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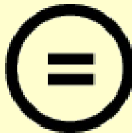

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Lactobacillus gasseri KBL697 prevents skin lesions in imiquimod-induced psoriasis-like mice by inducing regulatory T cell

락토바실러스 가세리 KBL697의 조절 T 세포 유도를 통한 이미퀴모드 유도 건선 동물모델의 병변 예방

2019년 2월

서울대학교 보건대학원

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// Abstract

Lactobacillus gasseri KBL697 prevents skin lesions in imiquimod-induced psoriasis-like mice by inducing regulatory T cell

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Psoriasis is a T cell-mediated chronic skin autoimmune disease. It is described by hyperproliferation and poor differentiation of epidermal keratinocytes. In this study, we aimed to identify the in vivo preventative effect of probiotic strain, *Lactobacillus gasseri* KBL697 which was selected by Raw 264.7 cell cytokine secretion assay in previous study. To figure out the effectiveness of *L. gasseri* KBL697 on imiquimod-induced psoriasis-like mice, we investigated the effect of imiquimod on the gut microbiota and immune response. In the gut, imiquimod reduced *Bacteroides* which is known as anti-inflammatory effect. In contrast *L. gasseri* KBL697 restored *Bacteroides* abundance to control mice in cecum. In addition, *L. gasseri*

KBL697 increased MHCII in CD11b+ CD11c+ dendritic cells (DCs) which is known as cell to induce regulatory T cell in the mesentric lymph nodes (MLNs) of imiquimod-treated mice by analyzing flow cytometry. Then, *L. gasseri* KBL697 group increased mRNA level of Foxp3 that is regulatory T cell transcription factor in colon and skin by using real time PCR. We also investigated the effect of *L. gasseri* KBL697 on inflammation and phenotype. *L. gasseri* KBL697 reduced the quantity of macrophage in the spleen of imiquimod-treated mice. *L. gasseri* KBL697 reduced proinflammatory and Th17-associated cytokines in the skin. In addition, psoriasis associated Psoriasis Area and Severity Index (PASI) score and dorsal thickness of imiquimod-treated mice were decreased. Taken altogether, these results suggest that *L. gasseri* KBL697 reduced proinflammatory and Th17-associated cytokines in the skin by increasing bacteroides and regulatory T cell activated by MHCII in CD11b+ CD11c+ DCs. It shows the possibility that *L. gasseri* KBL697 can be used as a preventive medicine for psoriasis.

Key words: Cytokines, Imiquimod, *Lactobacillus gasseri* KBL697, Foxp3, *Bacteroides*, Psoriasis, Dendritic cell, Regulatory T cell

Student No. 2017-22337

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I. Introduction

Psoriasis is a chronic inflammatory skin disease characterized by epidermal keratinocyte hyperplasia and massive leukocyte infiltration [1]. About 2-3% of the world's population is suffering, and it has been steadily increasing in industrialized countries recently [2]. However, the mechanism for the disease has not yet been fully identified. Recently, numerous studies have confirmed that T helper 17 (Th17) cells and the immune-derived cytokines these cells produce, including interleukin IL-17, IL-22, and IL-23, were detected in psoriatic skin lesions and serum that were involved in pathogenesis of psoriasis [3].

Fortunately, there are a variety of treatments for the health, including immunosuppressants, ointments, and steroids. However, if ointment is ineffective and inhibiting immunity, the side effects are serious [4-6]. Recently, research is moving on using probiotics as a safer and cheaper option. Probiotics are defined as “living microorganisms that, when administered in adequate amounts, confer health benefits to the host” [7]. Accumulating evidences propose that probiotics have been affected on the alleviation of various autoimmune diseases by changing gut microbiota community and immune cell, such as, atopic dermatitis [8], inflammatory bowel disease [9], encephalomyelitis [10], rheumatoid joint pain [11], asthma [12], and type 1 diabetes [13]. The human gut is colonized by more than one hundred trillion microbes, and contains upwards of 1,000 bacterial species [14]. Particular subsets of microbiota have been appeared to

differently manage immune function. *Bacteroides fragilis* through creation of polysaccharide A can induce regulatory T cell development and control Th1/Th2 balance [15, 16], and *Clostridium* species prompt regulatory T cell improvement [17], while Segmented filamentous microorganisms coordinate Th17 cell separation [18]. The *lactobacillus spp.* also increased the regulatory T cell, weakening the Th17 cell's function, and thus preventing autoinflammation from occurring [19]. Several types of Th17 autoinflammation, such as asthma, psoriasis, inflammatory bowel disease mice models also used lactobacillus to relieve diseases [9, 12, 20].

The relationship between microbes and immune cells is mainly mediated by the antigen presenting cells (APC) which contain dendritic, macrophage, and B cells in the gut [21]. For this reason, Th1, 2, 17, and regulatory T cells are stimulated by APC cell and each immune response occurs. This immune response in the gut affects the entire immune system by changing gut immune response or microbial metabolism [22]. However, it is not fully understood how the probiotics, such as *Lactobacillus*, *Bifidobacterium*, affect microbiota and immune cells to prevent or treat autoimmune diseases, especially in psoriasis which is skin related chronic inflammatory disease.

In this study, we applied imiquimod on mice. Imiquimod is a sort of immunomodulating drug by binding to Toll-like receptor 7. It was first affirmed by Food and Drug Administration (FDA) for the topical treatment of outside genital and perianal warts in 1997, and now is additionally used to treat shallow basal cell carcinoma and actinic keratosis [26-28]. However, imiquimod may cause certain symptoms identified with irritation, such as skin flaking, blisters, burning sensation, scaling or skin redness. Many

reports have been shown that continuous topical use of imiquimod cream in mice causes similar histological and phenotypic characteristics, including erythema, scaling and epidermal thickening. Also imquimod generates the inflammatory infiltrates of neutrophils, T cells and dendritic cells like human psoriasis [29]. It has been confirmed that the mice model treated imiquimod has similar results to that of humans psoriasis with regard not only to the development of the lesions via the IL-23/IL-17A axis. So we selected imiquimod-induced psoriasis-like mice model to reveal the effect of *L. gasseri* KBL697 (We will call it 697) which has a potent effect of increasing IL-10/TNF-a, IL-10/IL-6 ratio in Raw 264.7 cell line in previous study [23] on psoriasis in mice. We also investigated the changing of cecal microbiota and immune cell response in the gut on imiquimod-induced mice to identify the mechanism how to reduce the skin inflammation by 697.

II. Materials and Methods

1. Mice

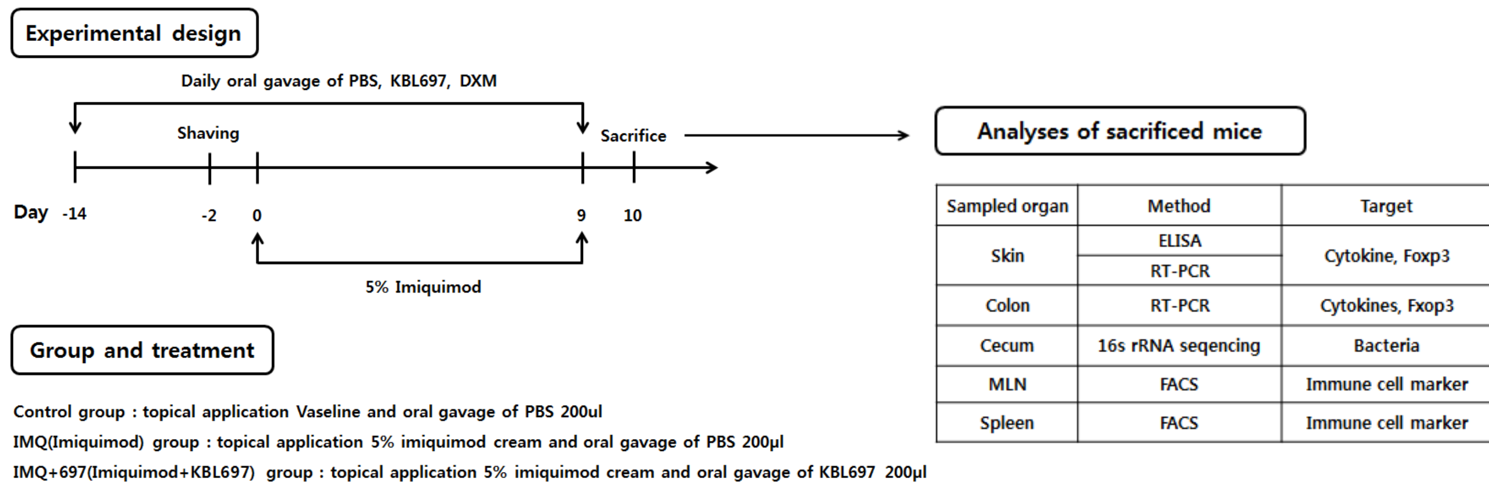
Female Balb/c mouse (6 weeks; 16-18 g) were purchased from Orient Bio Inc. (Seongnam, Korea). The mice were housed at the facility of Research Institute Public Health in Seoul National University (South District, Seoul). All experimental animals were used in this study according to a protocol approved by the Institutional Animal Care and Use Committee of Seoul National University (IACUC).

