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Original Article

The Effect of Microbial Extracts on the Cell Activation and Inhibition Associated with Atopic Dermatitis

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Atopic dermatitis (AD) is an inflammatory, relapsing, chronic skin disease and lesions in AD are frequently colonized with *Staphylococcus aureus* (*S. aureus*). Activation of T cells and IgE production by staphylococcal enterotoxins B (SEB) plays a crucial role in the pathogenesis of AD. *Enterococcus faecalis* (*E. faecalis*) is a nonpathogenic bacterium and produces the probiotic products that have been shown to have inhibitory effects on inflammatory responses. In present study, we carried out to assess the anti-inflammatory role of lyzed *E. faecalis* against the damaging effects of SEB on AD related immune responses. Furthermore, we attempted to determine whether the co-cultured lyzed *E. faecalis* can influence the colonization of *S. aureus*. As a result, we identified the effect of *E. faecalis* lysate as a potent therapeutic agent for atopic dermatitis (AD). *E. faecalis* lysate reduces the productions of total IgE and cytokines of AD-related immune cells in response to SEB stimulation. The proliferation of *S. aureus* was also inhibited by *E. faecalis* lysate. In conclusions, *E. faecalis* lysate may improve the skin-defense system disturbed by atopic condition, and may prevent subsequent secondary infection of *S. aureus* and development of AD.

Key Words: Atopic dermatitis, Staphylococcal enterotoxins B, *Enterococcus faecalis*, IgE, cytokines

INTRODUCTION

Atopic dermatitis (AD) is an important common chronic disease and also known as atopic eczema. AD patients often have dry and scaly skin that span the whole body and have intensely itchy red, splotchy, raised lesions in the folded region of skin (Boguniewicz and Leung, 2011). AD is caused by a variety of environmental factors that interact with susceptibility genes in AD. These factors include allergens, stress and secondary microbial infections (Baker, 2006).

In many AD patients, *Staphylococcus aureus* (*S. aureus*) can frequently colonize in skin lesions, and can contribute to persistent skin inflammation in AD patients by secreting various toxins (Baker, 2006). *S. aureus* secretes staphylococcal superantigens (SsAgs), including staphylococcal enterotoxin A (SEA), staphylococcal enterotoxin B (SEB) and toxic shock syndrome toxin-1 (TSST-1) (Pinchuk et al., 2010). In these SsAgs, SEB induces the marked activation of T cells and other immune cells (Skov et al., 2000). Activated T cells produce various cytokines and the cytokines function as important factors in these disease processes (Baker, 2006). A number of cytokines typically increase in allergic disease and have important effects on the activation of other inflammatory cells, dysregulation of skin barrier proteins and induction of IgE production (Guzik et al., 2005; Leung, 2013). Persistent *S. aureus* colonization on the skin of AD is also associated with higher IgE levels. Higher IgE levels produced by B cells may contribute to an increased susceptibility to bacterial infections (Guzik et al., 2005).

In contrast, resident nonpathogenic bacteria seem to stabilize the immune system of surface organs such as the

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skin (Roll et al., 2001; Rakoff-Nahoum et al., 2004). The nonpathogenic bacteria may restore the balance between the immune responses of pathogenic and nonpathogenic bacteria and may have the potential to alleviated inflammatory responses in damaged lesion.

Enterococcus faecalis (*E. faecalis*) is a Gram-positive, nonpathogenic bacterium inhabiting the gastrointestinal tracts of humans. Lysate of *E. faecalis* is known as a probiotic product and has been shown to have inhibitory effects on allergen-induced local accumulation of eosinophils and anaphylaxis (Shimada et al., 2003; Shimada et al., 2004).

In present study, to investigate the effects of nonpathogenic bacteria on atopic dermatitis, we carried out to assess the anti-inflammatory role of lyzed *E. faecalis* against the damaging effects of SEB on AD related immune responses. Furthermore, we attempted to determine whether the co-cultured lyzed *E. faecalis* can influence the colonization of *S. aureus*.

