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FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

Mixture of probiotics alleviates the symptoms of atopic dermatitis through recovering immune balance in mice

복합 유산균의 면역 회복에 의한 아토피성 피부염 마우스의 증상 완화

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By

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농 학 박 사 학 위 논 문

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Abstract

Mixture of probiotics alleviates the symptoms of atopic dermatitis through recovering immune balance in mice

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Atopic dermatitis (AD) is a chronic inflammatory skin disease, seen mostly in children causing eczema often together with intense itching. AD can be caused by genetic factors, immune system dysfunction and/or environmental factors in conjunction with the uncontrolled permeability of the skin. AD is caused by excessive T helper (Th) 2 responses, which induce immunoglobulin (Ig)E. Hypersensitivity of Th2 cells to AD is also associated with increased risk of other allergy diseases such as asthma. There are variety of therapies available to relieve AD, including emollients, anti-inflammatory agents and inhibitors. However, excessive use of these therapies causes side effects such as skin thinning, purpura, telangiectasias, drowsiness and edema. Therefore, for a fundamental atopic treatment, a particular strategy to maintain the continuous immune balance seems to be necessary. The use of probiotics could be such strategy for overcoming AD.

However, selection of probiotics and their combination are important task when consider AD treatment. Therefore, to overcome AD, it is necessary to understand the exact immune mechanism induced by each probiotic.

In the first study, the immunomodulatory capacity of Duolac ATP, a mixed formulation of probiotics, was examined. Results showed that the expression of programmed death-ligand (PD-L)1 was significantly upregulated on dendritic cells (DCs) treated with Duolac ATP. Furthermore, the anti-inflammatory cytokines, interleukin (IL)-10 and transforming growth factor (TGF)-β were both upregulated when BMDCs were treated with Duolac ATP. The percentage of proliferated regulatory T cells (Tregs) was enhanced when $CD4^+$ T cells were co-cultured with Duolac ATP-treated DCs on plates coated with anti-CD3/CD28 antibodies. Intriguingly, IL-10 secretion from $CD4^+$ T cells was also observed. The AD symptoms, histologic scores, and serum IgE levels in spontaneous mutation AD mice (Nc/Nga) were significantly decreased after oral treatment with Duolac ATP. Moreover, the Th1-mediated response in AD-induced mice treated with oral Duolac ATP showed upregulation of IL-2 and IFN-γ as well as of downstream signaling molecules T-bet, STAT1, and STAT4. Conversely, Duolac ATP suppressed Th2 and Th17 responses in AD-like mice, as evidenced by the downregulation of GATA3, C-maf, IL-4, IL-5, and IL-17. Additionally, Duolac ATP increased the number of Tregs found at Peyer's patches (PP) in AD mice. These results suggest that Duolac ATP modulates DCs to initiate both Th1 and Treg responses in AD mice.

In the second study, the mechanism of alleviation of AD by YK4, a probiotic mixture consisting four strains of *Lactobacillus* and *Bifidobacterium*, was unveiled through intestinal galectin-9 production and immune cells analysis. The results showed that administration of YK4 in AD mouse alleviates symptoms of AD by regulating Th2-mediated response. YK4 inhibited the expression of skin thymic stromal lymphopoietin and serum IgE to near normal concentration. YK4 administration also resulted in decrease in IL-4 producing $CD4^+$ T cells whereas increased Tregs population in PP and mesenteric lymph node (mLN). Moreover, YK4 induced an increase in interferon-gamma (IFN-γ) producing $CD4^+$ T cells in spleen. Furthermore, the proportion of $CD103^+$ DCs in mLN and spleen was significantly increased in repetitive treatment of skin irritants AD mice administered with YK4 when compared to AD mice. Expression of galectin-9 in the intestine was significantly increased in AD mice administered with YK4. The expression of CD44, a receptor of galectin-9, together with PD-L1 was significantly up regulated on BMDCs when treated with YK4. Furthermore, the anti-inflammatory cytokine, IL-10 was upregulated when BMDCs were treated with YK4. The percentage of proliferated Tregs was enhanced when $CD4^+$ T cells were co-cultured with YK4-treated BMDCs. Galectin-9 appeared to be partially contributed to the proliferation of Tregs.

In summary, in the spontaneous mutation mouse model, Duolac ATP regulated IL-10 and TGF-β expression and allowed DCs to become functionally tolerant and potentially induce Treg differentiation.

Furthermore, Duolac ATP regulated transcription factors and cytokines to drive naïve T cell differentiation toward Th1 lineages. In the skin irritation mouse model, YK4 induced expression of IL-10 and IL-12 in DCs, which inhibited Th2 responses by inducing Tregs differentiation. Furthermore, YK4 regulated intestinal galectin-9 and $CD103^+$ DCs to drive naïve T cell differentiation toward Th1 and Tregs. Taken together, these results suggest that the probiotic mixture, Duolac ATP and YK4 have therapeutic potential to prevent AD symptoms and may act as an immunomodulator for AD patients.

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Keywords: Probiotics, atopic dermatitis, dendritic cell, T cell balance, galectin-9

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Chapter 1. Review of Literature

List of Abbreviations

AD	Atopic dermatitis
BCL-3	B-cell lymphoma 3-encoded protein
BM	Bone marrow
BMDCs	Bond marrow-derived DCs
BSA	Bovine serum albumin
CCL	CC chemokine ligand
CCR	Chemokine receptor
CD	Cluster of differentiation
cDNA	Complementary DNA
CFU	Colony forming unit
COX	Cyclooxygenase
CRD	Carbohydrate-recognition domain
CTV	CellTrace™Violet
DCs	Dendritic cells
DNA	Deoxyribonucleic acid
DNCB	2,4-denitrochlorobenzene
dNTP	Deoxyribose containing nucleoside triphosphates
DTT	Dithiothreitol
ELISA	Enzyme-linked immunosorbent assay

FcεR1	Immunoglobulin-ε receptors
Foxp3	Forkhead box protein P3
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GATA3	GATA binding protein 3
GM-CSF	Granulocyte macrophage-colony stimulating factor
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HDM	House dust mite
HRP	Horseradish peroxidase
IDO	Indoleamine 2,3-dioxygenase
IEC	Intestinal epithelial cell
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
JAK	Janus kinase
LCs	Langerhans cells
mAbs	Monoclonal antibodies
MAPK	Mitogen activated protein kinases
MHC	Major histocompatibility complex
mLN	Mesenteric lymph node
mRNA	Messenger RNA
NFκB	Nuclear factor-kappa B
Ova	Ovalbumin

OX40L	OX40 ligand
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PDE4	Phosphodiesterase-4
PD-L	Programmed death-ligand
PI3K	Phosphatidyl inositol 3-kinase
PMA	Phorbol 12-myristate 13-acetate
PP	Peyer's patches
qPCR	Quantitative PCR
RBC	Red blood cells
RNA	Ribonucleic acid
SCORAD	Scoring of Atopic Dermatitis
Smad	Small mothers against decapentaplegic
Src	Serine-threonine kinase
STAT	Signal transducer and activator of transcription'
T-bet	T-box transcription factor TBX21
tDCs	Tolerogenic DCs
TGF	Transforming growth factor
Th	Helper T cell
TIM-3	T cell immunoglobulin mucin-3
TLR	Toll-like receptor
TMB	Tetramethylbenzidine

TNF	Tumor necrosis factor
Tregs	Regulatory T cells
TSLP	Thymic stromal lymphopoietin

Chapter 1

Review of Literature

1. Atopic dermatitis

1.1. Characteristics and symptoms

Atopic dermatitis (AD), also known as atopic eczema or allergic eczema, is a chronic inflammatory disorder of skin leading to its deformities in the structure and barrier function [1-3]. Thus, major symptoms of AD are destruction of epidermis of the skin and increased excessive itching and eczema. Itching can lead to scratching, and thus worsening symptoms and increasing the risk of skin infection [1-3]. The location of the onset of AD varies with age. In infants, scalp, face, neck, hands and feet are usually affected by AD [1-3]. Children generally have AD in the curved part of the skin such as elbow and back of the knee. In adolescence and adulthood, hands and feet are commonly affected areas [4,5]. The incidence of AD has steadily increased over the past 30 years, with about 15-20% of children and 2-10% of adults suffering from this disease in developed countries [4,5]. Most children with atopic dermatosis develop spontaneously before puberty. However, some children with atopic symptoms that disappear during adolescence may recur in adults [4,5]. Like other allergic diseases, AD is mainly caused by excessive immune responses of T helper (Th) 2 cells that induce immunoglobulin (Ig) E [2,4,6]. Hypersensitivity of Th2 cells to AD is also associated with increased risk of other inflammatory diseases such as arthritis and inflammatory bowel

disease [6].

1.2. Pathogenesis and immune responses of atopic dermatitis

1.2.1. Pathogenesis

Although the cause of AD is not yet fully understood, genetics, immune system dysfunction and environmental factors can be associated with the permeability of the skin [7]. Many genes are known to be associated with AD, among which the filaggrin gene is the best known genetic risk factor for AD. About 10% of people have mutations in the filaggrin gene, and more over about 50% of people with atopic dermatitis have mutations in this gene [8]. Family history have been also reported [9]. Children whose parents have AD are reported to be three to five times more likely to have AD than those who do not. Immune system dysfunction also caused AD [1,4]. When the pH of the skin changed due to excessive water loss in the epidermis, the overgrowth of bacteria such as *Staphylococcus aureus*, on the skin surface overgrow and triggered the immune response [10]. Potential environmental risk factors including excessive UV exposure, dry climatic conditions, and unbalanced eating habits are known to be potential causes of AD [11].

1.2.2. Immune responses

When AD occurs, it caused problems in the primarily skin epidermis. The epidermis is divided into four areas, namely stratum corneum, granular layer, spinous layer, and basal layer (Figure 1-1) [5,12].

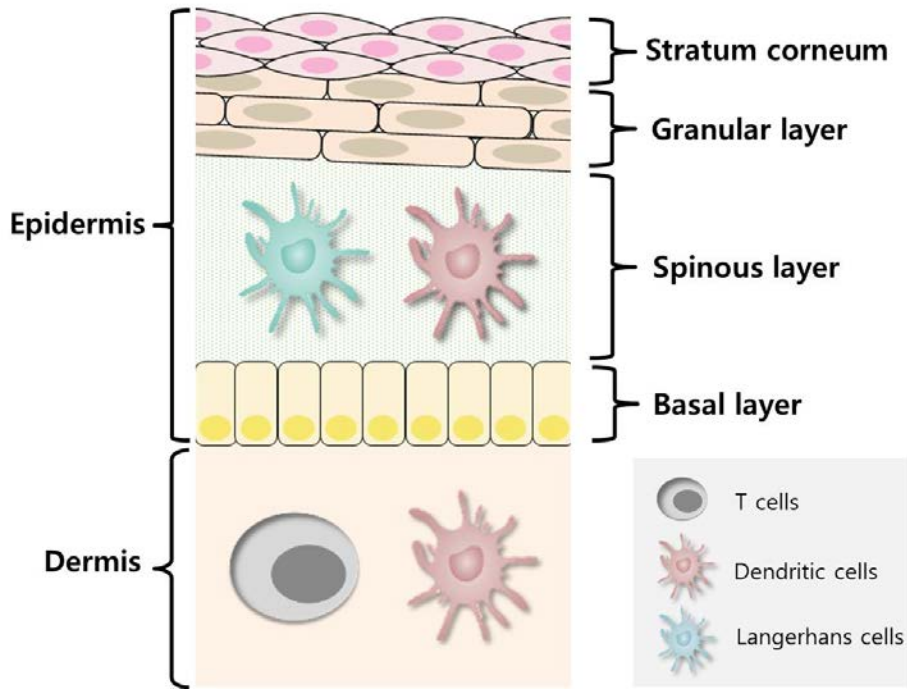

Figure 1-1. Structure of skin and immune cell distribution.

The skin is largely divided into epidermis and dermis. Then the epidermis is divided into four regions such as stratum corneum, granular layer, spinous layer, and basal layer. Dendritic cells and langerhans cells is distributed in the spinous layer. Dendritic cells and T cells are distributed in the dermis.

AD caused the increased of pH of the skin surface to make the skin dry, causing collapse of the stratum corneum [10]. Then, granular layer is exposed to the outside, which allows various antigens such as *S. aureus* and allergens to infiltrate the skin [13]. These antigens stimulate keratinocytes, which was constitutive cells of granular layer, to induce the production of chemokines such as CC chemokine ligand (CCL) 17 and CCL22, and interleukin (IL)-1β, IL-33 and thymic stromal lymphopoietin (TSLP). CCL17 and CCL22 bind to chemokine receptor (CCR) 4 and recruit the cells expressed CCR4, particularly T cells, to the skin. IL-1β, IL-33 and TSLP accelerated the differentiation and inflammatory responses of dendritic cells (DCs) and langerhans cells (LCs) in spinous layer [5]. In particular, IL-33 and TSLP stimulated cutaneous sensory neurons directly to deepen the itch of the skin, promoting skin collapse [14]. TSLP also induced and activated the expression of OX40 ligand (OX40L) in dermal DCs. Activated DCs bound to OX40L receptors on naïve $CD4^+$ T cells and induced differentiation into Th2 cells, which were known to regulate skin inflammatory responses by producing IL-4 and IL-13 [15,16]. In particular, IL-4 activated signal transducer and activator of transcription (STAT) 6 to inhibit the differentiation of keratinocytes. IL-4 also induced B cell activation and proliferation, in accordance with induction of IgE class switching [17]. IgE bound to immunoglobulin-ε receptors (FcεR1) of LCs to promote allergen uptake and initiated T cell-mediated delayed

hypersensitivity. IgE also bound to FcεR1 on mast cells and promoted the secretion of granules, especially histamine that promoted vasodilation and the recruitment of immune cells and exacerbated skin inflammation (Figure 1-2) [18].

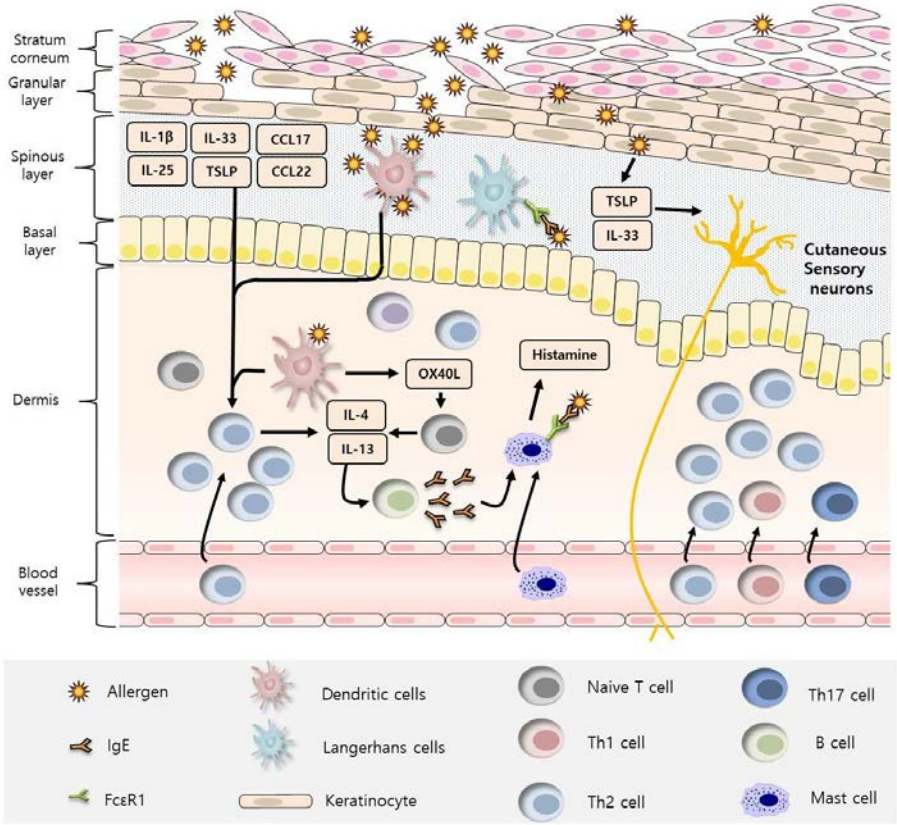

Figure 1-2. Cellular and molecular immunologic mechanism of atopic dermatitis.

AD breaks down the skin, causing external antigens to penetrate, leading to the activation of skin cells and various immune cells. This worsens the symptoms of AD.

1.3. Animal models for atopic dermatitis

Various animal models were developed to characterize human AD for the better understanding of the causes and results of AD, and therefore providing therapeutic strategies. A mouse models for AD induced by genetic modification or repetitive skin irritation are well studied [19]. Spontaneous mutation resulted in one of the genetically modified mice in which some genes related to AD are modified. Transgenic mouse was another type of genetically modified mouse that designed to overexpress selective cytokine (Table 1-1). Skin irritation was known to induce AD in mouse by repetitive treatment of skin irritants such as certain proteins, antigens and chemical reagents [19-21]. These AD mouse models (Table 1-1) mostly induced excessive Th2 responses and developed AD-like skin changes in the dermis showing high density of mast cells and eosinophils [19-21].

Table 1-1. Mouse models for atopic dermatitis.

Classification		Characteristics	Ref
Genetic modification	**Spontaneous mutant**	Nc/Nga mouse (Chromosome 9 mutant)	22
	Transgenic	IL-4 overexpressing mouse	23
		IL-18 overexpressing mouse	24
		TSLP overexpressing mouse	25
Repetitive skin irritation		Ova (protein) stimulation	26
		HDM (allergen) stimulation	27
		DNCB (chemical reagents) stimulation	28

TSLP, thymic stromal lymphopoietin; Ova, ovalbumin; HDM, House dust mite; DNCB, 2,4-dinitrochlorobenzene.

Among these, a model similar to the AD occurring in the general population without genetic diseases was a repetitive treatment of skin irritant. House dust mite (HDM) allergen was known as one of the most frequent factors that cause human AD [27]. Mice subjected to repeated skin irritation by HDM allergen exhibited dermatitis due to epidermal hyperplasia. $CD4^+$ and $CD8^+$ T cells were infiltrated to the skin of these mice and systemic excessive Th2 responses were observed [27]. Hapten such as 2,4-dinitrochlorobenzene (DNCB) were also commonly used to induce allergic contact dermatitis [28]. When DNCB was exposed to mouse skin for 2 weeks or more, the skin inflammation migrated to a Th2 dominating response similar to human AD [28]. Challenges to repetitive DNCB have resulted in increased epidermal hyperplasia and reduced expression of the skin differentiation proteins such as filaggrin[1], loricrin[2] and involucrin[3] [28]. Studies of various AD mouse models have led to a better understanding of the pathogenesis of human AD and the potential for AD treatment.

1.4. Therapeutics for atopic dermatitis and their limitation

There are variety of therapies available to relieve the AD, including hydration and recovery of skin barriers by the use of emollients, anti-

[1] Filaggrin: Filament-related protein that binds to the keratinous fibers of epithelial cells

[2] Loricrin : Protein component of the cornified cell envelope

[3] Involucrin: Substrate protein present in keratinocytes of epidermis

microbial agents, and anti-inflammatory agents [29]. The goals for the therapeutic were to reduce itching, inhibit inflammation, and restore skin barrier function [29]. Emollients were widely recommended because they were not only effective but also safe for skin moisturization. The emollient was known to actively bind the epidermal barrier by tying or pulling the stratum corneum water, reducing the severity of the disease [30].

Topical corticosteroids (TCSs) had an anti-inflammatory effect and intermittent used reduces the recurrence of the disease [31]. TCSs were reducing *S. aureus* levels and restoring skin barrier function. However, it was important to note that excessive use of TCS caused side effects such as skin thinning, purpura, telangiectasias, and growth retardation [32]. Various inhibitors were also used in the treatment of AD. The Janus kinase (JAK) /STAT pathway is used by multiple cytokines and growth factors for signaling in AD [33]. Tofacitinib, inhibiting JAK1 and JAK3, blocks Th2 response and induced Th1 response. Phosphodiesterase-4 (PDE4) inhibitors were also used as potential treatments for AD [34]. PDE4 has been shown to directly attenuate inflammation by inhibiting degradation of cAMP resulting in a decrease in levels of tumor necrosis factor (TNF)-α, IL-12 and IL-21 [35]. Another topical application, SB011, contained the DNAzyme hgd40 targeting GATA3, a major regulator of Th2-induced immune responses, which effectively prevents the differentiation of Th2 cells [36]. Most of the inhibitors are effective in short-term treatment, however they are not recommended because a long-

term use can cause side effects including immune irregularities [37]. Some inhibitors were causing side effects such as drowsiness and edema [30]. Therefore, for a fundamental atopic treatment, a particular strategy to maintain the continuous immune balance seems necessary. Recently, probiotics have been suggested and studied as such strategy for overcoming AD.

