이학박사학위논문

피부 염증에 대한 *Actinidia arguta* 수용성 추출물 PG102 의 면역 조절 활성 분석

Molecular Characterization of Immunomodulatory Activities of PG102, a Water-Soluble Extract from *Actinidia arguta*, in Skin Inflammation

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ABSTRACT

Molecular Characterization of Immunomodulatory Activities of PG102, a Water-Soluble Extract from *Actinidia arguta*, in Skin Inflammation

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PG102 is a water-soluble extract from *Actinidia arguta*, commonly known as a hardy kiwifruit. It has previously shown to contain potent anti-inflammatory and anti-oxidative activities both *in vitro* and *in vivo*. Based on previous works done in our laboratory, it was hypothesized that it might possess ameliorating effects on psoriasis. In particular, my thesis research was focused on anti-inflammatory effects of PG102 on psoriasis-like skin inflammation in the mouse model and human keratinocyte cell line, HaCaT. I also attempted to identify fraction containing related biological activities.

First, the effects of PG102 on imiquimod-induced psoriasis-like skin inflammation model were tested. Topical application of PG102 suppressed increase in epidermal thickness and IL-17A levels in draining lymph node and serum. In HaCaT keratinocyte cell line, PG102 was found to downregulate expressions of various neutrophil-chemotactic chemokines and antimicrobial peptides such as CXCL1, IL-8, S100A8/9 and hBD-2 when cells were stimulated with mixture of five inflammatory cytokines. These effects were mediated by inhibition of NF-kB and STAT signaling pathway as evidenced by data from Western blot. The effects of PG102 on neutrophil chemotaxis were further investigated using migration assay and immunohistochemistry. The results indicated that PG102 might ameliorate symptoms of psoriasis via suppression neutrophil chemotaxis.

Interleukin 37 (IL-37) is an immunomodulatory cytokine that suppresses

inflammation in various cell types and disease models. Another detailed biological investigation was carried out to study the effect of PG102 on IL-37, a potent anti-inflammatory cytokine, in HaCaT cells. In HaCaT cells, siRNA-mediated knockdown of IL-37 significantly augmented expression of antimicrobial peptides when the cells were exposed to inflammatory cytokines. These results indicated that upregulation of IL-37 might be a potential approach to alleviate psoriasis. It was found that treatment with PG102 effectively increased expression of IL-37 at both RNA and protein levels and these effects were mediated by activating ERK, p38 and Smad3 pathways. PG102 also promoted colocalization of phospho-Smad3 and IL-37 which is necessary for the anti-inflammatory effects of IL-37. These results suggested that PG102 might exert anti-inflammatory effects, in part, through modulation of IL-37.

Lastly, I tried to identify compounds or fractions responsible for anti-psoriatic effects of PG102. PG102 extracts were fractionated using chloroform, ethyl acetate, butanol and water. Each fraction was tested for its effects in psoriasis-like skin inflammation model and HaCaT cells. It was found that the holistic bioactivity of PG102 was distributed into each fraction, without much specificity. Among four fractions, ethyl acetate fraction possessed stronger antimicrobial peptide-suppressing activities, whereas water fraction possessed higher chemokine-suppressing activities than other two fractions *in vitro*. However, none of the fractions were more effective than total PG102 in terms of anti-psoriatic effects. These results demonstrated that the combinatorial effects of different fractions of PG102 might lead to the bioactivity of PG102 observed in mouse psoriasis-like skin inflammation model and M5-stimulated HaCaT cells.

In conclusion, PG102 showed therapeutic effects on imiquimod-induced psoriasis-like skin inflammation model. These effects might be mediated directly by suppressing inflammatory signaling cascades NF-κB, STAT signaling pathways and neutrophil chemotaxis and indirectly by upregulating anti-inflammatory cytokine IL-37

in keratinocytes. The anti-psoriatic effects of total PG102 was the strongest and none of the fractions showed dominant bioactivities. Taken together with previous findings, PG102 may be developed as a safe and effective agent for the treatment of various inflammatory skin diseases.

Keywords: Actinidia arguta, botanical drug, fractionation, PG102, psoriasis, keratinocyte, IL-37

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ABBREVIATIONS

AMP	Antimicrobial peptides
CCR	CC chemokine receptor
CXCL	C-X-C motif ligand
CXCR	CXC chemokine receptor
DC	Dendritic cell
dLN	Draining lymph node
DMSO	Dimethyl Sulfoxide
EA	Ethyl acetate
EGF	Epidermal growth factor
ERK	Extracellular signal–regulated kinases
EtOH	Ethanol
FBS	Fetal bovine serum
FDA	Food and Drug Administration
GWAS	Genome-wide association studies
H&E	Hematoxylin & Eosin
hBD	Human β-defensin
HPLC	High performance liquid chromatography
IC50	Half maximal inhibitory concentration
IL	Interleukin
IMQ	Imiquimod
IPI	Imiquimod-induced psoriasis-like skin inflammation
JNK	c-Jun N-terminal kinases
LPS	Lipopolysaccharide

Ly6G	Lymphocyte antigen 6 complex, locus G
MAPK	Mitogen-activated protein kinase
MHC	Major histocompatibility complex
NET	Neutrophil extracellular trap
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
PASI	Psoriasis Area Severity Index
PEG	Polyethylene glycol
PBMC	Peripheral blood mononuclear cells
PMA	Phorbol 12-myristate 13-acetate
SALT	Skin-associated lymphoid tissue
SBE	Smad binding element
SIGIRR	Single Ig IL-1 related receptor
siRNA	Small interfering RNA
Smad3	Mothers against decapentaplegic homolog 3
SNP	Single nucleotide polymorphism
STAT	Signal transducer and activator of transcription
TDW	Triple distilled water
TGF-β	Transforming growth factor β
Th17	T helper 17 cell
TLR	Toll-like receptor
TNF	Tumor necrosis factor
UVB	Ultraviolet B

Chapter I

Introduction

1. Overview of the skin immune system

Skin is the first line of defense, protecting body from a wide range of stimuli such as pathogens, physical and chemical insults. Since the establishment of the concept of skin-associated lymphoid tissue (SALT) in 1983, many researches have focused on the immunological functions of skin – aside from being a simple physical barrier, skin comprises a complex immune system with a wide variety of cell types actively participating in immune homeostasis and responses [1].

Skin is divided into two main compartments: the epidermis and the dermis. Dermis harbors a great cell diversity such as dermal dendritic cells (DCs), $CD4^+$ T cells, mast cells, fibroblast and macrophages (Fig. 1). On the other hand, keratinocytes are the major cell type of the epidermis, constituting more than 90% of epidermis and minor cell types include Langerhans cell, $CD8^+$ T cells and melanocytes [2, 3]. Indeed, keratinocytes, located at the outermost surface of skin, have substantial roles in regulating and initiating immune responses, contributing to various skin diseases. It is now widely accepted that the crosstalk between keratinocytes and immune cells is essential for tissue homeostasis [4].

In the epidermis, there are two main ways of how keratinocytes interact with other immune cells. First, keratinocytes may act as immune sentinels at the front line – they detect various external stimuli through Toll-like receptors (TLRs) [5]. In response, they produce immune mediators such as antimicrobial peptides (AMPs), cytokines and chemokines to activate and recruit immune cells [6, 7]. The immune cells, in turn, secrete cytokines that induce keratinocytes to produce even more inflammatory mediators, creating a positive feedback loop. Secondly, keratinocytes may act as non-professional antigen-presenting cells by expressing major histocompatibility complex (MHC) class II molecules [8]. They can either present epitopes of antigen directly to memory $CD4^+$ or $CD8^+$ T cells or induce tolerance of T cells [9].

