Essential Roles of Monocytes in Stimulating Human Peripheral Blood Mononuclear Cells with *Lactobacillus casei* To Produce Cytokines and Augment Natural Killer Cell Activity

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We examined the effect of a probiotic strain, *Lactobacillus casei* strain Shirota, on cytokine production and natural killer (NK) cell activity in human peripheral blood mononuclear cells (PBMC). The cellular mechanisms of immunoregulation by *L. casei* strain Shirota were also investigated. *L. casei* strain Shirota stimulated PBMC to secrete interleukin-12 (IL-12), gamma interferon (IFN-γ), tumor necrosis factor alpha (TNF-α), and IL-10. However, depletion of monocytes from PBMC eliminated the induction of these cytokines. *L. casei* strain Shirota was phagocytosed by monocytes and directly stimulated them to secrete IL-12, TNF-α, and IL-10. IFN-γ production was diminished by the addition of anti-IL-12 antibody to the PBMC cultures. Purified T cells, but not NK cells, produced IFN-γ effectively when stimulated with *L. casei* strain Shirota in the presence of monocytes, indicating that monocytes triggered by *L. casei* strain Shirota help T cells to produce IFN-γ through secreting IL-12. In addition, NK cell activity and CD69 expression on NK cells increased after cultivation of PBMC with *L. casei* strain Shirota. When monocytes were depleted from PBMC, *L. casei* strain Shirota did not enhance NK cell activity. These results demonstrate that monocytes play critical roles in the induction of cytokines and following the augmentation of NK cell activity during the stimulation of human PBMC with *L. casei* strain Shirota.

Increasing interest has been paid to the beneficial functions of lactobacilli in addition to their importance in the preparation process of fermented foods such as yogurt and cheese (5, 29). Lactobacilli and bifidobacteria are now recognized as the most popular probiotics, which are defined as live microbial food ingredients beneficial to human health (31). It has been shown that some strains of probiotic lactobacilli are effective in reducing the incidence of cancer and infectious diseases, ameliorating inflammatory bowel diseases, and preventing allergies in experimental animal models and in humans (1, 13, 28, 32). Although the detailed mechanisms of these beneficial effects exerted by lactobacilli have not yet been clarified, their effects on the host immune system are suggested.

Cytokines induced by lactobacilli are considered to play key roles in immunoregulation. Several studies have revealed that some specific strains of lactobacilli can induce proinflammatory cytokines such as interleukin-1 (IL-1), IL-6, IL-12, tumor necrosis factor alpha (TNF-α), and gamma interferon (IFN-γ) as well as anti-inflammatory cytokines such as IL-10 and transforming growth factor β (4, 26, 38). In these cytokines, IFN-γ and IL-12 potently augment the functions of macrophages and NK cells, which may be a possible mechanism of their anticarcinogenic and anti-infectious activity (3, 37). On the other hand, induction of IL-10 and transforming growth factor β by lactobacilli is assumed to participate in the down-regulation of inflammation, since these cytokines can inhibit the functions of macrophages and T cells and promote the development of regulatory T cells (18).

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The immunoregulatory functions of *Lactobacillus casei* strain Shirota, a well-known probiotic strain, have been extensively studied using in vitro and in vivo murine models. *L. casei* strain Shirota stimulates murine macrophages to secrete IL-12, which induces T cells to produce IFN-γ (17), and *L. casei* strain Shirota-induced IL-12 promotes the differentiation of naive CD4+ T cells into Th1 cells (33). Administration of *L. casei* strain Shirota to mice enhanced the production of IL-12, TNF-α, and IFN-γ and augmented NK cell activity, leading to the prevention of influenza virus infection and cancer (10, 11, 36, 39).

