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Lactobacillus gasseri OLL2809 inhibits development of ectopic endometrial cell in peritoneal cavity via activation of NK cells in a murine endometriosis model

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Abstract We have previously reported that peritoneal administration of interleukin-12 (IL-12) suppresses development of ectopic endometriotic lesions via activation of natural killer (NK) cells in a mouse endometriosis model. *Lactobacillus gasseri* OLL2809 is one of a probiotic lactobacillus that has been selected on an ability to stimulate production of IL-12 from murine splenocytes. In this study, we examined whether the oral administration of heat-killed *L. gasseri* OLL2809 suppressed development of endometriosis. Administration of *L. gasseri* OLL2809 for 21 consecutive days resulted in reduction of the development of ectopic endometriotic lesions in an extent similar to IL-12. Although obvious effects of *L. gasseri* OLL2809 on the peritoneal cytokine levels, population of peritoneal cells as well as cytotoxicity of splenic NK cells, gene expression analysis of the peritoneal cells revealed enhancement in the transcription of IL-2 and natural killer cell triggering receptor 1 genes. Therefore, it was suggested that *L. gasseri* OLL2809 suppressed development of endometriosis via activation of NK cells.

Keywords Endometriosis · IL-12 · *Lactobacillus gasseri* · Natural killer cell

Abbreviations

IL	Interleukin
IFN	Interferon
NK	Natural killer
Th	Helper T cells

Introduction

Endometriosis is a common chronic gynecological disorder associated with pelvic pain and infertility. It is characterized by the presence of uterine endometrial tissue outside of the normal location. It has been established that impaired immunosurveillance systems in peritoneal cavity cause endometriosis in women of reproductive age (Giudice and Kao 2004). Somigliana et al. have shown that peritoneal administration with interleukin-12p70 (IL-12) to experimental endometriosis mice resulted in suppression of development of endometriotic lesions (Somigliana et al. 1999). IL-12 is well known to induce Th1 proliferation followed by activation of NK cells by secreting Th1-cell derived cytokines such as IL-2 and interferon-gamma (IFN-γ) (Chan et al. 1992; Zhou et al. 2003). We recently observed suppressive effect of IL-12 on development

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of ectopic endometriotic lesions and found it was due to activation of natural killer (NK) cells because anti-IL-2 receptor β chain treatment resulted in attenuation of splenic cytotoxicity of NK cells concomitant with suppression of development of endometriotic lesions (Itoh et al. 2011).

Lactobacillus gasseri OLL2809 is one of a probiotic lactobacillus that has been selected on the basis of its immunostimulatory activity (Sashihara et al. 2006, 2007). We previously reported that the oral administration of heat-killed *L. gasseri* OLL2809 effectively enhances production of IL-12 in splenocytes ex vivo and shifts the Th1/Th2 balance from Th2-dominant immunity toward Th1-dominant immunity (Sashihara et al. 2008; Gotoh et al. 2009). Therefore, we hypothesized that heat-killed *L. gasseri* OLL2809 suppressed development of endometriotic lesion in a murine endometriosis model. In this study, we examined whether the oral administration of heat-killed *L. gasseri* OLL2809 suppressed development of endometriosis.

Materials and methods

Mice

Female, 6-week old specific pathogen-free BALB/c mice that have been undergone ovariectomy at an age of 5 weeks were purchased from Japan SLC (Shizuoka, Japan) and were maintained on a standard diet (CRF-1 diet; Oriental Yeast, Tokyo, Japan). The mice were housed in groups of four in a temperature- and humidity-controlled room, at 21 ± 2 °C and 55 ± 15% humidity, on a 12/12 h reversed light/dark cycle. Food and tap water were provided ad libitum. The experimental protocols were approved by the Animal Care Committee of the Division of Research and Development, Meiji Dairies Corporation.

Induction of endometriosis

Induction of endometriosis was performed according to a method reported by Somigliana et al. with slight modifications (Somigliana et al. 1999). Briefly, endometriotic lesions were induced on day 0 by inoculating finely chopped endometrial fragments corresponding to about 50% (about 15 mg) of the endometrial tissue from both uterine horns of syngenic mice as described below into the peritoneal space. Mice challenged with endometrium have been subjected to ovariectomy through a small vertical midline laparotomic incision on day -7 (at an age of 5 weeks) and injected with oestrogens depot in castor oil (Wako, Osaka, Japan; 100 μg/kg *i.m.*) on days -7, 0, 7, 14. Ovariectomy plus oestrogen supplementation was performed in order to abrogate differences related to the stage of the oestrous cycle.