MATERIALS AND METHODS

Strains and cell lines

The strains of *S. aureus* and *E. faecalis* were purchased from American Type Culture Collection (Rockville, MD, USA). Human B lymphoblast cell line, IM-9 cell, was purchased from Korean Cell Line Bank (Seoul, Korea). Human acute T leukemia cell line, Jurkat T cell, was purchased from American Type Culture Collection (USA).

Cell culture

IM-9 cells and Jurkat T cell were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated FBS, penicillin (100 U/ml), and streptomycin (100 μg/ml) (Life Technologies, Inc., USA), and were incubated at 37℃ in 5% CO_2 incubator.

Preparation of bacterial lysate from *E. faecalis*

E. faecalis was cultured for 24 hours at 37℃, in a broth medium containing 2% glucose, 2% yeast extract, 2% meat extract and 4% K_2HPO_4. *E. faecalis* cells were harvested by centrifugation, washed three times with distilled water (DW) and *E. faecalis* lysate was prepared by freeze/thawing the bacterial suspension three times, while sonicating the suspension between each cycle. The lysates were sterilized by passing through a 0.2 μm pore size sterile cellulose acetate membrane syringe filter.

MTT assay

MTT assay was performed to determine cell proliferation using the cell proliferation kit (Roche, Penzberg, Germany). After treatment of SEB or *E. faecalis* lysate, the cells in 100 μl of the culture medium were respectively plated into a 96-well culture plate. 10 μl of MTT solution was added in each well. After incubation of the plate at 37℃ for 4 h, 100 μl of solubilization solution was added to each well. After 24 h incubation, the absorbance was measured using an ELISA reader (Bio-Tek Instruments, Winooski, VT) at 550 nm.

Enzyme-linked immunosorbent assay (ELISA)

Protein concentrations in the supernatant of the cells were measured with a sandwich ELISA using OptEIA™ set human total IgE, IL-4, IL-5, IL-1β and TNF-α (BD bioscience, USA) according to the manufacturer's instructions. All assays were performed in triplicate. The concentration of each protein was calculated from the standard curve.

Measurement of bacterial proliferation

S. aureus was cultured on nonselcective medium, brain heart infusion (BHI) broth (BBL microbiology systems, USA) and incubated in an aerobic environment at 37℃ for 6 hours. After the incubation of *S. aureus*, 1 μg/ml of *E. faecalis* lysate was added in BHI broth. *S. aureus* treated with *E. faecalis* lysate was incubated in an aerobic environment at 37℃ for 24 hours. For measurement of bacterial proliferation, the turbidity of bacterial suspensions was measured with a spectrophotometer at a wavelength of 600 nm.

Statistical analysis

All data are expressed as the means ± S.E.M. Data were analyzed by Student's *t* test using the SPSS statistical