Randomized clinical trials revealed that oral ingestion of *L. casei* strain Shirota prevented the recurrence of superficial bladder cancer and the development of colorectal tumors with moderate or higher atypia (2, 12), and an epidemiological survey showed that the habitual intake of fermented milk containing *L. casei* strain Shirota reduced the risk of bladder cancer (27). Moreover, the intake of fermented milk containing *L. casei* strain Shirota restored NK cell activity in subjects with relatively low NK cell activity (20, 24, 25). Those results suggest that orally ingested *L. casei* strain Shirota may enhance innate immunity and suppress the occurrence of cancer. Previous findings obtained from the studies using murine models suggested that proinflammatory cytokines, such as IL-12, induced by *L. casei* strain Shirota might promote the augmentation of NK cell activity in humans. However, little information concerning the effects of *L. casei* strain Shirota on cytokine production by human immunocompetent cells is available. Considering the clinical applications of *L. casei* strain Shirota in disease prevention, it will be of great importance to reveal the cellular mechanisms by which *L. casei* strain Shirota induces cytokines and modulates the host immune system.

In the present study, we examined the effect of *L. casei* strain...
Shirota on cytokine production and NK cell activity in human peripheral blood mononuclear cells (PBMC). The data demonstrate that monocytes are essential for L. casei strain Shirota to induce the production of IL-12, IFN-γ, TNF-α, and IL-10 and to augment NK cell activity in human PBMC.

MATERIALS AND METHODS

Bacteria. The probiotic strain Lactobacillus casei Shirota was originally isolated from human intestine and maintained at the Yakult Central Institute for Microbiological Research (Tokyo, Japan). Lactobacillus acidophilus ATCC 4356 and Bifidobacterium breve ATCC 15700 were obtained from the American Type Culture Collection (Rockville, MD). L. casei strain Shirota and L. acidophilus were cultured in Lactobacilli-MRS broth (BD, Sparks, MD), and B. breve was cultured in GAM broth (Nissui, Tokyo, Japan) supplemented with 1% glucose at 37°C for 20 h. Cultured bacteria were washed with sterile, distilled water, heated at 100°C for 30 min, and then lyophilized. We used heat-killed bacteria in the present study, because a preliminary experiment showed that heat-killed and X-ray-irradiated (10,000 rad) L. casei strain Shirota induced similar amounts of cytokines in PBMC cultures.

Subjects. Twenty-nine healthy Japanese volunteers aged 24 to 57 years (42.2 ± 8.7 years; 26 males and 3 females) were enrolled in this study. We explained the aim, significance, and risk of the study to the volunteers, and informed consent was obtained from all the subjects. Experiments were carried out in accordance with the guidelines of the Helsinki Declaration and with the approval of the ethical committee overseeing clinical studies at the Yakult Central Institute.

Preparation of PBMC and cell fractions. PBMC were isolated from fresh whole blood by Ficoll-Conray (Lymphosepar I; Immuno-Biological Laboratories, Takasaki, Japan) density gradient centrifugation. Monocytes, T cells, and NK cells were purified from PBMC by magnetic cell sorting with anti-CD14, anti-CD3, and anti-CD56 microbeads (Miltenyi Biotech, Bergish Gladbach, Germany), respectively. The purity of monocytes (CD14+ cells), T cells (CD3+ cells), and NK cells (CD56+ cells) in each fraction were confirmed by flow cytometry to be more than 92%, 98%, and 93%, respectively. Monocyte-depleted, T-cell-depleted, and NK-cell-depleted fractions were prepared by magnetic cell sorting, and the resultant cell fractions contained less than 1% CD14+ cells, 4% CD3+ cells, or 1% CD56+ cells. PBMC were X irradiated at 3,000 rad and used as monocyte-enriched cell fractions, because X irradiation destroys lymphocyte functions, leaving monocyte functions hardly damaged.