Uterine samples to be inoculated were obtained from syngenic mice used as donor mice. Similarly to the challenged mice, donor mice were ovariectomized and oestrogen-treated on day -7. On day 0, one donor mouse was killed for every two mice to be challenged with endometrium. Both uterine horns were removed using an aseptic technique and subsequently placed in a sterile Petri dish containing sterile normal saline. Then, uterine samples were gently peeled in order to detach the uterine muscle from the endometrium. Following this procedure, they were transferred to a glass slide and finely chopped using two blunt scalpels. Endometrial fragments were suspended in 0.3 mL of sterile saline per challenged mouse and inoculated into the peritoneal cavity.

Throughout the experiment period (21 days), the mice were orally given heat-killed *L. gasseri* OLL2809 by gastric gavage at a dose of 2 mg/body/day (approximately 5×10^8 cells/mg) in 0.5 mL distilled water. Murine recombinant IL-12 (Wako) was administrated *i.p.* by daily injections of 0.15 μg/0.4 mL for 5 days, from day - 2 through day 2. Control and other mice received only the vehicles following the same procedure.

Assessment of endometriotic lesions

On day 21 after endometrium challenge, mice were sacrificed, the abdomen was inspected and endometriotic lesions were carefully excised from the surrounding tissue in order to assess their weight and surface area. The surface areas were measured with a caliper and calculated. When the lesion had a cyst appearance, measures were taken after aspiration of the cyst. The operator was blinded for the different conditions.

Microorganism

Lactobacillus gasseri OLL2809 was cultured in Lactobacilli MRS broth (Becton–Dickinson, Sparks,

MD) at 37 °C for 18 h. After fermentation, the cells were harvested in a refrigerated centrifuge (10,000 × *g*, 15 min) and washed twice with saline solution followed by 1 wash with water. The cells were resuspended in distilled water, heat-killed at 75 °C for 60 min, and lyophilized.

Measurement of cytotoxicity of NK cells

The cytotoxicity of splenic NK cells was assessed using flow cytometry (Johann et al. 1995); YAC-1 cells were used as the target cells, and 0.5×10^6 YAC-1 cells/mL were labeled with 2.5 μg/mL of 3,3'-dioctadecyloxacarbocyanine perchlorate (Sigma–Aldrich, St. Louis, MO) by overnight incubation at 37 °C. The cells were washed 3 times with RPMI 1640 medium, and resuspended at a concentration of 2.5×10^6 cells/mL. Murine splenocyte effector cells (1×10^6 cells/well) were added to the target cells at 2.5×10^4 cells/well (40:1 ratio) in a total volume of 200 μL/well in a 96-well, round-bottomed plate (Corning, Corning, NY). Samples were centrifuged (30 × *g*, 1 min), and incubated for 4 h at 37 °C in a humidified 5% CO_2-air atmosphere. Fifteen minutes before the end of the incubation, 20 μL of propidium iodide (0.5 mg/mL in PBS; Sigma–Aldrich) was added to each well to label the dead cells. The level of target cell lysis was determined using a FACSCalibur flow cytometer with CellQuest software (BD Biosciences, Flanklin Lakes, NJ), and the cytotoxicity of NK cells was expressed as the percentage of effector cell-specific lysis.

Gene expression

Total RNA was extracted from the peritoneal cells using a Trizol reagent (Invitrogen, Calsbad, CA). Total RNA (1 μg) was used for synthesis of the first strand of cDNA using a PrimeScript RT reagent Kit (Takara Bio, Shiga, Japan). Real-time quantitative PCR were performed with an ABI prism 7300 (Applied Biosystems, Foster City, CA). The oligo-nucleotide primer pair for *Il-2* was synthesized as described elsewhere (Overbergh et al. 2003). *Ncr1* was analyzed using a TaqMan probe (Applied Biosystems). The fluorescence intensity of each target gene was normalized with that of glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) amplified under identical conditions.

Statistical analysis

Data were expressed as the mean ± SD. Statistical differences between the groups ($n = 7$ per group) were analyzed by a one-way analysis of variance (ANOVA) for parametric data followed, if justified, by Fisher's protected least significant difference (PLSD) test. For non-parametric data, Steel–Dwass' multiple comparison test was performed. Differences were considered significant when the *p*-value for the effect was less than 0.05.

Results and discussion

Effect of *Lactobacillus gasseri* OLL2809 on endometriosis

Administration of *L. gasseri* OLL2809 resulted in significant suppression of both total weight and total area of endometriotic lesions as compared with the control group, and the extent of the suppression was similar to the IL-12 group (Fig. 1). Therefore, it was found that oral administration of *L. gasseri* OLL2809 was effective in reducing the development of ectopic endometriotic lesions in mice.