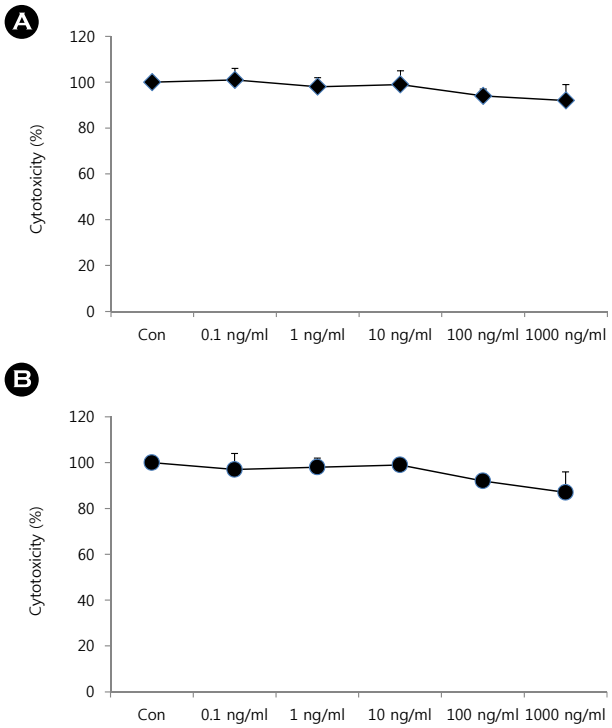

Fig. 1. The cytotoxic effect of SEB on IM-9 cell line and Jurkat T cell. IM-9 cells (A) and Jurkat T cells (B) were incubated for 24 hours in the absence and presence of SEB (0.1, 1, 10, 100 and 1,000 ng/ml). The cytotoxicity of SEB on these cells was measured by performing MTT assay. The data are expressed as the relative ration to the absorbance of the untreated cell (Con), which was set at 100%. All data are expressed as the means ± SD in three individual experiments.

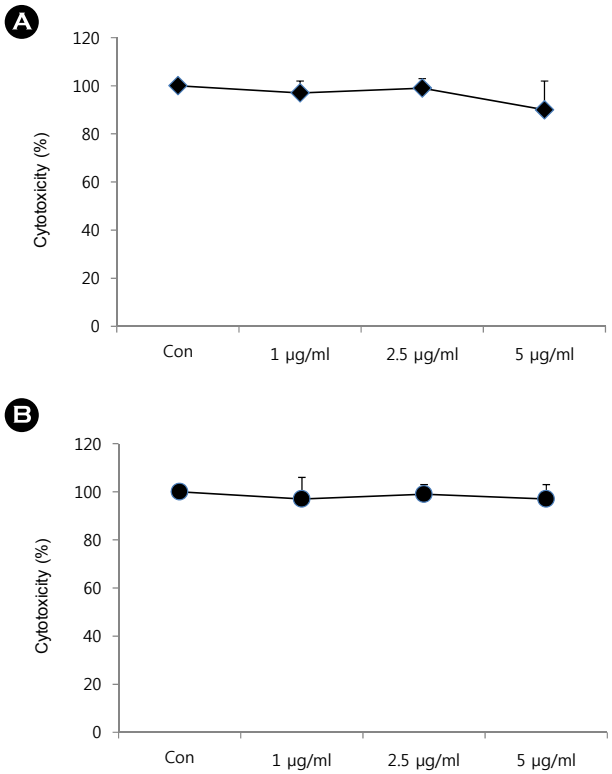

Fig. 2. The cytotoxic effect of *E. faecalis* lysate on IM-9 cell line and Jurkat T cell. IM-9 cells (A) and Jurkat T cells (B) were incubated for 24 hours in the absence and presence of *E. faecalis* lysate (Ef-L) (1, 2.5 and 5 μg/ml). The cytotoxicity of Ef-L on these cells was measured by performing MTT assay. The data are expressed as the relative ration to the absorbance of the untreated cell (Con), which was set at 100%. All data are expressed as the means ± SD in three individual experiments.

software package, Version 10.0 (SPSS Inc., Chicago, IL). A *p* value less than 0.05 was considered significant.

RESULTS

E. faecalis lysate decreases SEB-induced total IgE levels in IM-9 cells, the human B cell line

To find out the optimal concentrations of SEB and *E. faecalis* lysate for *in vitro* efficacy test, cytotoxicity of SEB and *E. faecalis* lysate was tested. We first determined the cytotoxic effect of SEB on IM-9 cells and Jurkat T cells. After addition of SEB in IM-9 cells and Jurkat T cells, cytotoxicity was detected using the MTT assay. We found that SEB at concentration up to 1,000 ng/ml did not affect the viability of IM-9 cells and Jurkat T cells (Fig. 1). *E. faecalis* lysate also was examined the cytotoxicity on IM-9 cells and Jurkat T cells. *E. faecalis* lysate at concentration up to 5 μg/ml has no cytotoxic effect on the IM-9 cells and Jurkat T cells (Fig. 2). So based on this results, 1,000 ng/ml of SEB and 5 μg/ml of *E. faecalis* lysate was applied to all experiments.