Cell cultures. To analyze cytokine production, unfractionated PBMC (2 × 10^6 cells) or fractionated cells (the numbers of cells seeded are described in the figure legends) were cultured with L. casei strain Shirota (0.1 to 100 μg/ml) in 200 μl of AIM-V medium (Gibco BRL, Rockville, MD) in a round-bottomed 96-well culture plate (Nunc, Roskilde, Denmark). Supernatants were collected on day 3 for determination of IL-12, TNF-α, and IL-10 levels or on day 6 for determination of IFN-γ levels, unless otherwise indicated. In the neutralization study, mouse anti-human IL-12 monoclonal antibody (10 μg/ml) (clone 8.6; BD PharMingen, San Diego, CA) or control mouse immunoglobulin G1 (FN50; BD Pharmingen) and phycoerythrin-conjugated anti-human CD56 antibody (clone B159; BD Pharmingen) were added to the cultures containing L. casei strain Shirota (1 μg/ml) at the beginning of culture. To examine the effect of bacteria on NK cells, PBMC or fractionated cells (2 × 10^6 cells) were cultured in the absence or presence of bacteria (1 μg/ml) in 3 ml of AIM-V medium in a flat-bottomed 12-well culture plate (Nunc) for 6 days. Thereafter, viable cells were counted and subjected to assay for NK cell activity and CD69 expression. Supernatants were collected on day 3 for determination of IL-12 levels.

Microscopic analysis. Purified monocytes (2 × 10^6 cells) were plated onto round 12-mm collagen type I-coated cover glasses (Asahi Techno Glass, Tokyo, Japan) in a 24-well culture plate (Nunc) in 2 ml AIM-V medium and cultured in the absence or presence of L. casei strain Shirota (10 μg/ml). The morphology of monocytes was observed on day 3 by light microscopy. To examine phagocytosis of bacteria, the monocytes were harvested after 24 h of culture, rinsed with phosphate-buffered saline, fixed with methanol for 10 min, and then stained with Giemsa solution. Phagocytosis of L. casei strain Shirota by monocytes was observed macroscopically.

Assay of NK cell activity. NK cell activity of freshly isolated or bacterium-stimulated PBMC was measured by a chromium release assay using K562 target cells. K562 cells were labeled with 100 μCi Na₂⁵¹CrO₄ for 1 h. PBMC (1.56 × 10⁷ to 2.5 × 10⁶ cells) were placed into each well of a round-bottomed 96-well culture plate to which ⁵¹Cr-labeled K562 cells (5 × 10⁵ cells) were then added. After centrifugation at 150 × g for 5 min, cells were incubated in 200 μl of 10% fetal calf serum–RPMI 1640 medium (Sigma, St. Louis, MO) for 4 h. Spontaneous release was determined by incubating only target cells, and maximal release was measured by standing target cells in medium containing 1% Triton X-100. Supernatants were collected, and radioactivity released from lyzed target cells during incubation was measured. The percentage of specific lysis was calculated using the following formula: specific lysis (%) = (experimental release – spontaneous release)/(maximal release – spontaneous release) × 100. One lytic unit (LU) was defined as the cytotoxic activity giving 33.3% of maximal release (24). NK cell activity was expressed as either percent specific lysis at an effector/target ratio of 12.5 or LU/10⁶ cells.

ELISA for cytokines. Purified mouse anti-human cytokine monoclonal capture antibodies, corresponding biotinylated detection antibodies, and standard recombiant human cytokines were all obtained from Biosource International (Carpal, West Chester, PA). Purified mouse anti-human cytokine monoclonal capture antibodies, corresponding biotinylated detection antibodies, and standard recombiant human cytokines were all obtained from Biosource International (Camarillo, CA).

Statistical analysis. Differences in NK cell activity between groups were analyzed by the paired Student t test. Differences in cytokine production and CD69 expression between groups were analyzed by the Mann-Whitney U test.