Cytotoxicity of splenic NK cells

We have recently reported NK cells contribute to suppress development of endometriotic lesions from observations that IL-12 administration for 5 consecutive days, from day -2 through day 2, enhanced cytotoxicity of splenic NK cells at day 3, and that attenuating the cytotoxicity of NK cells by IL-2Rβ resulted in an abrogation of suppressive effect of IL-12 (Itoh et al. 2011). However, in this study, there was no significant difference in the cytotoxicity of splenic NK cells in all groups (Fig. 2), although it was slightly higher in the OLL2809 group. Regarding IL-12 group, the result was consistent with the previous study; which was probably because determination of the cytotoxicity was done on day 21 while IL-12 administration was done from day -2 through day 2, and the effect of IL-12 on the cytotoxicity was attenuated at the time of determination.

Analysis of peritoneal immune environment

We then investigated peritoneal cell population and peritoneal cytokine levels such as IL-1β and IL-2. The

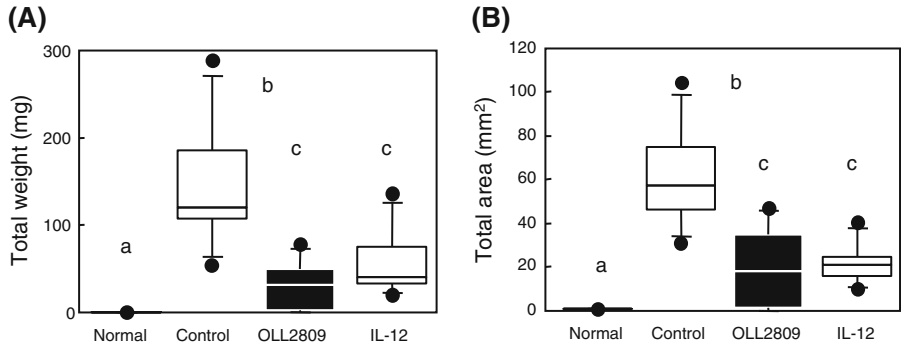

Fig. 1 Effect of *Lactobacillus gasseri* OLL2809 on the development of endometriotic lesions. Each value of total weight (A) and total surface area (B) of the endometriotic lesions is shown. The mean and SD of individual mice ($n = 7$ per group) are indicated beside each value. a-c: *Different letters* denote significant differences between groups ($p < 0.05$)

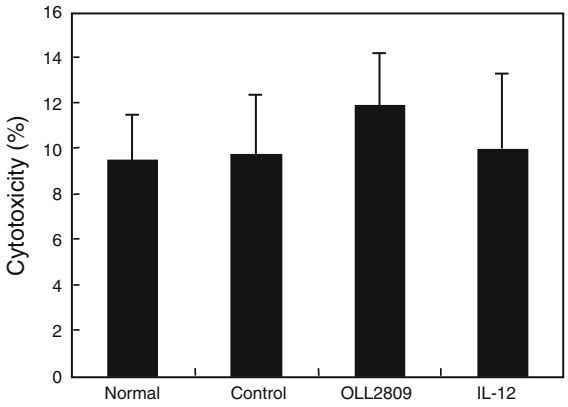

Fig. 2 Effect of *Lactobacillus gasseri* OLL2809 on the cytotoxicity of splenic natural killer cells. Splenocytes derived from each mouse were used as effector cells against YAC-1 target cells (E:T ratio = 40:1). Data are expressed as the mean with SD of individual mice ($n = 7$ per group)

results showed that there were no differences in the population and the absolute number of helper and cytotoxic T cells, B cells and NK cells (data not shown). The peritoneal cytokine levels were under detection limit in almost all individuals regarding IL-1β, IL-6, IL-2, IFN-γ and IL-12. Again, these results in the IL-12 group were consistent with those obtained in the previous observations (Itoh et al. 2011).

Gene expression analysis of peritoneal cells

To further analyze the effect of administration of *L. gasseri* OLL2809, the relative gene expression in peritoneal cells was determined. The results showed that relative *Il-2* expression levels were significantly lower in the control group as compared with the normal group, while the reduced expression levels were found to be restored to levels similar to the normal group in the OLL2809 and IL-12 groups (Fig. 3A). IL-2 is one of the Th1-derived cytokines that stimulates the proliferation and differentiation of B cells, T cells and NK cells (Anderson et al. 1993; Zhou et al. 2003) and has been shown to reduce endometriosis in rats (Velasco et al. 2006; Sel'kov and Pavlov 2007; Quereda et al. 2008). A significant impairment of IL-2 level of peritoneal fluid has also been demonstrated in endometriosis (Ho et al. 1996). In addition, the local administration of IL-2 in the cyst of endometriotic lesions showed suppressive effect of recurrence of endometriosis after GnRH analogues therapy (Acién et al. 2003). We have previously reported that not only IL-12 but also IL-2 and IFN-γ levels in the peritoneal lavage fluid were not detected on the next day after the final administration of IL-12, even though 0.15 μg/body of IL-12 was intraperitoneally administered for 5 consecutive days (the detection limit was 0.14 pg/mL) (Itoh et al. 2011). Therefore, the cytokine levels detected in the peritoneal fluid are thought not to be always parallel to the *Il-2* mRNA levels; this could be because the secreted cytokines were immediately removed after interaction with its specific receptor.