If the inflammation is not resolved in a timely manner, it may cause chronic

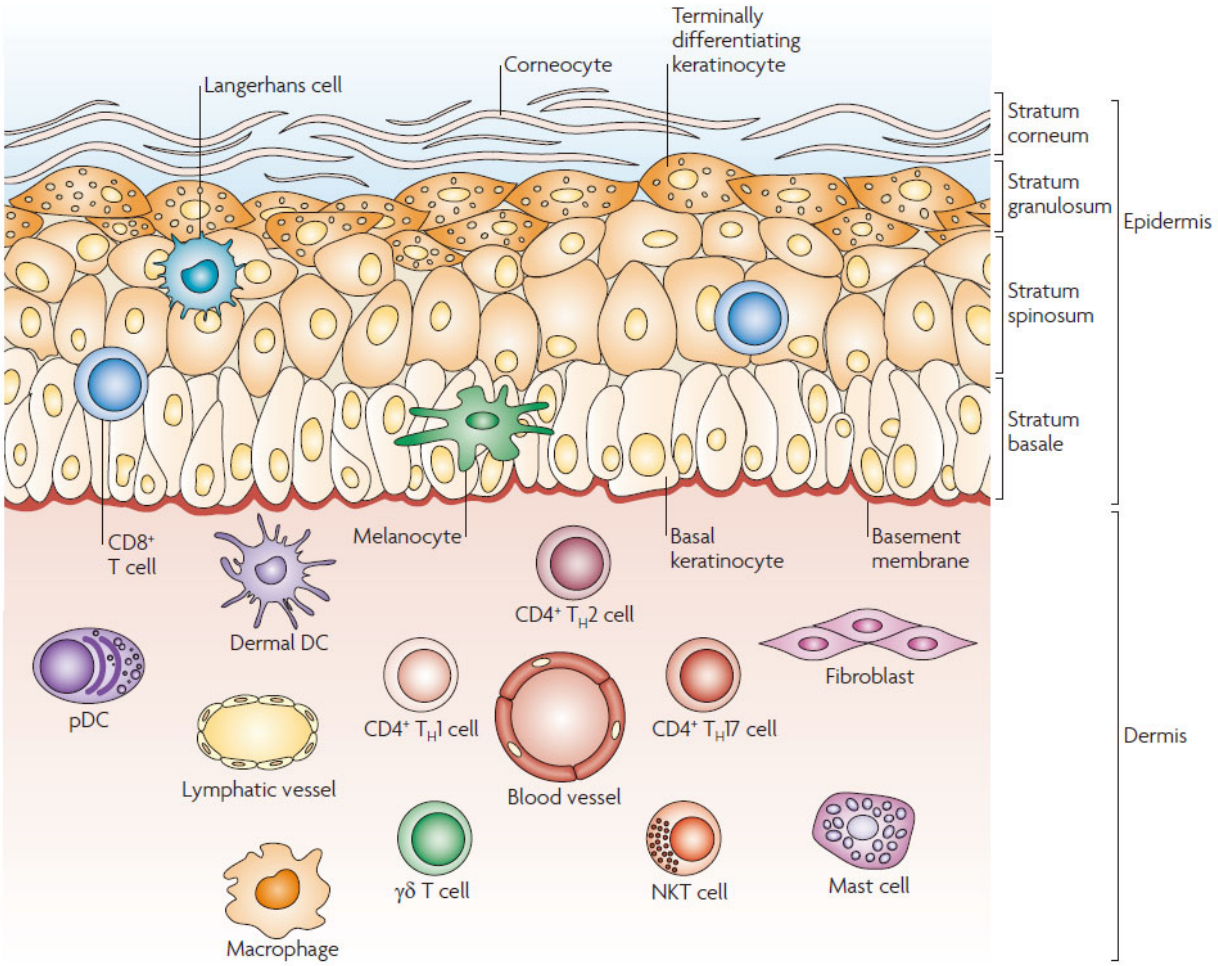


Fig. 1. Skin immune system. Skin is mainly divided into two compartments: epidermis and dermis. Keratinocytes are the main cell type in the epidermis while various cell types such as fibroblasts, dendritic cells (DCs), T cells and macrophages are present in the dermis. (Taken from FO Nestle et al., *Nature Reviews Immunology*, 2009).

skin diseases or autoimmunity. Conversely, inadequate immune response may cause infection or development of cancer. As subtle differences in skin microenvironments may result in cutaneous or even systemic defects, immune homeostasis, controlled largely by keratinocytes, is essential for healthy skin.

2. Psoriasis

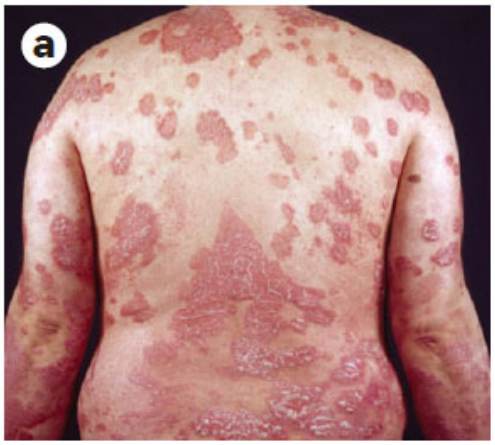
2.1. Background

Psoriasis is a chronic inflammatory skin disease characterized by red and scaly plaques with itching and burning sensations [10]. Psoriatic lesions show distinct histopathological features such as epidermal acanthosis, parakeratosis and hyperkeratosis (Fig. 2) [11]. Although psoriasis mainly affects skin, it is also associated with various comorbidities: psoriatic arthritis, metabolic syndromes and cardiovascular diseases [12]. These conditions together substantially impact the quality of life of patients along with placing them under socioeconomic and psychological burdens [13]. Psoriasis affects at least 125 million people globally, but there is no effective treatment method at present [14].

2.2. Pathobiology

Two central axes exist in the pathophysiology of psoriasis: keratinocytes/immune cells axis and IL-23/Th17 axis, although these two axes are essentially interconnected (Fig. 3). Previously, much of the focus on psoriasis research was on IL-23/Th17 axis but with the identification of keratinocyte-derived autoantigen in psoriasis, keratinocytes have been highlighted as one of the main players of the disease. It is now widely accepted that self-DNA released from the host cells forms complexes with AMP LL-37 produced from keratinocytes [15]. These complexes act as autoantigens of T cells; recent study has showed LL37-specific CD4+ and CD8+ T cells

A



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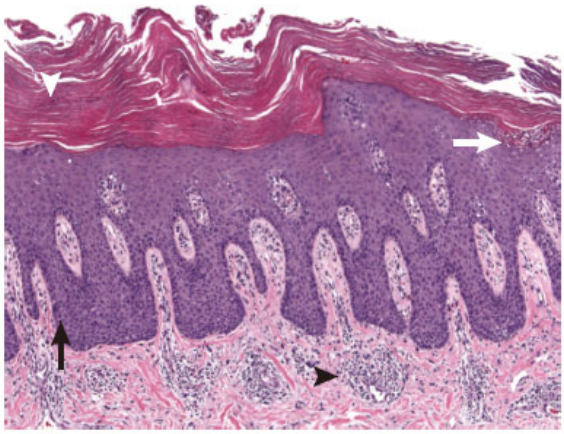
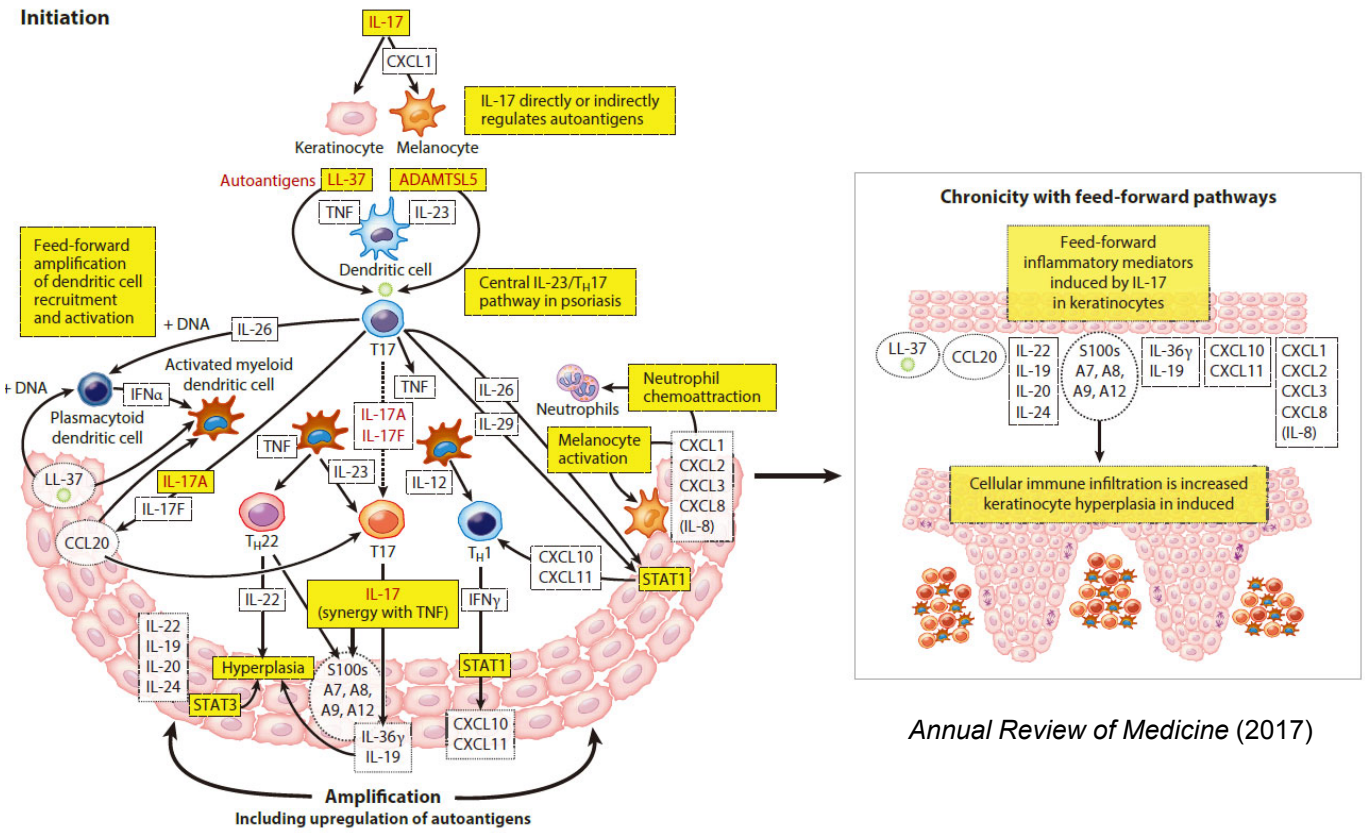