2. Bacterial strain, media, and growth conditions

697 was isolated from Korean women vagina. 697 was identified to the species level by sequencing of 16s rRNA. 697 was cultured in MRS broth (Thermo Fisher Scientific, USA) with L-cysteine hydrochloride (Sigma-Aldrich, USA) at 37°C for 18 hours. After being subcultured twice (1% v/v) from the stock, the bacterial cells were collected by centrifugation at 12,000 rpm for 3 minutes to remove the MRS broth, and washed twice in phosphate buffered saline (PBS) [23].

3. Imiquimod-induced psoriasis-like mice model and bacteria treatment

Mouse were shaved to expose dorsal skin by electric shaver. One mice were took a topical application on back skin and right ear by 62.5 mg vasaline for control group. Other groups of mice were received a topical application to back skin and right ear by 62.5 mg imiquimod cream (Aldara; 3M Pharmaceuticals, St Paul, MN, USA) daily for 10 consecutive days. For the 697 treatment, the mice were fed orally with 1×10^9 CFU/200 μl/day by oral gavage from day 0 to day 9, while the control and IMQ control groups of mice were fed with PBS for same period (Fig. 1) [20].



Sampled organ	Method	Target
Skin	ELISA	Cytokine, Foxp3
	RT-PCR	
Colon	RT-PCR	Cytokines, Fxop3
Cecum	16s rRNA seqencing	Bacteria
MLN	FACS	Immune cell marker
Spleen	FACS	Immune cell marker

Figure 1. Diagram depicting the design of imiquimod-induced psoriasis-like m model.

4. Evaluation of PASI score

To score the severity of aggravation of the back skin, a target scoring framework was created dependent on the clinical Psoriasis Area and Severity Index (PASI). Erythema, scaling, and thickening were scored freely on a scale from 0 to 4: 0, none; 1, slight; 2, moderate; 3, stamped; 4, exceptionally checked. The level of thickness was measured with a electronic caliper. The level of erythema was scored utilizing a scoring table with redness. The total score (erythema plus scaling plus thickening) filled in as a proportion of the seriousness of aggravation (scale 0 – 12) [3].

5. Histological analysis

To find out the thickening of epidermis and immune cell's infiltration, mouse's shaved back skin samples were collected and fixed in 10% neutral buffered formalin, and embedded in paraffin. The skin section were cut longitudinally into 5 μm sections and stained with haematoxylin and eosin (H&E). and observed under a microscope at 100X magnification [3].

6. Cecal DNA extraction and 16S rRNA gene sequencing

Mouse cecum was stored at –80 ℃ until used. To figure out the microbiota of cecum, Bacterial DNA was extracted from cecum using a QIAamp Stool Mini Kit (Qiagen, USA), according to the manufacturer's instruction. For 16S rRNA sequencing, the V4 region of the 16S rRNA gene was amplified using 16S rRNA-specific primers 341F and 806R which were barcoded to enable multiplexing of sequencing libraries. KAPA Library Quantification kit (KAPA Biosystems, USA) was used to quantify the amplicons. The amplicons were purified, pooled in equal quantities, and then sequenced on the Illumina Miseq platform [35].

7. Sequence analysis and bioinformatics

Sequence data were analyzed by Quantitative Insights into Microbial Ecology(QIIME) ver. 1.8.0 software package. The sequences were clustered into operational taxonomic units (OTUs) at 97% identity. Alpha-diversity indexes (Chao1) were analyzed from OTU table. Taxa profiles were visualized as cumulative bar graph by excel 2010 (Micro Software, USA) [35].

8. Fluorescence-activated cell sorting (FACS)

To analyze the immune cell's population of systemic and gut immune organ, Splenocytes and mesenteric lymph nodes (MLNs) were harvested and minced using a cell strainer (70 μm, Corning, USA) in cold FACS buffer (2% inactivated FBS, 0.1% Sodium azide, 1 mM EDTA; in 50 mL PBS). Cell suspensions were centrifuged 1500 rpm for 5 min at 4 ℃. Splenocytes were re-suspended in red blood cell (RBC) lysis buffer for 1 – 2 min. MLNs and splenocytes were washed with 9ml of FACS buffer and centrifuged. Viable cell numbers were counted by adding Trypan blue and diluted with FACS buffer to 5.0×10^5 cells/well in 96 well plate. Intracellular staining was performed according to manufacturer's instructions (BD Biosciences, USA). Cells were stained with PE-conjugated with anti-CD11b which captures monocytes, PE-Cyanine7-conjugated with anti-CD11c wich captures DCs, FITC-conjugated with anti-F4/80 which captures macrophages, APC-conjugated with anti-B220 which captures B cells, APC-conjugated anti-MHCII (All the antibodies were from eBioscience, USA). Flow cytometry data were acquired by FACSVerseTM (BD Bioscience, USA) and analyzed by FlowJo (TreeStar, USA).

9. Enzyme linked immunosorbent assay (ELISA)

After sacrifice, skin tissues were cut by 1cm x 1cm in -80℃ until used. The skin tissue were homogenized with solution [43]. IFN-γ which is a representative cytokine of Th1 type cell and IL-17 which is a representative cytokine of Th17 type cell. Proteins were quantified with ELISA kit, according to the manufacturer's instructions (BD Biosciences, USA).

10. Quantitative real-time polymerase chain reaction (qRT PCR)

For RNA quantification, tissues were homogenized using a TissueLyser II (Qiagen, USA) in Lysis buffer (Easy spinTM Total RNA Extraction Kit, iNtRON biotechnology, South Korea). RNA was extracted according to manufacturer's protocol (Easy spinTM Total RNA Extraction Kit, iNtRON Biotechnology, South Korea). cDNA was synthesized with the High Capacity RNA-to-cDNA kit (Applied Biosystems, Thermo Fisher Science, USA). To estimate expression of various genes, cDNA was amplified using Power SYBR Green PCR Master Mix (Applied Biosystems, Thermo Fisher Science, USA) with primers (Table 1.) according to manufacturer's instructions. GAPDH was measured as a normalizer for each sample. Thermo cycling conditions were hold at 95 ℃ for 10 min, 40 cycles of PCR stage (denaturation at 95 ℃ for 15 min, annealing and extension at 60 ℃ for 1 min), and continuous melt curve stage at 95 ℃ for 15 min and 60 ℃ for 1 min. The $2^{-\Delta\Delta Ct}$ method was used for relative quantification of gene expression [20].

Table 1. List of primers for gene expression analysis

Name	Primer sequence(5’->3’)
V4_F	GCC AGC AGC CGC GGT AA
V4-R	GAC TAC CAG GGT ATC TAA T
GAPDH_F	GGA GCG AGA TCC CTC CAA AAT
GAPDH_R	GGC TGT TGT CAT ACT TCT CAT GG
IL-1β_F	AAG GGC TGC TTC CAA ACC TTT GAC
IL-1β_R	ATA CTG CCT GCC TGA AGC TCT TGT
IL-6_F	TGC CAT TGC ACA ACT CTT TTC T
IL-6_R	TCG GAG GCT TAA TTA CAC ATG TTC
IL-10_F	GGT TGC CAA GCC TTA TCG GA
IL-10_R	ACC TGC TCC ACT GCC TTG CT
IL-17A_F	TTT TCA GCA AGG AAT GTG GA
IL-17A_R	TTC ATT GTG GAG GGC AGA C
IL-17F_F	GAG GAT AAC ACT GTG AGA GTT GAC
IL-17F_R	GAG TTC ATG GTG CTG TCT TCC
IL-22_F	GAA GGC TGA AGG AGA CAG TGA AA
IL-22_R	GTT CCC CAA TCG CCT TGA
IL-23_F	AAT AAT GTG CCC CGT ATC CA
IL-23_R	CAT GGG GCT ATC AGG GAG TA
Foxp3_F	ACT CGC ATG TTC GCC TAC TT
Foxp3_R	GGC GGA TGG CAT TCT TCC A
TGF-β_F	GGT TCA TGT CAT GGA TGG TGC
TGF-β_R	TGA CGT CAC TGG AGT TGT ACG G

11. Statistical analysis

All statistical analyses were performed using Prism 5 (GraphPad Software, San Diego, Ca, USA). Statistical significance was measured using one-way ANOVA test. Pos-hoc analysis was analyzed by Tukey. In all graphs, data were presented as mean ± standard deviation (SD). Statistical significance was given as #P <0.05, ##P <0.01, ###P <0.001 vs control group and *P-value < 0.05, **P-value < 0.01, ***P-value < 0.001 vs IMQ control group.

