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Administration of plasmacytoid dendritic cell-stimulative lactic acid bacteria enhances antigen-specific immune responses

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ABSTRACT

Lactic acid bacteria (LAB) have been reported to have beneficial effects on protective immunity against viruses and pathogenic bacteria by activating innate immune cells such as dendritic cells (DC) or macrophages. However, little is known about whether LAB contributes to antigen-specific immune responses. Because plasmacytoid DC (pDC) links innate and acquired immunity, here we investigated whether the pDC-stimulative LAB, *Lactococcus lactis* strain Plasma (LC-Plasma), influences antigen-specific immune responses. In *in vitro* co-culture experiments, LC-Plasma enhanced the expression of MHC class I and II, and CD80 and CD86 on both pDC and conventional DC, and this enhancement was abolished by treatment with a Toll-like receptor 9 antagonist. A subsequent *in vitro* study showed that LC-Plasma increased antigen-specific T cell responses via DC activation. In mice, oral administration of LC-Plasma in combination with intraperitoneal antigen administration enhanced the percentage of antigen-specific $CD8^+$ T cells and the amount of antigen-specific IgG. Furthermore, continuous intake of LC-Plasma increased T helper 1 responses, which contribute to antigen-specific cellular and humoral immune responses. Taken together, these results reveal that the oral intake of pDC-stimulative LAB enhances antigen-specific immune responses.

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1. Introduction

The innate immune response is the first line of defense against pathogens and leads to acquired immune responses [1]. Among the innate immune cells, dendritic cells (DC) are the major antigen-presenting cell type, and they initiate acquired immune responses by presenting antigens to T cells. DC activate T cells through three DC-derived signals: an antigen-specific signal mediated by major histocompatibility complex (MHC) class I or II associated with peptides derived from antigens; a co-stimulatory signal such as CD80 or CD86 expressed on DC; and a polarizing signal mediated by the secretion of cytokines such as interferons (IFNs) or interleukins (ILs) [2]. DC are classified into two major subsets: conventional DC (cDC), which are considered antigen-presenting cells [3]; and plasmacytoid DC (pDC), which differ from cDC in terms of their ability to produce large amounts of type I IFN in response to the recognition of pathogens through Toll-like receptor 7/9 (TLR-7/9) [4–8].

Lactic acid bacteria (LAB) are one of the most commonly used food supplements for human health, and various beneficial effects of LAB, such as improving intestinal conditions and host metabolism, have been reported [9,10]. In addition to the physiological effects of LAB, potent and diverse immune-modulatory effects have been reported [11]. Oral intake of some strains of LAB has been reported to affect innate immune cells and protect the host against pathogenic bacterial or viral infection [12,13]. In addition, specific strains of LAB have been reported to ameliorate allergy symptoms by acting on cDC and suppressing T helper 2 (Th2) responses and serum IgE elevation in a mouse model of allergy [14,15]. Although the effect of LAB on innate immune cells such as cDC or macrophages has been well studied, how LAB affects antigen-specific immune responses is poorly understood.

We previously reported that *Lactococcus lactis* strain Plasma (LC-Plasma, also known as *Lactococcus lactis* subsp. *lactis* JCM 5805) stimulates pDC to induce IFN-α production via the TLR-9 signaling pathway [16]. Recently, it was reported that pDC also participate in antigen-specific immune responses by activating various immune cells such as T cells and B cells. Studies suggest that pDC induce antigen-specific anti-tumor or anti-viral immune responses that

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synergize with cDC through direct communication between pDC and cDC [17–19]. Moreover, an analysis of pDC-depleted mice demonstrated that pDC are associated with the initiation of $CD4^+$ T cell or $CD8^+$ T cell responses [20]. In addition to antigen-specific T cell immunity, pDC also differentiate naïve B cells into antibody-producing plasma cells via IFN-α [21,22]. Thus, cDC as well as pDC might elicit antigen-specific immune responses via direct or indirect mechanisms.

Given the above observations, the aim of the present study was to determine whether the pDC-stimulative LAB LC-Plasma affects antigen-specific immune responses, in addition to stimulating the innate immune system.

2. Materials and methods

2.1. Mice

Six-week-old C57BL/6J mice were purchased from Charles River Japan (Kanagawa, Japan). OT-I CD8-Tg mice specific for OVA peptide 257-264 and OT-II CD4-Tg mice specific for OVA peptide 323-339 were bred and housed in laboratory animal facility in Kirin Company. All animals were maintained in a specific pathogen-free facility. All animal experiments were performed in accordance with the guidelines for the care and use of laboratory animals of Kirin Company. The studies were approved by the Committee for Animal Experimentation Kirin Company. All efforts were made to minimize animal suffering.

2.2. LAB strains

The LAB strains tested in this study were purchased from collections held at the Japan Collection of Microorganisms (JCM) and American Type Culture Collection (ATCC). Culture of LAB strains has been previously described [16]. Cultured LAB strains were washed twice with sterile distilled water, heat-killed at 100 °C, lyophilized, and suspended in phosphate-buffered saline.