2. Probiotics

2.1. General characteristics

Probiotics are live microorganisms that had beneficial effects on host health and potential for disease prevention and treatment [38]. Probiotics are usually commensal lactic acid bacteria that can be ingested through a variety of foods that found in dairy products and fermented foods. Among various probiotics, *Lactobacillus, Bifidobacterium* and *Saccharomyces* have been widely studied and commonly used in humans and animals [39]. Microbial flora plays an important role as a part of intestinal mucosal immune system. Especially, probiotics competed with harmful microorganisms to prevent pathogens from adhering to the epithelium [40]. For example, *Lactobacillus rhamnosus* and *L. plantarum* were able to inhibit the adherence of enteric pathogenic *Escherichia coli* in the gastrointestinal tract [41]. Probiotics also enhanced the survival of intestinal epithelial cell (IEC) and improved the barrier function [42]. The most important aspects of probiotics are beneficial to human health by activating and matured intestinal immune systems [42,43].

2.2. Probiotics as intestinal epithelial cell modulators

IECs provided a physical and chemical barrier between the intestinal

lumen and the lamina propria [44]. Probiotics can modulate function of IECs in a variety of ways including directly stimulating the surface of IECs and indirectly producing soluble proteins. Furthermore, probiotics could improve the barrier function by controlling tight junctions of IECs [45]. Various transmembrane proteins such as zonula occludens (ZO)-1, Claudin-1 and Occludin were involved in the formation of tight junctions [44]. Probiotics could induce the expression of transmembrane proteins through direct toll-like receptor (TLR) stimulation. For example, *L. rhamnosus* and *L. plantarum* stimulated TLR2 signaling, leading to increased expression of ZO-1 and Occludin in IECs and epithelial permeability [46]. In addition, *L. casei* activated p50/p50 NFκB homodimers through the activation of B-cell lymphoma 3-encoded protein (BCL-3) to prevent the production of inflammatory cytokines [47]. It has been suggested that soluble proteins *L. rhamnosus GG* (LGG) p40 and LGGp75 of probiotics were involved in the survival of IECs. The LGGp40 and LGGp75 bound to epithelial growth factor receptor (EGFR) on IECs and stimulate serine-threonine kinase (Src) that activated phosphatidyl inositol 3-kinase (PI3K), resulting in the prevention of apoptosis [48]. The ability of probiotics to regulate cell death and function of IECs could be a useful strategy to prevent immune collapse due to various inflammatory diseases (Figure 1-3).

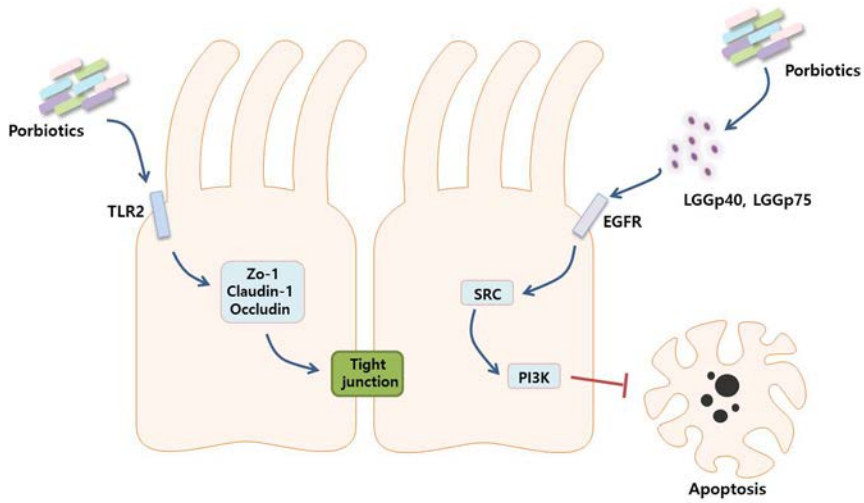

Figure 1-3. Probiotics modulate the function of intestinal epithelial cells.

Probiotics induce the expression of ZO-1, Claudin-1 and Occludin on intestinal epithelial cells through TLR2 stimulation. In addition, probiotics produced LGGp40 and LGGp75, which activates SRC through EGFR and activates PI3K to block intestinal epithelial cells apoptosis.

2.3. Immunological roles of probiotics

Probiotics mainly interact directly with the IECs but some reach to the lamina propria through M cells and interact with immune cells to regulate gastrointestinal immune system [49]. DCs in the lamina propria layer was known to be the main cell that recognizes probiotics [50]. DCs are one of the antigen-presenting cells that can most effectively induce a primary immune response against pathogens as well as maintain tolerance to self-antigens [51]. DCs are also known to played a key role in bridging innate and adaptive immune responses [52]. DCs are stimulated through TLR signaling dependent on the type of stimulus. Several probiotic strains were known to regulate the function and characteristics of DCs [53]. Certain probiotics, such as *L. casei,* induced the production of IL-12, IL-6 and TNF-α by DCs through the activation of STAT1 and STAT3, thereby supporting inflammatory responses [54]. On the other hand, *L. reuteri* inhibited the production of these cytokines and neutralized the inflammatory response [55]. In addition, IRT5, a mixture of probiotics consisting *of L. casei, L. acidophilus, L. reuteri, B. bifidum*, and *Streptococcus thermophiles,* induces indoleamine 2,3-dioxygenase (IDO) and cyclooxygenase (COX)-2 expression in DCs via TLR2 stimulation and helped to suppress inflammatory responses by producing IL-10 and transforming growth factor (TGF)-β [56]. Specifically, DCs that were specialized for inhibiting inflammation, called tolerogenic DCs (tDCs), and $CD103^+$ DCs played a similar role in the intestinal immunity [57].

$CD103^+$ DCs inhibited naive $CD4^+$ T cell differentiation to Th2 cells and, at the same time, induced the differentiation of regulatory T cells (Tregs) through the production of IL-10 and TGF-β [57]. *B. breve* induced the production of IL-10 in DCs through TLR2/MyD88 signal transduction and promoted the differentiation of Tregs [58]. In addition, *L. salivarius* induced IL-10-dependent development of $CD103^+$ DCs and Tregs [59]. In conclusion, certain probiotic bacterial strains could regulate intestinal homeostasis by promoting the induction of Tregs through DCs (Figure 1-4).

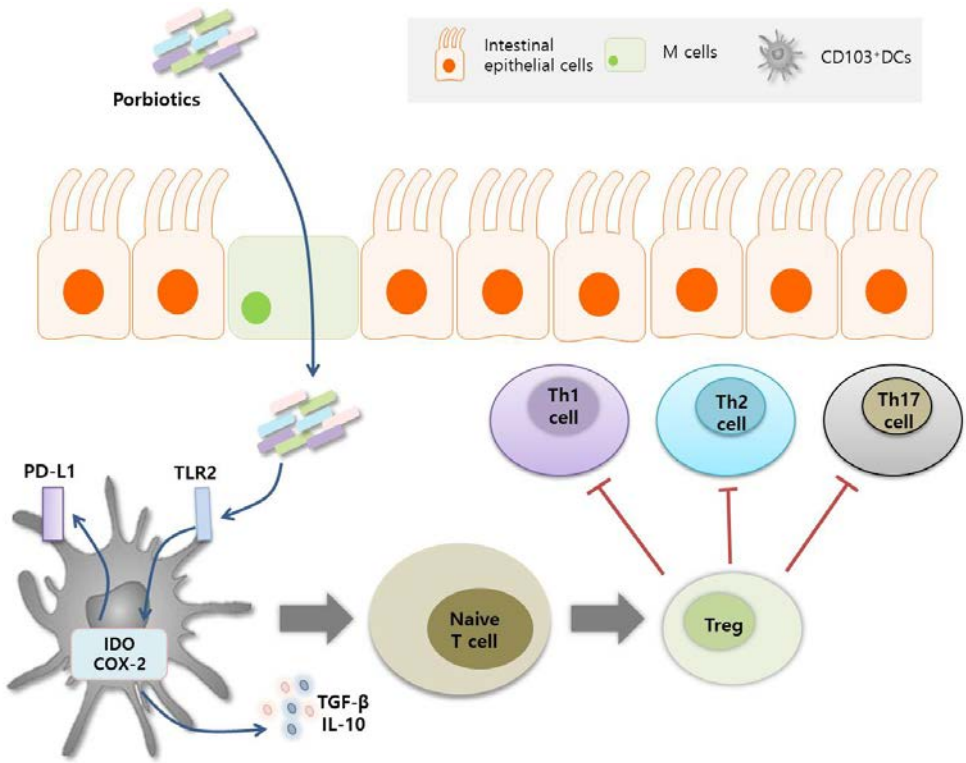

Figure 1-4. Probiotics modulate the Dendritic cells and T cell differentiation.

Probiotics induced the expression of IDO and COX-2 through TLR2 stimulation and induced the expression of PD-L1 in $CD103^{+}$ DC, while enhancing TGF-β and IL-10 production. These DCs and cytokines induced the differentiation of naive T cells into Tregs and prevented the differentiation of Th1, 2 and 17.

2.4. Probiotics in the treatment for atopic dermatitis

The collapse of immune balance due to excessive Th2 response is the major cause of AD. Certain probiotics are known to improve the symptoms of disease by controlling the Th2 immune response [1,2,4]. There is a number of studies that have identified the potential efficacy of probiotics in the treatment of AD [60,61]. *L. plantarum* showed prevention effect in AD symptom together with the reduction of IFN-γ and IL-4 level as well as proportion of eosinophil [62]. *L. salivarius* showed the reduction of the Th2 cytokine production together with the maintenance of Th1 cytokine production relieved AD symptoms [63]. *Weissella cibaria* induced a population of Tregs in mesenteric lymph node (mLN) and reduced the production of the Th2 cytokines, IL-4, IL-5 and IL-13 [64]. Moreover, *W. cibaria* attenuated epidermal thickening and AD-like skin lesions with reduction of serum IgE levels [64]. Oral treatment of IRT5 induced tDC and Treg differentiation with up-regulation of IL-10 and TGF-β level in AD mice that eventually mitigates symptom of AD [56]. These various probiotics also have shown clinical efficacy in AD. 8-weeks treatment of *L. fermentum* in 6-18 months old infants showed a significantly reduced severity of AD including Scoring of Atopic Dermatitis (SCORAD) [65]. When *L. acidophilus*, *B. lactis* and fructo-oligosaccharide were supplemented to children, It showed the significant attenuation in SCORAD (33.7%), infant dermatitis quality of life (33%),

dermatitis family impact (35.2%) scores [66]. ProBiotik® pur, a mixture of *L. salivarius, L. casei, L. acidophilus*, and *B. bifidum* showed an effective reduction in SCORAD, IgE, IL-6, IL-5, and IFN-γ levels with unchanged TNF-α, IL-10, IL-2, and IL-4 expression levels in children [65]. It is important to note that not all probiotics had a such effect on AD. Importantly, even the same strain may have different efficacy depending on the combination or the target. The infant study with *L. paracasei* or *B. lactis* showed no significant difference in SCORAD and other AD related parameters compared with placebo groups [67]. Although, *L. rhamnosus* showed alleviation of symptoms of AD syndrome including SCORAD score in IgE-sensitized infants, the cocktail of probiotic mixture containing the *L. rhamnosus* with *Propionibacterium freudenreichii*, *B. breve* had no effect on the allergic diseases [68]. Thus, previous studies suggested that probiotics are not always showing effective immunomodulatory response against AD. In other words, the efficacy of AD treatment of probiotics are depending on the selection and combination of strains. Therefore, to overcome atopy, it is necessary to study the exact immune mechanism induced by each probiotics. Moreover, research on the ideal combination of probiotics is essentially required.

3. Galectin

3.1. Characteristics and members of galectins

Galectin is non-glycosylated soluble proteins of lectin family that binds to glycan, especially β-galactosides [69,70]. Galectins have carbohydrate-recognition domain (CRD) sequence motif and are widely distributed [69,70]. There are mainly three types of mammalian galectins dependent on structural difference. Proto type galectins are galactin-1, -2, -5, -7, -10, -11, -13, -14, and -15, while tandem repeat types are galectins-4, -6, -8, -9 and -12. Galectin-3 is the only chimera type [70,71]. Proto type galectins have single CRD and exist as monomers or homodimers. Tandem repeat types contained two different CRDs occur within a single polypeptide. Two different CRDs separated by a linker of up to 70 amino acids. Chimera type contained a single CRD connected to a non-lectin amino-terminal domain, which is rich in glycine and tyrosine residues. In humans, only Galectin-1, -2, -3, -4, -7, -8, -9, -10, and -12 are identified. Galectin-5 and -6 are found in mouse whereas galectin-11, -14 and -15 are found in sheep and goats (Figure 1-5) [70,71].

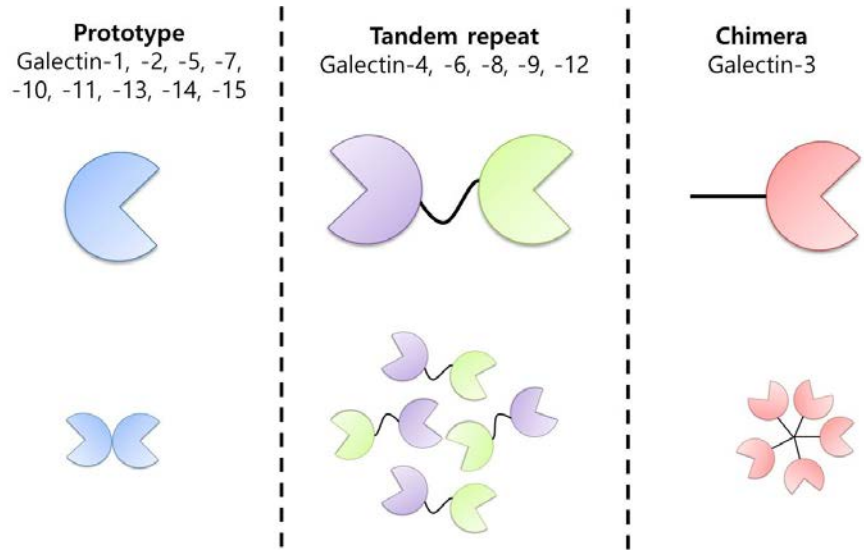

Figure 5. Three types of galectins.

Galectins are divided into proto type, tandem repeat type and chimera type based on the structure.

Galectins are synthesized in the cytoplasm of free ribosomes, and the synthesized galectins are detected in the cytoplasm and nucleus as well as extracellular space [72]. Galectins caused cell adhesion through cross-linking glycan on neighboring cells [73]. Galectins could also form galectin–glycoprotein lattices at the cell surface that can activate functional signaling pathways of endocytosis, host–pathogen interactions, and homeostasis of immune cells [72]. Galectins are known to be expressed in a variety of cells and tissues. Among them, galectin-1, -3 and -9 were relatively well known and studied [74]. Galectin-1 is secreted by various immune cells such as macrophages, DCs, neutrophils, T cells. It was also produced in thymus, muscles, neurons and kidneys [69,70]. Galectin-1 bound to CD2, CD3, CD7, CD43 and CD45 on T cells and induced apoptosis [75]. Galectin-1 also bound to neuropilin-1 on vascular endothelial cells, activating VEGFR-2 signaling and regulating migration [76]. Galectin-3 is mainly produced in neutrophils, basophils, macrophages and DCs in immune cells and was produced in most of tissues [69,70]. Galectin-3 bound to CD7, CD29, CD45 and CD71 on T cells and induced apoptosis [77]. Galectin-3 also bound to CD98 on macrophages and differentiated it into alternative M2-type macrophages [78]. Galectin-9 is mainly secreted by kidney, thymus and intestine [69,70]. Galectin-9 bound to T cell immunoglobulin mucin-3 (TIM-3) on Th1 and Th17 cells and induced apoptosis [79]. Galectin-9 also bounds to TIM-3 and CD44 on DCs and monocytes to regulate cell

maturation and cytokine release [80]. Above all, Galectin-9, unlike other galectins, bounds to CD44 on Tregs and involved in immunosuppression (Table 1-2) [81].

Table 1-2. Main functional galectins for their source cells and known receptors.

Galectins	Major cell types	Receptors on immune cells
Galectin-1	Macrophages, DCs, Neutrophils, T cells, Muscle, Neurons, Kidney	CD45, CD43, CD7, CD2, CD3, GM1, Neuropilin 1
Galectin-3	Neutrophils, Basophils, Macrophages, DCs, Most of tissues	CD45, CD7, CD71, CD29, CTLA4, CD98
Galectin-9	Macrophages, DCs, Neutrophils, Eosinophils Kidney, Thymus, Intestine	TIM-3, CD44

3.2. Immune function of galectin-9

Galectin-9 has been suggested to regulate immune function in allergy mouse [82]. Galectin-9 is involved in the survival and differentiation of mouse T cells. T lymphocytes to be mature in the thymus, they should overcome positive and negative selection processes [83]. Galectin-9 bound to TIM-3 on $CD4^{-}CD8^{-}$ and $CD4^{+}CD8^{+}$ T cells and induces apoptosis from the thymus [83]. Galectin-9 also bound to the TIM-3 on Th1 and Th17 cells inducing intracellular $Ca2^{+}$ flux and promoting caspase-1 activation [84]. Moreover, galectin-9 bound directly to CD44 on Tregs and phosphorylates Smad3 to stabilize Foxp3 [85]. Stabilized Foxp3, then, promoted Tregs differentiation and produced IL-10 and TGF-β to maximize immunosuppression [85].

Galectin-9 inhibited differentiation of naïve T cells to Th17 cells and at the same time induced differentiation of Tregs *in vitro* [86]. Such ability to control T cell populations might be effective in attenuating autoimmune disease and prolonging allogeneic survival. Galectin-9 also affected the activity of DCs [87]. Galectin-9 bound to CD44 on DCs induced phosphorylation of the MAPK p38 and extracellular signal–regulated kinases (ERK)1/2, preventing their maturation and activation [87]. Galectin-9 also bound to TIM3 on DCs, preferentially producing IL-10, and initiated an adaptive immune response by synergizing with the activity of TLRs [80]. Galectin-9 was known to bind directly to the

immunoglobulin. Galectin-9 bound to carbohydrate moieties of IgE to prevent IgE-antigen complex formation [88]. Based on these immunomodulatory mechanism, galectin-9 has been recognized as a therapeutic to overcome in allergic diseases. Indeed, recombinant galactin-9 treatment improved clinical and immunological symptoms of allergic disease in a mouse model [89]. Some studied have reported that galactin-9 is produced in the intestine by probiotics [90]. When *B. breve* was fed to mouse with allergy induced with cow's milk, galectin-9 was produced in the intestinal cells and serum, leading to the differentiation of Th1 and Tregs [90]. Thus, galectin-9 induced Treg activity differently from other galectins and appeared to be involved in immune tolerance. Galectin-9 also showed direct and indirect immunomodulatory effects in various allergy mouse models. However, research on probiotics involved in the production and immunoregulation of galectin-9 is still in its early stages. Therefore, it is necessary to investigate whether galectin-9 is involved in the immune regulation of probiotics.

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Chapter 2

Dietary probiotic mixture, Duolac ATP reduces the symptoms in mice with atopic dermatitis

1. Introduction

Atopic dermatitis (AD) is a long lasting inflammatory skin disorder that affects the structural and barrier functions of the skin. Major symptoms of AD include excessive pruritus and eczema. Although the exact pathophysiology of AD is not yet fully understood, it can be caused by a combination of genetic, environmental, allergic, and microbial factors [91]. Like other allergic diseases, AD is caused by an immune response primarily Th2 cell hypersensitivity. A balance between Th1 and Th2 cells is important for disease induction and tolerance. In AD patients, overexpression of Th2 cytokines, including IL-4, IL-5, and IL-13, upregulate IgE production, resulting in eosinophil accumulation within the dermis [92,93]. Although steroid drugs are effective therapies, they have serious side effects, including dermal atrophy, acne, cataracts, growth retardation, and skin irritation. As such, long-term steroid treatment should generally be avoided [94,95]. Other alternative therapeutic agents are being investigated, including herbs, phytochemicals, vitamins, and probiotics [96].

Probiotics, which are non-invasive, non-pathogenic, Gram-positive bacteria with known health-promoting effects, are primarily found in fermented food and feed products [97,98]. An adequate quantity of probiotics can be helpful for host gut homeostasis, which is achieved through immune system modulation and the production of antimicrobial

agents that block the adhesion of pathogens and their toxins [99]. Recent studies have reported that probiotics are also effective in preventing allergic disorders in mice and humans [100-102]. *Lactobacillus plantarum* supplementation reduced the scoring atopic dermatitis (SCORAD) index in young atopic patients [62,100]. In another study, *L. casei* supplementation prevented the development of AD in NC/Ng mice [103]. However, the mechanism by which probiotics function is still not completely understood.

