such antigen-presenting cells (APC) as macrophages and dendritic cells (DCs) through the pattern recognition receptors (PPRs).16–18 Toll-like receptors (TLRs) might play a major role in this response. Kawashima et al. have reported that a certain strain of LAB induced IL-12 production in mouse peritoneal macrophages, and that this response partially depended on TLR-2.16 Ichikawa et al., however, have reported that several strains of LAB induced IL-12 via MyD88 but not TLR-2, 4, 9.17 Kaji et al. have revealed that some signals introduced by some bacterial components via TLR-2 reversed the predominant IL-12 production induced by certain Lactobacillus strains into predominant IL-10 production,18 although the immune-regulating mechanism of LAB remains controversial.

The mechanism of LAB for inducing Treg cells has also not yet been clarified. Jounai et al. have reported that several strains of LAB activated plasmacytoid DCs via TLR-9, and that those cells were involved in the induction of Treg cells,19 although the molecular mechanism for inducting Treg cells by those cells has not been investigated. The differentiation of Treg cells has been found to be induced by TGF-β20 while IL-6 has been reported to inhibit the TGF-β-induced differentiation of Treg cells.21 Retinoic acid has been reported to abrogate the inhibitive effect of IL-6,22 although the involvement of LAB in regulating those molecules has not been examined.

We therefore examined the effect of NRIC0380 on the expression of such molecules which are involved in the induction of Treg cells in vitro and in vivo. We show that NRIC0380 altered the functions of some intestinal
immune cells, including the aldehyde dehydrogenase (ALDH) activity of MLN DCs. DCs have been shown to be a major type of APC and were reported to be important for inducing immune responses against foods.23 Our findings from this present study will contribute to a better application of LAB to food products with anti-allergic and anti-inflammatory effects.

Materials and Methods

**Lactic acid bacteria (LAB).** *Lactobacillus plantarum* NRIC0380 was obtained from the Culture Collection Center of Tokyo University of Agriculture (NRIC, Tokyo, Japan). NRIC0380 was cultured in half-strength MRS broth under anaerobic conditions with a mixed gas (N₂/H₂/CO₂ = 8:1:1) at 30°C until the late exponential phase of growth. The half-strength MRS broth contained (g/L) glucose, 10; Bacto proteose peptone no. 3 (Becton Dickinson, Franklin Lakes, NJ, USA), 5; Lab-Lemco powder (Oxoid, Cambridge, UK), 5; Becto yeast extract (Becton Dickinson), 2.5; Tween 90, 0.5; K₂HPO₄, 1; sodium acetate-3H₂O, 2.5; triammonium citrate, 1; MgSO₄-7H₂O, 0.1; and MnSO₄·4H₂O, 0.025. The pH value was adjusted to 6.5 with HCl. NRIC0380 was harvested by centrifugation, washed with sterilized deionized water, lyophilized, and then stored at −20°C until needed. The bacterial cells were used after autoclaving.

**Mice.** Six week-old female BALB/c mice were purchased from Clea Japan (Tokyo, Japan). All the mice were maintained and used in accordance with the guidelines for the care and use of experimental animals of Tokyo University of Agriculture and Technology.

**Cell culture.** Single-cell suspensions from the mesenteric lymph node (MLN), PP and SPL were prepared as described in a previous report.23 To evaluate the effects of LAB on the functions of MLN cells, PP cells and SPL cells, these cells (2.5 × 10⁶ cells/mL) from BALB/c mice were incubated without or with NRIC0380 (1 μg/mL) in an RPMI1640 medium (Nissui Pharmaceutical, Tokyo, Japan) containing 10% heat-inactivated fetal calf serum (JRH Bioscience, Lenexa, KS, USA). The cells were collected from some wells of the culture plates after 24 h of incubation, and subjected to flow cytometry to analyze the ALDH activity and expression of B7-DC and B7-H1 in DCs. Each supernatant was also collected from the other wells after another 1 d of culture for use in cytokine assays.

**Oral administration of LAB.** BALB/c mice were orally administered by a feeding needle with NRIC0380 (2 mg) suspended in PBS. PBS alone was given to the control mice. The MLN cells, PP cells and SPL cells were prepared the following day and subjected to flow cytometry. The cells were also cultured for a cytokine assay in the absence of further bacterial stimulation.