Next, we tested the effect of *E. faecalis* lysate on total IgE production induced by SEB in IM-9 cells that secrete highly level of total IgE (Kimata, 1996). The cells were stimulated with SEB (5 μg/ml) and then the level of IgE production in the culture supernatant were measured by ELISA. Stimulation of SEB significantly increased IgE production compared to control in a dose dependent manner (Fig. 3A). LPS was treated as a positive control for the production of total IgE in IM-9 cells. Increased total IgE level was significantly reduced by 5 μg/ml of *E. faecalis*

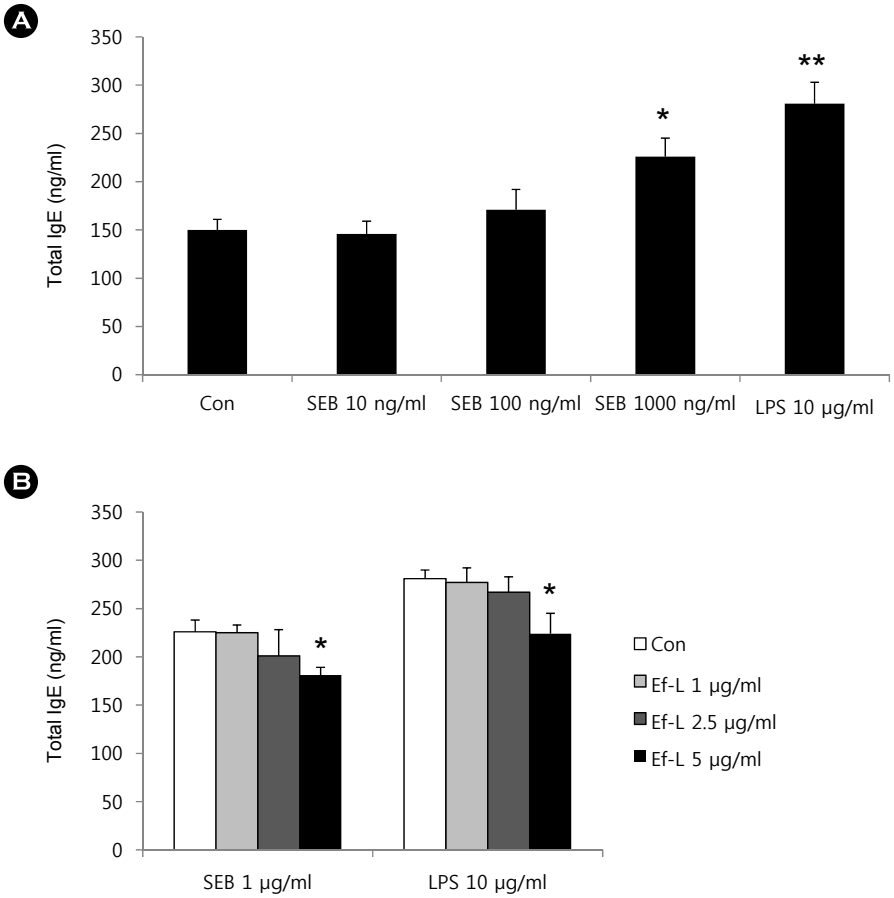

Fig. 3. The inhibitory effect of *E. faecalis* lysate on the IgE secretion by SEB-stimulated IM-9 cells. (A) IM-9 cells were incubated with SEB (10, 100 and 1,000 ng/ml) and LPS (10 μg/ml) for 72 h. The supernatant was collected and total IgE level was analyzed by ELISA. Data are expressed as the means ± SD. **P* < 0.05 and ***P* < 0.01 indicate a significant difference between the control and the SEB-/LPS- treated group. (B) IM-9 cells were pretreated with SEB (1 μg/ml) and LPS (10 μg/ml) for 12 h, respectively. After 12 hours, pretreated IM-9 cells were incubated with *E. faecalis* lysate (Ef-L) at the indicated concentration for 48 hours. The supernatant was collected and total IgE level was analyzed by ELISA. Data are expressed as the means ± SD. **P* < 0.05 indicates a significant difference between the SEB-/LPS- treated group and the *E. faecalis* lysate (Ef-L)-treated group.