DCs are antigen-presenting cells that can effectively induce a primary immune response to pathogens as well as maintain tolerance to self-antigens [51]. DCs play a key role in bridging innate and adaptive immune responses [51,52,104]. Depending on the stimulus, DCs can secrete cytokines and induce naïve T-cell differentiation toward Th1, Th2, Th17, or Treg lineages. Therefore, much attention has focused on the impact of DC priming by probiotics to modulate T cell responses [105]. Some probiotic strains, including *Lactobacillus* and *Bifidobacterium*, modulate the action of DCs to produce IL-10 and IL-12 along with the expression of co-stimulatory molecules [106,107]. Several Lactobacillus strains have been shown to inhibit T cell proliferation, induce IL-10 and TGF-β production, and modify Th1 and Th2 cytokine production in various models of autoimmune diseases [108]. In another study, *B. lactis* inhibited TGF-β production [109]. These findings suggest that probiotics should be carefully selected so that the resultant immune response is appropriate for the

desired clinical application.

Duolac ATP is a probiotic preparation containing four probiotics strains (*L. casei, L. plantarum, L. rhamnosus*, and *B. lactis*) and additives (polydextrose, fructooligosaccharide, glucooligosaccharide, and magnesium stearate). Duolac ATP has been previously evaluated for anti-inflammatory activity in a trinitrobenzene sulfonic (TNBS)-induced colitis model [110] and a DNCB-induced AD model [111]. However, these studies focused on the therapeutic effects of Duolac ATP through fragmentary indicators.

The aim of the present study was to evaluate the probiotics efficacy of Duolac ATP without additives. The effect of Duolac ATP on innate and adoptive immune systems was evaluated via BMDCs. Moreover, a transcription factor and cytokine are analysed from atopic mice to explore the mechanism by which Duolac ATP overcomes AD.

2. Materials and Methods

2.1. Animal

Female, 7-10-week old, BALB/c from Orient (Gapyeong, South Korea), or 4-week old NC/Nga mice from the Shizuoka Laboratory Animal Center (Tokyo, Japan) were purchased. The mice were randomized and housed in stainless steel cages in a controlled environment with a 12-h light-dark cycle. All the experimental procedures were carried out in accordance with the Animal Use and Care Protocol approved by the Institutional Animal Care and Use Committee (IACUC) at Seoul National University, Seoul, Korea (Approval No. SNU-170428-1).

2.2. Probiotics

Probiotic strains were obtained from Cell Biotech Co. Ltd (Gimpo, Korea) as a powder form, containing 1×10^{11} CFU/g. In this study, minor modification of the Duolac ATP was used. These Duolac ATP is composed of four different strains of probiotics (*Lactobacillus casei* CBT LC5 (KCTC12398BP), *L. plantarum* CBT LP3 (KCTC10782BP), *L. rhamnosus* CBT LR5 (KCTC12202BP), and *Bifidobacterium lactis* CBT BL3 (KCTC11904BP)) without additives such as prebiotics.

2.3. Generation and culture of BMDCs *in vitro*

Bone marrow (BM) cells were isolated from femurs of mice. Red blood cells were depleted using RBC-lysis buffer (Sigma-Aldrich, MO, USA) and BM cells were cultured in a complete RPMI with 20 ng/ml GM-CSF (Creagene, Korea). The complete RPMI was composed of RPMI-1640 supplemented with 10% fetal bovine serum, 20 mM HEPES, 1 mM sodium pyruvate, 220 nM 2-Mercaptoethanol, 100 μg/ml Gentamicin (all from Sigma-Aldrich). At day 0, BM cells were seeded at 3×10^6 cells/well in 6-well plate in 3 ml media, and 2 ml of fresh media was added at day 3. At day 5, a half of the culture supernatant was discarded, and 3ml of fresh media was added. At days 7, suspended BM cells were harvested and sorted by CD11c MicroBeads UltraPour kit (Miltenyi Biotec Inc., CA, USA). Suspended $CD11c^+$ BM cells that is BM-derived DCs (BMDCs). BMDCs were seeded at 2×10^5 cells/well in 96-well plate and stimulated with Duolac ATP or 100 ng/ml lipopolysaccharide (LPS) in a complete RPMI. After the incubation for 24h, the supernatant was collected for cytokine concentration.

2.4. *In vitro* $CD4^+$ T cell stimulation

$CD4^+$ T cells were isolated from mesenteric lymph node (mLN) from wild type mice using mouse CD4 T lymphocyte enrichment Set (BD Biosciences, CA, USA). $CD4^+$ T cells were labeled with CellTrace™ Violet (CTV) Cell Proliferation Kit (Thermo Scientific, Rockford, USA).

$CD4^+$ T cells (2x105 cells/well) were co-cultured with Duolac-treated BMDCs (2×10^4 cells/well) incubated on anti-CD3/CD28 mAbs (BD Biosciences)-coated 96-well plate. After the incubation for 72h, the cells were examined for proliferation of $Foxp3^+CD4^+$ T cells and the supernatant was collected and examined for IL-10 and IFN-γ concentration.

2.5. Mouse AD model

The back of NC/Nga mice were shaved and dorsal skin and ears were sensitized with house dust mite (HDM) extracts (Biostir, Japan) or DNCB (Sigma-Aldrich). For the HDM-induced AD mouse model, 100 mg of HDM extracts were treated twice a week for 3 weeks. After following the last treatment, mice were administered with PBS (200 μL/day) or Duolac ATP (2×10^9 CFU/200 μL/day) every day for 28 days. In case of DNCB-induced AD mouse model, 1% DNCB that was dissolved in acetone-olive oil (3:1) were treated twice a week for 3 weeks. After the last treatment, mice were administered with PBS (200 μL/day) or Duolac ATP (2×10^9 CFU/200 μL/day) three times a week for 28 days (Figure S2-3). At the end of the treatment of the both types of AD mice models, the mice were anesthetized with CO_2. Blood samples were collected by heart puncture into heparinized tubes. The sera were then collected by centrifugation for 10 min at 3,000 rpm and stored at -80°C,

until further use for ELISA. The mice, at the end of experiment, were sacrificed, and dorsal skin and ear samples were collected for histological analysis and TUNEL assay, respectively. Peripheral blood mononuclear cells (PBMCs), purified from blood of mice treated with/without Duolac ATP, were purified by density gradient centrifugation using Histopaque®-1077 (sigma-Aldrich), and stored at -80°C, until further use for qPCR and western blot. mLN and PP were taken and the distribution of immune cells was measured by flow cytometric analysis.

2.6. Histology

The dorsal skin was removed and fixed in a 4% paraformaldehyde (Sigma-Aldrich, MO, USA). The paraffin-embedded skin sections were heat immobilized, deparaffinized by immersing in xylene (Sigma-Aldrich), rehydrated using a graded series of ethanol, and washed with distilled water. The dorsal skin samples were then cut and subjected to hematoxylin and eosin (H&E) (Sigma-Aldrich) staining. Samples were then examined under the light microscopy (Leica Microsystems, Wetzlar, Germany) for histological evaluation. All clinical and histological evaluations were performed in a blinded manner.

2.7. TUNEL assay

To visualize DNA fragmentation, a marker of apoptosis, TUNEL

staining was performed using an In Situ Cell Death Detection Kit (Roche, Mannheim, Germany) according to the manufacturer's protocol. The sections were post-fixed with ethanol-acetic acid (2:1) and rinsed. The sections were then incubated with proteinase K (100 mg/mL), rinsed, and incubated in 3% H_2O_2, permeabilized with 0.5% Triton X-100, rinsed again, and incubated in the TUNEL reaction mixture. The sections were rinsed and visualized using Converter-POD with 0.03% 3,30-diaminobenzidine (DAB). Then, the sections counterstained with eosin were examined for TUNEL staining by using an optical microscope (Olympus BX53, Japan).

2.8. RNA isolation and qPCR

Total RNA was isolated from PBMCs by TRIzol® reagent (Life Technologies, Carlsbad, CA, USA). One microgram of RNA was reverse-transcribed in a 20μl reaction containing random primers (500 μg/ml), dNTP (10 mM), 5x first strand buffer, DTT (0.1M), Superscript III enzyme (200 U/μl) and RNase inhibitor (10 U/μl) (all from Invitrogen, Carlsbad, CA). RNA was reverse transcribed with HyperScript reverse transcription reagents (GeneAll Biotechnology, Seoul, Korea), and quantitative PCR (qPCR) was performed with the SYBR Green Supermix (iQ SYBR Green Supermix, Bio-Rad Laboratories, Hercules, CA, USA) on the LightCycler 480 Real-Time PCR System (Roche,

Indianapolis, IN, USA). This was then used to calculate the relative amounts of target mRNA in test samples. Quantities of all targets in test samples were normalized to the corresponding GAPDH levels. Primers for IL-2 (forward: 50-CCT GAG CAG GAT GGA GAA TTA CA-30, reverse: 50-TCC AGA ACA TGC CGC AGA G-30), IL-4 (forward: 50-ACA GGA GAA GGG ACG CCA T-30, reverse: 50-GAA GCC CTA CAG ACG TCA-3 0), IL-5 (forward: 50-GGG CTT CCT GCT CCT ATC TA-30, reverse: 50-CAG TCA TGG CAC AGT CTG AT-30), IL-10 (forward: 50-CAA CAT

ACT GCT AAC CGA CTC CT-30, reverse: 50-TGA GGG TCT TCA GCT TCT CAC-30), IL-17 (forward: 50-TCT GAT GCT GTT GCT GCT G-30, reverse: 50-ACG GTT AGA GGT AGT CTG AGG-30), IFN-γ (forward: 50- CAG CAA CAA CAT AAG CGT CA-30, reverse: 50-CCT CAA ACT TGG CAA TAC TCA-30), TGF-β (forward: 50- GTG TGG AGC AAC ATG TGG AAC TCT-30, reverse: 50-TTG GTT CAG CCA CTG CCG TA-30), GAPDH (forward: 50-CAT GGC CTT CCG TGT TCC TA-30, reverse: 50-CCT GCT TCA CCA CCT TCT TGA T-30) were synthesized from Bioneer Inc (Daejeon, Korea).

2.9. Western blot

Total protein was isolated from PBMCs by using RIPA buffer (Abcam, Cambridge, UK). The amount of proteins was quantified by BCA Protein

assay kit (Thermo Scientific, Rockford, US A), with bovine serum albumin (BSA) as a standard. Each protein sample was loaded onto 10% SDS-polyacrylamide gel and transferred to nitrocellulosemembrane (Schleicher & Schuell BioScience, Germany) for 90 min at 4°C, and blocked with 5% skim milk in TBST (1M Tris-HCl, 5M NaCl, 10% Tween-20) for 1 h at room temperature. The blots were incubated with anti-GATA3, -T-bet, -C-maf, -STAT1, -P-STAT1, -STAT4, -P-STAT4 or -beta-actin (all from Abcam) antibodies overnight at 4°C, incubated with anti-GATA3, pics/biochemistry-generated with goat anti-rabbit IgG-HRP antibody (Santa Cruz Biotechnology, USA) for 1 h at room temperature. The target protein was visualized with enhanced chemiluminescence system (GE Healthcare Life Sciences, USA), followed by analysis using ChemiDoc XRS (Bio-rad).

2.10. Enzyme-linked immunosorbent assay (ELISA)

The immunological response of the mice following HDM extract or DNCB-induced AD was monitored by measuring the serum levels of mouse IL-10, IL-12p40, TGF-β, IFN-γ (all from R&D Systems, USA) and IgE (BD Biosciences). Mouse TGF-β, IL-10 and IL-12p40 were measured from the supernatants taken after the BMDC cultured with Duolac ATP by using ELISA DuoSet kits (all from R&D Systems). Mouse IFN-γ was measured from supernatants taken after the $CD4^+$ T

cells treated with BMDCs supernatants by using ELISA DuoSet kit (R&D Systems). Briefly, 96-well microplate (Nunc) was pre-coated with 100 µl/well of capture antibody. After blocking with 1% BSA for 1 h at room temperature, 100 µl/well of supernatant along with the standard solution diluted in diluent buffer was added and incubated for 2 h at room temperature. After the wash PBS for three times, 100 µl/well of detection antibody was added and incubated for 2 h at room temperature, followed by addition of the Streptavidin-HRP in PBS. After the incubation for 20 min at room temperature, tetrame thyl benzidine (TMB, Millipore) was added to develop the color and then the reaction was stopped by adding 50 µl of 2M H_2SO_4. The absorbance at wavelength 450 nm was measured by a microplate reader (Molecular Devices)

2.11. Phenotypic and functional examination of immune cells by using flow cytometry analysis

In order to examine activation status of the cells, BMDCs were treated with Duolac ATP or LPS for 24h at 37°C. The cells were stained with anti-mouse CD86-FITC, PD-L1-PE, MHC II-PE-cy7, CD11c-APC (all from BD Biosciences) for 20 min at 4°C in the dark. To test the increase of Treg, CTV labeled $CD4^+$ T cells cultured with Duolac-treated BMDCs for 3 days were stained with anti-mouse CD4-PE (BD Biosciences). After surface staining, $CD4^+$ T cells were fixed and stained with anti-mouse

Foxp3-APC mAb (Biolegend, Dedham, MA) using FOXP3 Fix/Perm Buffer Set (Biolegend). *In vivo* examination, single cells from mLNs and PP were isolated from AD mice. Population changes of DCs and Tregs were examined as aforementioned. To analyze for subpopulation of Th cells, total mLN and PP cells were stimulated with PMA and ionomycin (Sigma-Aldrich) in the presence of brefeldin A for 4h. After the stimulation, the cells were stained with appropriate combination of anti-mouse CD11c-APC, CD4-bv605, Foxp3-APC, IFN-γ-PE, IL-4-bv605 and IL-17-APC-cy7 mAb (all from Biolegend). The cells were washed and the expression was examined using a FACSCanto II (BD Biosciences). All flow cytometric data acquired were analyzed with FlowJo software (Tree Star, Ashland, OR).

2.12. Statistical analysis

The levels of significance for comparison between samples were determined by Tukey's multiple comparison test by using GradPad InStat software (Ver 5.01, GraphPad). The data in the graphs were expressed as the mean ±SEM.

3. Results

3.1. Duolac ATP effectively induces regulatory immune responses by BMDCs

First, the harmful effects, if any, of Duolac ATP on BMDCs were examined. BMDCs were treated with various concentrations of Duolac ATP and their components. Regardless of the probiotic type, BMDC survival was significantly reduced when treated with over 2×10^6 CFU (Figure S2-1), suggesting that the optimal concentration is less than 2×10^6 CFU. Based on these results, Duolac ATP concentration was determined to be used at 2×10^5 CFU. BMDCs treated with Duolac ATP showed similar surface expressions of the co-stimulatory molecules CD86 and MHC II compared with untreated cells within the same intensity (Figure 2-1A) and percentage of cells (Figure 2-1B and Figure S2-2). However, Duolac ATP-treated BMDCs demonstrated a significantly higher expression of PD-L1 compared with untreated cells within the same intensity (Figure 2-1A) and percentage of cells (Figure 2-1B and Figure S2-2). Next, immunomodulatory cytokines were examined in the culture supernatant from BMDC treated with Duolac ATP. These results showed that Duolac ATP induced a significant amount of IL-10, an anti-inflammatory cytokine, at a rate 1.5-fold greater than cells treated with LPS (Figure 2-1C). TGF-β production was also increased when BMDCs were treated with Duolac ATP, whereas IL-

12p40 production was slightly lower than that of those treated with LPS (Figure 2-1C). Taken together, these results indicate that Duolac ATP effectively induced a regulatory immune response in BMDCs.

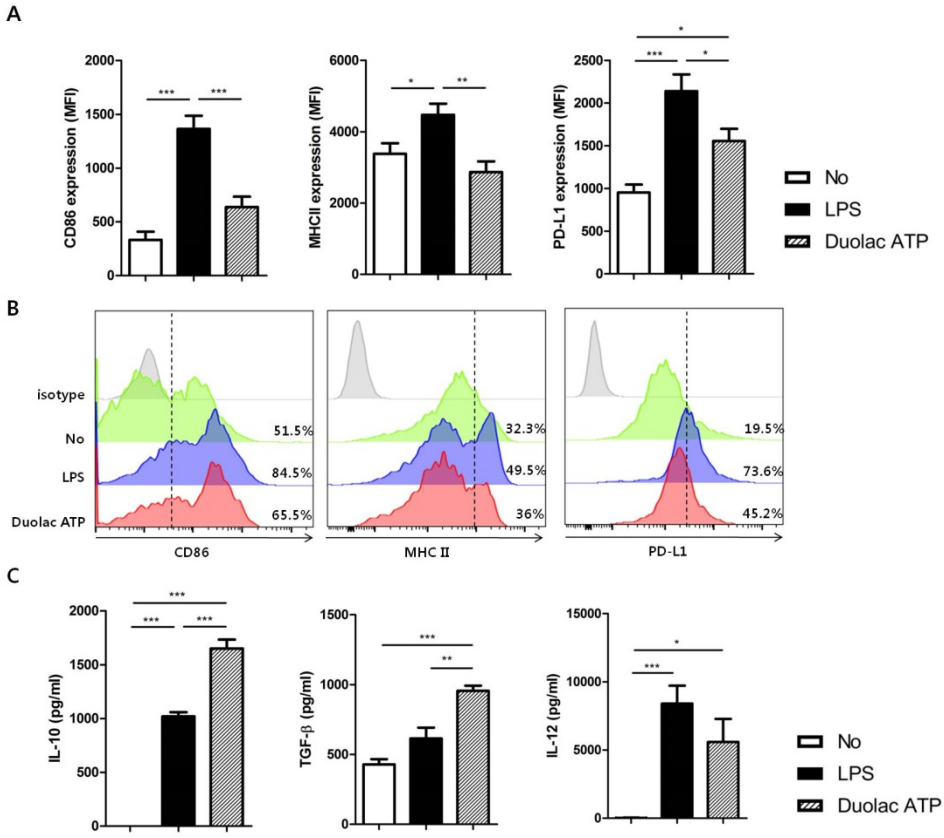

Figure 2-1. Duolac ATP induced regulatory molecules in BMDCs.

BMDCs were treated with LPS and/or 2×10^6 CFU of Duolac ATP for 24 hours. (A) The expression of surface markers and (B) a representative histogram of CD86, MHC II, and PD-L1 on BMDCs were measured by flow cytometry. (C) The expression of cytokines in the supernatants was measured by ELISA. Data are representative of at least three experiments. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ using one-way ANOVA with Tukey's multiple comparison test. Bars indicate mean ± SEM.

3.2. BMDCs treated with Duolac ATP promote proliferation of Tregs *in vitro*

DC is known to induce the differentiation of Tregs and to suppress excessive immune responses. Thus, whether Duolac ATP-treated DCs could induce Treg differentiation was investigated. $CD4^+$ T cells were co-cultured with Duolac ATP-treated BMDCs on an anti-CD3/CD28 mAbs-coated plate for 3 days. The results showed that BMDC-induced $CD4^+$ T cell proliferation was higher than that in the control group, but it was slightly reduced when co-cultured with Duolac ATP-treated BMDCs (Figure 2-2A). The ratio of $Foxp3^+$ Tregs in the proliferated $CD4^+$T cells was also significantly increased in the Duolac ATP-treated BMDC group compared to the group treated with BMDC alone or controls (Figure 2-2B). Immunomodulatory cytokines were then examined in the culture supernatant from $CD4^+$ T cells co-cultured with Duolac ATP-treated BMDCs. IL-10 was increased in a similar fashion to $Foxp3^+$ Treg proliferation. However, the expression of IFN-γ was not affected by Duolac ATP treatment (Figure 2-2C). Taken together, these results indicate that Duolac ATP-treated BMDCs were able to induce Treg proliferation with a unique profile of cytokine induction.

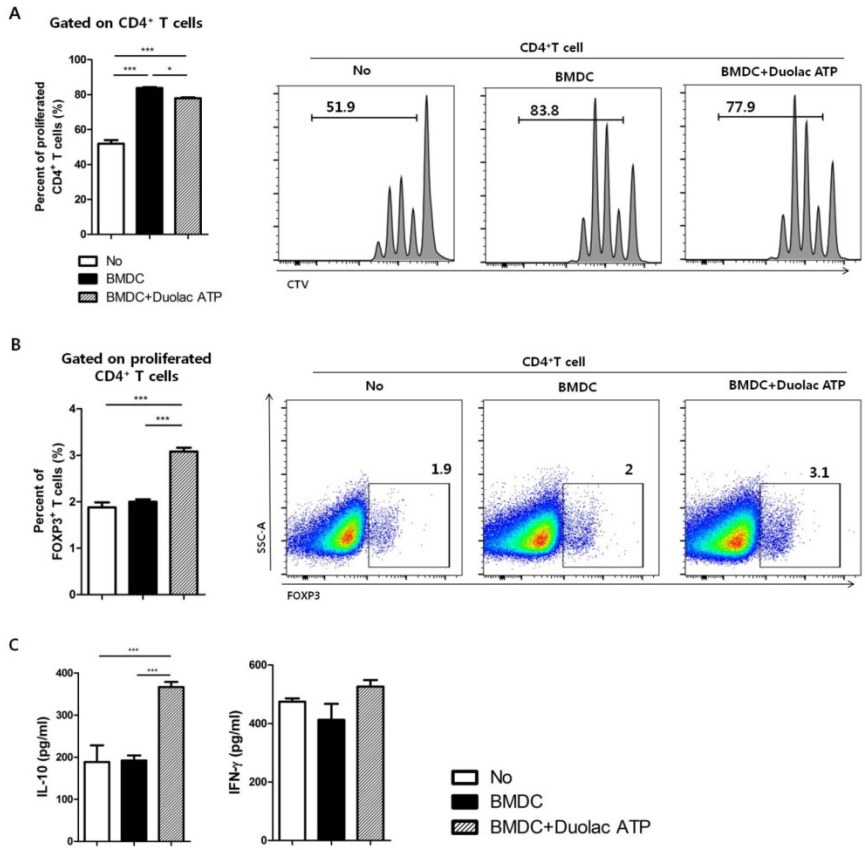

Figure 2-2. BMDC treated with Duolac ATP promotes proliferation of Tregs *in vitro*.