**ELISA.** The amounts of IL-6 and TGF-β in each supernatant were detected by ELISA. We used a Ready-Set-Go ELISA kit (eBioscience, San Diego, CA, USA) for measuring each cytokine. Each supernatant was used without an acid treatment for activating TGF-β. Our data suggested that DCs of MLN might be involved in the Treg induction by NRIC0380. We next examined the influence of NRIC0380 on the expression of ALDH in DCs from each immune organ on the next day of stimulation. Retinoic acid has been reported to induce Treg cells even in the presence of IL-6. Our results show that the percentage of ALDH-positive DCs of MLN, PP and SPL cells was also increased by stimulation with NRIC0380, although there was no statistical significance in the case of those cells.

**Results**

**NRIC0380 enhanced TGF-β production by MLN cells in vitro**

TGF-β has been found to induce the differentiation of naïve T cells into Treg cells. We therefore focused on the effect of NRIC0380 on TGF-β production by the cells of several immune organs. MLN, PP and SPL cells were incubated with the bacterial cells. Table 1 shows the amount of TGF-β in each supernatant on day 2. NRIC0380 significantly enhanced the TGF-β production by MLN cells. The production by PP and SPL cells was also increased by stimulation with NRIC0380, although there was no statistical significance in the case of those cells.

**NRIC0380 induced ALDH activity in DCs of MLN in vitro**

Our results show that NRIC0380 enhanced both TGF-β and IL-6 production. Although TGF-β induces Treg cells, it also induces Th17 cells in the presence of IL-6. We therefore could not determine the induction mechanism for Treg cells by NRIC0380 from these results. We then examined the influence of NRIC0380 on the expression of ALDH in DCs from each immune organ on the next day of stimulation. Retinoic acid has been reported to induce Treg cells even in the presence of IL-6. Our result shows that the percentage of ALDH-active DCs of MLN was significantly increased by NRIC0380 (Fig. 1). However, this effect was not apparent in DCs of the PP and SPL cells (Fig. 1).

**NRIC0380 enhanced the expression of B7-H1 in DCs in vitro**

Our data suggested that DCs of MLN might be involved in the Treg induction by NRIC0380. We next examined the influence of NRIC0380 on the expression of costimulatory molecules, which could induce Treg cells, on the surface of DCs on the next day of stimulation. Figure 2A shows that NRIC0380 enhanced the expression of B7-H1 on DCs of MLN, PP and SPL.

**Table 1. Effect of NRIC0380 on Cytokine Production in Vitro**

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>NRIC0380</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>TGF-β (pg/mL)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MLN</td>
<td>35.22 ± 22.44</td>
<td>82.17 ± 16.24</td>
<td>0.01</td>
</tr>
<tr>
<td>PP</td>
<td>55.18 ± 33.83</td>
<td>88.89 ± 49.29</td>
<td>0.24</td>
</tr>
<tr>
<td>SPL</td>
<td>20.57 ± 8.37</td>
<td>37.74 ± 29.99</td>
<td>0.25</td>
</tr>
<tr>
<td><strong>IL-6 (pg/mL)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MLN</td>
<td>26.74 ± 11.11</td>
<td>38.17 ± 11.77</td>
<td>0.21</td>
</tr>
<tr>
<td>PP</td>
<td>17.86 ± 11.47</td>
<td>31.38 ± 7.16</td>
<td>0.16</td>
</tr>
<tr>
<td>SPL</td>
<td>17.18 ± 5.9</td>
<td>40.29 ± 27.36</td>
<td>0.23</td>
</tr>
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</table>
On the other hand, NRIC0380 did not affect the expression of B7-DC on DCs from any organs (Fig. 2B). NRIC0380 induced ALDH activity in DCs of MLN in vivo as well as in vitro. We then confirmed our results obtained in the in vitro experiments by using an in vivo system. The TGF-β and IL-6 produced by MLN and PP cells tended to be enhanced by the oral administration of NRIC0380 (Table 2). Similar results were obtained from another independent experiment (data not shown). Our data clearly show that orally administered NRIC0380 significantly induced the ALDH activity in DCs of MLN, although this activity was not affected in DCs of PP and SPL (Fig. 3). However, there were no apparent effects on the expression of B7 in any of the groups tested (Fig. 4).