III. Results

1. 697 alleviated dorsal thickness and PASI scores in imiquimod-induced psoriasis-like mice

697 was originated from the healthy women vagina. This strain had effect on the anti-inflammation effect in Raw 264.7 cell. Also in Atopic dermatitis mouse model, this strain showed inhibitory effect on AD [23]. Bacteria and PBS were fed before treating imiquimod. After that, Mice were topically treated with imiquimod for 10 consecutive days, except for control group. control group treated with vasaline. The weight changes were checked every two days. Imiquimod-treated groups had effect on reducing their weights under 90% (Fig. S1). Oral treatment of 697 suppressed the development of imiquimod-induced psoriasis-like mice skin lesion (Fig. 2A). dorsal skin thickness also increased, when each group was treated by imiquimod. IMQ+697 group were significantly decreased from fourth day when compared to IMQ treated group (Fig. 2B). In addition, IMQ+697 group was significantly decreased right ear thickness compared to IMQ treated group (Fig. S2). Also, the PASI score of IMQ+697 was significantly reduced from fourth day when compared to IMQ treated group (Fig. 2C). In histological analysis by H&E staining, imiquimod treated groups had highly thickened epidermis compared to control group. In comparison with the IMQ treated group, IMQ+697 treated group tended to be decreased immune cell infiltration in dermis (Fig. 3).

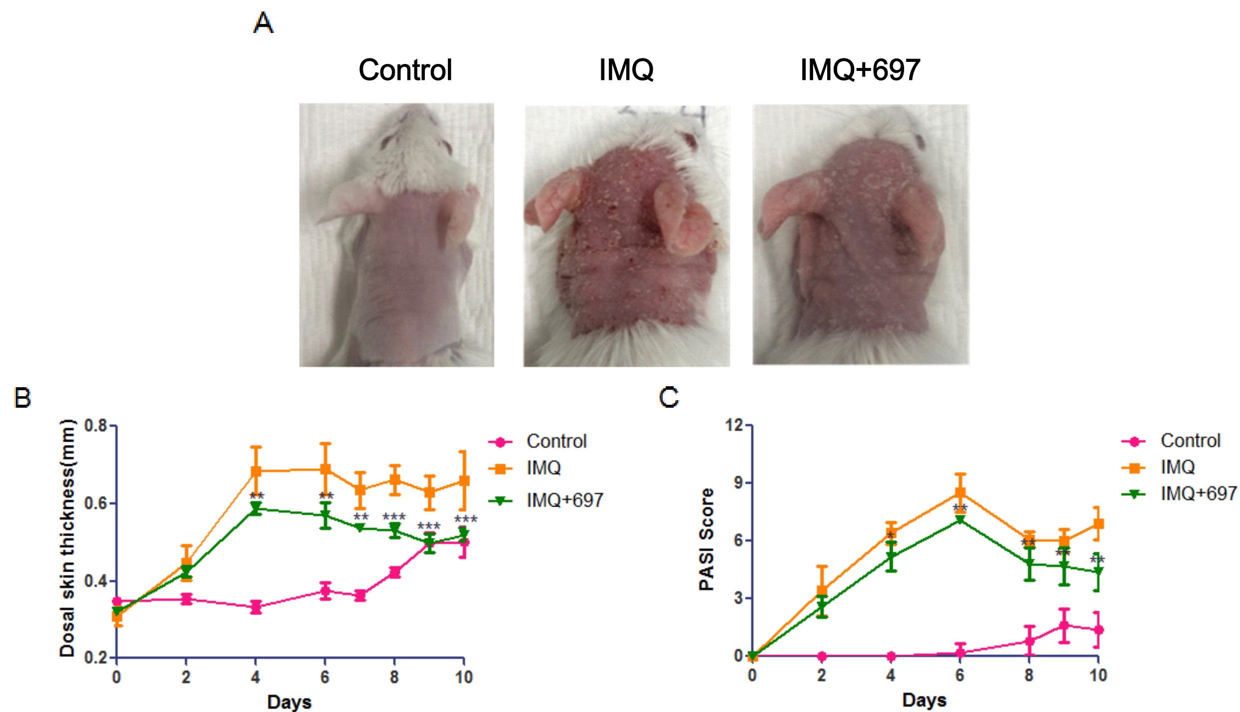
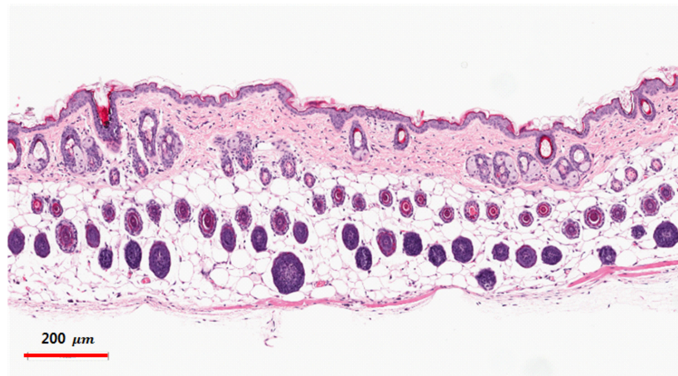


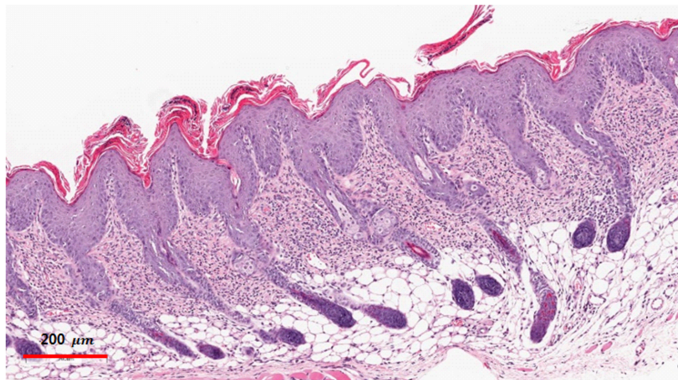
Figure 2. Imiquimod-induced psoriasis symptoms in Balb/c mice

Imiquimod-induced psoriasis-like mice model. (A) Phenotypical presentation of mouse back skin after 10 days' treatment. (B) The middle of dorsal skin thickness was measured for each group. (C) The scores of PASI are shown for each group. The data are shown as mean±S.D (N = 5 per each group). Significant differences are expressed as *P <0.05, **P <0.01, ***P <0.001 vs control group

Control



IMQ



IMQ+697

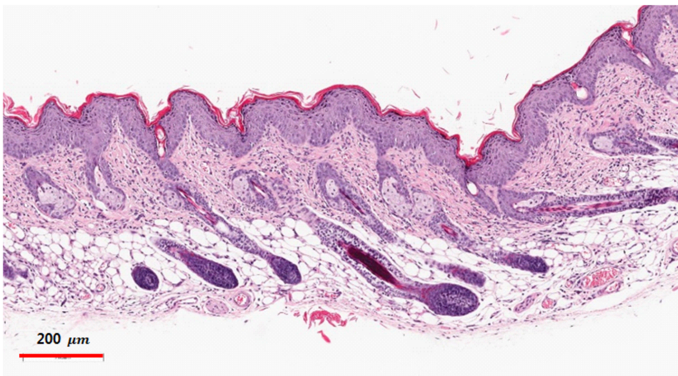


Figure 3. Histological changes in skin of Balb/c mice

Representative photomicrographs of hematoxylin and eosin-stained section of back skin tissue of the experimental mice (100X).