lysate in SEB- and LPS-stimulated IM-9 cells (Fig. 3B).

E. faecalis lysate inhibits SEB-induced cytokine production in Jurkat T cell, the human T cell line

To determine the other effect of *E. faecalis* lysate on AD-related mechanism, we examined the effect of *E. faecalis* lysate on various cytokine productions of Jurkat T cell that has the ability of cytokine release including IL-1β, TNF-α, IL-4 and IL-5 (Lee and Kim, 2011). The protein expressions of IL-1β, TNF-α, IL-4 and IL-5 in Jurkat cells were evaluated using ELISA. In Jurkat T cells, the levels of IL-1β, TNF-α, IL-4 and IL-5 were markedly enhanced by stimulation with SEB or LPS (Fig. 4). Although the expression level of IL-1β were not changed by the *E. faecalis* lysate (Fig. 4A), *E. faecalis* lysate considerably decreases the elevated levels of TNF-α, IL-4 and IL-5 in Jurkat cells (Fig. 4 B-D). However, *E. faecalis* lysate reduced TNF-α and IL-5 production following LPS stimulation ($P < 0.05$) (Fig. 4B and 4D).

E. faecalis lysate reduces the proliferation of *S. aureus*

Over 90% of the skin of AD patients was colonized with *S. aureus* (Manders, 1998). To examine the antibiotic effect of *E. faecalis* lysate against *S. aureus*, we detected the viability of *S. aureus* in the absence and in the presence of *E. faecalis* lysate. The viability of *S. aureus* was