BMDCs treated with Duolac ATP were co-cultured with $CD4^+$T cells for 3 days on an anti-CD3/CD28 mAbs-coated plate. The percentage of the proliferated (A) total $CD4^+$T cells and (B) proportion of $CD4^+Foxp3^+$ T cells among $CD4^+$T cells were analyzed by flow cytometry. At the same time, supernatants were harvested and examined for (C) the production of IL-10 and IFN-γ in the $CD4^+$ T cells using ELISA. Data are representative of at least three experiments. $*P< 0.05$, $***P < 0.001$ using one-way ANOVA with Tukey's multiple comparison test. Bars indicate mean ± SEM.

3.3. Amelioration of AD in mice treated with Duolac ATP

To determine the therapeutic properties of Duolac ATP *in vivo*, a mouse model for AD-like skin lesions was established. The NC/Nga mice were sensitized with HDM extract twice a week for 3 weeks. The mice were then administered phosphate-buffered saline (PBS) (200 μL/day) or Duolac ATP (2×10^9 CFU/day) for 4 weeks, as shown in Figure S2-3. No significant changes were found in the mice's physical habitus or spleen weight during the feeding period (Figure S2-4). Compared to the control group, the AD group showed severe atopic symptoms, such as itching, erythema/hemorrhage, edema, excoriation/erosion, and scaling/dryness, at week 4 (Figure 2-3A). Interestingly, however, the atopic symptoms of AD mice treated with Duolac ATP were less severe. In addition, weekly examination of ear thickness was performed after the induction of AD. Compared to the control group, the ear thickness of the AD group increased in a time-dependent manner. However, the AD group treated with Duolac ATP showed an attenuated increase in ear thickness (Figure 2-3B). AD is a chronic inflammatory skin disease that not only causes the aforementioned clinical symptoms but also increases epidermal and dermal thickness via activation and infiltration of immune cells [112]. To investigate the increase in dermal thickness, the skin was stained and measured the epidermis and dermis based on known disease indices. Epithelial hypertrophy and hyperkeratosis coincident with immune cell

infiltration were observed in the AD group (Figure 2-3C, left panel). Furthermore, some mice showed epidermal collapse with bleeding and extensive cartilaginous ulceration. The AD group treated with Duolac ATP had fewer histopathologic anomalies (Figure 2-3C right panel). Furthermore, by conducting a TUNEL assay, the degree of apoptosis could be examined. In the AD group, several apoptotic cells were observed, whereas this was less obvious in the AD group treated with Duolac ATP (Figure 2-3D). Serum IgE levels were significantly increased in the AD group compared to the control group, whereas the AD group treated with Duolac ATP showed a significant decrease (Figure 2-3E). These results suggest that Duolac ATP efficiently ameliorates the symptoms of AD and decreases overall serum IgE levels.

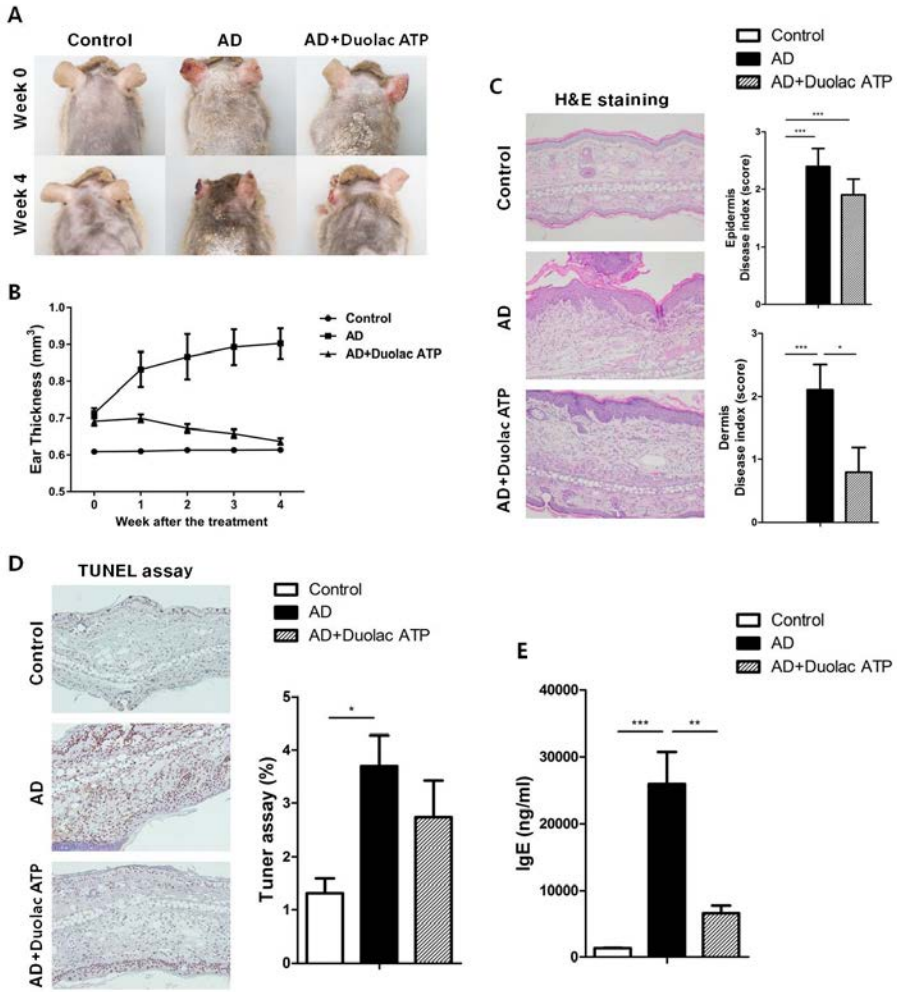

Figure 2-3. Amelioration of AD symptoms in mice treated with Duolac ATP.

NC/Nga mice were sensitized by exposing them to HDM extract twice a week for 3 weeks. PBS or Duolac ATP was then orally administered for 4 weeks. (A) Atopic symptoms and (B) ear thickness were scored every week for the last 4 weeks. At the end of the experiment, (C) the results of the histological analysis of cell infiltration by H&E staining (left panel, one representative from 10 samples) and the epidermis and dermis index

score (right panel) were examined. (D) TUNEL assay on dermis samples (left panel, one representative from 10 samples) and the percentage of the plot (right panel) are shown. (E) Blood samples were acquired, and serum IgE levels were measured by ELISA. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ using one-way ANOVA with Tukey's multiple comparison test. Bars indicate mean ± SEM.

3.4. Maintenance of systemic T cell balance in AD mice treated with Duolac ATP

To investigate the transcription factors associated with Th1 and Th2 cell differentiation in AD mice treated with Duolac ATP. T-bet (Figure 2-4A and B), STAT1 (Figure 2-4A and C), and STAT4 (Figure 2-4A and D) are factors that induce Th1 cell differentiation; these were all expressed at a significantly higher rate in PBMCs from AD mice treated with Duolac ATP compared to untreated AD mice. It was further noted that Th2 differentiation factors GATA3 (Figure 2-4A and E) and C-maf (Figure 2-4A and F) were increased in PBMCs from AD mice. These results suggested that Duolac ATP led to the balance between Th1 and Th2 cells in AD mice by preferentially increasing the release of Th1 differentiation factors.

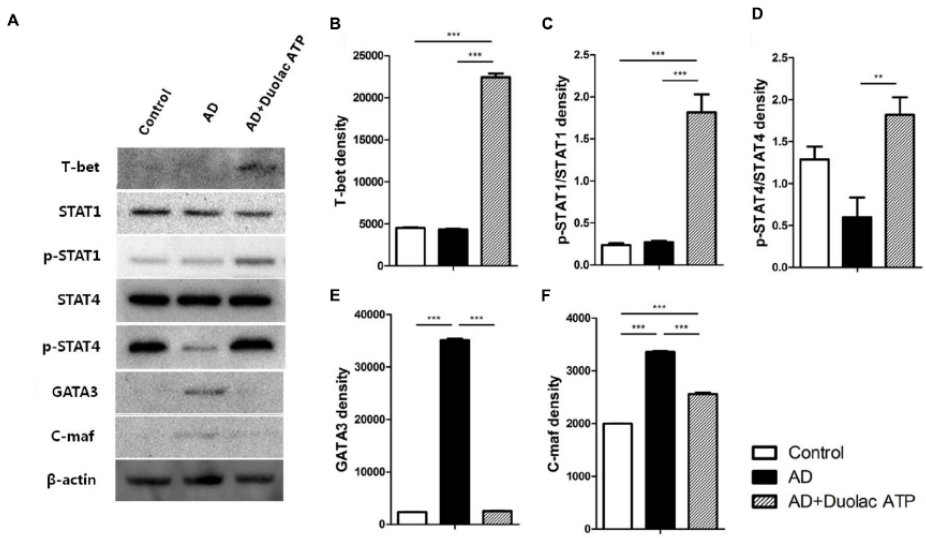

Figure 2-4. Expression changes on transcriptional factors involved in the maintenance of T cell balance in AD mice administered Duolac ATP.

Nc/Nga mice (n=6 per group) were sensitized by exposing them to HDM extracts twice a week for 3 weeks. They were then subjected to oral administration of Duolac ATP. PBMCs collected at week 4 were used to make lysates, which were used to examine the expression of transcriptional factors using Western blotting. (A) The expression of T-bet, STAT1, p-STAT1, STAT4, p-STAT4. GATA3, C-maf, and beta-actin was used as an internal control. Density was measured using a densitometer for semi-quantitation for (B) T-bet, (C) STAT1, (D) STAT4, (E) GATA3, and (F) C-maf. $^{**}P < 0.01$, $^{***}P < 0.001$ using one-way ANOVA with Tukey's multiple comparison test. Bars indicate mean ± SEM.

In addition, the mRNA expression of Th1 and Th2 cytokines was examined in PBMCs from AD mice given Duolac ATP. Higher rates of IL-4 (Figure 2-5A) and IL-5 (Figure 2-5B) expression was found in AD mice, as was expected from a typical Th2 response. Furthermore, IL-17 appeared to be increased in AD mice (Figure 2-5C). However, AD mice treated with Duolac ATP showed a decrease in Th2 and Th17, with a concurrent increase in IL-2 (Figure 2-5D) and IFN-γ (Figure 2-5E and Figure S2-6A). mRNA expression of IL-10 was also increased in AD mice treated with Duolac ATP, whereas there was no change in TGF-β (Figure 2-5F and G). These results indicate that Duolac ATP can induce Th1- but not Th2- or Th17-driven responses.

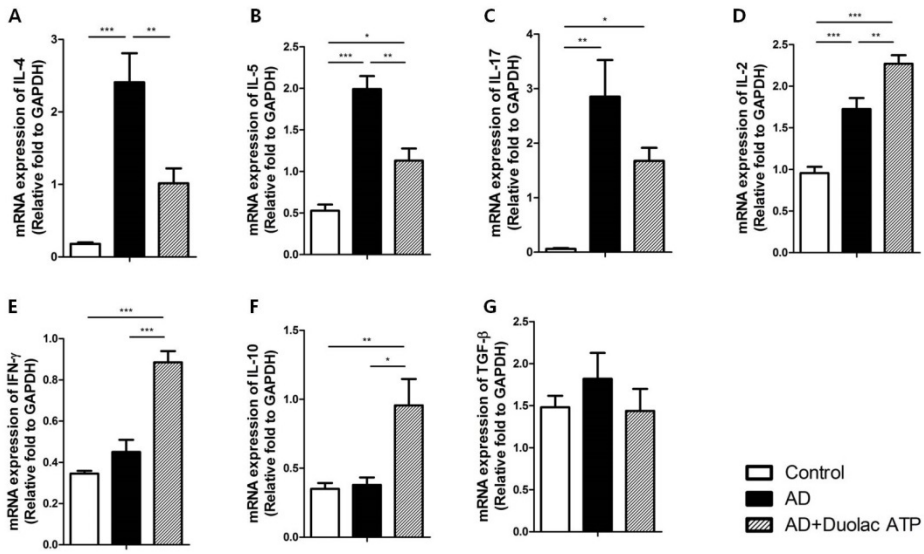

Figure 2-5. mRNA expression of cytokine levels from PBMCs in HDM-sensitized Nc/Nga mice treated with Duolac ATP.

Nc/Nga mice (n=6 per group) were sensitized by exposing them to HDM extracts six times over 3 weeks. The mice were then subjected to oral administration with Duolac ATP. PBMCs were collected at week 4, and RNA was extracted for cDNA synthesis. qPCR was performed to examine the mRNA expression of (A) IL-4, (B) IL-5, (C) IL-17, (D) IL-2, (E) IFN-γ, (F) IL-10, and (G) TGF-β. Relative fold changes of target genes were compared with that of the housekeeping gene, GAPDH. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ using one-way ANOVA with Tukey's multiple comparison test. Bars indicate mean ± SEM.

3.5. Maintenance of intestinal T cell balance in AD mice treated with Duolac ATP

To determine whether Duolac ATP has an effect on the balance of intestinal immune cells, mLN and PP were examined from AD mice treated with Duolac ATP. The proportion of DCs in the mLN and PP was slightly increased in AD mice given Duolac ATP (Figure 2-6A). Although the subpopulation of DCs did not change significantly, the number of $CD11b^{+}CD103^{-}$ DCs that induce atopic inflammation decreased slightly after treatment with Duolac ATP in PP (Figure S2-6B). Moreover, the number of $CD4^{+}$ T cells in mLN and PP slightly decreased when Duolac ATP was administered (Figure 2-6B). It is well known that Tregs increase in response to a Th2-driven allergic reaction and that they are responsible for maintaining functional tolerance in an AD mouse model [113]. Thus, the ratio of Tregs in mLN and PP was measured from AD mice with and without Duolac ATP treatment. Compared with PP, mLN had a relatively high proportion of Tregs but no change was found in any treatment group (Figure 2-6C). In, PP however, Treg differentiation was increased in AD mice treated with Duolac ATP compared to control and untreated AD groups. Then, the other subtypes of helper T cells were examined by testing the expression IFN-γ (Th1), IL-4 (Th2), and IL-17 (Th17) in $CD4^{+}$ T cells after PMA/ionomycin stimulation. In mLN and PP, Th1 cells reduced by AD induction were not affected by Duolac ATP

treatment. In addition, Th2 cells increased more in the Duolac ATP treated group than in the control and untreated AD groups. (Figure 2-6E, F). These results suggest that Duolac ATP induced both Th2 and Treg responses in intestinal immunity, but not Th1 responses as seen in the PBMCs.

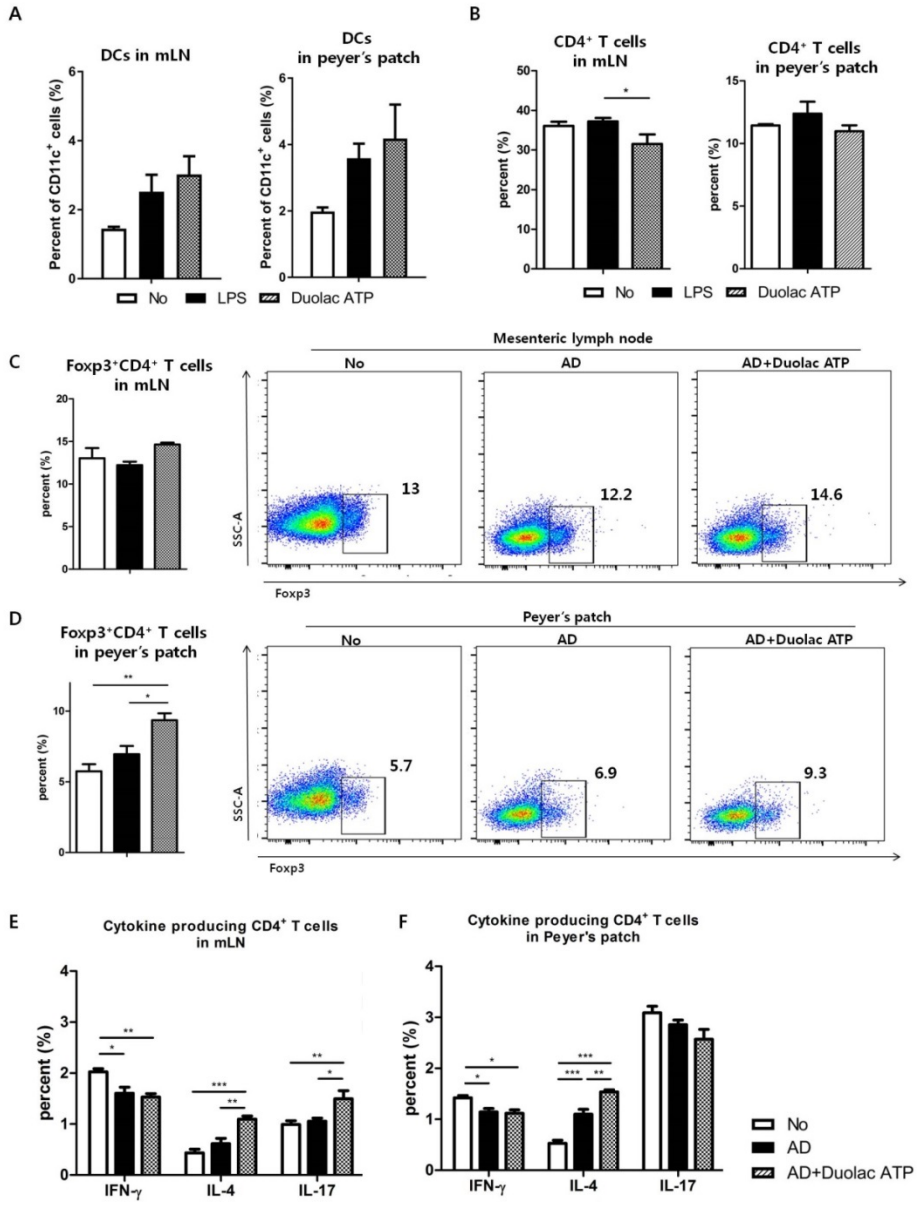

Figure 2-6. Composition of immune cells from mLN and PP in DNCB-sensitized Nc/Nga mice treated with Duolac ATP.

Nc/Nga mice were sensitized by exposing them to DNCB twice a week for 3 weeks. They were then subjected to oral administration of Duolac ATP. mLN and PP collected at week 4 were used to make single cells, which were used to examine the composition of immune cells. The

percentages of (A) DCs, (B) $CD4^+$ T cells, and $Foxp3^+CD4^+$ T cells from (C) mLN and (D) PP were examined using flow cytometry. mLN and PP cells were stimulated with PMA/ionomycin in the presence of brefeldin A for 4 hours. IFN-γ, IL-4, and IL-17 producing $CD4^+$ T cells from (E) mLN and (F) PP were examined after intracellular staining using flow cytometry. Data are representative of at least three experiments. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ using one-way ANOVA with Tukey's multiple comparison test. Bars indicate mean ± SEM.

4. Supplementary Figures

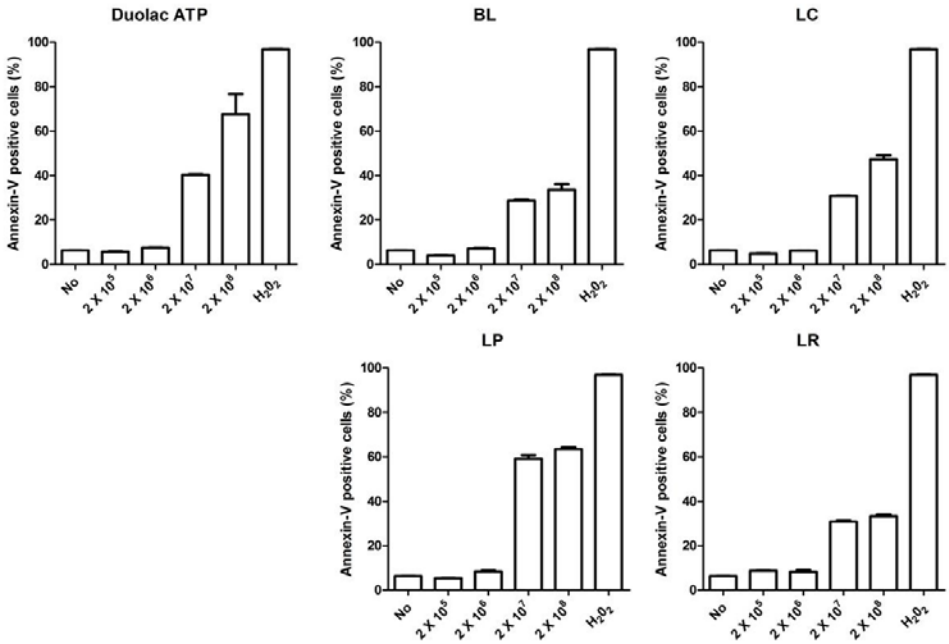

Figure S2-1. Apoptosis of BMDCs treated with various probiotics that comprise Duolac ATP.