Fig. 2. Clinical manifestations of psoriasis. (A) Photo of patient with severe psoriasis. It is characterized with red and scaly plaques. (B) H&E staining of psoriatic lesion. Thickened epidermis is stained in dark purple color. Infiltration of immune cells is indicated by black arrows. (Taken from Greb et al., *Nature Reviews Disease Primer*, 2016).



Annual Review of Medicine (2017)

Fig. 3. Pathobiology of psoriasis. ***Left*: *Initiation phase of psoriasis***. Keratinocytes stimulated by pro-inflammatory cytokines release LL37 which binds to self-DNA and acts as autoantigen that induces secretion of IL-23 from dendritic cell (DCs) and subsequent differentiation of Th17 cells. Th17 cells secrete IL-17A and other cytokines which stimulate keratinocytes and lead to *Right: chronic phase of psoriasis*. The feed-forward inflammatory mediators induced by IL-17 stimulates keratinocytes, leading to production of excessive chemokines and antimicrobial peptides. (Taken from Kim and Krueger, *Annual Review of Medicine*, 2017).

were found in two-thirds of patients with moderate-to-severe psoriasis [16]. Besides LL-37, keratinocytes release other AMPs such as β-defensin 2 or S100A8/9 or chemokines, which promote chemotaxis of neutrophils [17, 18]. Some of the main inflammatory mediators released by psoriatic keratinocytes are listed in Table I.

LL-37/self-DNA complex also has the capacity to stimulate plasmacytoid DCs (pDCs), resulting in interferon-α (IFN-α) production from pDCs. This event subsequently leads to the production of interleukin 23 (IL-23) by dermal DCs and differentiation of T helper cell 17 (Th17), another central axis of psoriasis [19]. Th17 cells secrete various inflammatory mediators such as IL-17 and IL-22, which induces increased production of AMPs, chemokines by keratinocytes and hyperproliferation of keratinocytes [20]. Thus, feed-forward mechanism, constitutively amplifying inflammatory cascades between keratinocytes and immune cells, causes psoriasis to be a chronic and relapsing disease.

Genetic elements are also indispensable components in understanding the disease etiology. Genome-wide association studies (GWAS) has revealed multiple genetic loci associated with the disease and these regions are referred as Psoriasis susceptibility loci (PSORS) [21]. Genes of most of the inflammatory mediators mentioned above are all located in PSORS; for instance, single nucleotide polymorphism (SNP) of *IL-23R* gene, which plays crucial role in IL-23/Th17 pathway was found to be associated with the disease and lies in PSORS7 segment of the chromosome [22]. Gene variants of components of NF-κB signaling, the key pathway in pro-inflammatory responses, also showed high association with psoriasis [23]. These studies have clearly showed the roles of genetic elements of psoriasis and the basis of genetic predisposition of the disease.

Despite ongoing arguments on whether innate or adaptive immune system is the main culprit of the disease onset, it is certain that psoriasis is a dynamic and multifactorial disease involving interactions of various cell types and mediators, leading

Chemokine	Receptor/Role in psoriasis
CXCL1	CXCR2/neutrophil chemotaxis
CXCL3	CXCR2/monocyte, neutrophil chemotaxis
CXCL5	CXCR2/neutrophil chemotaxis
CXCL8 (IL-8)	CXCR1, CXCR2/neutrophil chemotaxis
CCL17	CCR4/T cell chemotaxis
CCL20	CCR6/lymphocyte chemotaxis
Antimicrobial peptide	**Target cells/Role in psoriasis**
β-defensin 2	CCR2/monocyte, neutrophil chemotaxis
β-defensin 3	CCR2/monocyte, neutrophil chemotaxis
CAMP (LL-37)	FPR/eosinophil, neutrophil chemotaxis CXCR2/neutrophil chemotaxis
S100A7 (Psoriasin)	RAGE/chemotaxis of CD4+ T cells, macrophages, immune cell activation
S100A8/A9	RAGE/Upregulates C3 complement, immune cell activation

Table I. Inflammatory mediators released from psoriatic keratinocytes. CXCL, CXC motif ligand; CCL, C-C motif ligand; CAMP, cathelicidin antimicrobial peptide; FPR, formyl-peptide receptor; S100 calcium binding protein; RAGE, receptor for advanced glycation end products

to disease manifestation. The rapid advances in understanding the pathophysiology of psoriasis has spurred developments of various treatment options but at the same time, more factors must be taken into consideration for designing novel agents.

2.3. Therapeutic approaches and psoriasis drug market

Currently, there is no cure for psoriasis, though various treatment options are available to manage symptoms of psoriasis and they are often used in combinations for maximal effects (Table II). Topical glucocorticoids are still the mainstay of treatment for mild-to-moderate psoriasis as they are highly-accessible, effective and relatively cheap [24]. However, due to the broad immune-suppressing effects of glucocorticoids, they can cause various side effects such as skin atrophy and infection from long-term use [25]. Vitamin D analogues such as calcipotriol are sometimes utilized in place of corticosteroids or in combination with corticosteroids but these are associated with skin irritation and hypercalcemia [26]. Phototherapy is another treatment option for the patients with large affected area. Using ultraviolet B (UVB) radiation that does not include carcinogenic wavelengths, it is administered 2-3 times per week to induce apoptosis of inflammatory cells while increasing secretion of anti-inflammatory cytokines [27, 28].

Recent advances in the understanding of psoriasis pathobiology have resulted in the development of high-specificity and high-efficacy biologics. Biologics are administered to the moderate-to-severe psoriasis patients and those who does not respond to topical agents or phototherapy any more. The most recent biologics under clinical trial are IL-23p19 antibodies, which are under phase III clinical trials and they were shown to be even more effective than IL-17 antibodies, as reflected in PASI 90 (reduction of >90% of affected area) response rates at week 12 [29, 30]. As IL-23 is the upstream cytokine that induces differentiation of Th17 cells and production of IL-17 by these cells, one can easily assume that targeting upstream molecules is a more effective

Drug	Mechanism of action	Sales ($, year)	Cost for maintenance ($, time period)
Topical agents			
Corticosteroids	Broad immunosuppressor	N/A	164 (12 months) [32]
Vitamin D analogs	Immune modulation, induces keratinocyte differentiation	N/A	296 (12 months) [32]
Biologics			
Adalinumab	Anti-TNF antibody	2.05 billion (2014)	18,224 (3 months) [33]
Infliximab	Anti-TNF antibody	214 million (2014)	22,372 (3 months) [33]
Ustekinumab	Anti IL-12 and IL-23	1.94 billion (2014)	30,336 (3 months) [33]
Secukinumab	Anti IL-17A	1.1 billion (2016)	32,823 (3 months) [33]
Others			
Phototherapy	Apoptosis of inflammatory cells, stimulates production of anti-inflammatory cytokines	N/A	5000 (3 months) [33]

Table II. Main treatment options for psoriasis. IL, interleukin; N/A, not assessed; TNF, tumor necrosis factor.

way in treating disease. However, they have been associated with increased risk of opportunistic infections and immunogenicity, not to mention the high costs [31-33]. Another drawback of biologics is the loss of efficacy after a long-term use due to generation of anti-drug antibodies, a phenomenon known as biologic fatigue. This phenomenon was observed in biologics for psoriasis as well, with the frequency up to 32% in patients who received infliximab [34].