In addition, expression of *Ncr1* was significantly higher in the OLL2809 group compared with the control group (Fig. 3B). *Ncr1*, which is also known as Ly94 or NKp46 in humans (Pessino et al. 1998), encodes one of an activation marker protein expressed only on NK cells (Biassoni et al. 1999;

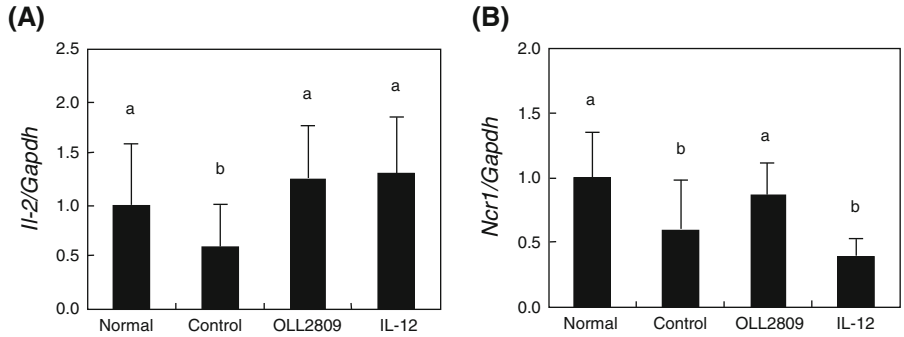

Fig. 3 Relative mRNA expression of *Il-2* (A) and Natural cytotoxicity triggering receptor 1 (*Ncr1*) (B) in peritoneal cells. Data are quantitatively presented following normalization of the glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) levels and are expressed as the mean with SD of individual mice ($n = 7$ per group). *Different letters* denote significant differences between groups ($p < 0.05$)

Walzer et al. 2007). Although the cellular ligand for NCR1 and the signaling pathway has not yet been identified, the expression is shown to be directly involved in eradication of virus-infected and tumor cells (Gazit et al. 2006). On measuring the relative expression of the mRNA levels, cDNA was prepared from equal amount of total RNA extracted from equal number of peritoneal cells. Moreover, cell population analysis of the peritoneal cells revealed no significant difference in the number of NK cells (data not shown). Therefore, although the obvious phenotypic effects of oral administration of *L. gasseri* OLL2809 on the cytotoxicity of NK cells were not observed, the cytotoxicity was implicated to be enhanced as was found in the gene expression analysis.

In this study, we demonstrated that oral administration of a heat-killed *L. gasseri* OLL2809 suppressed ectopic development of endometriotic lesions in a murine model. This is the first time that a potential probiotic Lactobacillus could reduce the development of endometriotic lesions. *L. gasseri* OLL2809 has been selected on the basis of its immunostimulatory activity from 20 of potential probiotic candidates (Sashihara et al. 2006). It stimulates production of IL-12 from antigen-presenting cells such as macrophage and dendritic cells (Sashihara et al. 2007; Sashihara et al. 2008). The immunostimulatory effect of the strain was also observed in a clinical study with patients with Japanese cedar pollinosis (Gotoh et al. 2009). In addition, the enhancement of expression of *Il-2* and *Ncr1* in the peritoneal cells was observed in this study. Therefore, it was deduced that *L. gasseri* OLL2809 reduced the development of ectopic endometriotic lesions via activation of Th1 cells and NK cells.

It has been reported that endometriosis is associated with an altered profile of intestinal microflora in rhesus monkeys. Bailey and Coe found that monkeys with endometriosis had lower *Lactobacilli* concentrations and higher Gram-negative bacteria concentrations compared with younger and healthy aged monkeys (Bailey and Coe 2002). Although the exact mechanisms linking endometriosis and the microflora has not been elucidated, they speculated that possibly proinflammatory mediators, such as interleukin (IL)-1β, IL-6, and tumor necrosis factor secreted by macrophages and monocytes affected the gut by suppressing gastric acid secretion and the motility, which in turn altered the profile of intestinal microflora. In this study, heat-killed *L. gasseri* OLL2809 was administered, yet the efficacy of the strain was observed, suggesting that it is effective even in the heat-killed form and that stimulation of the intestinal immune systems could reduce the inflammation of the disease. Further analysis will be required to elucidate the mechanism by which oral administration of *L. gasseri* OLL2809 could reduce development of ectopic endometriotic lesions.

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