2. Cecal microbiota was changed by imiquimod and 697

We analyzed cecum samples from each group by QIIME. Alpha diversity is the species diversity in the sample. Alpha-diversity indexes (Chao1) were analyzed each sequence per sample from OTU table for each group. Each group had no significant difference in alpha diversity (Fig. 4A). Principle coordinate analysis (PCA) using unweighted unifrac distance showed the differences of microbial community among experimental groups. IMQ+697 group had microbial community that was more similar to control group than IMQ group (Fig. 4B). Then, we analyzed the microbial composition of cecum from day 10 mice. *Bacteroidetes* and *Firmicutes* were most abundant in any other groups at phylum level (Fig. 5A), which is consistent with findings from previous studies [24]. We observed that IMQ treated group had less *Bacteroidetes* and more *Firmicutes* compared to control group. 697 treatment recovers the *Bacteroidetes* percentage in microbial composition (Fig. 5B, C).

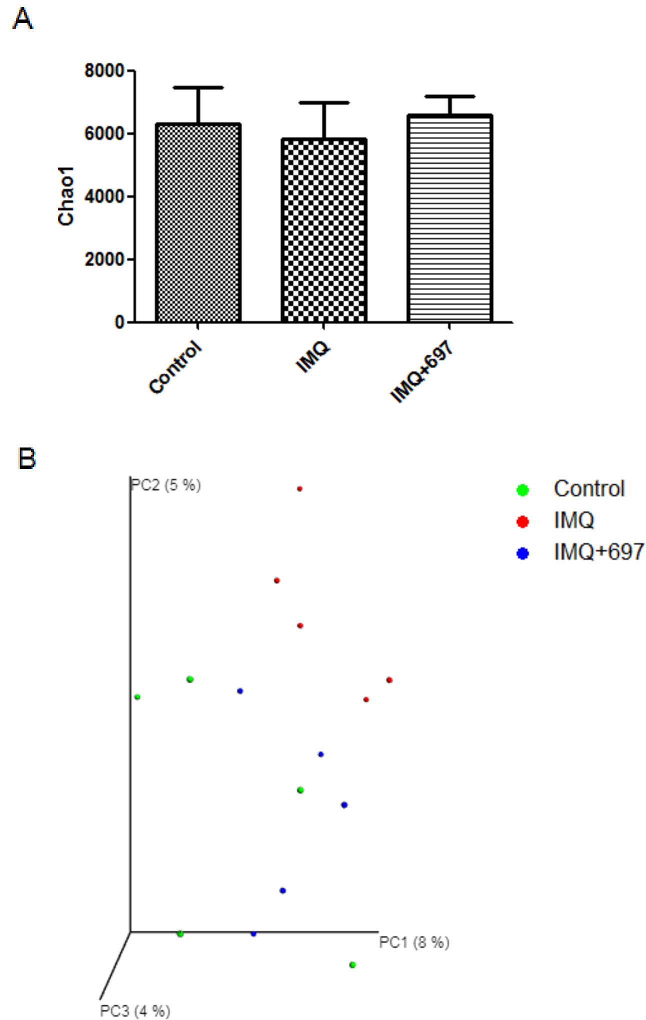


Figure 4. Alpha and beta diversity analysis of cecal microbiota

Diversity analysis of cecal microbiota in each group. (A) Alpha diversity analysis using the Chao1 data, when the sequence per sample is 22010 in rarefaction curves. (B) Beta diversity analysis using Unifrac distance of cecal microbiota among each groups. Each colored point represents a cecal sample from each groups. The data are shown as mean±S.D (N = 5 per each group). Significant difference is expressed as $*P < 0.05$ vs IMQ treated group

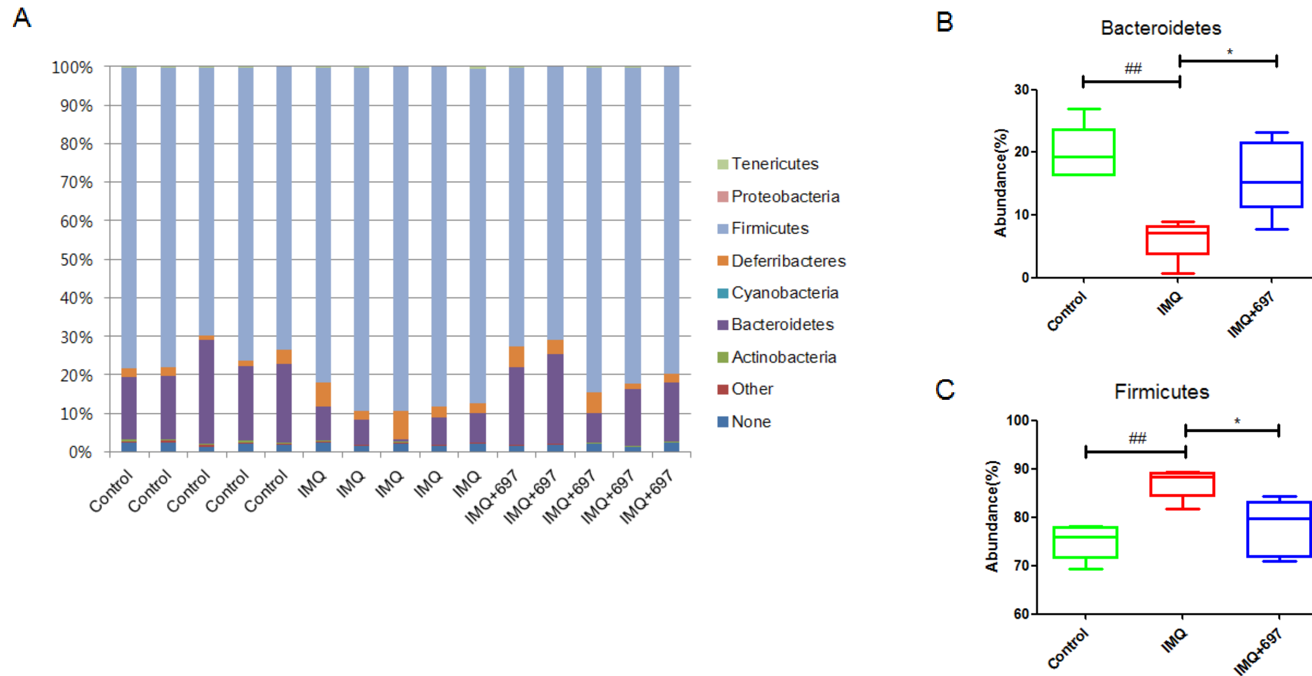


Figure 5. Relative abundance of cecal microbiota

Composition comparison of cecum. (A) Relative abundance of cecal microbiota of each sample at the phylum level. Each column represents pooled samples from individual experiments. (B) and (C) graphs represent two phylum group that is significantly different from control group. The data are shown as min to max. (N = 5 per each group). Significant differences are expressed as **P* <0.05, ***P* <0.01, ****P* <0.001 vs IMQ treated group; #*P* <0.05, ###*P* <0.001 vs control group

3. 697 increased the number of MHCII in CD11b+ CD11c+ DC in the MLNs of imiquimod-treated mice

To understand the effect of 697 underlying mechanism responsible for the alleviation of skin psoriasis in mice, we analyzed the CD11b+ CD11c+ DCs which has the ability to induce CD3+ CD25+ Foxp3+ regulatory T cell in MLNs by flow cytometry. In addition, MHCII+ CD11b+ CD11c+ DCs present antigenic peptides to naive CD4+ T lymphocytes. These DCs affects to differenciate Foxp3+ regulatory T cell from naive CD4+ T lymphocytes [25]. Surprisingly we found the increase of the percentage of MHCII in CD11b+ CD11c+ DCs which are shown to induce regulatory T cell by producing IL-10 cytokine [25] compared to IMQ treated group (Fig. 6A). In addition, the total number of MHCII in CD11b+ CD11c+ DCs were also increased compared to IMQ treated group (Fig. 6B). However, IMQ+697 group was not increased MHCII+ CD11b+ CD11c+ DCs compared to IMQ treated group as it was in MLNs in spleen (Fig. S3). These results may imply that 697 not induced to CD11b+ CD11c+ DCs in spleen but CD11b+ CD11c+ DCs in gut.