It was estimated that around $1.6 billion to $3.2 billion is spent per year to treat psoriasis in the U.S and the psoriasis drug market is projected to reach $9.02 billion by 2019, with high-cost biologics accounting for the majority of market shares [35]. As shown in Table II, the cost of biologics are exceptionally high compared to other treatment options and consequently, it may cause substantial economic burdens on the patients [32, 33]. At present, there isn't any botanical drug approved by FDA for psoriasis, although topical ointment of *Indigo Naturalis* is under phase II clinical trial [36]. Thus, a novel topical agent that can replace corticosteroids while also being low-cost and effective may be an attractive candidate to enter the psoriasis market.

3. Interleukin 37 (IL-37)

3.1. Biology of IL-37

IL-37, formerly known as IL-1F7, is an anti-inflammatory cytokine. Until 2001, it was called IL-1F7 as it was the 7th cytokine of IL-1 family based on sequence analysis but its function has remained elusive for a long period of time [37]. In 2010, its function as a fundamental inhibitor of innate immunity was first discovered and a new name IL-37 was assigned to this cytokine [38]. After classification of IL-37 as an anti-inflammatory cytokine along with IL-10 or TGF-β, many studies have been published, emphasizing its role and potential therapeutic effects in various diseases. However, studies on the general biology of IL-37, including its mechanism of action and signaling

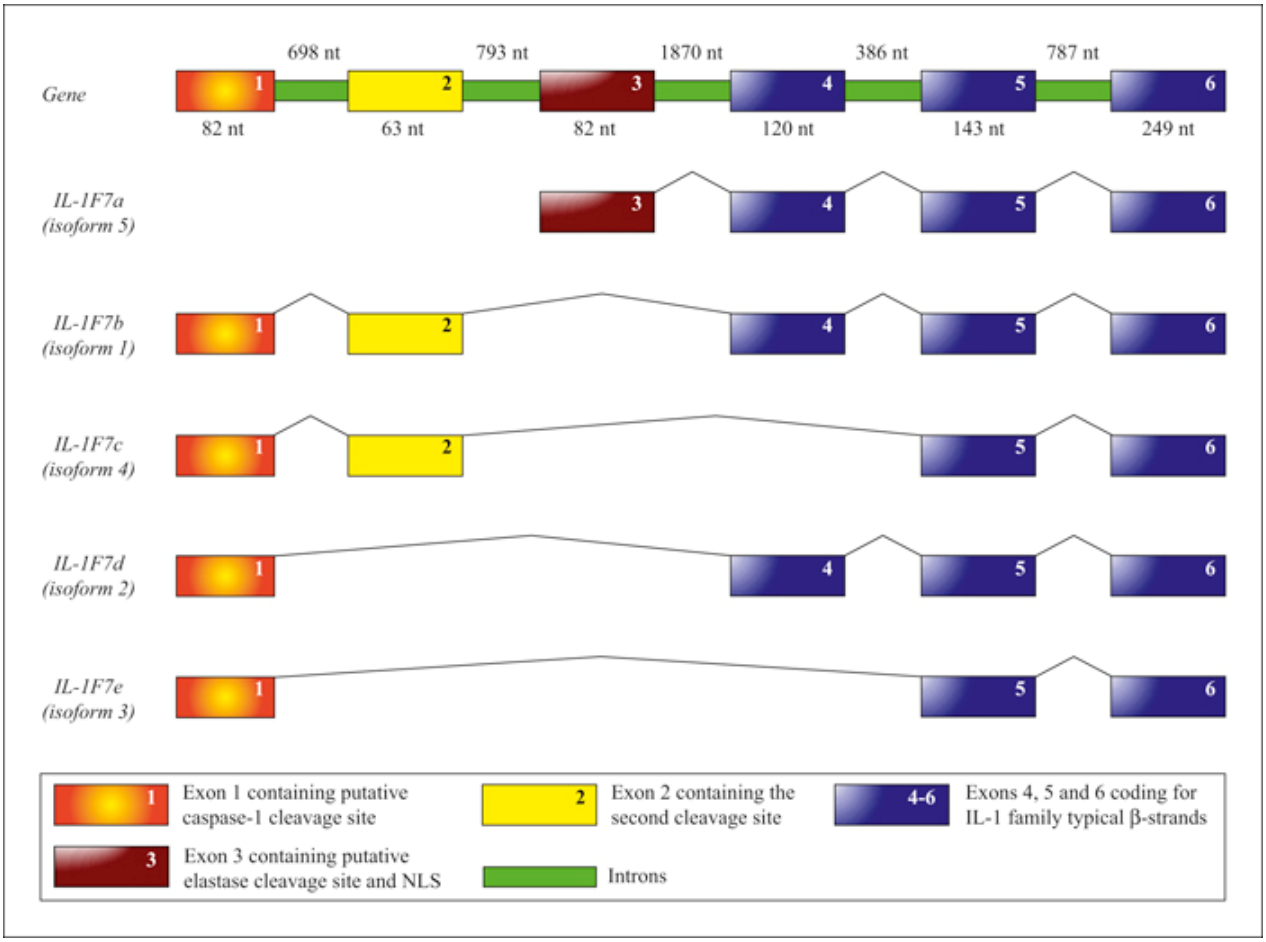


Fig. 4. Structure of IL-37. IL-37 is comprised of 6 exons which encode 5 isoforms, with IL-37b being the isoform with the largest molecular weight. IL-37 has a molecular weight of about 17~25 kDa and contains putative caspase-1 cleavage sites. Cleavage of inactive precursor activates IL-37. (Taken from Boraschi et al., *European Cytokine Network*, 2011)

pathway are limited.

IL-37 gene encodes 6 exons which is spliced into 5 different isoforms, namely IL-37a, IL-37b, IL-37c, IL-37d and IL-37e (Fig. 4) [39]. IL-37b is the isoform with the largest molecular weight and is studied most extensively as its biological function was discovered first [38]. Further studies have revealed anti-inflammatory function of IL-37a and IL-37d as well while biological function of IL-37c and IL-37e have not been discovered until today [40, 41].

IL-37 is detectable in low levels in healthy human tissues including liver, lung, bone marrow, skin, testis and colon [39, 42]. It can be induced by various inflammatory stimuli such as LPS, IL-1β, TNF as well as growth factors such as TGF-β and epidermal growth factor (EGF) [38, 43]. Upon stimulation, it is cleaved by caspase-1 and either translocates into the nucleus or secreted outside the cell [44]. In the nucleus, forms complex with Smad3 and suppresses transcription of inflammatory genes [38]. On the other hand, when mature form of IL-37 is released outside the cell, it can bind to IL-18Rα, IL-18BP to inhibit IL-18 pathway or single immunoglobulin IL-1 receptor related protein (SIGIRR) and IL-1R8 to carry out anti-inflammatory cascades [40]. Thus, IL-37 is a dual function cytokine with both intracellular and extracellular properties.