BMDCs were treated with various concentrations of probiotics ((Duolac ATP), *Bifidobacterium lactis* (BL), *Lactobacillus casei* (LC), *L. plantarum* (LP), or *L. rhamnosus* (LR)) for 24 hours. The percentage of apoptotic cells, the Annexin V-positive fraction, was measured in BMDC in for 24 hours. The concentration of BMDCs was 2×10^5 cells in all groups. Data are representative of at least three experiments.

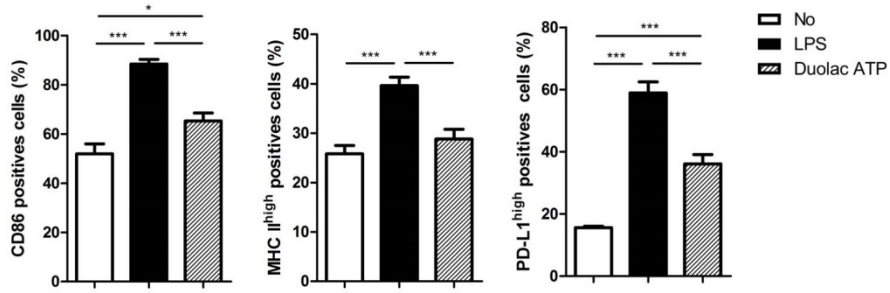

Figure S2-2. Percentage of surface molecules on BMDCs treated Duolac ATP.

BMDCs were treated with LPS or 2×10^6 CFU of Duolac ATP for 24 hours. The ratios of CD86-, MHC II-, and PD-L1-expressing cells in BMDCs were measured by flow cytometry. Data are representative of at least three experiments. $*P < 0.05$, $***P < 0.001$ using one-way ANOVA with Tukey's multiple comparison test. Bars indicate mean ± SEM.

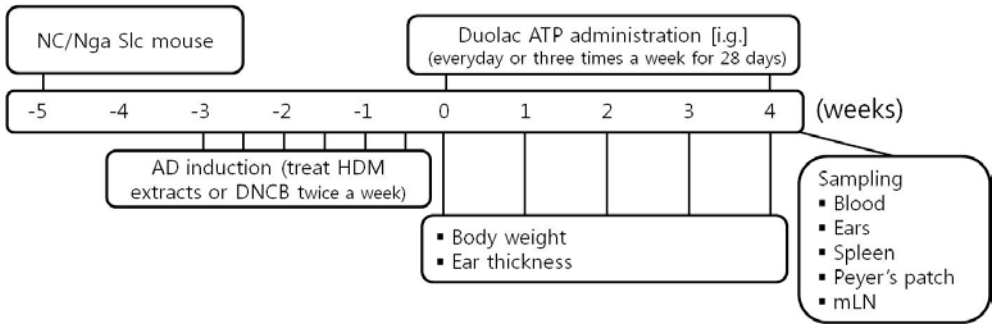

Figure S2-3. HDM extract and DNCB-induced mouse model of atopic dermatitis (AD)-like skin lesions and oral administration of Duolac ATP in NC/Nga mice.

NC/Nga mice were divided into three groups: (1) normal control (Control), (2) atopic dermatitis (AD), and (3) AD + Duolac ATP (2×10^9 CFU/day) (AD + Duolac ATP). To study the effect of Duolac ATP on AD, the dorsal skin and ears of the mice were treated with 100 mg HDM extracts or 1% DNCB six times over 3 weeks to induce AD-like skin lesions. Duolac ATP was administered following the last treatment with HDM extract at week 0. At week 4, the mice were sacrificed, and the blood, spleen, PP, and mLN were collected for immunological analysis and dorsal skin histological analysis.

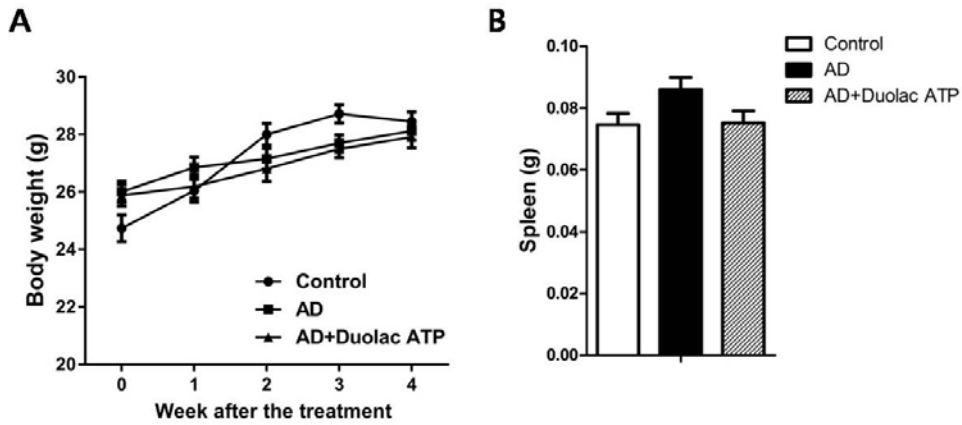

Figure S2-4. Body and spleen weight changes in the AD mouse model treated with Duolac ATP.

Atopic dermatitis was induced by exposing mice to HDM extracts for 3 weeks; mice were then administered Duolac ATP for 4 weeks. (A) Body weight was monitored weekly for 4 weeks. (B) The spleen was isolated and weighted at week 4.

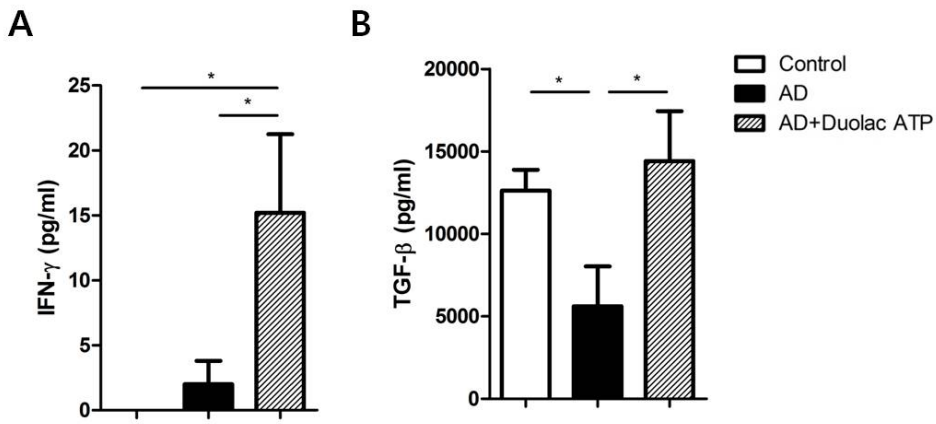

Figure S2-5. Cytokine expression changes in the AD mouse treated with Duolac ATP.

NC/Nga mice were sensitized by exposure to DNCB twice a week for 3 weeks. They were then orally administered PBS or Duolac ATP for 4 weeks. Blood samples were taken and serum (A) IFN-γ and (B) TGF-β levels were measured by ELISA. Data are representative of at least three experiments. *$P < 0.05$ using one-way ANOVA with Tukey's multiple comparison test. Bars indicate mean ± SEM

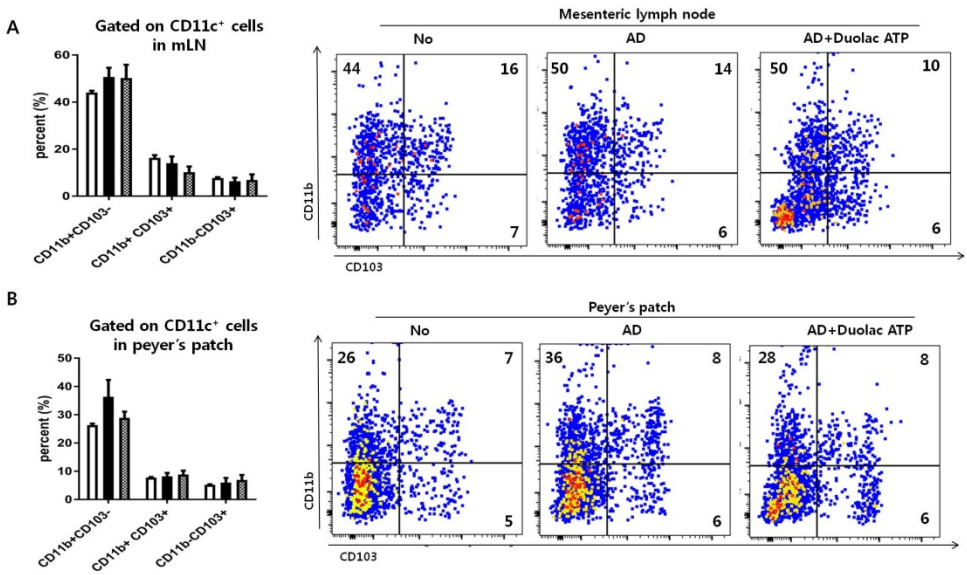

Figure S2-6. Subpopulation of DC from mLN and PP in the AD mouse treated with Duolac ATP.

NC/Nga mice were sensitized by exposing DNCB twice a week for 3 weeks. The mice were then orally administered PBS or Duolac ATP for 4 weeks. (A) mLN and (B) PP collected at week 4 were used to make single cells. The cells were gated on CD11c and subdivided based on CD11b and CD103 expression using flow cytometry. Data are representative of at least three experiments.

5. Discussion

AD is one of the allergic diseases that results in increased IgE levels and clinically manifests itself as pruritic skin lesions, dermal hyperkeratosis, and hyperplasia [91,114]. Recent studies have shown that certain probiotics can play a role in the treatment and prevention of AD [115,116]. Duolac ATP possesses anti-inflammatory properties in a DNCB-induced AD mouse model by modulating Th1 and Th2 cytokine expression in the blood. [111]. However, the exact mechanism by which probiotics contained in Duolac ATP regulates the immune system has not been well defined. In the present study, the regulatory role of Duolac ATP, consisting pure probiotic mixture without additive, for the mechanisms by which immune cells, transcription factors and cytokines have a protective effect against AD in mice are examined.

As expected, mice treated with HDM- or DNCB-induced AD showed an excessive Th2 response in the present study, a number of different strategies have been suggested in the management of AD, including the preferential differentiation of T cells toward a Th1 lineage/blocking Th2 action. The T cell response is critically regulated by major transcription factors for both Th1 (T-bet, STAT1, STAT4) and Th2 (GATA3, C-maf) responses [117,118]. IL-12 produced by antigen-presenting cells induces phosphorylation of STAT4, resulting in the subsequent production of IL-2 and IFN-γ by Th1 cells [119,120]. IFN-γ then induces the activation of T-bet

and STAT1, which, in turn, generate a positive feedback loop of the Th1 response [121]. Increased circulating T-bet concurrently inhibits the binding and activation of GATA3 to the IL-4 gene promoter along with other Th2-related gene promoter [122].

In one study, PBMCs were taken from allergic patients and treated with various probiotics, including *Lactobacillus plantarum, L. lactis*, and *L. casei*, resulting in reduced IL-4 secretion [123]. Treatment with *L. plantarum*, one of the components of Duolac ATP, decreased T-bet and GATA-3 expression in the mouse small intestinal lamina propria [124]. T-bet expression also decreased in human PBMCs when stimulated with a single-strain probiotic *B. lactis* [125]. A probiotic mixture called WIKIM28 decreased IL-4 and IL-5 levels, but not IFN-γ, in peripheral lymph node cells [64]. Administration of IRT5, another probiotic mixture, also reduced the levels of IL-4, IFN-γ and the TNF-alpha in mesenteric lymph nodes [126]. Our results demonstrated that Duolac ATP induced upregulation of phospho-STAT1 and T-bet while downregulating GATA3 in an AD mouse model. Furthermore, Duolac ATP increased IFN-γ and decreased IL-4 *in vivo*. These results suggest that the use of multiple probiotic strains can lead to different Th1 responses.

Another strategy for overcoming AD is to suppress the overall immune response through regulatory cytokines, such as IL-10 and TGF-β. These cytokines downregulate the activation of immune responses and therefore minimize the amount of tissue damage caused by inflammation in AD [127].

IL-10 is produced by a number of immune cells, including DCs, macrophages, monocytes, T cells, and B cells [128]. Microbial products stimulate pattern recognition receptors to increase the expression of mitogen-activated protein kinase kinase in DCs or macrophages, thereby increasing IL-10 production [129].

Lactobacillus casei and *L. rhamnosus* induce IL-10 production in allergic patients by regulating the composition of intestinal microbial flora [130]. IL-10 production in Th2 cells was suppressed by IL-4 and GATA3 [131,132]. Given that Duolac ATP decreased IL-4 and GATA3 expression while increasing STAT4 levels, it is unlikely that IL-10 was secreted in Th2 cells. On the other hand, Duolac ATP induced upregulation of IL-10 in BMDCs and AD mice, suggesting that Duolac ATP may drive IL-10 production from cells other than Th2 cells, such as DCs.

TGF-β is produced by many immune cells, and the TGF-β signal is an essential component in maintaining healthy immune tolerance [133]. It has been suggested that TGF-β can reduce allergic inflammation by inhibiting IgE synthesis in B cells and mast cell proliferation [134]. TGF-β is involved in the inhibitory action of *Lactobacillus acidophilus* on Salmonella typhimurium-induced inflammation [135]. A mixture of probiotics, known as VSL#3, induced TGF-β and alleviated allergic inflammation by reducing the Th2 response in a mouse model [136]. Similarly, in this study,TGF-β was increased when BMDCs were treated

with Duolac ATP. TGF-β secreted from DCs may regulate the differentiation of Tregs [137]. TGF-β expression, which is slightly decreased in atopy, recovered to a normal level in mice after Duolac ATP treatment (Figure S2-5B). Moreover, Duolac ATP promoted Treg differentiation in PPs (Figure 2-5F). It is likely that Duolac ATP modulates immune cells in a manner similar to how tolerogenic DCs maintain immune tolerance.

Immune imbalances caused by atopic reactions induce epidermal inflammation and hyperplasia [19,91]. It has been suggested that oral administration of *Enterococcus faecalis* downregulates the production of Th1 and Th2 cytokines to attenuate skin hyperplasia [138]. In the present study, Duolac ATP reduced IgE production by decreasing Th2 and increasing the Th1 response, thereby preventing skin inflammation and hyperplasia.

The present study showed that Duolac ATP induced Th1 responses and suppressed Th2 responses in PBMCs, while it induced Th2 response in the intestine. The intestinal Th2 response could not only restore mucus secretion and intestinal tissue formation but also prevent parasite infections such as helminthes [139]. Th2 response also helped cure Crohn's disease, an acute intestinal disorder caused by excessive Th1 and 17 responses [140]. In the previous studies, Duolac ATP alleviate symptoms in acute inflammatory bowel disease model [110]. However, additional studies are needed to determine whether Duolac ATP affects ulcerative colitis and colorectal cancer due to intestinal Th2 response.

In summary, the present study shows that Duolac ATP regulated IL-10 and TGF-β expression and allowed DCs to become functionally tolerant and potentially induce Treg differentiation. Furthermore, Duolac ATP regulated transcription factors and cytokines to drive naïve T cell differentiation toward Th1 lineages. Taken together, these results indicate that Duolac ATP shows great preventive potential in the management of AD symptoms and could serve as a future immunomodulatory agent for AD patients.

This chapter will be published elsewhere

as a partial fulfillment of Han wool Kim's Ph.D. program

Chapter 3

Dietary probiotic mixture, YK4 regulates immune balance in mice with atopic dermatitis

1. Introduction

AD is one of the most common chronic inflammatory skin diseases seen mostly in children, although it can occur at all ages. The main symptom of AD is the destruction of the stratum corneum and the increase of eczema and itching. AD is known to be caused by complex interactions between genetic factors and extrinsic allergens [1,141]. When the atopic dermatitis occurs, scratching the skin owing to severe itching causes a damage to the skin epidermal barrier followed by a dysregulation of immunoregulatory proteins such as IL-1, IL-25, IL-33 and TSLP in skin epithelial cells [142,143]. These immunomodulatory proteins are known to initiate and promote Th2 cell-mediated immune responses [144]. Activated Th2 cells release type 2 cytokines, such as IL-4, IL-13 and IL-31 and stimulate B cells to isotype switch of IgM to IgE [145,146]. Then, increased IgE production causes eosinophil accumulation in the dermis [147].

The immune imbalance induces the increase of inflammation in the skin and exacerbate the symptoms of AD. Moreover, AD patients have an increased risk of other atopic diseases including asthma, allergic rhinitis and food allergies [148]. Several drugs under development are aimed at improving itch-associated symptoms with AD, and an anti-inflammatory or epithelial barrier repair action. Monoclonal antibodies and protein inhibitors are under investigation to block the cytokine and its receptor signaling pathway and thereby to alleviate AD [149]. Although steroids are

widely accepted as the first choice for anti-inflammatory drug and an intermittent use can reduce the disease recurrence, a long-term drug therapy should be avoided due to side effects such as nausea, vomiting, diarrhea, skin thinning, and purpura [150]. Therefore, alternative therapies including herbs, plants, vitamins and probiotics have been recently studied [151,152].

Probiotics, beneficial Gram-positive bacteria, mainly helps to maintain a healthy digestive function including fermentation of dairy products and produces lactic acids as a by-product. Probiotics control intestinal homeostasis and immune responses through intestinal pH regulation, direct antigen suppression, and/or secretion of short-chain fatty acids [153,154]. Recently, the effect of DCs primed by probiotics to control T cell response has been reported [155,156]. DCs are antigen-presenting cells that play an important role in linking innate and adaptive immune responses [51,52]. Several probiotic strains are known to regulate the function of DCs, leading to the production of IL-10 and induction of the differentiation of Tregs [126,157]. Duolac ATP, a mixture of probiotic strains, modulates the expression of co-stimulatory molecules of DCs and downregulates the Th2 responses in the AD mouse model [158]. Mixed probiotic strains of *Lactobacillus* and *Bifidobacterium* reduced the atopic dermatitis index in young AD patients [101] and AD mouse model [159,160]. However, the action mechanism of probiotics is by far only partially understood.

Galectins are a soluble form of lectin that exhibit specific binding ability for beta-galactoside sugars [69]. Galectin-1, -2, -3, -4 and -9 are generally expressed in the intestinal areas [161] and seemingly involved in the regulation of intestinal homeostasis and immunity [162]. Especially, galectin-9 directly binds to CD44 and promotes Foxp3 expression of Tregs. Tregs have been implicated to suppress the excessive Th2 responses [163]. Indeed, recombinant galectin-9 treatment resulted in improved clinical and immunological symptoms of allergic diseases in mouse models [164]. Recently, *B. breve*, used as a dietary supplement, induced an elevation of serum galectin-9 in mouse and human [90]. However, the precise mechanism by which galectin-9 modulates immune cells in AD therapy is yet to be known.

Prior to this study, candidate probiotics were selected based on the production of IL-10 and IL-12 in bone marrow-derived DCs (BMDCs) among eight probiotic strains: *B. breve* (BR), *B. lactis* (BL), *L. acidophius* (LA), *L. casei* (LC), *L. plantarum* (LP), *L. paracasei* (LPC), *L. rhamnosus* (LR), *Pediococcus pentosaceus* (PP) (Figure S3-1). LA, LP, BL and BR were selected and combination of these candidate probiotic strains were fed to BALB/c mice for 21 days (Figure S3-2A). The generation of immunomodulating cytokines such as IL-10, IL-12, TNF-α and TGF-β were confirmed in serum. The amounts of serum IL-10, IL-12 and TNF-α were below the detection limits in all groups (data not shown). The mixture of four strains (BL/BR/LA/LP) showed higher expression of

serum TGF-β than the control or other mixture group (Figure S3-2B). Based on these preliminary results, I designed YK4, a mixture of four probiotic strains: *L.actobacillus acidophius, L. plantarum, B. breve*, and *B. lactis*. The aim of this study was to evaluate the effect of YK4 on the regulation of intestinal and systemic immune system by using AD mouse model in relation to galectin-9 expression and $CD4^+$ T cells.

2. Materials and Methods

2.1. Animal

Female, 6-9-week old, BALB/c mice were purchased from Orient (Gapyeong, South Korea). The mice were randomized and housed in stainless steel cages under the controlled environment with a 12h light-dark cycle. All the experimental procedures were carried out in accordance with the Animal Use and Care Protocol approved by the Institutional Animal Care and Use Committee at Seoul National University, Seoul, Korea (Approval No. SNU-170428-1-1).