### Table 2. Effect of Orally Administered NRIC0380 on Cytokine Production in Vivo

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Control</th>
<th>NRIC0380</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>TGF-β (pg/mL)</td>
<td>MLN 97.67 ± 18.85</td>
<td>113.3 ± 12.74</td>
<td>0.30</td>
</tr>
<tr>
<td></td>
<td>PP 109.9 ± 9.80</td>
<td>116.8 ± 7.75</td>
<td>0.40</td>
</tr>
<tr>
<td></td>
<td>SPL 95.35 ± 8.58</td>
<td>94.69 ± 6.76</td>
<td>0.92</td>
</tr>
<tr>
<td>IL-6 (pg/mL)</td>
<td>MLN 50.69 ± 21.40</td>
<td>68.13 ± 18.58</td>
<td>0.35</td>
</tr>
<tr>
<td></td>
<td>PP 60.78 ± 7.80</td>
<td>68.75 ± 15.68</td>
<td>0.47</td>
</tr>
<tr>
<td></td>
<td>SPL 60.68 ± 9.98</td>
<td>59.12 ± 13.33</td>
<td>0.88</td>
</tr>
</tbody>
</table>

BALB/c mice were administered with NRIC0380. MLN, PP and SPL cells were prepared 24 h later, and cultured in the absence of NRIC0380. Each supernatant was collected 2 d later, and the amounts of TGF-β and IL-6 were measured by ELISA. The results are representative of two independent experiments (n = 3).
Discussion

The objective of this study was to identify the mechanism for inducing Treg cells by orally administering NRIC0380. A full understanding of this mechanism would contribute to the use of NRIC0380 as a food material having the ability for preventing and treating allergies and inflammation. We particularly focused on the effect of NRIC0380 on the functions of DCs. Our results demonstrate that orally administered NRIC0380 altered the functions of intestinal DCs, suggesting that this effect might be involved in the ability of NRIC0380 to induce Treg cells.

It is known that LAB have anti-allergic and anti-inflammatory activity. IL-10 induced by LAB has been considered to be an important factor involved in this function. We have also found by using a murine allergy model that the oral administration of a strain of LAB (NRIC0380) induced Treg cells. Treg cells have been reported to inhibit the symptoms of allergic patients. Indeed, our findings also show that Treg cells induced by orally administered NRIC0380 had activity to inhibit the allergic symptoms in a murine model (a manuscript is now being prepared), indicating that the ability of LAB to induce Treg cells is also important for anti-allergic and anti-inflammatory activity. However, the mechanism of LAB for inducing Treg cells has not previously been investigated. TGF-β is a critical cytokine for inducing the differentiation of naïve T cells into Treg cells. It has been reported that TGF-β induced Foxp3 expression in CD4+CD25− T cells and that the peripheral T cells expressing Foxp3 were decreased in Tgft−/− mice. Our results show that NRIC0380 enhanced TGF-β production by MLN and PP cells in vitro and in vivo. In particular, the enhanced TGF-β production by MLN cells which had been stimulated with NRIC0380 in vitro was statistically significant. These results indicate that the enhanced production of TGF-β by those cells might be one of the mechanisms for inducing Treg cells by NRIC0380. However, our results also show that stimulation with NRIC0380 enhanced IL-6 production by those cells, as well as that of TGF-β. TGF-β has been reported to induce Th17 in the presence of IL-6 by inducing the expression of RORyt. It is therefore important to consider the balance of TGF-β and IL-6 to evaluate the mechanism of LAB for inducing Treg cells, although it is not known which balance induces Treg or Th17. We cannot conclude from these observations that the enhanced production of TGF-β induced by NRIC0380 was involved in Treg induction.

Retinoic acid has been reported as a critical factor for inducing Treg cells, other than the TGF-β/IL-6 balance. Retinoic acid is a carboxylic acid and known as a vitamin A derivative. Recent studies have revealed that intestinal DCs expressed ALDH and produced retinoic acid. Retinoic acid is produced from retinol via retinal, and these conversions are mediated by two types of enzyme. The first step is mediated by several types of alcohol dehydrogenases, and the latter step, by retinal dehydrogenases. Most cells express some alcohol dehydrogenases, but the expression of retinal dehydrogenases is restricted to such cell types as intestinal DCs. Retinoic acid produced in intestinal lymphoid organs has been reported to imprint gut-homing specificity on intestinal naïve T cells. Moreover, the importance of retinoic acid for inducing Treg cells has also been clarified. It has been reported that retinoic acid induced Treg cells even under the condition for inducing Th17 of inhibiting RORyt expression. Our results indicate that NRIC0380 induced ALDH activity in DCs of MLN, suggesting that the enhanced production of retinoic acid overcame the effect of IL-6 and induced Treg cells in MLN of the mice that had been fed with NRIC0380.