RESULTS

L. casei strain Shirota stimulates PBMC to secrete various cytokines. PBMC were prepared from six subjects were cultured with various concentrations of L. casei strain Shirota for various time periods, and the levels of IL-12, TNF-α, IL-10, and IFN-γ in the supernatants were measured. As shown in Fig. 1,
all cytokines tested were detected in the supernatants of PBMNC stimulated with *L. casei* strain Shirota, although the absolute amounts of cytokines varied from subject to subject, and the dose-response curves and time courses of cytokine production were different for all cytokines. The optimal dose of *L. casei* strain Shirota to induce IL-12 and IFN-γ was 1 to 10 μg/ml, and these cytokines were induced only weakly at a high dose (100 μg/ml) of *L. casei* strain Shirota (Fig. 1A). On the other hand, a high dose of *L. casei* strain Shirota induced TNF-α and IL-10 more effectively than low doses (0.1 to 1 μg/ml). The results showed that IL-12 was detected as early as day 1, and the level of IL-12 continued to be of a similar degree until day 6 (Fig. 1B). The level of IFN-γ was lower on day 1 and gradually increased until day 6. In contrast, the level of IL-10 was highest on day 1 and decreased thereafter. TNF-α was also produced on day 1, and the level of TNF-α changed thereafter in a variable way.

Monocytes play critical roles in cytokine production of PBMNC triggered by *L. casei* strain Shirota. We prepared monocytes (CD14+ cells) from PBMNC by immunomagnetic separation and cultured them in the presence of *L. casei* strain Shirota. Monocytes phagocytosed *L. casei* strain Shirota and markedly spread (Fig. 2A and B). In contrast, monocytes simply adhered in the absence of *L. casei* strain Shirota (Fig. 2C).

Cytokine responses of monocytes and monocyte-depleted PBMNC were compared. Monocytes, but not monocyte-depleted PBMNC, secreted IL-12, TNF-α, and IL-10 in response to *L. casei* strain Shirota (Fig. 3). On the other hand, monocytes and monocyte-depleted PBMNC could hardly produce IFN-γ, indicating that monocytes and other cells cooperate to produce IFN-γ more efficiently.

**FIG. 2.** Monocytes phagocytose *L. casei* strain Shirota and change in morphology. Purified monocytes (2 × 10^5 cells) were cultured on collagen type I-coated cover glasses with *L. casei* strain Shirota (10 μg/ml) for 24 h and stained with Giemsa solution (A). Monocytes were cultured in the presence (B) or absence (C) of *L. casei* strain Shirota for 72 h. Phagocytosis of bacteria by monocytes and morphology of monocytes were observed by light microscopy. Original magnifications, ×1,000 (A) and ×200 (B and C).

T cells produce IFN-γ through IL-12 secretion by *L. casei* strain Shirota-triggered monocytes. The involvement of IL-12 in IFN-γ production was examined. As shown in Fig. 4, the addition of anti-IL-12 antibody to the PBMNC cultures con-
taining \textit{L. casei} strain Shirota markedly diminished the production of IFN-\(\gamma\), proving that IL-12 is indispensable for outstanding production of IFN-\(\gamma\).

Next, the type of cell that produced IFN-\(\gamma\) was identified. Depletion of T cells, but not of NK cells, from PBMNC resulted in the disappearance of IFN-\(\gamma\) production (Fig. 5). Although purified T cells alone did not produce IFN-\(\gamma\) in response to \textit{L. casei} strain Shirota, IFN-\(\gamma\) was produced when T cells were cultured in the presence of \textit{L. casei} strain Shirota together with irradiated PBMNC or purified monocytes (Fig. 5 and 6). In contrast, only low levels of IFN-\(\gamma\) were produced by NK cells even in the presence of irradiated PBMNC or monocytes. These results suggest that T cells mainly produce IFN-\(\gamma\) in the presence of IL-12 secreted by monocytes during stimulation of PBMNC with \textit{L. casei} strain Shirota.