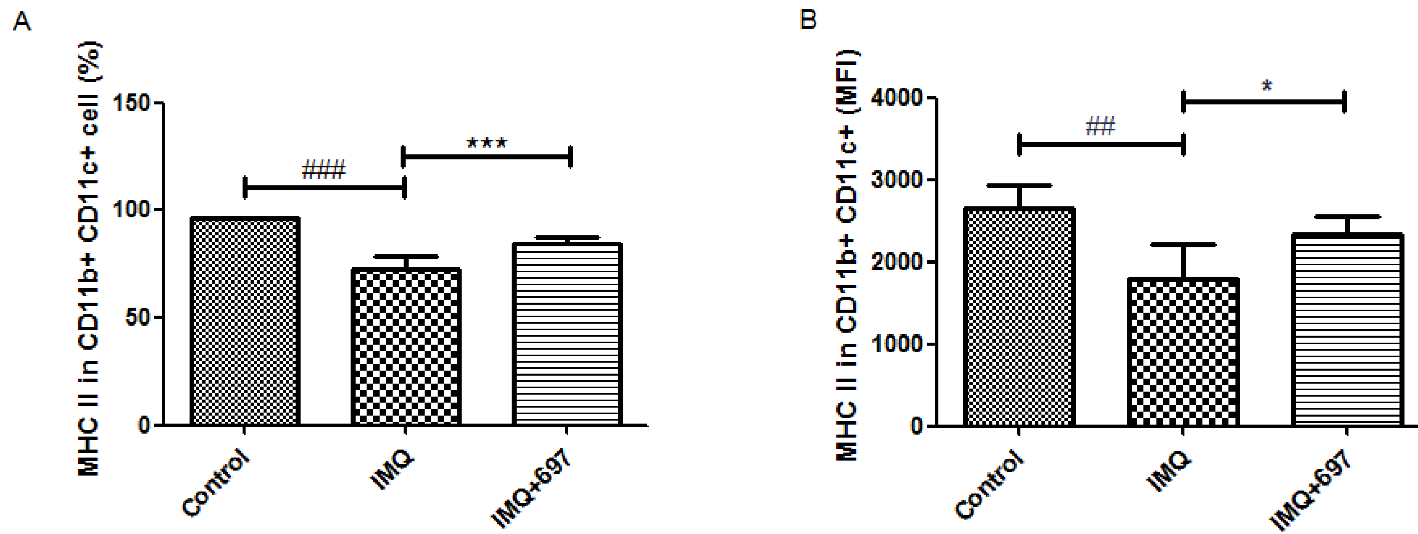


Figure 6. MHC II of CD11b+ CD11c+ DC in MLNs of imiquimod-treated mice

(A) Percentage of MHC II in CD11b+ CD11c+ DCs. (B) The total number of MHC II in CD11b+ CD11c+ cells. (C) Representative histogram of each group. The data are shown as mean±S.D (N = 5 per each group). Significant differences are expressed as **P* <0.05, ****P* <0.001 vs IMQ control group; ##*P* <0.01, ###*P* <0.001 vs control group

4. 697 increased Foxp3+ transcription factor in colon

To identify the effect of 697 on inflammatory and anti-inflammatory cytokines including Foxp3 transcription factor, we researched expression of the following genes which were compared using real-time PCR in colon tissue: IL-1b, IL-17A, IL-10, Foxp3 (Fig. 7A-D). Imiquimod-treated groups were increased inflammatory cytokine such as IL-1b, IL-17A compared to control group in colon (Fig. 7A, B). In addition anti-inflammatory cytokine, IL-10, was also increased compared to control group (Fig. 7C). The most interesting thing was that IMQ+697 group's mRNA expression level of Foxp3 transcription factor was increased compared to the other groups (Fig. 7D).

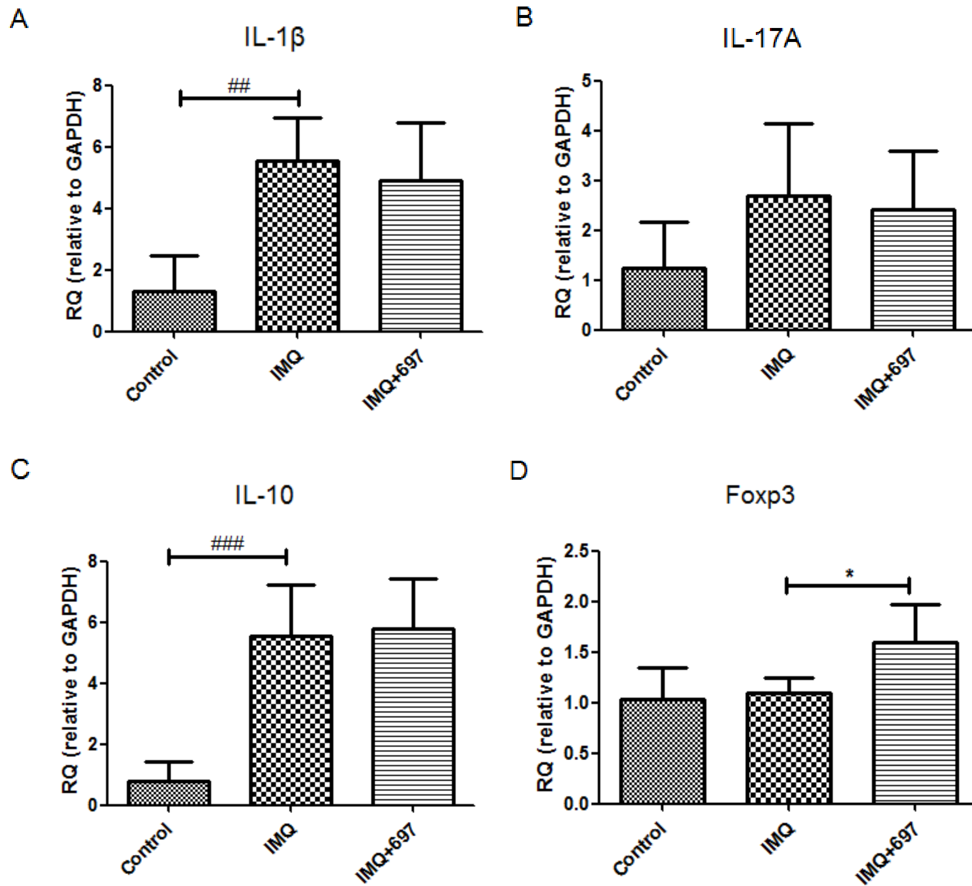


Figure 7. mRNA level of cytokines and Foxp3+ transcription factor in colon

Relative expression of IL-1β (A), IL-17A (B), IL-10 (C), Foxp3 (D) were checked by RT-PCR. The data are shown as mean±S.D (N = 5 per each group). Significant differences are expressed as *P <0.05, ***P <0.001 vs IMQ treated group; #P <0.05, ###P <0.001 vs control group

5. 697 reduced the number of macrophage in the spleen of imiquimod-treated mice

To further determine whether orally treated 697 can affect systemic immune responses, we investigated the spleen weight/body weight ratio that is a indicator for splenomegaly. Imiquimod treated groups increased as twice as control group. However, 697 had no significant effect on reducing splenomegaly (Fig. S5). Then, we examined the myeloid cell population by flow cytometry in spleen (Fig. 8A). CD11b+ CD11c- represents the monocytes, neutrophil, NK T cell. Imiquimod treated groups were significantly increased CD11b+ CD11c- cells in spleen. It means that imiquimod caused systemic inflammatory response. In addition, F4/80 in CD11b+ CD11c- cells were increased (Fig. 8B). F4/80 in CD11b+ CD11c- cells is a biomarker to macrophage. However, IMQ+697 group was decreased CD11b+ CD11c- cells and macrophages compared to IMQ treated group in spleen (Fig. 8A, B). It means that IMQ+697 group had less systemic inflammation compared to IMQ treated group. In MLNs, imiquimod treatment increased F4/80 in CD11b+ CD11c- macrophage cells. However, IMQ+697 group had no effect on reducing F4/80 in CD11b+ CD11c- macrophage cells as it did in spleen (Fig. S4).