2.2. Probiotics

Probiotic strains were obtained from Cell Biotech Co. Ltd (Gimpo, Korea) as a powder form, containing 1×10^{11} CFU/g. YK4, used in the present study, is composed of four different strains of probiotics: *L. acidophius* LA1 (KCTC11906BP), *L. plantarum* CBT LP3 (KCTC10782BP), *B. breve* CBT BR3 (KCTC12201BP), and *B. lactis* CBT BL3 (KCTC11904BP).

2.3. Mouse with atopic dermatitis model

The protocol to induce atopic dermatitis (AD)-like skin lesion in

mouse model was modified from Lim et al [64]. The dorsal hair of the mouse was shaved and, the skin was sensitized with 1% 2,4-dinitrochlorobenzene (DNCB; Sigma-Aldrich, St. Louis, MO, USA) twice for the first week, and 0.2% DNCB for three times in the second week. In order to maintain the atopy, 0.2% DNCB was applied once a week for 3-6 weeks. After the last treatment, mice were administered with phosphate buffered saline (PBS) (200 μl/day) or YK4 (1×10^9 CFU/200 μl/day) for three times a week. At the end of the treatment, the mice were anesthetized by using CO_2. Then, blood samples were taken from the mice and dorsal skin, intestines, spleen, mLN and Peyer's patches (PP) were extracted.

2.4. Dermatitis index

The severity of dermatitis score was evaluated once a week after DNCB treatment. Scores of 0 (none), 1 (mild), 2 (moderate), and 3 (severe) were examined for each of the four symptoms: erythema/hemorrhage, scarring/dryness, edema and excoriation/erosion. The sum of the individual scores indicating clinical severity was taken as the dermatitis score [112].

2.5. Sample preparation

Blood samples were collected by eye bleeding into heparinized tubes.

The sera were then collected by centrifugation for 10 min at 1,500 g and stored at -80° C, until further use. The mice were sacrificed at the end of the experiment. Spleen and large intestine were collected and the length was measured to determine the visual examination and inflammatory responses. Dorsal skin, and small and large intestines were collected for the mRNA expression of galectin-9. Spleen, mLN and PP were taken and the composition of immune cells was measured by flow cytometric analysis.

2.6. Generation and culture of BMDCs

Bone marrow (BM) cells were isolated from femurs of mice. Red blood cells were depleted using RBC-lysis buffer (Sigma-Aldrich) and BM cells were cultured in a complete RPMI with 20 ng/ml GM-CSF (Creagene, Seongnam, Korea). The complete RPMI was composed of RPMI-1640 supplemented with 10% fatal bovine serum, 20 mM HEPES, 1 mM sodium pyruvate, 220 nM 2-mercaptoethanol, 100 μg/ml gentamicin (all from Sigma-Aldrich). BM cells were seeded at 3×10^6 cells/well in a 6-well plate in 3 ml media, and then 2 ml of fresh media was added at day 3 and 5. At day 6, a half of the culture supernatant was carefully discarded, and 3 ml of fresh media was added. At day 7, suspended BM cells were harvested and sorted by CD11c MicroBeads UltraPure kit (Miltenyi Biotec, Bergisch Gladbach, Germany).

Suspended $CD11c^+$ BM cells (i.e., BM-derived DCs, BMDCs) were seeded at 2×10^5 cells/well in 96-well plate and stimulated with YK4 (2×10^6 CFU/well) and/or 1 μg/ml of recombinant mouse galectin-9 (R&D Systems, Minneapolis, MN, USA) in a complete RPMI. After the incubation for 24h, the supernatant was collected for the examination of cytokine concentration.

2.7. *In vitro* $CD4^+$ T cell stimulation

$CD4^+$ T cells were isolated from mLN from wild type mice using mouse $CD4^+$ T lymphocyte enrichment Set (BD Biosciences, San Jose, CA, USA). The $CD4^+$ T cells were labeled with CellTrace™ Violet (CTV) Cell Proliferation Kit (Thermo Fisher Scientific, Waltham, MA, USA). $CD4^+$ T cells (2×10^5 cells/well) were co-cultured with BMDCs (2×10^4 cells/well) that had been treated with YK4 (2×10^5 CFU/well) and/or galectin-9 (1 μg/ml), on anti-CD3 mAbs (BD Biosciences)-coated (2 μg/ml) 96-well plate. After the incubation for 72h, the cells were examined for proliferation of $Foxp3^+CD4^+$ T cells by using flow cytometry and the supernatant was collected and examined for IL-10 and IFN-γ concentration by using ELISA.

2.8. RNA isolation and qPCR

Total RNA was isolated from dorsal skin and intestines by TRIzol®

reagent (Thermo Fisher Scientific). One microgram of RNA was reverse-transcribed in a 20 μl reaction containing random primers (500 μg/ml), dNTP (10 mM), 5× first strand buffer, DTT (0.1 M), Superscript III enzyme (200 U/μl) and RNase inhibitor (10 U/μl) (all from Thermo Fisher Scientific). RNA was reverse transcribed with HyperScript reverse transcription reagents (GeneAll Biotechnology, Seoul, Korea), and quantitative PCR (qPCR) was performed with the iQ SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA) on the LightCycler 480 Real-Time PCR System (Roche, Mannheim, Germany). This was then used to calculate the relative amounts of target mRNA in test samples. Quantities of all targets in test samples were normalized to the corresponding GAPDH levels. Primers for mouse galectin-9 (forward: 5'-CAG CAC CCC TGG ACA GAT GT -3', reverse: 5'-ATG GAC TTG GAC GGG TAA AGC-3'), mouse TSLP (forward: 5'-TAC TAT ACT CTC AAT CCT ATC CCT G-3', reverse: 5'-ACT TCT TGT GCC ATT TCC TG-3'), mouse GAPDH (forward: 5'-CTC CAC TCA CGG CAA ATT CA -3', reverse: 5'-GCC TCA CCC CAT TTG ATG TT -3') were synthesized from Bioneer Inc. (Daejeon, Korea).

2.9. Enzyme-linked immunosorbent assay (ELISA)

The immunological response of the mice with DNCB-induced AD was monitored by measuring the levels of serum IL-4, IL-10, IL-12p40,

IL-17, TGF-β, IFN-γ (all from R&D Systems) and IgE (BD Biosciences). Using the ELISA DuoSet kit (R&D Systems), mouse IL-6, IL-10 and IL-12p40 were measured in stimulated BMDCs supernatant, and IL-4, IL-10, IL-17 and IFN-γ were measured in stimulated T cells supernatant *in vitro*. Briefly, 96-well microplate (Thermo Fisher Scientific) was pre-coated with 100 µl/well of capture antibody. After blocking with 1% BSA for 1 h at room temperature, 100 µl/well of supernatant along with the standard solution diluted in diluent buffer was added and incubated for 2 h at room temperature. After the wash with PBS for three times, 100 µl/well of biotinylated detection antibody was added and incubated for 2 h at room temperature. Then, the plate was washed with PBS for three times and 100 µl of Streptavidin-HRP in PBS was added. After the incubation for 20 min at room temperature, tetramethylbenzidine (TMB, Merck Millipore, Burlington, MA, USA) was added to develop the color and then the reaction was stopped by adding 50 µl of 2M H_2SO_4. The absorbance at wavelength 450 nm was measured by a microplate reader (Molecular Devices, San Jose, CA, USA).

2.10. Phenotypic and functional examination of immune cells by using flow cytometry

In order to examine the activation status of the cells, BMDCs were treated with YK4 and/or galectin-9 for 24h at 37°C. The cells were

stained with anti-mouse CD44-FITC, CD86-FITC, PD-L1-PE, MHC II-PE-cy7, OX40L-APC, CD11c-APC (all from BD Biosciences) for 20 min at 4°C in the dark. To test the change of Tregs, CTV labeled $CD4^+$ T cells cultured with YK4 and/or Galectin-9-treated BMDCs for 3 days were stained with anti-mouse CD25-FITC, CD4-PE (BD Biosciences). After surface staining, $CD4^+$ T cells were fixed and stained with anti-mouse Foxp3-APC mAb (Biolegend, San Diego, MA, USA) using Foxp3 Fix/Perm Buffer Set (Biolegend). *In vivo* examination, spleen, mLNs and PP were isolated from the mice and single cells were prepared. Population changes of DCs were examined after the staining with combination of anti-mouse CD103-bv421, MHC II-PE-cy7 and CD11c-APC (all from BD Biosciences) for 20 min at 4°C in the dark. To examine the preferential subpopulation of $CD4^+$ T cells, splenocytes, and mononuclear cells from mLN and PP were stimulated with 50 ng/ml of of phorbol 12-myristate-13-acetate (PMA) and 750 ng/ml of ionomycin (Sigma-Aldrich) in the presence of brefeldin A (Sigma-Aldrich) for 4h. Then, the cells were stained with appropriate combination of anti-mouse CD4-bv605, IFN-γ-PE, IL-4-bv605 and IL-17-APC-cy7 mAb (all from Biolegend). The cells were washed and the expression of fluorescence was examined using a FACS Canto II (BD Biosciences). All flow cytometric data acquired were analyzed with FlowJo software (Tree Star, Ashland, OR, USA).

2.11. Statistical analysis

The levels of significance for the comparison between *in vivo* samples were determined by Tukey's multiple comparison test by using GradPad InStat software (Ver 5.01, GraphPad). The data were expressed as the mean ± SEM. *In vitro* data were evaluated using *Student's t-test*, and $P < 0.05$ was considered as statistically significant.

3. Results

3.1. Amelioration of AD in mice treated with YK4

To investigate the therapeutic properties of YK4 *in vivo*, a mouse model with AD-like skin lesion was established. As shown in Figure 3-1A, the mice were sensitized with DNCB for 2 weeks and then administered intragastrically with PBS or 1×10^9 CFU of YK4. AD group showed severe atopic symptoms including erythema/hemorrhage, edema, excoriation/erosion, and scaling/dryness. The oral administration of YK4 significantly ameliorated the severity of AD-like skin lesion compared to the AD group (Figure 3-1B). No significant change was found on the visual and physical examination of the AD mice based on the length of spleen and large intestine at the end of feeding period (Figure S3-3A, B). It has been suggested that the expression of TSLP in keratinocytes is one of the main features of AD [165]. To investigate the induction of skin inflammation, the mRNA expression of TSLP was measured in the skin. The result showed that TSLP in the skin of AD group was significantly increased, whereas it kept a normal level in the AD group treated with YK4 (Figure 3-1C). Excessive production of serum IgE is a typical characteristic of AD symptom in accordance with the induction of a Th2-type immune response [141,166]. The results showed that serum IgE levels were significantly increased in the AD group

compared to that in control group, whereas the AD group administered with YK4 showed a significantly low IgE (Figure 3-1D). These results suggest that YK4 effectively inhibits the expression of TSLP and IgE, thereby ameliorating the symptoms of AD.

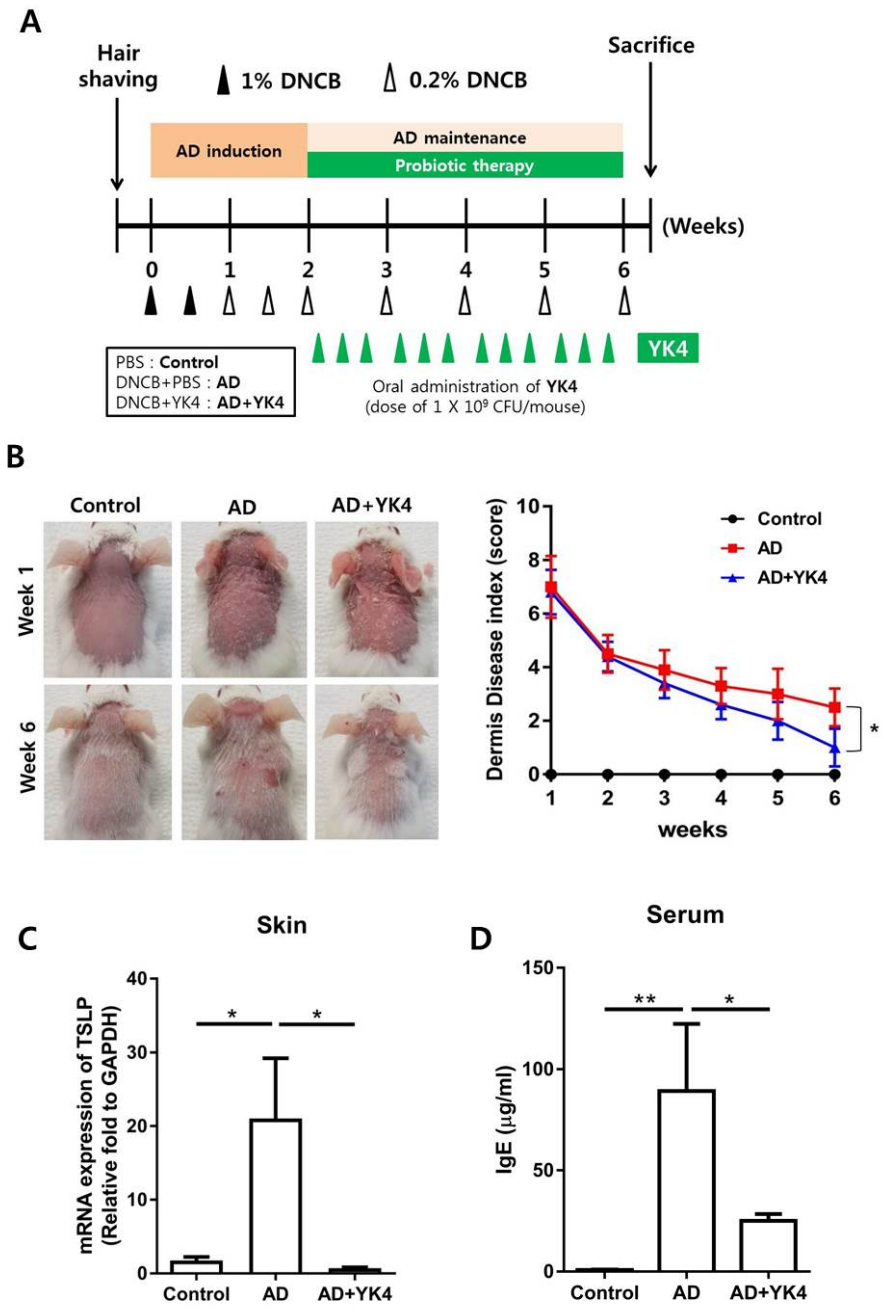

Figure 3-1. Amelioration of AD-like symptoms in mice treated with YK4

(A) Schematic diagram of DNCB-induced atopic dermatitis (AD) mouse model of AD-like skin lesions and oral administration of YK4 in BALB/c

mice. DNCB was applied to the dorsal skin of BALB/c mouse as described in the Materials and Methods. The mice were divided into three groups: (1) PBS control (Control), (2) DNCB+PBS (AD), and (3) DNCB+YK4 (1×10^9 CFU/day, AD+YK4). (B) AD-like skin lesions were evaluated by visual observation. Scoring for the dermatitis index was performed as explained in the Materials and Methods. (C) Dorsal skin was also collected at week 6, and RNA was extracted for cDNA synthesis. qPCR was performed to examine the mRNA expression of TSLP. Relative fold changes of target genes were compared with that of the housekeeping gene, GAPDH. (D) Blood samples were acquired, and serum IgE levels were measured by ELISA. *P <0.05, ** P <0.01 using one-way ANOVA with Tukey's multiple comparison test. Bars indicate mean ± SEM.

3.2. YK4 administration induces a decrease in Th2 response coincident with an increase in Tregs *in vivo*

AD is triggered by the hypersensitivity of Th2 response, which can be overcome by rebalancing $CD4^+$ T cell subsets [158]. To determine whether YK4 administration affects intestinal and systemic T cell response, subpopulation of $CD4^+$ T cells in mLN, PP and spleen by using gating strategy shown in Figure 3-2A were examined in AD mice with/without YK4 administration. The result showed that the proportion of $CD4^+$ T cells in the PP was significantly increased in AD mice administered with YK4, but no changes in mLN and spleen were observed (Figure 3-2B). Next, the other subtypes of helper T cells was examined by testing the intracellular expression of IFN-γ, IL-4, and IL-17 in $CD4^+$ T cells. The ratio of Th1 to Th17 cells was not changed while the ratio of Th2 cells decreased significantly in the PP and mLN from the AD mice administered with YK4. On the other hand, Th1 response in the spleen was increased in the AD mice administered with YK4 (Figure 3-2C), suggesting potential counteract to the decrease of Th2 response. It is well known that Th2-induced allergic reactions could be suppressed by Tregs [126]. Thus, the population change of Tregs in PP, mLN and spleen was examined from AD mice administered with YK4. Population of Tregs in PP and mLN was increased in AD mice administered with YK4 compared to control and AD groups, while no change was found in the

spleen (Figure 3-2D).

Cytokine production in atopic disease is one of the indicators that indirectly examine the systemic immune response. For instance, IL-10 and TGF-β affect the differentiation and function of Tregs [167]. Therefore, serum cytokines were measured to assess their possible relationship between YK4 and $CD4^+$ T cell responses. The results showed that amounts of serum IFN-γ, IL-4, IL-10 and IL-17 were below the detection limits in all groups (data not shown). However, the level of serum TGF-β significantly decreased at the onset of AD, which was recovered upon administered with YK4 (Figure 3-2E). These results indicate that YK4 induces a decrease in Th2-type response coincident with an increase in Tregs in the intestine and induction of serum TGF-β expression.

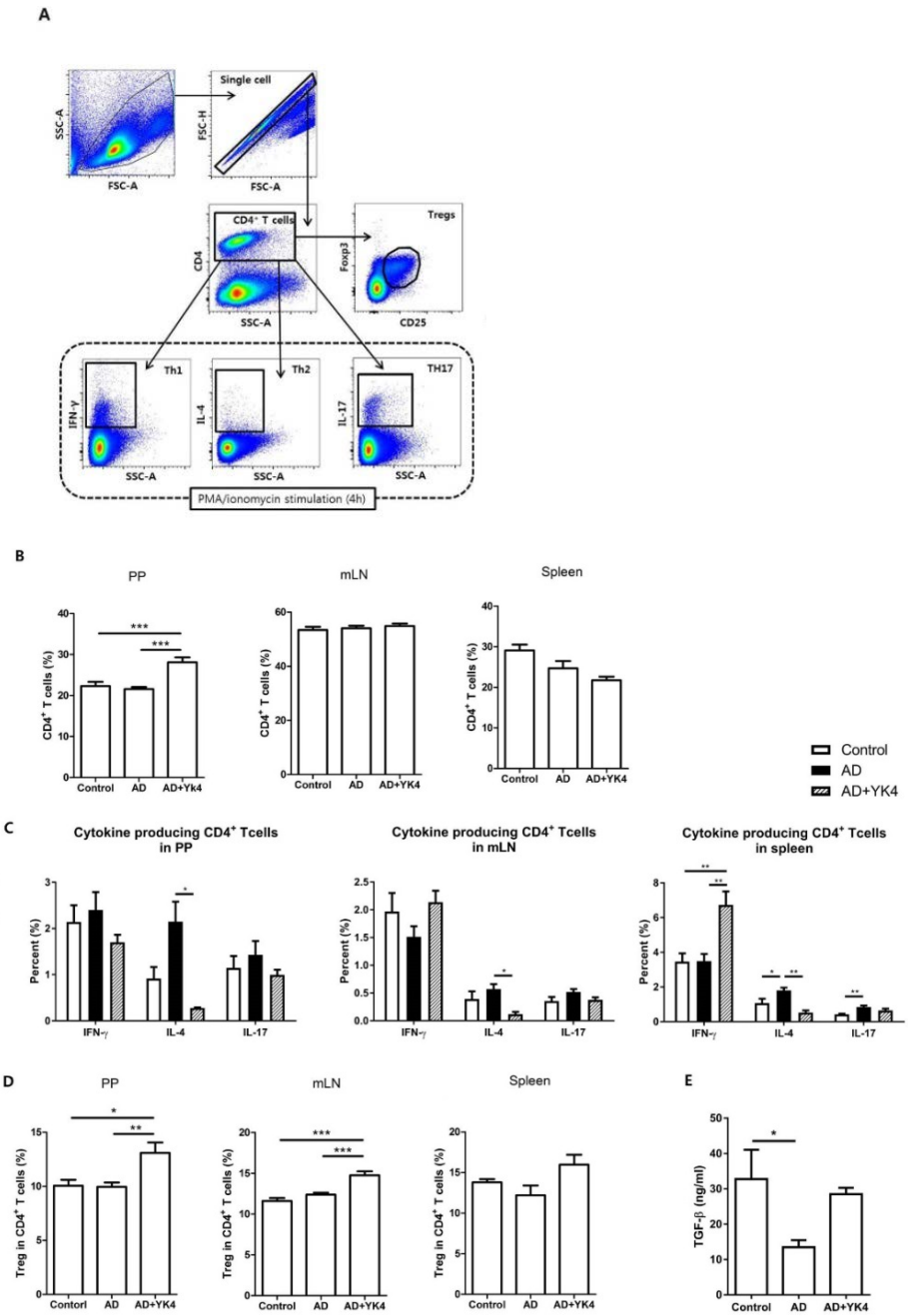

Figure 3-2. Characterization of CD4$^+$ T cells from PP, mLN and spleen in DNCB-sensitized BALB/c mice treated with YK4.