\textit{L. casei} strain Shirota promotes the activation of NK cells. The effects of \textit{L. casei} strain Shirota and other bacteria that are often used in dairy foods on NK cells were investigated. PBMNC were cultured with \textit{L. casei} strain Shirota, \textit{L. acidophilus}, or \textit{B. breve} at a concentration of 1 \(\mu\)g/ml for 6 days, and viable cells were then collected. The cultured cells as well as freshly isolated cells (Fresh) were assayed for NK cell activity (Fig. 5). Al-

FIG. 5. IFN-\(\gamma\) is produced mainly by T cells. PBMNC, T-cell-deple
ted cells (T-de), and NK cell-depleted cells (NK-de) were cultured with \textit{L. casei} strain Shirota (1 \(\mu\)g/ml) at a density of \(2 \times 10^5\) cells/well. Purified T cells and NK cells at a density of \(1 \times 10^5\) cells/well were cultured with \textit{L. casei} strain Shirota in the absence (–) or presence of irradiated (3,000 rad) PBMNC (Irr-PBMNC) (\(2 \times 10^5\) cells/well). The levels of IL-12 on day 3 and IFN-\(\gamma\) on day 6 in culture supernatants were determined by ELISA. Data are expressed as means ± SD of individual values for four subjects. Each symbol represents values of individual subjects. *, \(P < 0.05\) versus the PBMNC group; #, \(P < 0.05\) versus the irradiated-PBMNC group.

FIG. 6. Monocytes are essential for IFN-\(\gamma\) production by T cells. PBMNC (\(2 \times 10^5\) cells/well) were cultured with \textit{L. casei} strain Shirota (1 \(\mu\)g/ml). Purified monocytes (Mono) (\(1 \times 10^5\) cells/well) were cultured with \textit{L. casei} strain Shirota in the absence (–) or presence (+) of purified T cells or NK cells (\(1 \times 10^5\) cells/well). The levels of IL-12 on day 3 and IFN-\(\gamma\) on day 6 in culture supernatants were determined by ELISA. Data are expressed as means ± SD of individual values for four subjects. Each symbol represents values of individual subjects. *, \(P < 0.05\) versus the PBMNC group; #, \(P < 0.05\) versus the Mono group.

FIG. 7. \textit{L. casei} strain Shirota and other bacteria induce IL-12 secretion, augment NK cell activity, and enhance CD69 expression on NK cells. PBMNC were cultured in the absence (Med) or presence of \textit{L. casei} strain Shirota (LcS), \textit{L. acidophilus} (La), or \textit{B. breve} (Bb) at a concentration of 1 \(\mu\)g/ml for 6 days, and viable cells were then collected. The cultured cells as well as freshly isolated cells (Fresh) were assayed for NK cell activity (Fig. 5 and 6). In contrast, only low levels of IFN-\(\gamma\) were produced by NK cells even in the presence of irradiated PBMNC or monocytes. These results suggest that T cells mainly produce IFN-\(\gamma\) in the presence of IL-12 secreted by monocytes during stimulation of PBMNC with \textit{L. casei} strain Shirota.

\textit{L. casei} strain Shirota promotes the activation of NK cells. The effects of \textit{L. casei} strain Shirota and other bacteria that are often used in dairy foods on NK cells were investigated. PBMNC were cultured with \textit{L. casei} strain Shirota, \textit{L. acidophilus}, or \textit{B. breve} for 6 days, and NK cell activity and CD69 expression on NK cells were analyzed. Although the NK cell activity of PBMNC almost disappeared after cultivation without bacteria, the addition of \textit{L. casei} strain Shirota augmented NK cell activity and induced CD69 expression on NK cells (Fig. 7). Although \textit{L. acidophilus} and \textit{B. breve} also enhanced NK cell activity as well as CD69 expression on NK cells, the ability of these bacteria to activate NK cells was weaker than that of \textit{L. casei} strain Shirota. Furthermore, the ability of these three bacteria to augment NK cell activity seemed to correlate with their IL-12-inducing ability (Fig. 7).
Monocytes are essential in the augmentation of NK cell activity. In order to determine how *L. casei* strain Shirota augments NK cell activity, we depleted monocytes or T cells from PBMC and examined the effect of *L. casei* strain Shirota on NK cell activity. NK cell activity decreased in all cases except one (indicated by the open circle in Fig. 8) when monocyte-depleted PBMC were cultured with *L. casei* strain Shirota. Interestingly, after cultivation of T-cell-depleted PBMC even without *L. casei* strain Shirota, NK cell activity remained at a relatively high level. Furthermore, T-cell-depleted PBMC cultured with *L. casei* strain Shirota showed higher NK cell activity than did PBMC cultured without *L. casei* strain Shirota. These results suggest that monocytes, but not T cells, play critical roles in the *L. casei* strain Shirota-induced augmentation of NK cell activity.