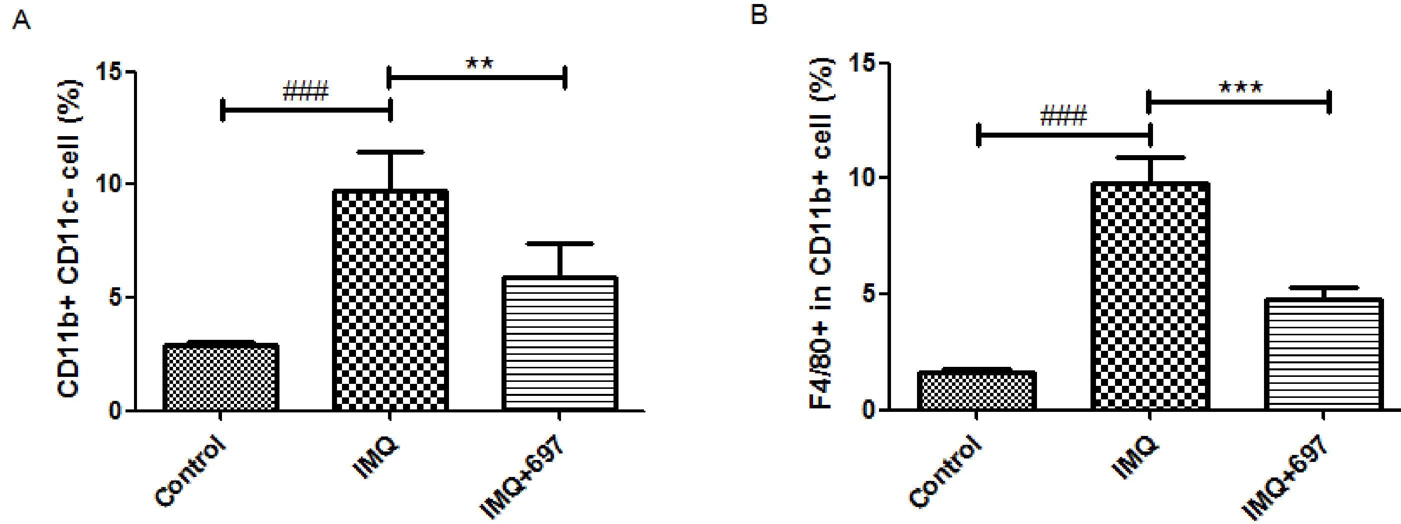


Figure 8. Macrophage in spleen of imiquimod-treated mice

(A) Percentage of CD11b+ cells in spleen (B) Percentage of F4/80 in CD11b+ cells in spleen. The data are shown as mean±S.D (N = 5 per each group). Significant differences are expressed as ***P* <0.01, ****P* <0.001 vs IMQ treated group; ###*P* <0.001 vs control group

6. 697 suppressed inflammatory cytokines in the skin lesions of imiquimod-treated mice by acting Foxp3+ regulatory T cell

Recently, cytokines have also been found to be involved in the induction of imiquimod-treated mice inflammation [3]. Therefore, to figure out the effect on reducing proinflammatory and Th17 cell-associated cytokine profiles, we performed ELISA in dorsal skin tissue. Proinflammatory and Th17-associated cytokine proteins, such as IFN-γ and IL-17, were increased by imiquimod. However, the cytokines of IMQ+697 group in the skin protein were significantly lower than IMQ treated group (Fig. 9A, B). In addition, we performed further study to identify the suppression effect of 697 on inflammation in imiquimod-treated mice by using real-time PCR. As shown in Fig. 10, 697 group were significantly decreased the level of IL-6 (proinflammatory cytokine), IL-17A, Il-17F and IL-22 (Th17-associated cytokines) compared to IMQ treated group (Fig. 10A, C). We also examined the mRNA level of regulatory T cell associated cytokine (TGF-β) and transcription factor (Foxp3) by using real-time PCR in skin lesion. The IMQ+697 group's Foxp3 transcription factor and TGF-β were significantly increased compared to the IMQ treated group (Fig. 10B).

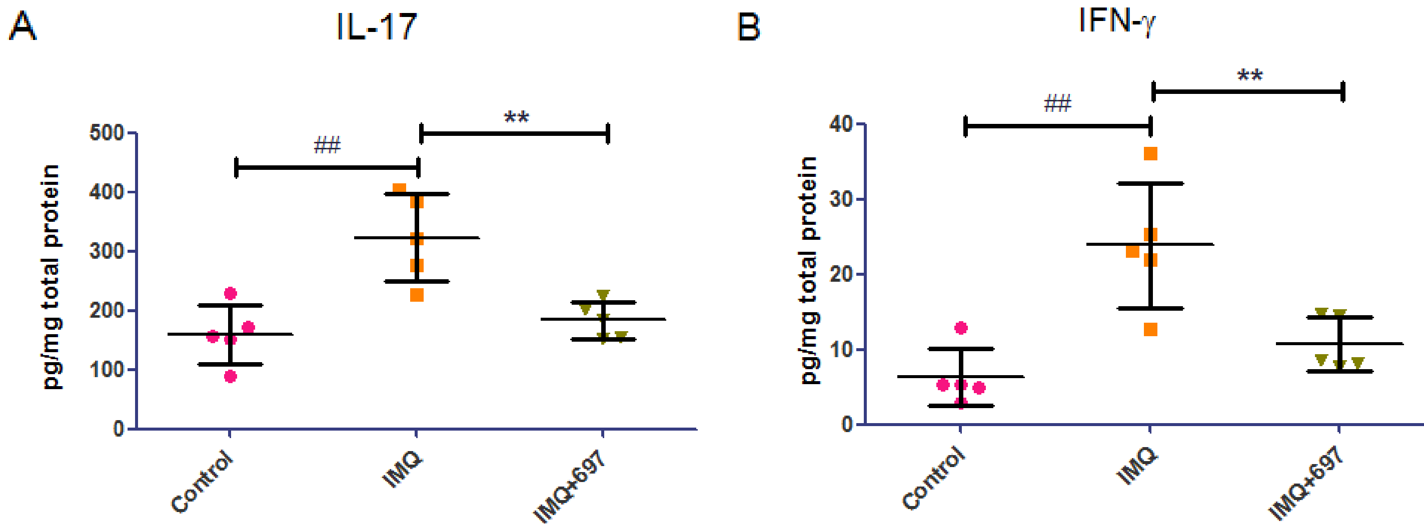


Figure 9. Protein level of skin inflammatory cytokines

The protein level of (A) IFN-γ and (B) IL-17 by ELISA in mice back skin. The data are shown as mean±S.D (N = 5 per each group). Significant differences are expressed as **P* <0.05 vs IMQ treated group; #*P* <0.05, ##*P* <0.01 vs control group

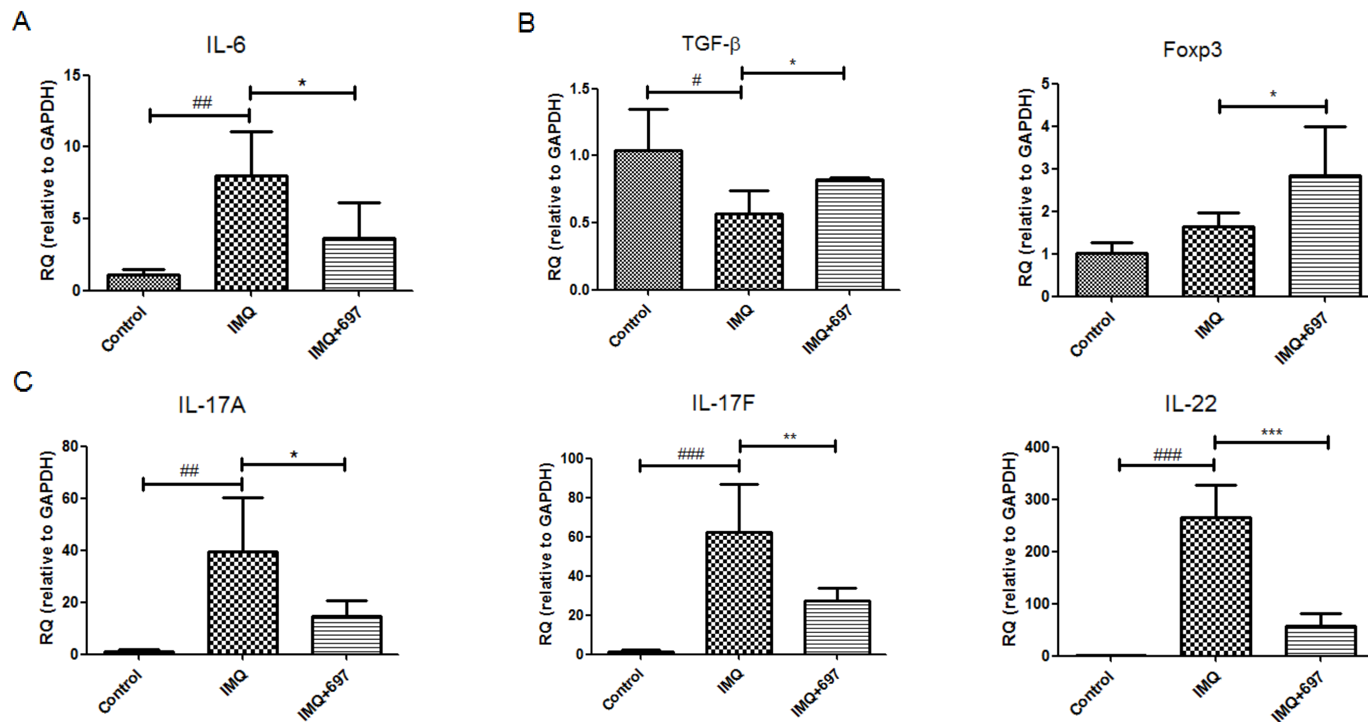


Figure 10. mRNA level of skin inflammatory cytokines and Foxp3 transcription factor of imiquimod-treated mice

mRNA level was measured by RT-PCR. (A) Th1 type of cytokines, (B) regulatory T cell type of mRNA related and (C) Th17 type of cytokines. The data are shown as mean±S.D (N = 5 per each group). Significant differences are expressed as **P* <0.05, ***P* <0.01, ****P* <0.001 vs IMQ treated group; ##*P* <0.01, ###*P* <0.001 vs control group