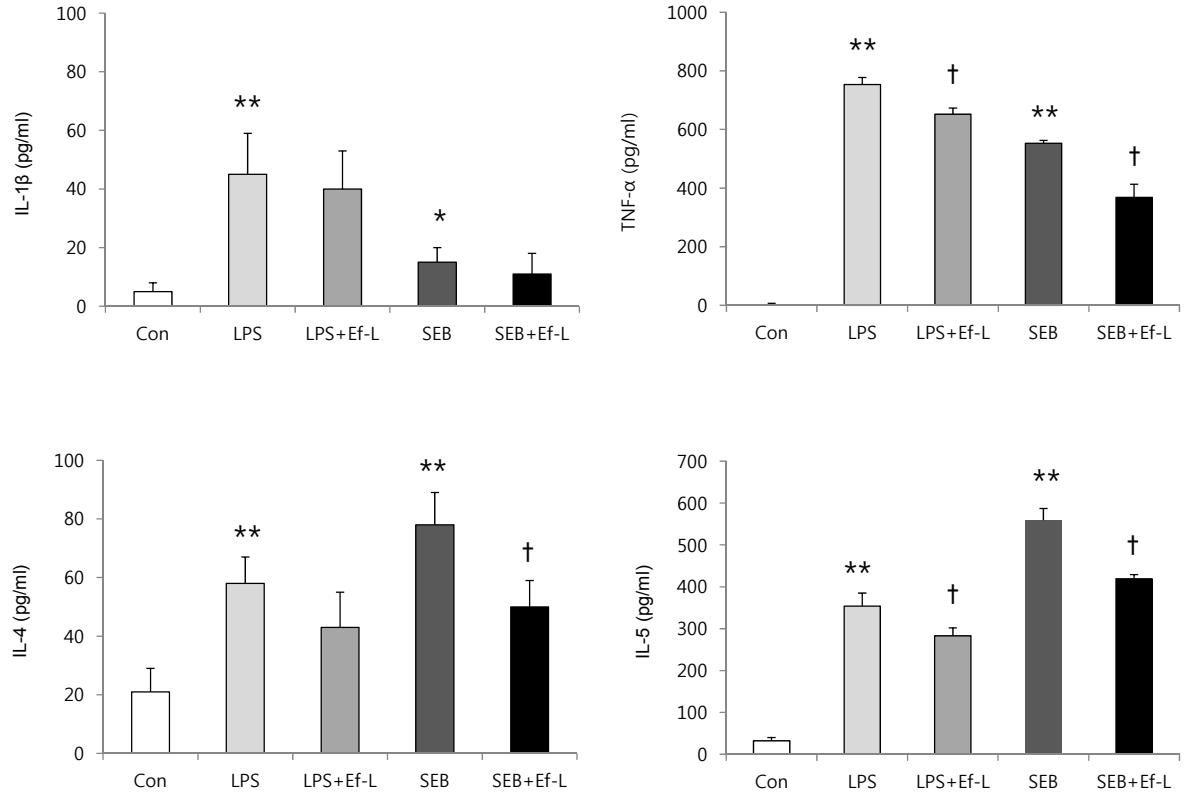

Fig. 4. The effect of *E. faecalis* lysate on the cytokine release induced by SEB in Jurkat T cell. (A) Jurkat T cells were pretreated with SEB (1 μg/ml) and LPS (10 μg/ml) for 1 h, respectively. After pretreatment, Jurkat T cells were incubated with 5 μg/ml of *E. faecalis* lysate (Ef-L) for 24 hours. The supernatant was collected and protein levels were analyzed by ELISA. Data are expressed as the means ± SD. **P* < 0.05 and ***P* < 0.01 indicate a significant difference between the control and the SEB-/LPS- treated group. †*P* < 0.05 indicates a significant difference between the SEB-/LPS- treated group and the *E. faecalis* lysate (Ef-L)-treated group.

measured the turbidity of *S. aureus* culture suspension with a spectrophotometer at a wavelength of 600 nm. As Shown in Fig. 5, optical densities at 12 and 24 h of culture growth were significantly decreased by the addition of *E. faecalis* lysate. These data indicate that *E. faecalis* lysate may have the antibacterial activity and may be involved in the inhibition of the *S. aureus* colonization.

DISCUSSION

In this study, we identified the effect of *E. faecalis* lysate as a potent therapeutic agent for atopic dermatitis (AD). *E. faecalis* lysate reduces the productions of total IgE and cytokines of AD-related immune cells in response to SEB stimulation. In addition, the proliferation of *S. aureus* was inhibited by *E. faecalis* lysate.

AD is a common chronic inflammatory skin disease and is associated with atopic conditions such as asthma and IgE-mediated allergy and whose skin lesions are characterized by a Th2 cell-mediated reaction to allergen. The severity of AD is increased by exposure to various environmental factors, especially microorganisms such as *S. aureus* (Baker, 2006). *S. aureus* is gram-positive bacteria and it can cause local infections of the skin, vagina and gastrointestinal tract, most of which are not life-threatening (Harris et al., 2002). However, *S. aureus* infects patients and colonizes with decreased immunity and produces a ragen of extracellular toxins, such as staphylococcal enteroxoxin A-E, toxic shock syndrome toxin-1 (TSST-1) and exfoliative A and B (Harris et al., 2002). In these toxins, SEB induced eczematous skin lesions and can subsequently induce the release of Th1/Th2 cytokines, including IL-1β, TNF-α, IL-4