3.2. IL-37 and diseases

The role of IL-37 in anti-inflammation was initially studied in LPS-induced endotoxemia model. IL-37tg mice showed weakened inflammatory responses compared to the wild-type mice and prevented septic shock [38]. Following this study, role of IL-37 was assessed in various models of inflammatory diseases and it was shown to alleviate symptoms of asthma, atherosclerosis, inflammatory bowel disease (IBD), insulin resistance, psoriasis and rheumatoid arthritis (RA) (Table III) [45-49]. Although overexpressing IL-37 expression or adding exogenous IL-37 all showed therapeutic effects, its expression profile was different in each disease. For instance, in IBD

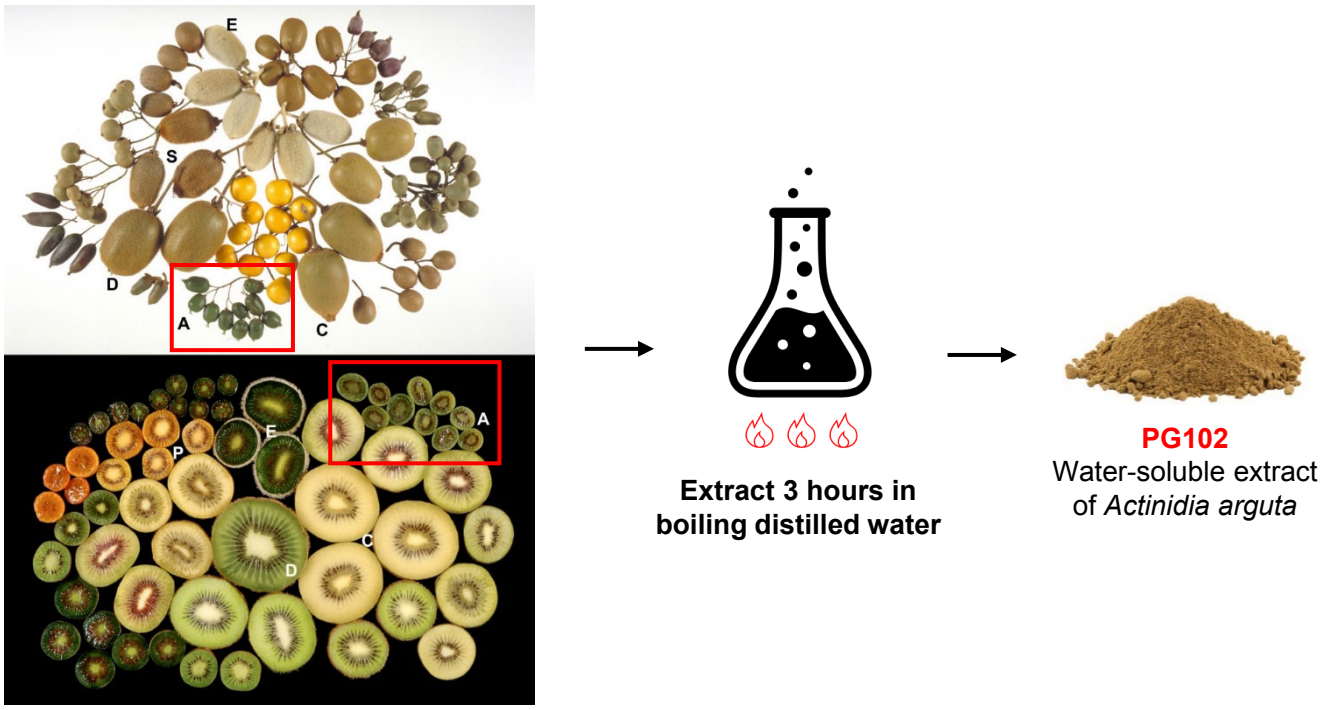
Disease	Model	Method of delivery	Effects	Reference (s)
Asthma	Ovalbumin-induced asthma	Recombinant hIL-37, intranasal	↓ IL-6, IL-1β, IL-17, TNF-α	[45]
Atherosclerosis	ApoE-deficient mice	Recombinant hIL-37, intraperitoneal	↑ Tregs, ↓ Th1, Th1	[46]
Endotoxic shock	LPS-induced shock	IL-37tg	↓ IL-6, IL-1β, IL-17, IFN-γ	[38]
Inflammatory Bowel Disease	DSS-induced colitis	IL-37tg	↓ T cells, ↑ IL-10	[48, 50]
Insulin Resistance	High-fat diet	IL-37tg	↓ IL-6, cytotoxic T cells, macrophages	[47]
Psoriasis	K14-VEGF-Tg mice	Plasmid, intravenous	↓ IFN-γ, S100A7, CXCL8, IL-6	[84]
Rheumatoid Arthritis	Collagen-induced arthritis	Adenovirus	↓ Th17 cells, IL-17, IL-6, IL-1β	[49]

Table III. Therapeutic effects of IL-37 in inflammatory diseases. LPS, lipopolysaccharide; K14, Keratin 14; VEGF, vascular endothelial growth factor; IL, interleukin; IFN, interferon; Th17, T helper 17 cell, CXCL, CXC motif ligand; S100 calcium binding protein

patients, IL-37 were highly expressed in colon of IBD patients and the expression level was positively correlated with the severity of disease [50]. Similar patterns were observed in RA patients – serum IL-37 levels were higher in RA patients than in healthy controls [51]. On the other hand, transcriptome analysis of psoriasis patient has shown that IL-37 was one of the most downregulated gene in the psoriatic lesion and serum IL-37 levels in sputum of asthmatic children were lower than those of healthy controls [52]. It can be implied that in some diseases, IL-37 is highly expressed to inhibit severe inflammation while in other diseases, its low expression may contribute to exacerbation of inflammation.

4. PG102

Compounds and extracts from plant sources have often been used to manage chronic inflammatory diseases, in place of conventional immunosuppressors as their safety profiles are well-established. *Actinidia arguta*, commonly known as hardy kiwifruit, is a widespread plant in northeastern Asia, including Siberia, northern China, Korea and Japan (Fig. 5). It is rich in flavonoids such as isoquercitrin, vitamin C and organic acids such as quinic acid [53, 54]. PG102 is a water-soluble extract of *Actinidia arguta* and has been shown to exert strong anti-oxidative and anti-inflammatory effects both *in vitro* and *in vivo*. Its extensive list of publications shows its potential as a safe and efficacious agent for various inflammatory diseases (Table IV). *In vitro*, it suppressed production of IL-4, IL-5 and IL-13 from A23187-stimulated RBL-2H3 cells while upregulating expression of anti-oxidative enzyme heme oxygenase 1 (HO-1) [55, 56]. *In vivo*, it ameliorated symptoms of asthma, spontaneous dermatitis, atopic dermatitis, food allergy and general allergy in respective animal models [55, 57-61]. In addition, a double-blind, randomized exploratory clinical study with 90 patients with atopy (serum IgE > 300 IU) proved both its efficacy and safety in human subjects as



A: Hardy kiwifruit, *A. arguta*
C: Golden kiwifruit, *A. chinensis*
D: Common kiwifruit, *A. deliciosa*

Fig. 5. Preparation of PG102. PG102 is a water-soluble extract prepared from edible part of *Actinidia arguta*, which is commonly known as hardy kiwifruit. (Taken from Crowhurst et al., *BMC Genomics*, 2008).

Disease	Model	Effects	Reference (s)
General allergy	OVA-sensitized mice	↑Th1, ↓Th2	[60]
Spontaneous Dermatitis	Nc/Nga mice	↓ IgE, IL-2, ↑IL-12, IFN-γ	[58]
Dermatitis	Mg-deficient hairless rat	↓ IL-4, IL-10, NO	[61]
Asthma	OVA-induced asthma	↓ IgE, IL-4, IL-5, ↑TGF-β	[55]
Food allergy	OVA-induced food allergy	↓ IL-6, MCP-1	[59]
Atopic dermatitis (AD)	House-dust mite induced AD	↑ Treg, ↓IL-4	[57]
Atopy	Human clinical study	↓ Serum IgE	[62]

Table IV. Summary of previously observed ameliorating effects of PG102 in various inflammatory diseases. OVA, ovalbumin; Th, T helper cell; IgE, immunoglobulin E; NO, nitric oxide; MCP, monocyte chemoattractant protein; TGF, transforming growth factor.

well [62].

5. Rationale and the purpose of this study

Since psoriasis is a chronic and recurring disease, safer and still effective therapeutics that can be used for long-term are in need. Based on previous reports on PG102 showing its potent anti-inflammatory effects on various cell types and skin disease models, it was speculated that it might possess ameliorating effects on psoriasis as well. In this thesis work, I initially tested the effects of PG102 on murine model of psoriasis and showed that topical application PG102 could suppress increase in epidermal thickness and biochemical parameters of skin inflammation. Based on these observations, I further investigated molecular mechanisms underlying anti-inflammatory effects of psoriasis in HaCaT keratinocyte cell line as keratinocytes might be the main effector cells responding to topical application of psoriasis.