IV. Discussion

We have previously shown that *L. gasseri* KBL697 isolated from Korean women vagina had a potent effect of increasing IL-10/TNF-a, IL-10/IL-6 ratio in Raw 264.7 cell line which is a macrophage cell line of mice [23]. IL-10/TNF-a, IL-10/IL-6 ratio are considered to be a good indicator of the anti-inflammatory effect of a probiotic strain [30]. In addition, *L. gasseri* have been reported to significant probiotic effects, including modulation of the intestinal microbiota [31], suppression of bacterial and viral pathogens [32], and regulation of the host immune response [33]. This previous results suggest that 697 can be used as medicine to autoimmune disease. In the present study, we investigated the effect of *L. gasseri* KBL697 on skin lesions in imiquimod-induced psoriasis-like mice model. 697 significantly reduced dorsal thickness and PASI scores in imiquimod-treated mice (Fig. 2). Also, 697 inhibited immune cell infiltration compared to IMQ treated group in histological aspect (Fig. 3). We figured out that 697 appeared to inhibit skin inflammation in imiquimod-treated psoriasis-like mice.

Next, we investigated gut microbiota research to find out why skin inflammation was reduced by 697. The mice models are increasingly used to describe the role and function of diseases which is related to microbiota. The mice cecum is additionally substantial in respect to its aggregate gastrointestinal (GI) tract and is a vital site for the fermentation of plant materials and in addition for the generation of nutrient K and B, which mice reabsorb through coprophagy [34]. Modifications in gut microbiota composition have been related with numerous human pathologies. In

psoriasis-like mice, antibiotics-treated mice in neonatal life which is increased abundance of family *Lachnospiraceae* in gut had more susceptibility to psoriasis [35]. In adult mice treated antibiotics, they increased *Clostridiales* and *Erysipelotrichales* at orders with more severe psoriatic pathogenesis than control group [36]. These bacterial classes that is known as inducing Th17 cell are involved in *Firmicutes*. In this study, 697 group's beta diversity tended to recover control group. In microbial composition, 697 group increased *Firmicutes* at phylum level. It means that 697 reduces proinflammatory inducing bacteria. Also, 697 increased *Bacteroides* at phylum level (Fig. 4, 5). *Bacteroides* includes *Bacteroides fragilis* that have Polysaccharide A which is known as anti-inflammatory substance by inducing regulatory T cell [15, 16]. These results consistent with other psoriasis human patients research which indicated *Firmicutes/Bacteroides* ratio were increased than healthy human in the psoriasis human patient study [37].

In MLNs, 697 increased the number of MHCll CD11b+ CD11c+ DCs (Fig. 6). The microbiota changes may affect to activate MHCll CD11b+ CD11c+ DCs in the gut. As we mentioned, *Bacteroides fragilis* have Polysaccharide A which is known as anti-inflammatory substance by inducing immune cells [15, 16]. MLNs is the important site for nutrients and microbial substances entering the lymph fluid in the intestinal lamina propria [38]. The cecal composition ratio of a particular bacterial strain determined the numbers of viable bacteria of this strain translocating to the MLNs. [39]. MHCll CD11b+ CD11c+ DCs are shown to induce regulatory T cell by producing IL-10 cytokine [25]. As a result, Foxp3 transcripition

factor of regulatory T cell was increased in colon tissue (Fig 7). These regulatory T cells can circulate all the immune system. So it affected to other organ too. Foxp3 transcripition factor was also increased in the skin (Fig 10).

These increasment of foxp3+ regulatory T cell inhibited proinflammatory cytokines (Fig. 10). Psoriasis main pathogenic mechanism is IL23/17 axis. In the previous studies, IL17 and IL-23 antibody significant effect on psoriasis mice model and human study [40]. In the aspect of inflammatory suppression effect of 697, 697 alleviated IL-1 b and IL-6 that are proinflammatory cytokines and IL-17A, F and IL-22 that is related to Th17 cell cytokine (Fig. 10).

In the aspect of systemic immune response, spleen is the biggest immune organ that best represents the state of the entire immune system. [41]. Spleen also serves as a important reservoir of monocytes that are recruited during inflammation [42]. In this study, imiquimod-treated mice group appeared splenomegaly. However, 697 did not reduced the spleen weight (Fig. S5). But we found out that 697 group reduced CD11b+ F4/80+ macrophage in spleen (Fig. 8). It means that 697 group had less systemic inflammation because more macrophages in the spleen means more inflammatory reactions [42]. So, this result represents that 697 have a effect on suppression of proinflammation and Th17 cell associated cytokines in all the body parts including skin.

In conclusion, these results suggest that 697 can reduce proinflammatory and Th17-associated cytokines in the skin by increasing *Bacteroides which have a anti-inflammatory effect and regulatory T cell which is* activated by

MHCII in CD11b+ CD11c+ DCs. It shows the mechanism how to inhibit skin inflammation by probiotics. Also, it shows possibility that 697 can be used as a preventive medicine for psoriasis. However, further investigation is needed to figure out the specific effector molecules and which microbes activate MHCII in CD11b+ CD11c+ DCs.

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VI. Supporting Information

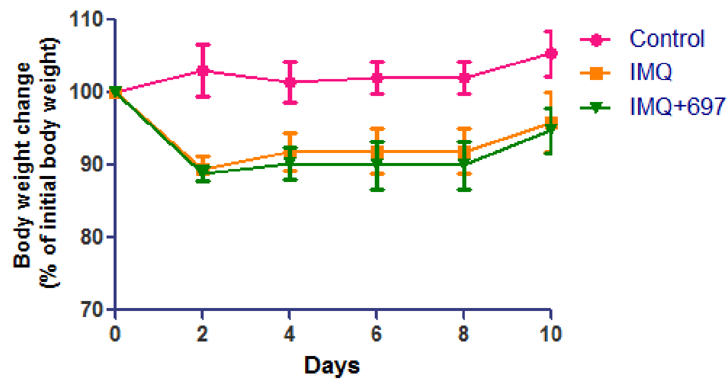
Supplementary figure 1. The weight changes are shown for each group during 0 – 10 days.

Supplementary figure 2. Right ear thickness are shown for each group during 0-10 days.

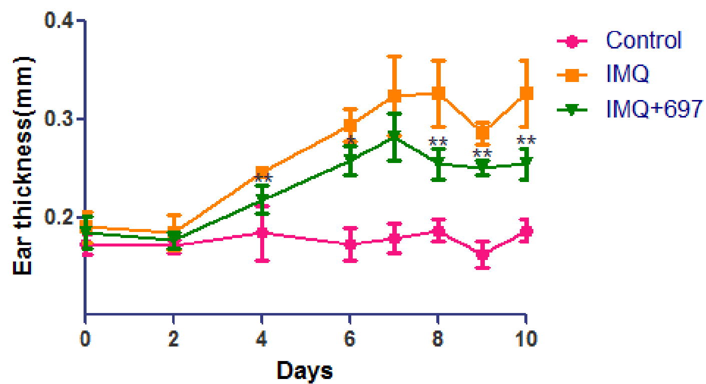
Supplementary figure 3. MHCII of CD11b+ CD11c+ DC in spleen of imiquimod-treated mice