We have not confirmed whether orally administered NRIC0380 was taken up by MLN. However, Berg et al. have reported the translocation of orally administered bacteria to MLN in a gnotobiotic mouse model. Hiramatsu et al. have also reported that a strain of Bifidobacterium was found in MLN and PP after its oral administration when using conventional mice. These reports strongly support our afore-mentioned hypothesis.

It has been reported in a previous study that Bifidobacterium longum AH1206 also enhanced the expression of some genes involved in the production of retinoic acid by PP cells. However, our results show no apparent effect of NRIC0380 on the expression of ALDH in DCs of PP. Although we are not sure of the reason for this result, the effect on DCs in PP might have been below the detectable limit with our system, because our results show that the percentage of ALDH-active DCs in PP was much less than of those cells in MLN. Our findings might also indicate that the effect of orally administered bacteria varied depending on the strain. DCs in PP express RLADH1, while those in MLN express RALDH2. These genes might be distinctly regulated. Different strains of bacteria would introduce different signals into DCs, and respectively affect the expression of different types of gene.

The percentage of ALDH-active DCs obtained from the in vitro experiment was generally lower than that in the in vivo experiment, especially for MLN DCs. The expression of ALDH in DCs might have been regulated by several factors, and a specific environment in MLN would be required for DCs to obtain a high level of ALDH expression. Indeed, the percentage of ALDH-positive DCs in PP was substantially lower than that in MLN, suggesting that the expression of ALDH might have decreased during the overnight culture, due to some particular factors for maintaining ALDH expression being missing from the culture. One candidate for such factors is retinoic acid. It has been reported that the expression of ALDH was enhanced by retinoic acid itself, the level of retinoic acid in the culture being considered to be less than that in the body.

B7-H1 and B7-DC have been found as ligands for PD-1 which is a T cell surface molecule inducing cell death. It has been reported that mice deficient in B7-H1 and/or B7-DC failed to generate Treg cells and to be rendered orally tolerant, suggesting that these mole-
cules might have been involved in the induction of Treg cells by NRIC0380. Our results from the in vitro experiment demonstrate that NRIC0380 enhanced the expression of B7-H1 on DCs, while the expression of B7-DC was not affected. Although B7-H1 and B7-DC are known to have similar functions in the induction of oral tolerance, the expression of each of these molecules is independently regulated; for example, B7-H1 can be induced by IFN-γ in various types of cell, while B7-DC is preferentially induced by IL-4 and IL-13 in DCs.37) These observations lead us to suggest from our results that the enhanced expression of B7-H1 on the surface of DCs but not of B7-DC would also have been involved in Treg induction by NRIC0380 as well as the induction of ALDH activity. However, no enhancement of B7-H1 expression by the oral administration of NRIC0380 was apparent for any organ in the in vivo experiment, although it cannot be discounted that even an insignificant level of change in B7-H1 could be effective for inducing Treg cells. Indeed, we observed slightly enhanced B7-H1 expression in the mice that had been fed with NRIC0380 in two independent experiments (Fig. 4 and data not shown).

DCs from each organ in our experiments seemed to consist of a single population in respect of the level of B7-H1 expression. However, only part of DCs from each organ expressed B7-DC. These results are consistent with the study by Fukaya et al.38) We therefore analyzed the percentage of B7-DC-positive cells as well as the intensity of B7-DC expression (evaluated by the median fluorescence intensity) on B7-DC-positive DCs with the results shown in Figs. 2 and 4. These results show that NRIC0380 had no effect on the percentage of B7-DC-positive cells, nor on the intensity of B7-DC expression (data not shown).

Taken together, our results indicate that NRIC0380 was likely to have induced Treg cells in MLN. A previous study has also shown MLN to be an important site for inducing suppressive T cells,39) which strongly supports our results. On the other hand, we have reported in our previous study that an oral administration of NRIC0380 increased the percentage of Treg cells in both PP and SPL,12) suggesting that Treg cells induced in MLN might have migrated to the systemic organs as well as to the intestinal lymphoid organs. Orally administered LAB could be taken into our body mainly through M cells on the surface of PP. The bacterial cells or those components could then be transferred to MLN and affect DCs there. A further study to investigate how NRIC0380 affects the functions of MLN DCs is now in progress in our laboratory. A complete understanding of the mechanism of LAB for inducing Treg cells is important to be able to use them as a valuable food material with immune-regulatory activity.

References


Induction of ALDH Activity in DCs by LAB