Anti-inflammatory effects of many natural compounds or botanical drugs can be achieved directly suppressing pro-inflammatory signaling pathways and indirectly by upregulating anti-inflammatory molecule. While screening genes affected by treatment with PG102, I have observed upregulation of IL-37, a recently discovered anti-inflammatory cytokine. As many studies have shown beneficial effects of this cytokine in the context of inflammation, I investigated the signaling pathways involved in the upregulation of IL-37 by PG102 in HaCaT cells.

Finally, fractionation of PG102 was performed in an effort to identify active fraction with concentrated bioactivity of PG102. Anti-inflammatory effects of total PG102 and 4 other fractions were tested *in vivo* psoriasis model and *in vitro* cell culture system. My thesis research focused on understanding the molecular mechanisms of PG102 underlying its anti-inflammatory effects as well as the relationship between its chemical composition and bioactivities.

Chapter II

Materials and Methods

1. Cell culture and reagents

1.1. Preparation of PG102

Hardy kiwifruit *Actinidia arguta* was purchased from a company specializing in this fruit (Hurst's Berry Farm, McMinnville, OR) and was identified by Plant DNA Bank in Korea (Seoul, Korea). PG102 was prepared as previously described [55, 60]. Briefly, the dried fruit was extracted in boiling distilled water for 3 hours, followed by filtration (no. 2; 110 mm, Whatman International Ltd., Kent, UK). The filtered extract was concentrated by rotary evaporator and lyophilized. This extract was designated as PG102 and its quality was controlled as previously described [55, 60]. Further quality control was employed by measuring the ability of PG102 to suppress IL-8 production in cytokine-stimulated HaCaT cells and IC_{50} value was compared to that of the reference batch. PG102 (batch #2) stocks were prepared at a concentration of 200 mg/ml in distilled water (DW), stored at -80°C and used throughout this study.

1.2. Chemical reagents

M5 cytokine mix was made by combining recombinant human TNF-α, Oncostatin M, IL-1α, IL-17A and IL-22, purchased from BioLegend (San Diego, CA). ERK inhibitor U0126, p38 inhibitor SB and Smad3 inhibitor SIS3 were obtained from Selleckchem (Houston, TX). Chemical inhibitor stocks were prepared at 50 mM and for all of the experiments, the concentration of DMSO in the cell cultures were lower than 0.1%.

1.3. HaCaT cell culture

Human keratinocyte cell line HaCaT was purchased from CLS Cell Lines Service GmbH (Eppelheim, Germany) and cultured in Dulbecco's modified Eagle's medium (Thermo Fisher, Waltham, MA) supplemented with 10% fetal bovine serum (FBS; Corning, Corning, NY) and antibiotics (100 U/mL penicillin and 100 mg/mL

streptomycin) at 37°C in a 5% CO_2 humidified incubator. Cells at passage 3 to 5 were used throughout the experiment.

1.4. HL-60 cell culture

Human promyelocytic cell line HL-60 was purchased from American Type Culture Collection (ATCC; Manassas, VA) and cultured in Iscove's Modified Dulbecco's Medium (IMDM; ATCC) supplemented with 20% FBS and antibiotics (1% penicillin/streptomycin) at 37°C in a 5% CO_2 humidified incubator.

2. Molecular techniques

2.1. Measurement of cell proliferation

Cells were seeded at a density of 5 x 10^4 cells/well in 24-well cell culture plates overnight (n=3). Cells were then incubated with M5 and PG102 at designated concentrations for 24 and 48 hours. After incubation, cell proliferation was assessed by CellVia WST-1 assay according to the manufacturer's protocol (Young In Frontier, Seoul, Korea).

2.2. RNA extraction and quantitative real-time polymerase chain reaction (RT-PCR)

Cells were seeded at a density of 2 x 10^5 cells/well in 12-well cell culture plates overnight (n=3) and then treated with M5 and PG102 at designated concentrations for designated time periods. Total RNA was isolated from cells using RNAiso (Takara Bio, Shiga, Japan) according to the manufacturer's protocol and complementary DNA (cDNA) was synthesized using AMV reverse transcriptase (Takara Bio) and oligo dT primers (Qiagen, Valencia, CA). Quantitative RT-PCR of each cDNA was performed using SYBR Premix Ex TaqTM (Takara Bio) and Thermal Cycler Dice Real Time System (Takara Bio) with the following protocol: 30 sec at 95 °C, followed by 40 cycles of 5 sec at 95 °C and 30 sec at 60°C. RNA levels were normalized by the level of HPRT

and the relative changes in gene expression were calculated as $2^{-\Delta\Delta Ct}$ method. Primer sequences used in this study are as follows:

Gene	Forward (5'-3')	Reverse (5'-3')
hBD-2	GGTGTTTTTGGTGGTATAGGC	AGGGCAAAAGACTGGATGACA
hBD-3	TGAAGCCTAGCAGCTATGAGGATC	CCGCCTCTGACTCTGCAATAA
hCXCL1	AATCCTGCATCCCCCATA	TGTCTCTCTTTCCTCTTCTGTTCCT
hCXCL5	TGCGTTGCGTTTGTTTACAG	GAAAAGGGGCTTCTGGATCA
hCXCL8 (IL-8)	ATGACTTCCAAGCTGGCCGTG	TTATGAATTCTCAGCCCTCTTCAAAAACTTCTC
hHPRT	TATGGCGACCCGCAGCCCT	CATCTCGAGCAAGACGTTCAG
hIL-37	GGACAAAGTCATCCATCCCTTC	GAGCCCACCTGAGCCCTATAA
hS100A8	GGGAATTTCCATGCCGTCT	CCTTTTTCCTGATATACTGAGGAC
hS100A9	CAGCTGGAACGCAACATAGA	TCAGCTGCTTGTCTGCATTT
mCXCL1	CACCTCAAGAACATCCAGAGCT	CAAGCAGAACTGAACTACCATCG
mDEFB4	GCTTCAGTCATGAGGATCCAT	CTTGCTGGTTCTTCGTCTTTT
mHPRT	CACAGGACTAGAACACCTGC	GCTGGTGAAAAGGACCTCT
mIL-17F	ACCCGTGAAACAGCCATGGTCAAG	CCCATGGGGAACTGGAGCGG
mMIP-2	TTGCCTTGACCCTGAAGCCCCC	GGCACATCAGGTACGATCCAGGC
mS100A8	AAATCACCATGCCCTCTACAAG	CCCACTTTTATCACCATCGCAA
mS100A9	ATACTCTAGGAAGGAAGGACACC	TCCATGATGTCATTTATGAGGGC

2.3. Enzyme-linked immunosorbent assay (ELISA)

Mouse IL-17A, human S100A8/A9 heterodimer, CXCL1, CXCL5 and IL-8 (R&D Systems, Minneapolis, MN) and β-defensin 2 (Peprotech, Rocky Hill, NJ) in cell culture supernatants were measured by using commercially available enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's instructions.

2.4. Western blot analysis

Cells were seeded at a density of 1 x 10^6 cells/plate on 60 mm cell culture dish or 4.5 x 105 cells/plate on 6-well cell culture plate overnight. Cells were incubated with M5 or PG102 at designated concentrations for designated time periods and total cell lysates were extracted with CytoBuster™ (Merck, Darmstadt, Germany) mixed with PhosSTOP™ and cOmplete™ Protease Inhibitor Cocktail (Roche, Basel, Switzerland). Total protein contents in the cell lysates were determined by BCA assay Kit and after reconstituting in sample buffer, 10 micrograms of protein samples were subjected to SDS-PAGE on Bolt™ 10% Bis-Tris Plus Gels (Invitrogen). Proteins were transferred onto a PVDF membrane (Merck) and the membrane was incubated in 5% skim milk in 0.1% TBST at room temperature for 1 hour to block nonspecific binding. The membrane was then incubated with antibodies specific for phospho-STAT3 (#9134, #9145), STAT1 (#9167), p65 (#3033), and STAT3 (#4904), STAT1 (#9172), p65 (#8242), IκB-α (#9242) (1:1000; Cell Signaling Technology, Danvers, MA) and β-actin (A5441; Sigma-Aldrich) overnight at 4°C followed by incubation with horseradish peroxidase-conjugated secondary anti-mouse or anti-rabbit IgG (1:100,000; Sigma-Aldrich) at room temperature for 1 hour. The blot was developed by Immobilon ECL HRP substrate (Merck) and visualized by exposure to autoradiography film.