DNCB-induced AD mouse was fed with YK4 and PP, mLN and spleen were collected at week 6. Single cells, produced from each tissue, were

used to examine the characterization of $CD4^+$ T cells. (A) Gating strategy for the subtype of $CD4^+$ T cells. (B) The percentages of total $CD4^+$ T cells from PP, mLN and spleen were examined by using flow cytometry. (C) Single cells from PP, mLN and spleen were stimulated with PMA/ionomycin in the presence of brefeldin A for 4h. IFN-γ, IL-4, and IL-17 producing $CD4^+$ T cells from PP, mLN and spleen were examined after intracellular staining by using flow cytometry. (D) The percentages of $Foxp3^+CD25^+CD4^+$ T cells from PP, mLN and spleen were examined by using flow cytometry. (E) Blood samples were taken and serum TGF-β levels were measured by ELISA. Data are representative of at least three experiments. $*P < 0.05$, $**P < 0.01$. $***P < 0.001$ using one-way ANOVA with Tukey's multiple comparison test. Bars indicate mean ± SEM.

3.3. YK4 administration induces an increase in $CD103^+$ DCs *in vivo*

DCs are one of the most important antigen presenting cells for the differentiation of naïve T cells into specific subset of T cells. In particular, $CD103^+$ DCs are known to induce Tregs differentiation in the gastrointestinal tract [168]. In the present study, the proportion of $CD11c^+MHCII^+$ DCs and $CD103^+$ DCs in AD mice administered with YK4 was examined by using the gating strategy shown in Figure 3-3A. The proportion of DCs in the PP, mLN and spleen was significantly increased in AD mice administered with YK4 when compared to those of AD mice (Figure 3-3B). Moreover, the increase of $CD103^+$ DCs in mLN and spleen was pronounced (Figure 3-3C). Taken together, these results suggest that $CD103^+$ DCs increased in AD mice when administered with YK4.

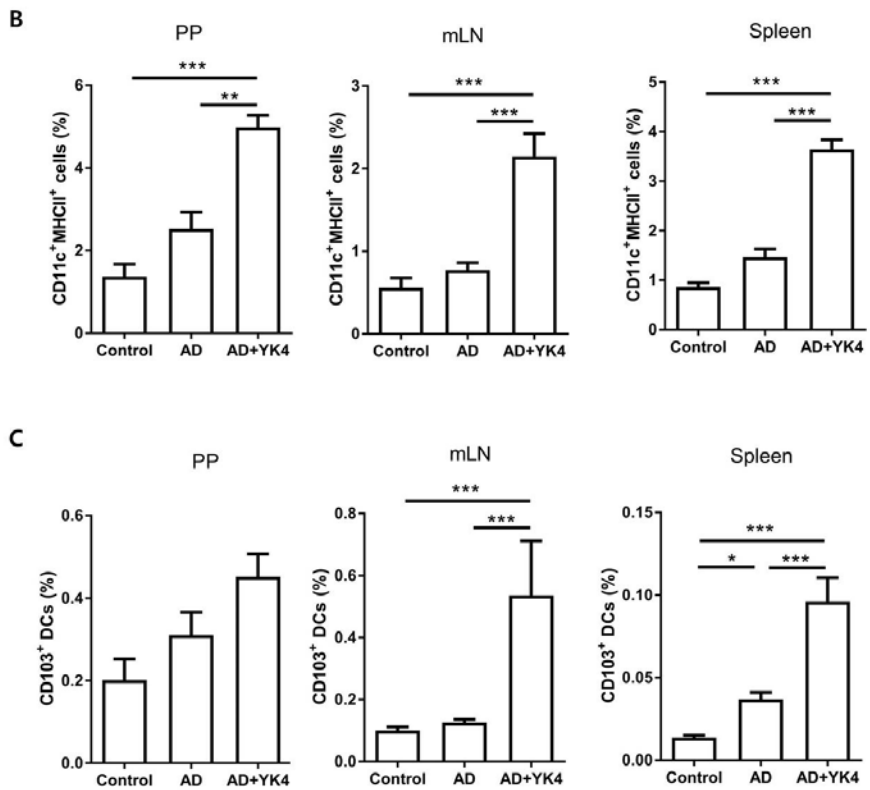

Figure 3-3. Composition of dendritic cells from PP, mLN and spleen in DNCB-sensitized BALB/c mice treated with YK4.

DNCB-induced AD mice were fed with YK4. PP, mLN and spleen were collected at week 6. Single cells from each tissue were used examine the proportion of dendritic cells (DCs). (A) Gating strategy for

subtype of DCs. The percentages of (B) $CD11c^{+}MHC\ II^{+}$ DCs and (C) $CD103^{+}$ DCs from PP, mLN and spleen were examined using flow cytometry. Data are representative of at least three experiments. *P <0.05, **P <0.01. ***P <0.001 using one-way ANOVA with Tukey's multiple comparison test. Bars indicate mean ± SEM.

3.4. Galectin-9 at intestine appears to be associated with alleviation of AD symptom

Galectin-9 is known to regulate the immune response via modulation of DCs with sequential differentiation of Tregs [80,169]. The expression of galectin-9 was examined in the intestine to assess whether it plays a part in the suppression of AD-like symptom. The results showed that there was no difference on the expression of galectin-9 in the small and large intestines between control and AD groups (Figure 3-4). However, galectin-9 expression was significantly increased in AD mice administered with YK4 (Figure 3-4). These results suggest that YK4 induces an increase of galectin-9 in the intestine suggesting potential involvement of galectins on the change of $CD103^+$ DCs and Tregs in AD mice.

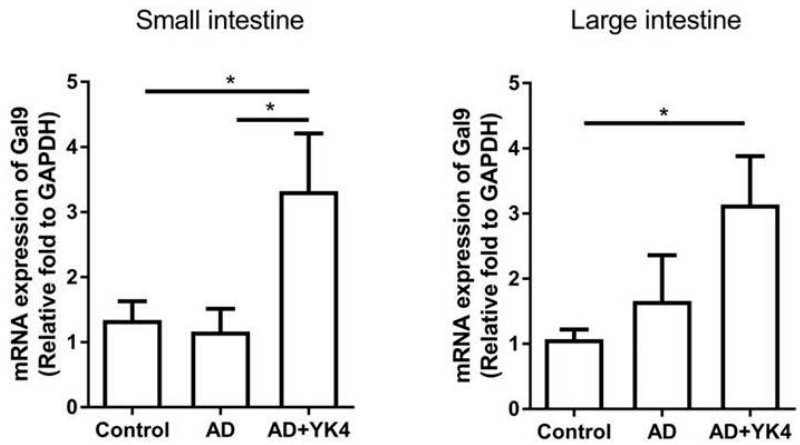

Figure 3-4. Expression of galectin-9 from intestine in DNCB-sensitized mice treated with YK4.

DNCB-induced AD mice were fed with YK4. Small and large intestines were collected at week 6, and RNA was extracted for cDNA synthesis to examine the mRNA expression of galectin-9 (Gal9). Relative fold changes of target genes were compared with that of the housekeeping gene, GAPDH. **P* <0.05using one-way ANOVA with Tukey's multiple comparison test. Bar indicates mean ± SEM.

3.5. YK4 effectively induce regulatory immune responses by BMDCs

Next, to determine how YK4 and galectin-9 affect DCs, I investigated whether YK4 and/or galectin-9 affects the survival of BMDCs. The results showed that the survival of BMDCs was affected neither by YK4 at the dose lower than 2×10^7 CFU (Figure S3-4A) nor by galectin-9 lower than 10 μg/ml (Figure S3-4B). Based on these results, the concentration of YK4 at 2×10^6 CFU and galectin-9 at 1 μg/ml were used. First, the expression of CD44, galectin-9 receptor on BMDCs was examined. While the treatment with galectin-9 alone did not change the expression of CD44 on BMDCs, significant increase was observed when the cells were treated with YK4 alone or together with galectin-9 (Figure 3-5A). Galactin-9-treated BMDCs showed a similar surface expression of CD86 as compared to the cells untreated, while it was slightly increased with YK4 treatment. It was noting that MHC II expression was decreased when the cells were treated with both YK4 and galectin-9 (Figure 3-5A). The expression of OX40L, known as the ligand for the amplification of Th2 cell differentiation, was not affected by galactin-9 and/or YK4 treatment in BMDCs (Figure 3-5A). PD-L1, responsible for the immunosuppressive response, was increased when the cells were treated with YK4 alone or together with galectin-9 (Figure 3-5A). Immunomodulatory cytokines were examined in the culture supernatant

from BMDCs treated with YK4 and/or galectin-9. The results showed that expression of proinflammatory cytokine, IL-6 was increased in BMDCs treated with YK4, which was slightly increased further when treated with both YK4 and galectin-9 (Figure 3-5B). Anti-inflammatory cytokine, IL-10 was also increased in BMDCs treated with YK4 while galectin-9 treatment with/without YK4 did not affect its expression (Figure 3-5B). IL-12p40, increased in BMDCs treated with YK4, was slightly decreased when treated together with galectin-9 (Figure 3-5B). Collectively, these results suggest that YK4 makes BMDCs functionally tolerant.

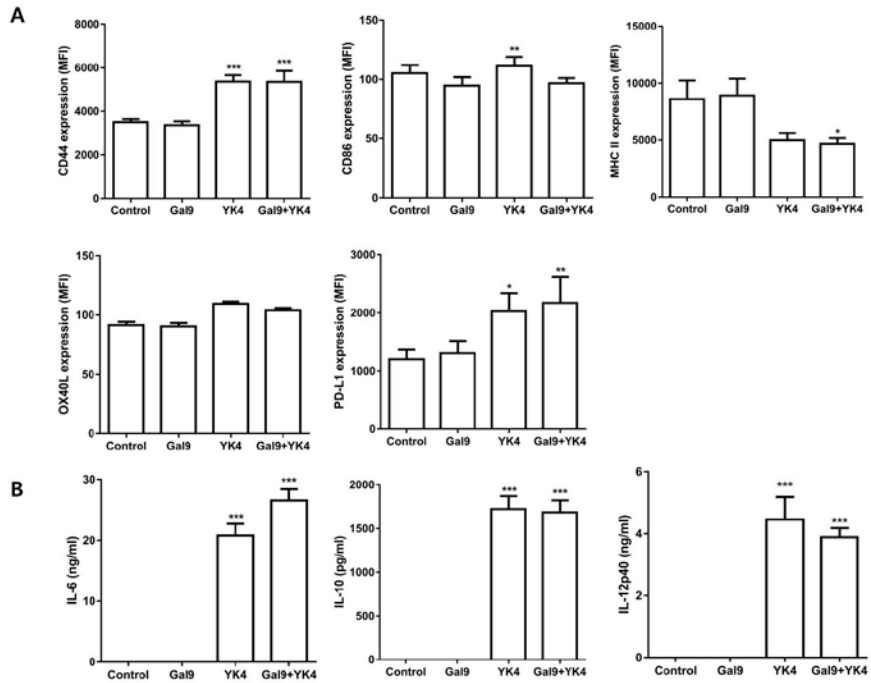

Figure 3-5. Changes of regulatory molecules in BMDCs treated with YK4 and/or galectin-9.

BMDCs were treated with galectin-9 and/or 2×10^6 CFU of YK4 for 24 h. (A) The expression of CD44, CD86, MHC II, OX40L and PD-L1 on BMDCs was measured by flow cytometry. (B) The expression of cytokines in the supernatants was measured by ELISA. Data are representative of at least three experiments. **P* <0.05, ***P* <0.01, ****P* <0.001 using *Student's t-test*, compared to the non-treated control group (Control).

3.6. YK4 and galactin-9 induced proliferation of Tregs and increase of immunomodulatory cytokines

Tregs are the main cell type for tolerance induction and suppressing the excessive immune response. I investigated whether BMDCs treated with YK4 and/or galectin-9 could induce Treg proliferation. $CD4^+$ T cells were isolated from mLN and co-cultured on anti-CD3 antibody-coated plate with BMDCs that had been treated with YK4 and/or galectin-9. The results showed that galectin-9-treated BMDCs failed to inhibit proliferation of $CD4^+$ T cells whilst YK4-treated BMDCs inhibited $CD4^+$ T cell proliferation (Figure 3-6A). Next, the proportion of Tregs among $CD4^+$ T cells was examined after co-culture with BMDCs. The proportion of Tregs increased when co-cultured with YK4-treated BMDCs, which increased even more when cultured with YK4 and galectin-9-treated BMDCs (Figure 3-6B). To further confirm the activation of $CD4^+$ T cells, the level of immunomodulatory cytokines was investigated in the supernatant of $CD4^+$ T cells co-cultured with YK4 and/or galectin-9-treated BMDCs. Galectin-9 alone did not affect the expression of immunomodulatory cytokines in $CD4^+$ T cells (Figure 3-6C). However, the expression of IL-4 was reduced while IL-10 and IL-17 were significantly increased in $CD4^+$ T cells when treated with YK4 alone or together with galectin-9 (Figure 3-6C). These results suggest that YK4 alone or together with galectin-9-treated BMDCs promoted IL-10

production and Tregs proliferation resulting in inhibition of $CD4^+$ T cell activities.

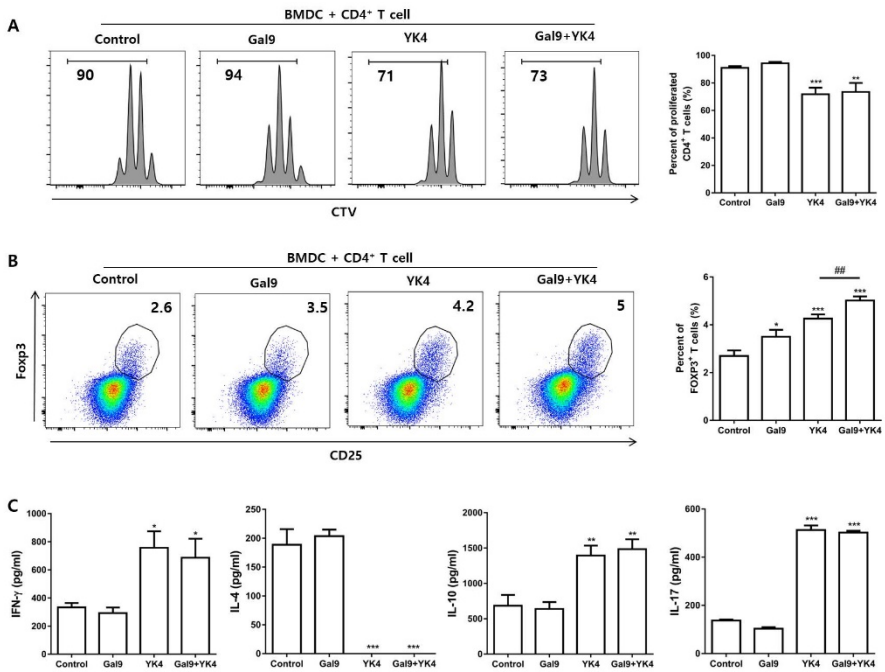

Figure 3-6. BMDCs treated with YK4 and galectin-9 promote Tregs proliferation and immunomodulatory cytokine production.

BMDCs treated with YK4 and/or galectin-9 were co-cultured with $CD4^+$ T cells for 3 days on anti-CD3mAbs-coated plate. The percentage of the proliferated (A) total $CD4^+$ T cells and (B) proportion of $CD4^+CD25^+Foxp3^+$ T cells among $CD4^+$ T cells were analyzed by flow cytometry. At the same time, supernatants were harvested and examined for (C) the production of IFN-γ, IL-4, IL-10 and IL-17 in the $CD4^+$ T cells using ELISA. Data are representative of at least three experiments. **P* <0.05, ***P* <0.01, ****P* <0.001 using *Student's t-test*, compared to the non-treated control group (Control). ##*P* <0.01 using *Student's t-test*, when (YK4) and (Gal9+YK4) group were compared.

4. Supplementary Figures

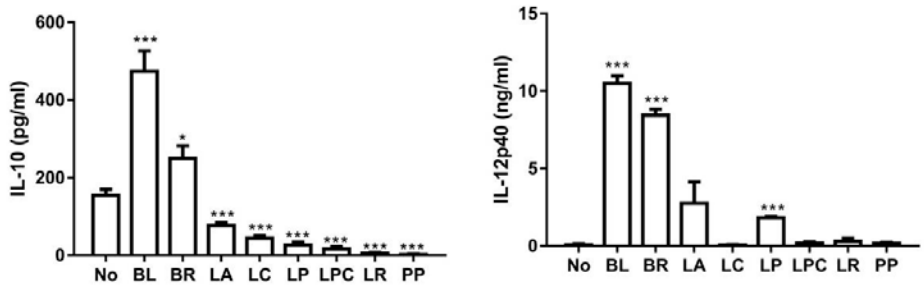

Figure S3-1. Changes of cytokine secretion in BMDCs treated with candidate probiotics.

BMDCs were treated with 2 × 10^6 CFU of candidate Probiotics (*B. breve* (BR), *B. lactis* (BL), *L. acidophius (LA), L. casei (LC), L. plantarum* (LP), *L. paracasei* (LPC), *L. rhamnosus* (LR), *Pediococcus pentosaceus* (PP)) for 24 h. The expression of cytokines in the supernatants was measured by ELISA. Data are representative of at least three experiments. **P* <0.05, ****P* <0.001 using *Student ′s t-test*, compared to the non-treated control group (No).

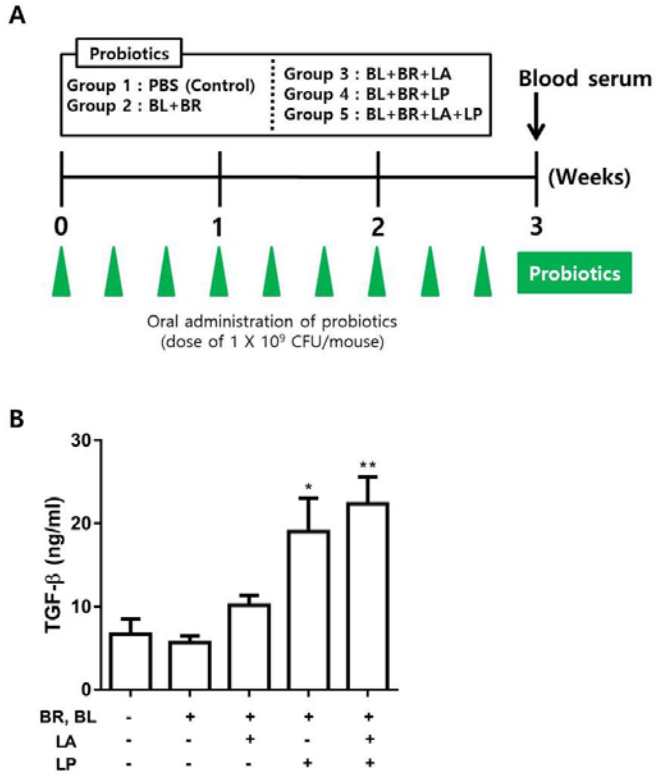

Figure S3-2. Cytokine secretion in BALB/c mice administrated with combination of candidate probiotic

(A) Schematic diagram of oral administration of combination of candidate probiotic (*L. acidophius* (LA), *L. plantarum* (LP), *B. breve* (BR), and *B. lactis* (BL)) in BALB/c mice. The mice were divided into five groups: (1) PBS (Control), (2) BL+BR, (3) BL+BR+LA, (4) BL+BR+LP and (5) BL+BR+LA+LP. **(B)** Blood samples were taken and serum TGF-β levels were measured by ELISA. Data are representative of at least three experiments. **P* <0.05, ***P* <0.01 using *Student's t-test*, compared to the non-treated control group.

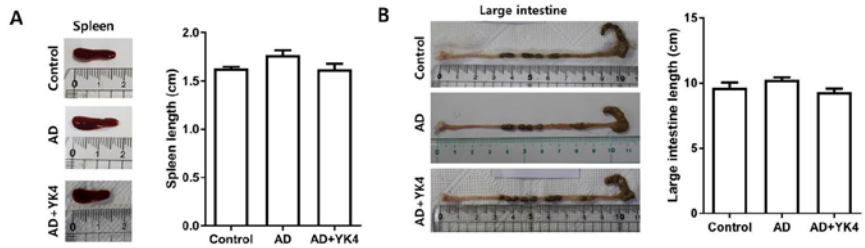

Figure S3-3. YK4 treatment does not induce inflammation in the spleen and large intestine.