**DISCUSSION**

Cytokine induction by a probiotic *Lactobacillus* strain, *L. casei* strain Shirota, and associated cellular mechanisms were investigated using healthy human PBMC. The data showed that *L. casei* strain Shirota was phagocytosed by monocytes and directly stimulated them to secrete not only proinflammatory cytokines such as IL-12 and TNF-α but also an anti-inflammatory cytokine, IL-10. Although it is still unknown how the production of IL-12 and IL-10 is regulated in monocytes triggered by *L. casei* strain Shirota, the different patterns of dose-response curves and time courses of IL-12 and IL-10 production suggest that different molecular mechanisms act in the induction of these two cytokines. It has been previously reported that other *Lactobacillus* strains preferentially induce either IL-12 or IL-10 under different conditions using human and murine immunocompetent cells (4, 9). These properties of lactobacilli may be concerned with their immunoregulatory activities to augment the innate immune defense and to down-regulate inflammation.

Studies of cell separation and reconstitution revealed that *L. casei* strain Shirota induced IFN-γ production of T cells through IL-12 secretion by monocytes. NK cells produced only low levels of IFN-γ when cultured with *L. casei* strain Shirota in the presence of monocytes (Fig. 6). It has been believed that NK cells as well as T cells produce large amounts of IFN-γ with the help of monocytes/macrophages and dendritic cells during the early phases of innate resistance to infections (37). Moreover, it was previously reported that NK cells mainly secreted IFN-γ when PBMC were stimulated with *Staphylococcus aureus* (40). Esin et al. (6) previously showed that NK cells could respond directly to *Mycobacterium bovis* bacillus Calmette-Guérin and produced IFN-γ when monocytes were depleted from PBMC. In the case of the response to nonpathogenic *Lactobacillus johnsonii*, NK cells secreted IFN-γ effectively in the presence of monocytes (7). Our study showed that the contribution of NK cells to IFN-γ production was much smaller than that of T cells when PBMC were cultured with *L. casei* strain Shirota, but cytotoxic activity and CD69 expression of NK cells were augmented by *L. casei* strain Shirota. Therefore, it is of great interest to unveil how IFN-γ production, cytotoxic activity, and CD69 expression of NK cells are differentially stimulated by pathogens and probiotics.

*L. casei* strain Shirota prompted monocytes to produce IL-12 more effectively in the presence of T cells or NK cells than in the absence of these cells (Fig. 6). It is well known that both stimulation of CD40 on monocytes/macrophages by CD40 ligand (CD154) on T cells and IFN-γ secreted by T cells and NK cells enhance IL-12 production by monocytes/macrophages (37). Karlsson et al. (14, 15) presented similar results indicating that *Lactobacillus plantarum* prompted human monocytes to secrete IL-12 effectively only when they were cultured in the presence of other mononuclear cells, including T cells, or supplemented IFN-γ. For the effective production of IL-12 by monocytes, *L. casei* strain Shirota is also considered to require other cells such as T cells and NK cells, which may play their roles through cell contact-based stimulation and IFN-γ secretion. Unexpectedly, depletion of either T cells or NK cells from PBMC resulted in the increase of IL-12 production in response to *L. casei* strain Shirota stimulation (Fig. 5). Although the mechanisms of the increase in IL-12 production are not clear, the following explanation might be possible. While relative proportions of T cells and monocytes in freshly isolated PBMC were 59.1% ± 5.4% and 10.2% ± 6.9%, respectively, depletion of T cells from PBMC increased the proportion of monocytes by at least twofold, which might lead to the increase in IL-12 production. In the case of depletion of NK cells from PBMC, enhanced IFN-γ production by the remaining T cells might contribute to the increase in IL-12 production by monocytes.