2.5. Neutrophil chemotaxis assay

To differentiate HL-60, cells were cultured in medium containing 1.3% DMSO for 5 days, as previously reported [63]. Differentiated HL-60 (dHL-60) cells were washed twice with serum free medium and chemotaxis assays were performed using 3 μm CytoSelect™ Cell Migration Assay Kit (Cell Biolabs, San Diego, CA), according to the manufacturer's instructions. Briefly, 2 x 10^5 cells/well were seeded onto the upper membrane chamber and cell culture supernatants from HaCaT cells incubated with M5 and various concentrations of PG102 were added to the bottom wells. After 2 hours, migrated cells in the bottom wells were lysed and fluorescence was read at 480 nm/520

nm using FlexStation 3 microplate reader (Molecular Devices, San Jose, CA).

2.6. siRNA-mediated gene knockdown

For siRNA-mediated knockdown of *IL37*, 5 x 10^4 cells (n=3) were seeded onto 12-well plate overnight. After replacement of culture medium, Silencer Select control siRNA and IL37 siRNA (ThermoFisher) were added with RNAiMAX transfection reagent (Invitrogen, Waltham MA), followed by 48 hours incubation. Cells were washed with PBS and incubated with cytokines for 24 hours for further analysis.

2.7. Immunofluorescence staining

HaCaT cells were seeded in 4-well Lab-Tek II Chamber Sliders (Nunc, Rochester, NY) at a density of 5 x 104 cells/well overnight. Thereafter, cells were incubated with PG102 for designated time points, followed by cold phosphate-buffered saline (PBS) wash and fixation with 4% paraformaldehyde. Each slide was blocked in 5% donkey serum and 10% FBS for 1 hour at room temperature. The slides were then incubated with antibodies specific to IL-37 (1:200, ThermoFisher) and phospho-Smad3 (1:200, Abcam) overnight at 4°C. This was followed by 1 hour incubation with the respective secondary antibodies and mounted with Vectashield 4',6-diamidino-2-phenylindole (DAPI) mounting medium. Digital confocal imaging was performed and analyzed using Carl Zeiss LSM 700 confocal microscope and software.

2.8. Statistical analysis

The data are presented as the mean ± standard deviation (SD) of triplicate measurements for *in vitro* experiments and mean ± standard error of the mean (SEM) for *in vivo* experiments. Each experiment was repeated at least three times, independently. Data analysis was performed using the GraphPad Prism version 6.0 (GraphPad Software, San Diego, CA). Comparisons with other experimental groups

were analyzed by either one-way analysis of variance (ANOVA) followed by the Bonferroni post-hoc test. P-values less than 0.05 were considered statistically significant.

3. Animal Studies

3.1. Mice and treatment

7-week-old female BALB/c mice were obtained from Samtako Bio Korea (Osan, Korea) and housed at 23±2 °C with a 12-h light/dark cycle and free access to standard chow and water. 3 days prior to treatment, back skin of mice were shaved using electronic shaver and Niclean shaving cream (Ildong Pharmaceuticals, Korea). Mice were topically administered with 62.5 mg of 5% Imiquimod (IMQ) cream (Aldara; 3M Health Care Limited, Loughborough, UK) along with 50 μl of dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO) or different fractions of PG102 reconstituted in DMSO (100 mg/kg) for 6 consecutive days. The control mice were treated with DMSO only. Mice were sacrificed and skin tissue samples, lymph nodes and blood were collected for further analysis. All experimental procedures were performed in compliance with the guidelines set by the Seoul National University Institutional Animal Care and Use Committee (Approval Number: SNU-150923-1-2).

3.2. Scoring severity of skin inflammation

Erythema and scaling were scored blindly based on the clinical Psoriasis Area and Severity Index (PASI). Photos of back skin taken at day 6 were distributed randomly to three people for scoring erythema and scaling on a scale from 0 to 4: 0, none; 1, slight; 2, moderate; 3, marked; 4, very marked and the scores were averaged. The level of erythema was scored using a scoring table with red taints. Back skin thickness was measured every day, from day 0 before treatment, and on day 6, before

sacrificing mice using a micrometer (Mitutoyo, Japan). The cumulative score (erythema plus scaling plus thickening) served as a measure of the severity of inflammation.

3.3. Cell isolation and stimulation

Axillary and inguinal lymph nodes were aseptically removed from each mouse. Lymph nodes in each experimental groups were pooled and single cell suspensions from lymph nodes were obtained as previously described [64]. Isolated cells were cultured at 1 x 10^6 cells/well with 50 ng/ml phorbol 12-myristate 13-acetate (PMA) and 500 ng/ml ionomycin (Sigma-Aldrich) and after 24 hours, culture supernatants were collected for further analysis.

3.4. Tissue preparation and immunohistochemistry (IHC)

Back skin tissues of mice were cut and kept in RNAlater™ Stabilization Solution (Invitrogen, Waltham, MA) until use for total RNA extraction or fixed with 10% neutral buffered formalin (Sigma-Aldrich) for at least 24 hours and staining was performed in Korean Pathology Technical Center (KP&T, Cheong-ju, Korea). Briefly, samples were embedded in paraffin and sectioned into 3 μm-thick sections, followed by staining with hematoxylin-eosin (H&E). For immunohistochemistry (IHC), sections were incubated with anti-mouse Ly6G antibody (1:1000; Abcam, Cambridge, UK) followed by incubation with biotinylated secondary antibody and developed with 3, 3'-diaminobenzidine (DAB). Samples were analyzed with microscope (Olympus, Tokyo, Japan) and random fields were acquired for each slide.

4. Fractionation

PG102, prepared as indicated in 1.1, was dissolved at 1:50 ratio by mass in distilled water (4 g PG102 in 200 ml DW). PG102 in DW was added to separatory

funnel and equal volume of water-saturated butanol. The mixture was shaken vigorously and the funnel was left at room temperature overnight for phase separation. The top layer (water fraction) was collected and the bottom layer (n-butanol fraction) was further fractionated serially using chloroform and ethyl acetate (EA). Each fraction was subjected to concentration using rotary evaporator and stock solutions in DMSO were prepared for further use.

Chapter III

Molecular characterization of anti-psoriatic effects of PG102

1. Background

Psoriasis is a chronic inflammatory disease with complex etiology involving multiple factors. Recent developments of single-target therapies such as IL-17A monoclonal antibody and IL-23 monoclonal antibody are shown to be effective in managing psoriasis yet the compliance rate is generally low and cause tremendous cost burdens [65, 66]. Because multiple mediators are involved in psoriasis, broad immunosuppressors such as topical glucocorticoids are still the mainstay of treatment for psoriasis as they are effective, highly accessible and cheap yet they are associated with various side effects [25]. Current treatment methods are limited and there is a strong need for the development of safer and efficacious agents.

PG102 has been shown to contain strong anti-inflammatory and anti-oxidative activities, alleviating symptoms of spontaneous dermatitis and atopic dermatitis in respective animal models by regulating the expression of chemokines and cytokines and by promoting differentiation of regulatory T cells [55, 57, 60]. Its efficacy and safety were not limited to animal studies – exploratory human clinical study involving 90 asymptomatic subjects with atopy (serum total IgE > 300 IU/mL) had shown its potential as a safe immunosuppressive agent [62]. However, its effects on IMQ-induced psoriasis-like skin inflammation (IPI) model and keratinocyte cell line have not been studied. In this chapter, the effects of PG102 on murine IPI model and the molecular mechanism of anti-inflammatory effects of PG102 were investigated.

2. Results

2.1. Standardization and establishment of in vitro bioassay system of PG102

To establish batch-to-batch consistency of extracts prepared from *A. arguta*, its quality has been standardized as described previously [55, 60]. First, the contents of two marker compounds citric acid and quinic acid were quantified using high performance

liquid chromatography (HPLC) (Fig. 6). Only the extracts containing these compounds within standard range (19.0 ~ 29.0 mg/g for citric acid; 14.0 ~ 22.0 mg/g for quinic acid) were chosen. As shown in Fig. 7, two different batches of PG102 were compared with the reference batch. Since batch 3 contained citric acid and quinic acid out of standard range, it has been discarded.