2.3. Bone marrow-derived DC culture

The preparation of bone marrow-derived DC (BMDC) has been previously described [16]. After 7 days of culture, BMDC were washed with RPMI 1640 medium and then incubated at a density of 1×10^6 cells/ml with 10 μg/mL of LC-Plasma or *Lactobacillus rhamnosus* strain GG (ATCC 53103) for 24 h. To block TLR-9 signaling, a TLR-9 antagonist (InvivoGen, CA, USA) was added with LAB. Treated cells were analyzed by FACS, and cell culture supernatants were analyzed by enzyme-linked immunosorbent assay (ELISA).

2.4. FACS analysis

Cells were stained with the following fluorescent dye-conjugated anti-mouse antibodies or tetramers: MHC I-FITC (28-14-8) (eBioscience, CA, USA), MHC II-FITC (M5/114.15.2) (eBioscience), CD8-FITC (KT15) (MBL, Nagoya, Japan), CD11b-PE (M1/70) (eBioscience), CD80-APC (16-10A1) (eBioscience), CD86-APC (GL1) (eBioscience), CD4-APC (RM4-5) (eBioscience), CD3e-APC-Cy7 (145-2C11) (BD Pharmingen, CA, USA), B220-APC-Cy7 (RA3-6B2) (BD Pharmingen), CD11c-PE-Cy7 (N418) (eBioscience), 7-AAD (BD Pharmingen) and H-$2D^b$ Influenza PA Tetramer-SSLENFRAYV-PE (MBL). After staining, the cells were washed twice with FACS buffer (0.5% BSA in PBS buffer) and suspended in 4% paraformaldehyde (Wako, Osaka, Japan) for FACS analysis. Cell populations were analyzed by using a FACS Canto II (BD Biosciences, NJ, USA) coupled with FCS Express software (De Novo Software, CO, USA).

2.5. ELISA

The concentration of cytokines in cell culture supernatants was measured by commercially available ELISA kits. A mouse IFN-α ELISA kit was purchased from PBL Assay Science (NJ, USA), and mouse IFN-γ, IL-2, and IL-4 Ready-SET-GO kits were purchased from eBioscience. Anti-PA antibodies in mouse plasma were measured as follows. MaxiSorp ELISA plates (Nunc, Roskilde, Denmark) were coated with 20 μg/ml of PA (MBL) at room temperature overnight. After washing and blocking, collected plasma was added and incubated at room temperature for 2 h. HRP-conjugated anti-mouse IgG, IgG2a, and IgE (Bethyl, TX, USA) were used for detection. The substrate was TMB (eBioscience), and the reaction was stopped by adding 1 M phosphoric acid. Absorbance was measured at 450 nm.

2.6. Spleen assay

Splenocytes were prepared as previously described [16]. Prepared splenocytes were cultured at a density of 1×10^6 cells/ml with LC-Plasma and 10 μM OVA peptide 257-264 (MBL) for OT-I-derived splenocytes or 10 μM OVA peptide 323-339 (MBL) for OT-II-derived splenocytes. After 48 h of cell culture, the supernatants were collected and analyzed by ELISA.

2.7. Co-culture study

$CD8^+$ T cells from the spleens of OT-I mice and $CD4^+$ T cells from the spleens of OT-II mice were isolated by using a $CD8^+$ or $CD4^+$ T Cell Isolation kit (Miltenyi Biotec, CA, USA), respectively. Bone marrow-derived DC (BMDC) were treated with 1 μM OVA 257-264 or 1 μM OVA 323-329 and LC-Plasma at 37 °C for 24 h. BMDC treated with OVA 257-264 were co-cultured with $CD8^+$ T cells from OT-I mice, and BMDC treated with OVA 323-329 were co-cultured with $CD4^+$ T cells from OT-II mice for 48 h. After 48 h, the supernatants were collected and analyzed by ELISA.

2.8. In vivo antigen-specific immune response study

Six week-old C57BL/6 mice were acclimatized for 1 week with free access to water and a basic diet, AIN93G (Oriental Yeast, Tokyo, Japan). Mice were divided into two groups, each consisting of eight mice. Control groups were fed AIN93G, and test groups were fed AIN93G plus heat-killed LC-Plasma at 1 mg/mouse/day. Two weeks later, 10 μg of Influenza PA peptide (MBL) emulsified in CFA (Sigma) was injected intraperitoneally into each mouse. Oral administration of LC-Plasma was continued throughout the experiment. Twelve days after immunization, mice were sacrificed and their spleens and blood were collected. To measure the ratio of antigen-specific $CD8^+$ T cells in the spleen, splenocytes were stained with a tetramer as described above. Splenocytes were also re-stimulated with PA peptide for 48 h and the cell culture supernatants were analyzed for IFN-γ or IL-4 by ELISA. To determine the amount of antigen-specific antibodies in the blood, antigen-specific antibodies in plasma were evaluated by ELISA.

2.9. Statistical analysis

All values are expressed as mean ± SEM. Data from the cytokine production assays and flow cytometry analysis *in vitro* and *in vivo* were analyzed by one-way ANOVA, followed by Tukey-Kramer's test or Student's t-test. All statistical analyses were performed by using the Ekuseru-Toukei 2012 software program (Social Survey Research Information, Tokyo, Japan). A P value of <0.05 was considered statistically significant.

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