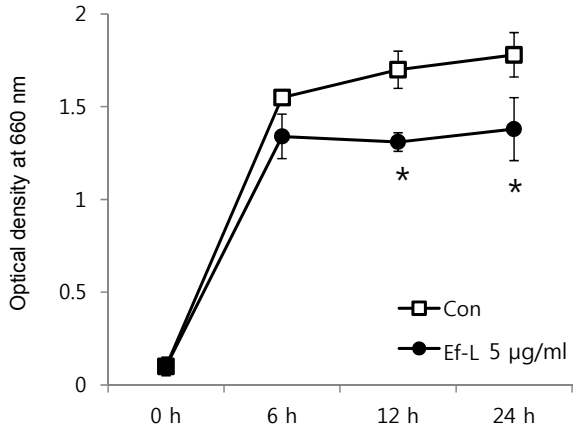

Fig. 5. The effect of *E. faecalis* lysate on the proliferation of *S. aureus*. After *S. aureus* strain was inoculated to the BHI medium, *S. aureus* was incubated for the indicated time in the absence (Con) and presence of *E. faecalis* lysate (Ef-L). The proliferation of *S. aureus* was measured by reading of optical density of BHI medium at a wavelength of 660 nm. ELISA. Data are expressed as the means± SD. $*P < 0.05$ indicates a significant difference between the control and the *E. faecalis* lysate (Ef-L)-treated group at the same time.

and IL-5, from immune cells. These processes can down-regulated skin barrier proteins in keratinocytes (Jiao et al., 2013). SEB even can experimentally induce the dermatitis to normal skin or normal-looking skin in AD patients (Strange et al., 1996). In the AD patients, SEB also induced the production of anti-SEB IgE, and enhanced secretion of total IgE (Sohn et al, 2003; Savinko et al., 2014). In this study, SEB induced the secretion of total IgE in IM-9 cells and the production of cytokines, including IL-1β, TNF-α, IL-4 and IL-5, in Jurkat T cells (Fig. 3 and 4).

Interestingly, some normal flora produces the probiotic supplementations that could modulate the response of immunity and could alleviate the symptoms of various diseases (Madden et al., 2005). *E. faecalis* is part of the normal flora of humans and is commonly found in the vaginal and intestinal regions of humans. In other reports, sufficient amounts of lysed *E. faecalis* FK-23 (LFK) have inhibitory effects on allergen-induced local accumulation of eosinophils and active cutaneous anaphylaxis in mouse models (Shimada et al., 2003; Shimada et al., 2004). Eosinophils are maturated and recruited by Th2 cytokines that are produced by Th2 cells. Th2 cells release various cytokines, including interleukin (IL)-2, IL-3, IL-4, IL-5 and IL-13. IL-4 and IL-13 play important roles in IgE switching in B cell. IL-5 activates eosinophils and activated eosinophils induce the release of proinflammatory mediators (Kips, 2001). In this study, *E. faecalis* lysate reduces the productions of total IgE in IM-9 cells and the release of cytokines in Jurkat T cells. These results indicate that *E. faecalis* lysate may exert immunoregulator effects on immune cells and can be involved as the anti-inflammatory agent in the pathogenesis of AD.

E. faecalis lysate was also inhibited the proliferation of *S. aureus* (Fig. 5). *E. faecalis* is known as a lactic acid-producing bacterium and also produce hydrogen peroxide, which is known to be directly cytotoxic to various microorganisms (Brosnahan AJ et al., 2013). Lactic acid is a secreted factor that is known to interfere with colonization by pathogens. Thus, *E. faecalis* lysate may prevent the colonization of *S. aureus* in the skin of AD patients.

In conclusion, we demonstrated the inhibitory effect of *E. faecalis* lysate against SEB-induced immune response and the proliferation of *S. aureus*. Therefore, *E. faecalis* lysate may improve the skin-defense system disturbed by atopic condition, and may prevent subsequent secondary infection of *S. aureus* and development of AD. These characteristics of *E. faecalis* lysate can also make the immune system more efficient.

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