Cultivation of PBMC with *L. casei* strain Shirota resulted in the augmentation of NK cell activity, for which monocytes were required (Fig. 8). The comparative analysis of *L. casei* strain Shirota with *L. acidophilus* and *B. breve* showed that the ability of these bacteria to augment NK cell activity seemed to correlate with their IL-12-inducing ability. We found that the addition of an anti-IL-12 antibody reduced the ability of *L. casei* strain Shirota to augment NK cell activity (K. Takeda et al., unpublished observation). These findings support the idea that IL-12 secreted by monocytes in response to *L. casei* strain Shirota is important for the augmentation of NK cell activity. Haller et al. (7) previously showed that *L. johnsonii* promoted the effective activation of NK cells, including enhanced CD69 expression and IFN-γ secretion, when NK cells were cultured.
with monocytes. Those authors indicated that costimulatory signals via CD80 and CD86 on monocytes, as well as IL-12, were important for the full activation of NK cells. It remains to be elucidated whether L. casei strain Shirota-stimulated monocytes might contribute to the enhancement of NK cell activity through cell contact-dependent costimulation.

Interestingly, the depletion of T cells from PBMCN inhibited a decrease in NK cell activity during cultivation in the absence of L. casei strain Shirota. The following possibilities can be proposed. One possibility is that T cells might give suppressive signals to NK cells when these cells are cultured without L. casei strain Shirota. Alternatively, the depletion of T cells from PBMCN increased the relative proportions of monocytes as well as NK cells, as mentioned above, which might provide an increased opportunity for cell-to-cell contact between monocytes and NK cells. Under these conditions, monocytes might be able to maintain NK cell activity without specific stimulation. Although most data indicate the importance of monocytes in the activation and maintenance of NK cell activity, PBMCN from one out of six subjects responded to L. casei strain Shirota to augment NK cell activity after depletion of monocytes, suggesting that other mechanisms not mediated by monocytes may be involved in NK cell activation. In fact, it was reported previously that L. johnsonii acted on purified NK cells directly to enhance the expression of CD25 (8) and that M. bovis bacillus Calmette-Guérin augmented NK cell activity even when monocytes were depleted from PBMCN (6).

The results obtained in this study clearly indicate that human immunocompetent cells, similar to murine cells, can respond to L. casei strain Shirota and that L. casei strain Shirota has a better potential to induce IL-12 and augment NK cell activity than other bacteria tested. The better potential revealed by this in vitro study cannot directly provide evidence that L. casei strain Shirota is an effective probiotic strain to modulate the immune systems (16, 19, 21, 23, 34). These immunoregulatory activities, probably through normalizing dysregulated host immune defenses (10, 22) and inhibit the development of allergies, autoimmune diseases, and inflammatory bowel diseases, probably through normalizing dysregulated host immune systems (16, 19, 21, 23, 34). These immunoregulatory functions of L. casei strain Shirota have not yet been confirmed in humans. Human clinical trials or epidemiological surveys will be necessary to confirm these beneficial functions of L. casei strain Shirota.

In the present study, we demonstrate that monocytes are essential for L. casei strain Shirota to induce various cytokines and to augment NK cell activity during in vitro stimulation of human PBMCN. We hope that the findings provide helpful information on the cellular mechanisms of immunoregulation by probiotics in view of the application of these bacteria in controlling certain clinical diseases. Macrophages and dendritic cells in the gut, however, rather than monocytes in the circulation are likely to be the primary target cells for probiotics when they are orally administered. Recent reports have shown that intestinal resident macrophages and dendritic cells are down-regulated for the production of proinflammatory cytokines in response to bacterial stimulation (30, 35). Moreover, Karlsson et al. (15) previously showed that cytokine responses of human monocytes to L. plantarum or Bifidobacterium adolescentis were changed when they differentiated into dendritic cells. To better understand the immunomodulating functions of probiotics, further studies examining the responses of mucosal macrophages and dendritic cells to L. casei strain Shirota and other probiotics remain to be done.

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