DNCB-induced AD mouse was fed with YK4. The (A) spleen and (B) large intestine were isolated at week 6. The length was measured by visual inspection to determine the inflammatory response.

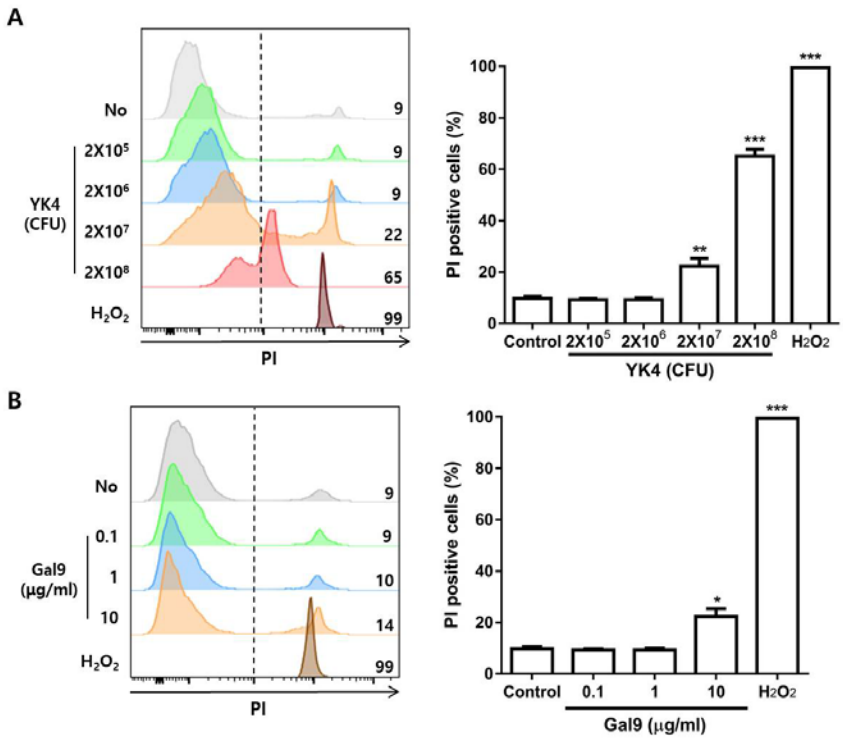

Figure S3-4. Apoptosis of BMDCs treated with YK4 and galectin-9.

BMDCs (2×10^5 cells) were treated with various concentration of (A) YK4 and (B) galectin-9 for 24 h. Then, the percentage of apoptotic cells, the propidium iodide positive fraction, was measured. H2O2 treatment was used as a positive control. Histogram of the results is shown. Data are representative of at least three experiments. **P* <0.05, ***P* <0.01, ****P* <0.001 using *Student's t-test*, compared to the non-treated control group (Control).

5. Discussion

Probiotics are living microorganisms that can be ingested in a form of dairy and fermented foods. Although their colonization at the intestine is controversial, it has been suggested in humans and animals for the regulation of intestinal immune homeostasis [43,170]. Despite numerous studies on allergies, protective effects and exact mechanism of probiotics remain unclear. In the present study, YK4, a probiotics mixture with potent anti-inflammatory properties, was examined its regulatory role against AD. Furthermore, this study elucidated the mechanism of overcoming AD by which YK4 and galectin-9 regulate $CD4^+$ T cells through DCs and Tregs.

Generally, when skin epidermal barrier is damaged by AD, TSLP is produced by keratinocytes. TSLP promotes activation of DCs and induces the expression of OX40L on the DCs surface [171]. The activated DCs together with IL-4 induce differentiation of naive T cells to become Th2 cells. These Th2 cells contribute to the generation of IgE in B cells [172,173]. As expected, in our study, DNCB-induced AD mice showed a skin barrier disruption and inflammation due to excessive Th2 response followed by increase of serum IgE and skin TSLP levels. Previous studies indicated that probiotics inhibit TSLP and IgE production to control AD symptoms [174]. Due to the oral administration of probiotics, the relationship between intestinal and systemic immune response is still

controversial issue. It has been suggested that certain probiotics induce the intestinal $CD4^+$ T cell population in specific subset and alleviate AD symptoms. For instance, a probiotic mixture, Duolac ATP is known to downregulte Th2 cells in mLN and to systemically inhibit Th2 responses [158]. Treatment with *L. plantarum*, one of the components of YK4, downregulated Th2 cells in the intestinal lamina propria [157]. Similarly, in the present study, IL-4-producing Th2 cells decreased not only in mLN and PP but also in spleen in DNCB-induced AD mice fed with YK4. These findings suggest that YK4 may inhibit TSLP and IgE production by controlling intestinal and systemic Th2 cells.

Several strategies have been proposed for managing Th2 response in AD, including the preferential differentiation of $CD4^+$ T cells toward Th1 cells. Th1-type response is known to counteract against Th2 responses, thereby inhibiting the progression of inflammation in AD [175]. Cytokines play an essential role in determining the direction and function of $CD4^+$ T cell and its differentiation by activating STAT [176]. IL-12, predominantly produced in antigen-presenting cells and phosphorylates STAT4 in $CD4^+$ T cells, plays an important role in the differentiation of naïve T cells into Th1 cells [177]. Phosphorylated STAT4 promotes T-bet expression and induces the production of IFN-γ, which again activates STAT1 in $CD4^+$ T cells to further stabilize T-bet [177]. This inhibits the expression of GATA3 and prevents $CD4^+$ T cells to differentiate into IL-4 producing Th2 cells [178]. *L. paracasei* has been shown to induce IL-12 production in DCs

via TLR9 signaling to inhibit Th2 response [179,180]. In addition, the probiotics mixture, Duolac ATP, activates T-bet to induce Th1 response and inhibits GATA3, thereby blocking the Th2 response in AD mice model [158]. Expression of T-bet and GATA3 was reduced in the small intestine after *L. plantarum* treatment in the mouse small intestinal lamina propria [157]. WIKIM28, one of the *W. cibaria* strains, decreased IL-4 in peripheral lymph node cells without affecting IFN-γ in peripheral lymph node cells [64]. These results suggest that depending on the type and combination of probiotics, it may contribute to a specific T cells response. YK4 in the present study induced IL-12 expression in DCs, which would turn directly to affect the activity of STAT4 in $CD4^+$ T cells. With this mechanism, YK4 increased IFN-γ and suppressed IL-4 production in $CD4^+$ T cell through DCs. Furthermore, YK4 induced Th1 cell activation that produced IFN-γ and inhibited IL4-producing Th2 cells in spleen of AD mouse. These results suggest that YK4 induces differentiation of Th1 cells and downregulates Th2 response in spleen through IL-12 production of DCs.

Another mechanism to suppress Th2 response would be Tregs to regulate unwanted inflammatory responses [181]. Like other $CD4^+$ T cells, Tregs are also stabilized and differentiated though specific cytokine signals such as TGF-β and IL-10 [182]. TGF-β activates Smad3 in Tregs and stabilizes Foxp3 that induces TGF-β and IL-10 production at the same time as differentiation, thereby suppressing unwanted immune

responses. [183]. Certain probiotics can induce these immunosuppressive cytokines and promote the generation of $Foxp3^+$ Tregs. For example, administration of WIKIM28 induced the differentiation of Tregs and the production of IL-10 in AD mice [64]. Our study also showed that YK4 increased the expression of TGF-β, which was reduced to AD, to normal levels and increased the population of intestinal $Foxp3^+$ Tregs. As an action mechanism, tDCs in particular, are essential to induce the differentiation of Tregs [184]. Intake of probiotics can be recognized by TLRs on $CD103^+$ DCs that are abundant in the small intestine [185]. Then, activated tDCs produce suppressive cytokines including IL-10 and TGF-β, and cell surface inhibitory molecules, PD-L1 and PD-L2 [186]. Certain probiotics, such as *B. bifidum*, induced increase of $CD103^+$ DCs in colonic lamina propria [187]. Indeed, oral administration of YK4 in the present study induced upregulation of $CD103^+$ DCs in mLN. Furthermore, DCs treated with YK4 induced increase of IL-10 and PD-L1 expression. Interestingly, when YK4-treated DCs were co-cultured with $CD4^+$ T cells, the frequency of Tregs markedly increased. These results suggest that YK4 induces the activity of tDCs and affects the differentiation and activity of intestinal Tregs, thereby suppressing the intestinal Th2 response.

Probiotics may directly regulate immune cells, but they can regulate the Th2 response indirectly by activating intestinal epithelial cells [188]. When the intestinal epithelial cells are activated by probiotics, effector

molecules including chemokines and galectins are produced [189]. In particular, galectin-9 is known to play a role in regulating AD through modulation of immune response [190]. The ingestion of dietary supplements with *Bifidobacterium breve* increased galectin-9 levels in intestinal epithelial cells causing the prevention of allergic symptoms [90]. Likewise, the expression of intestinal galectin-9 was observed to increase in AD mice administered with YK4. Galectin-9 binds directly to carbohydrate moieties of IgE to prevent IgE-antigen complex formation and mast cell degranulation. Moreover, galectin-9 binds to CD44 of DCs preventing their maturation and activation by inhibiting STAT1 activation [80]. In our study, galectin-9 did not directly affect the activity of DCs. However, when galectin-9 and YK4 were treated together, the expression of IL-12 was decreased in DCs. It is likely that activation of STAT1 by YK4 stimulation should precede the inhibitory effect of galectin-9 in DCs. The inhibition of IL-12 through galectin-9 also modulates the YK4-induced Th1 response. It is also known that galectin-9 binds to CD44 on Tregs and phosphorylates Smad3 to stabilize Foxp3 followed by secretion of IL-10 and TGF-β [162]. A number of reports suggested a direct impact of galectin-9 on T cells, however these studies have not considered the importance of T cell-to-DC interaction for the best outcome of T cell activity. Thus, in the present study, BMDCs was used rather than anti-CD28 mAbs to activate CD28 on $CD4^+$ T cells. The result showed that galectin-9 did not cause BMDCs to become tolergenic. Instead, treatment

of galectin-9 together with YK4 induced proliferation of Tregs and promoted IL-10 production. It is likely that TLR stimulation such as YK4 treatment in DCs may be essential for galectin-9 to affect $CD4^+$ T cells.

In summary, our results showed that YK4 induced expression of IL-10 and IL-12 in DCs that inhibited Th2 responses by inducing Th1 and Tregs differentiation. Furthermore, YK4 regulated intestinal galectin-9 and $CD103^+$ DCs to drive naïve T cell differentiation toward Tregs. Taken together, the probiotic mixture, YK4 has a therapeutic potential to prevent AD symptoms and might be act as an immunomodulator for AD patients.

Chapter 4

General Conclusion

Atopic dermatitis (AD) is a chronic inflammatory skin disorder with a complex etiology encompassing immunological responses. Recent studies have demonstrated the role of certain probiotics in the treatment and prevention of AD. However, it is not well defined for the mechanism of probiotics that regulate the immune system.

In the present study, the effect of Duolac ATP and YK4, a selected mixture of probiotics, supplementation on AD and its mechanisms were investigated. Both probiotic mixtures upregulated PD-L1 and IL-10 expression in dendritic cells (DCs) to become functionally tolerant and potentially induce intestinal Treg differentiation. In particular, YK4 induces intestinal galectin-9 production and thus contributes to intestinal $CD103^+$ DC and Treg differentiation and response. Both probiotic mixtures similarly regulated DCs function, but YK4-treated DCs induce IFN-γ in $CD4^+$ T cells, while Duolac ATP-treated DCs do not increase IFN-γ production. IFN-γ is a cytokine that has a complementary relationship with IL-4, and this difference is predicted to be that YK4, unlike Duolac ATP, may play a role in suppressing intestinal Th2 response. In addition, IL-4, IL-5 and IL-13 produced by type 2 innate lymphoid cells may have influenced the differentiation of intestinal Th2 cells. Thus, despite some other intestinal responses of both probiotic mixtures, they inhibited the systemic Th2 response and induced the Th1 response at the same time. Furthermore, they relieve AD symptoms (Figure 4-1).

In conclusion, these results show that the probiotic mixture, Duolac ATP and YK4 possesses preventive potential against AD and could serve as an effective immunomodulatory agent in AD patients.

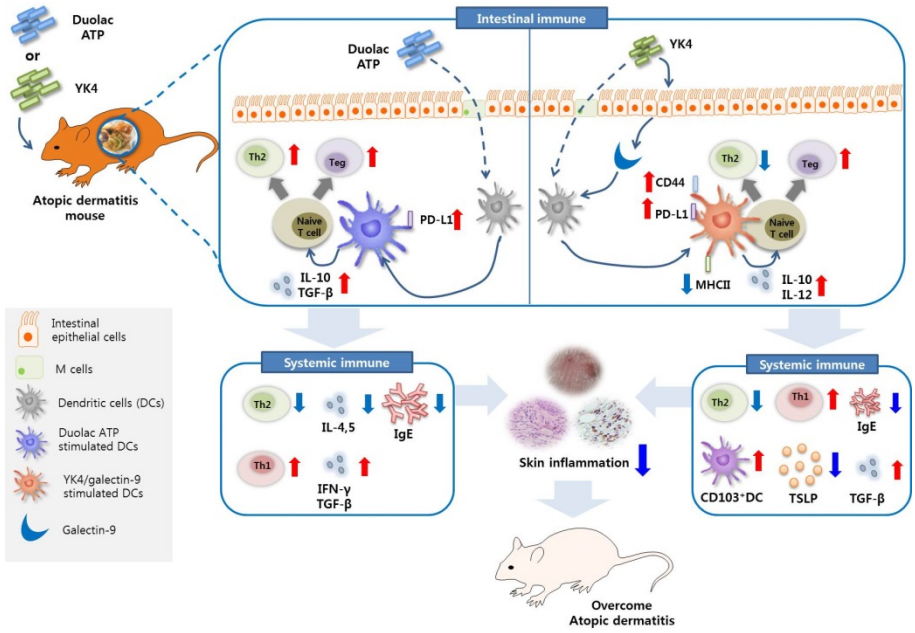

Figure 4-1. A possible immunological mechanism of Duolac ATP and YK4 in AD-like symptomatic mice.

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Summary in Korean

아토피 피부염은 염증성 피부질환으로 T helper (Th) 2 세포의 과도한 면역반응에 의해 생기는 만성 알레르기 질환이다. 아토피 피부염이 발병하면 가려움증으로 인해 피부를 긁게 되며, 이는 피부 손상과 피부 염증반응을 유도 하여 질환을 더욱 악화 시킨다. 선진국의 유아 15-20%, 성인 2-10%가 아토피 피부염으로 고통 받고 있으며, 유전적인 요인과 환경적인 요인으로 인한 면역 불균형이 아토피 피부염의 주요 원인으로 알려져 있다. 가족 중 알러지 질환을 지닌 사람이 있거나, 선천적으로 표피 세포의 필라그린 (Filaggrin) 단백질의 FLG 유전자의 돌연변이가 생길 경우 아토피 피부염이 발병 할 확률이 올라간다. 또한 환경적으로 급격한 온도 및 습도의 변화, 자외선 노출, 스트레스, 세균 감염을 통해서도 아토피 질환이 발병할 수 있다.

아토피가 발병하면, 피부 각질층이 붕괴 되면서 항원에 피부세포가 자극을 받게 된다. 자극을 받은 피부 세포는 흉선 간질 림포포에틴 (Thymic stromal lymphopoietin, TSLP)를 생성하게 되며, 이는 피부 내피의 수지상 세포의 활성을 유도한다. 활성화된 수지상 세포는 OX40L의 발현이 증가하며 이는, naive $CD4^+$ T 세포를 Th2 세

포로 분화하도록 도와준다. Th2 세포는 IL-4와 IL-13의 생성을 유도하며, 이는 B 세포를 분화를 유도 시킴과 동시에 면역글로블린 (Ig)의 종류 변환을 도와 IgE 생성을 유도한다. IgE는 알러젠과 결합하게 되면 비만세포의 FcεR1에 결합하여 히스타민의 생성을 유도한다. 이와 같은 과도한 Th2 반응은 피부 붕괴를 더욱 가속화 시킴과 동시에 아토피 증상을 더욱 악화 시킨다.

기존의 치료제는 피부 건조, 염증을 완화 시키는 방법, 특정 면역 신호 전달 또는 염증유발 사이토카인을 억제하는 방법으로 연구 되어왔다. 하지만 이와 같은 치료법은 단기간 사용시 치유 효과는 있으나, 장기간 사용시 졸음, 부종, 자반병 등과 같은 부작용이 유발되는 것으로 알려진다. 따라서 이상적인 아토피의 치유를 위해서는 과도한 면역을 조절함과 동시에, 지속 사용이 가능한 안전성 확보할 수 있는 물질에 대한 연구가 필요하다.

최근 유산균의 면역조절 능력을 바탕으로 다양한 알러지 질환 치료제로서의 가능성이 활발하게 연구되고 있다. 하지만, 유산균에 의한 면역 조절 기전 연구는 아직 미흡하며, 아토피 피부염에 대한 치유 효과는 유산균의 종류 및 조합에 따라 다르게 나타나므로, 여전히 논란의 대상이 되고 있다.

본 연구의 첫 번째 파트에서는, 유산균 혼합물인 Duolac ATP의 면역 조절 기전을 확인하였다. 시험관내 실험을 통해 Duolac ATP는

수지상 세포의 programmed death-ligand (PD-L) 1의 발현을 증가 시켰다. 또한 수지상 세포에서 면역 억제 사이토카인인 인터루킨 (interleukin, IL)-10의 생성과 변환성장인자 (Transforming growth factor, TGF)-β의 생성을 유도 하였다. 나아가 Duolac ATP를 처리한 수지상 세포와 $CD4^+$ T세포와 공동 배양을 하였을 때, 조절 T세포 (Treg)의 군집이 증가함을 확인하였다. 시험관내 실험의 Duolac ATP의 면역 조절 기전 능력을 토대로, 자연적으로 유전 질환이 있는 마우스 모델 (Nc/Nga)을 대상으로 Duolac ATP의 아토피 치료 효능을 확인 하였다. Duolac ATP는 아토피 마우스의 Th1세포 전사인자인 T-bet, STAT-1, STAT-4의 발현과 IL-2, 인터페론 (IFN)-γ 의 생성을 유도하였다. 나아가 Th2 세포의 전사인자인 GATA-3, c-maf의 발현과 IL-4, IL-5의 생성을 감소 시켰다. 더욱이 파이어 판 (Peyer's patches, PP)에서 Treg의 군집이 증가함을 확인하였다.

두번째 파트에서는, 일반 마우스 (BALB/c)에 반복적인 DNCB 자극을 통해 아토피를 유발하였고, 독자적으로 개발한 유산균 혼합물인 YK4의 효능을 확인하였다. YK4는 피부의 TSLP의 발현을 감소시킴과 동시에 혈 중 IgE 생성을 감소 시켜 아토피 피부염 질환을 호전 시켰다. 나아가 전신 면역 조직인 비장에서 Th1세포의 생성을 유도 하고, 장 면역기관인 PP와 장간막 임파절 (mesenteric lymph node, mLN)의 Treg의 군집을 증가 시켰다. 나아가 비장, PP,

mLN에서 면역 억제 $CD103^+$ 수지상 세포의 증가를 유도하고 아토피 피부염으로 생성된 Th2 군집을 감소시켜주었다. 또한, YK4는 장내 갈락틴-9의 생성을 유도하였다. 아토피 피부염 극복에 있어 갈락틴-9, YK4, 면역세포의 관계를 규명하고자 실험관내 실험을 수행하였다. YK4는 수지상세포에서 PD-L1, IL-10, IL-12의 생성을 유도하였으며, YK4를 처리한 수지상 세포는 $CD4^+$ T 세포와 공동 배양을 하였을 때, Treg의 군집을 증가시켰다. 또한 갈락틴-9는 YK4와 함께 처리시 수지상세포를 통한 Treg 군집의 분화를 도와주었다.

이러한 결과는, 유전자 변형 마우스 모델에서 유산균 혼합물인 Duolac ATP는 수지상세포의 활성 조절을 통해 Th1, Treg의 반응을 유도하여 혈 중 IgE 생성을 저하시켰고, 피부 염증을 억제하여 아토피 피부염을 극복하였다. 또한 일반 마우스 모델에서 유산균 혼합물인 YK4는 장내 갈락틴-9 생성을 통해 수지상세포의 면역 조절 능력을 극대화 시키며 Th1, Treg의 반응을 유도하였다. 나아가 Th2 반응을 억제 시켜 아토피 피부염을 극복함을 밝혀냈다.

종합하면, 본 연구는 아토피 질환의 치료 후보 물질로서 유산균 혼합물인 Duolac ATP와 YK4를 제시하였다. 두 유산균 모두 수지상세포를 통해 T세포 반응을 효율적으로 조절 하였고, 특히 YK4는 장내 갈락틴-9의 발현을 유도하였다. 이는 향 후 아토피 피부염과 같은 과도한 Th2 면역 질환의 극복 기전을 제시함과 동시에 치료

제로서의 가능성을 시사한다.