Supplementary figure 4. Macrophage in MLNs of imiquimod-treated mice

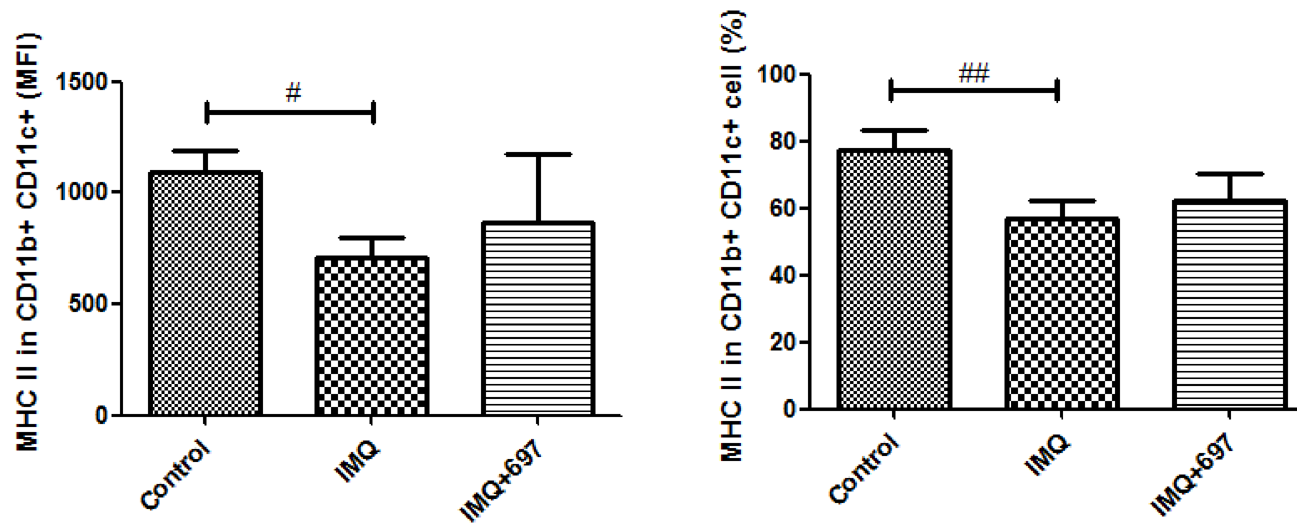
Supplementary figure 5. Spleen weight/Body weight ratio (%) of imiquimod-treated mice



Supplementary figure 1. The weight changes are shown for each group during 0 – 10 days. No significant difference compared to IMQ treated group in body weight change (% of initial body weight) was observed among the experimental groups. (N = 5 per group)

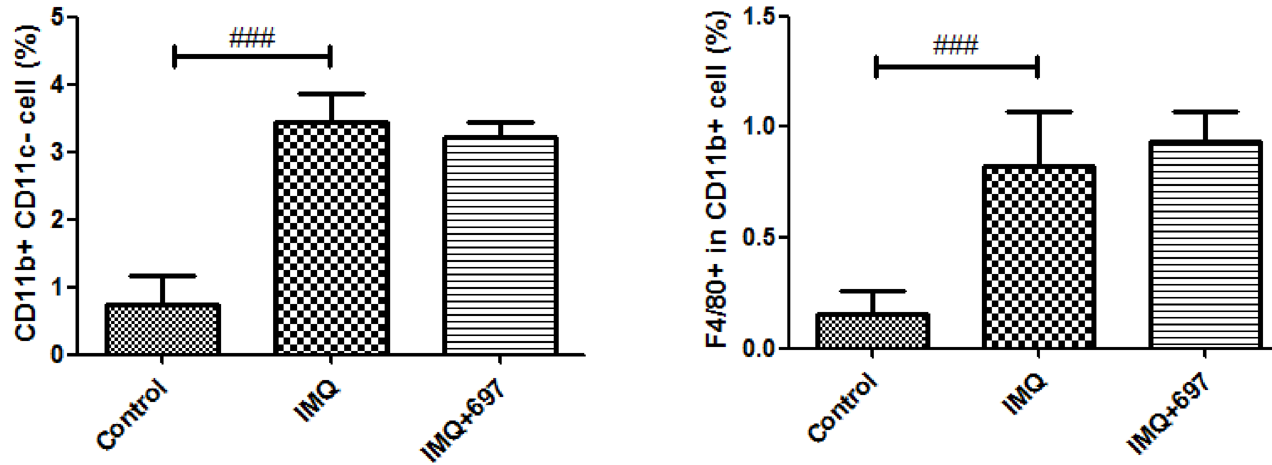


Supplementary figure 2. Right ear thickness are shown for each group during 0-10 days. We treated 12.5mg imiquimod to right ear. The ear thickness was measured by caliper. The data are shown as mean±S.D (N = 5 per each group). Significant differences are expressed as *P <0.05, **P <0.01, ***P <0.001 vs IMQ treated group.



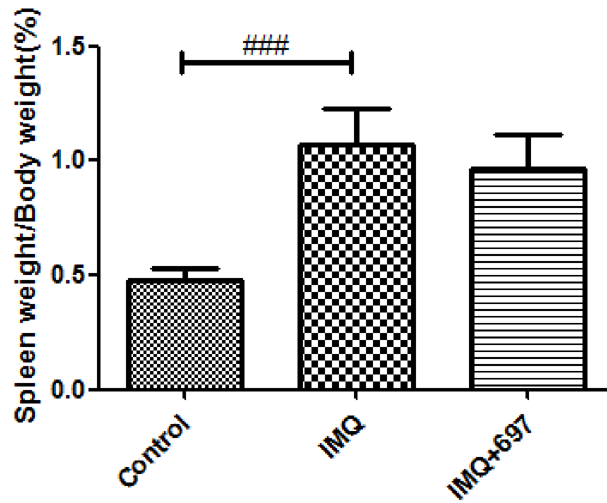
Supplementary figure 3. MHC II of CD11b+ CD11c+ DC in spleen of imiquimod-treated mice

Percentage of MHC II in CD11b+ CD11c+ DCs, the total number of MHC II in CD11b+ CD11c+ cells and representative histogram of each group. The data are shown as mean±S.D (N = 5 per each group). Significant differences are expressed as #*P* <0.05, ##*P* <0.01 vs control group.



Supplementary figure 4. Macrophage in MLNs of imiquimod-treated mice

Percentage of CD11b+ cells and percentage of F4/80 in CD11b+ cells in spleen. The data are shown as mean±S.D (N = 5 per each group). Significant differences are expressed as ###*P* <0.001 vs control group



Supplementary figure 5. Spleen weight/Body weight ratio (%) of imiquimod-treated mice

Spleen were collected after sacrifice. We measured spleen by electric scale. The data are shown as mean±S.D (N = 5 per each group). Significant differences are expressed as ###*P* <0.001 vs control group

국문초록

락토바실러스 가세리 KBL697의 조절 T 세포 유도를 통한 이미퀴모드 유도 건선 동물모델의 병변 예방

서울대학교 보건대학원

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박효인

지도교수 고 광표

건선은 T 세포가 매개하는 만성피부 자기면역질환으로 Th17 세포가 주로 염증 반응을 일으키는 것으로 알려져 있다. 락토바실러스가 건선 질환을 예방 및 치료한다고 보고되고 있으나, 그 기전에 대해서는 보고된 바가 없다. 본 연구에서는 이전의 실험에서 Raw 264.7 세포의 IL-10 cytokine 분비 연구를 통해 항염증성 기능을 가진 락토바실러스 가세리 KBL697을 선별하였다. KBL697의 건선 예방 효과를 확인하기 위해 이미퀴모드로 유발된 건선 Balb/c 마우스 모델을 사용하였다. 우리는 KBL697의 치료 기전을 확인하기 위해 장미생물군에 미치는 영향과 면역 반응에 대해 조사했다. 장미생물군에서 항염증 효과를 가진다고 알려진 박테로이데스를 정상상태로 회복시켰다. 면역 반응에서는 KBL697을 처리한 그룹에서 조절 T 세포를 유도한다고 알려진 CD11b+ CD11c+ MHCII+ 수지상세포를 증가시켰다. 그리고 KBL697을 처리한 그룹은 대장 및 피부에서 조절 T세포의 전사 인자인 Foxp3의 mRNA 발현 수준을 높였다. 마지막으로 염증반응에 대해서 살펴보았을 때, KBL697을 처리한 그룹은 이미퀴모드 처리 마우스 비장의 대식세포 수를 줄여 전체 면역 시스템을 완화하는 것을 확인하였다. 또한 KBL697을 처리한 그룹은 피부에서 Th17과 연결된 사이토카인을 감소시켰다. 이러한 염증완화를 통해 이미퀴모드 처리 마우스의 PASI 점수와 등두께도 감소하였다. 이러한 결과는 KBL697이 박테로이데스와 CD11b+ CD11c+ MHCII+ 수지상세포를 증가시켜 조절 T 세포의 분화를 촉진함으로써 피부에서 염증반응 완화시키는 것을 보여준다. 이는 KBL697의 건선 예방 및 치료로 사용될 가능성을 보여준다.

주요 단어: 사이토카인, 이미퀴모드, 락토바실러스 가세리 KBL697, Foxp3, 박테로이데스, 건선, 수지상세포, 조절 T 세포

학번: 2017-22337