In addition, a cell-based bioassay was employed using IL-8, which is a more relevant biomarker of psoriasis than IL-4 [67]. Keratinocyte cell line HaCaT cells were stimulated with a cytokine mixture called M5 (consisting of IL-1α, IL-17A, IL-22, TNF-α and Oncostatin M) to mimic conditions of psoriatic keratinocytes [68]. When HaCaT cells were treated with M5 and PG102, IL-8 level in the supernatant was decreased in a dose-dependent manner by PG102. The half maximal inhibitory concentration IC50 of batch 2 was 0.987 mg/ml, similar to that of the reference batch (1.16 mg/ml) (Fig. 7). Although the IC50 value of batch 3 was not significantly different from that of the reference batch, it was not used as its contents of marker compounds did not fall within the standard range. All batches of PG102 did not have any cytotoxic effects in any of the concentrations used in these experiments (Fig. 8). The contents of marker compounds citric acid and quinic acid of PG102 and IC50 values of many different batches of PG102 had been remarkably similar. The use of two quality control assays ensured not only its batch-to-batch chemical composition but also its biological activities.

2.2. Anti-psoriatic effects of PG102 in Imiquimod (IMQ)-induced psoriasis-like skin inflammation (IPI) model

2.2.1. Establishment of route of administration, vehicle and concentration for PG102 in vivo

The effects of PG102 were investigated in the IMQ-induced psoriasis-like skin inflammation model. Aldara cream, containing TLR7 agonist IMQ, has been used to

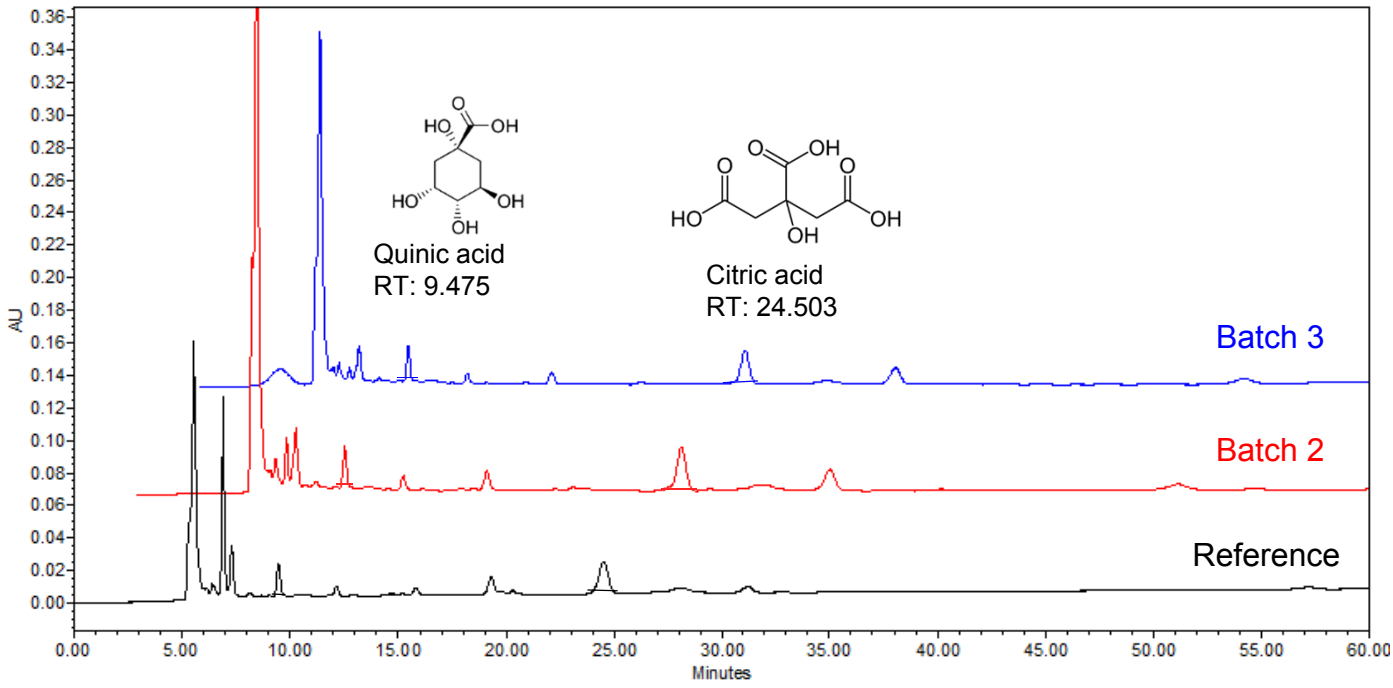
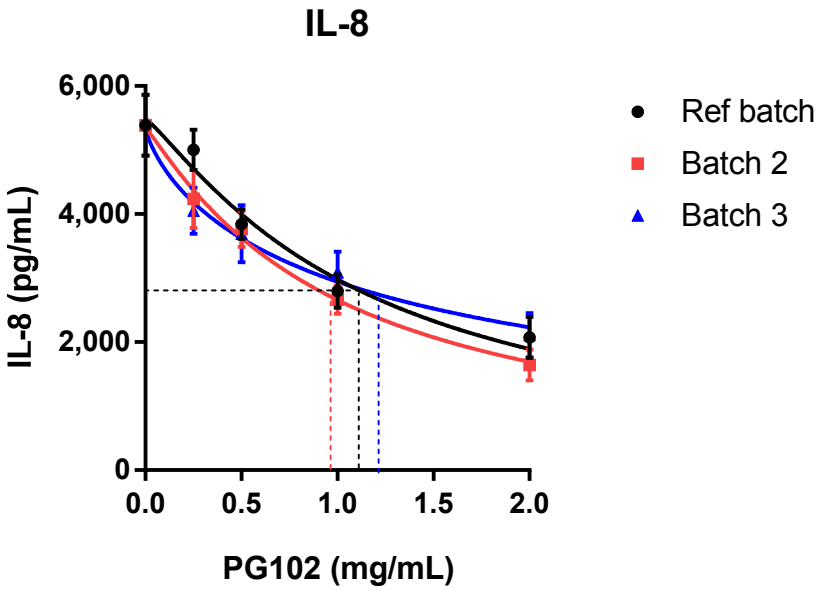


Fig. 6. HPLC fingerprint of PG102. Marker compounds quinic acid (retention time: 9.475 min) and citric acid (retention time: 24.503 min) in the reference batch, batch 2 and batch 3 were quantified by HPLC.



Batch No.	Citric acid (mg/g)	Quinic acid (mg/g)	IC_{50} (mg/ml)
Ref	21.51	14.12	1.16
2	26.93	16.15	0.987
3	25.31	33.69	1.29

Fig. 7. Quality control of PG102. Different batches of PG102 were subjected to IL-8 bioassay in HaCaT cells and the contents of two marker compounds, citric acid and quinic acid, were quantified. IC_{50} value of each batch was calculated using GraphPad Prism Software.

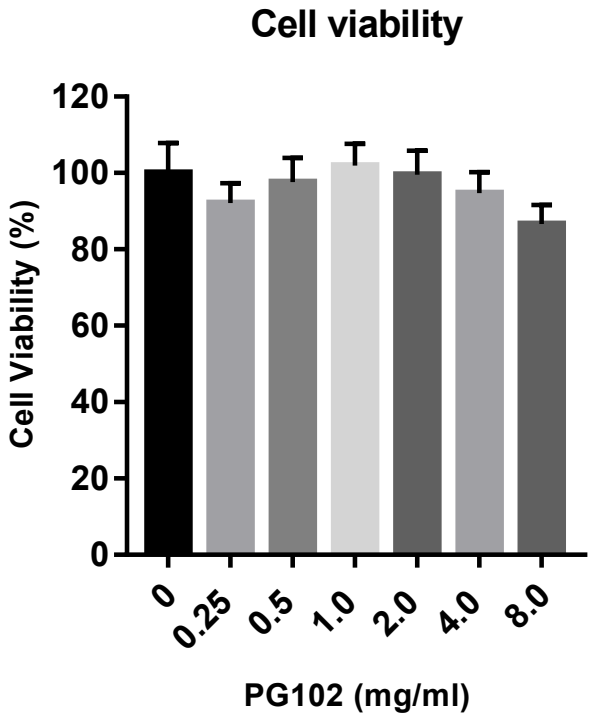


Fig. 8. Effects of PG102 on cell viability of HaCaT cells. HaCaT cells were treated with PG102 of various concentrations for 48 hours and cell viability was measured by WST-1 assay.

induce psoriasis-like inflammation characterized by increase in epidermal thickness, erythema and scaling [69]. In order to test the most efficient route of administration for PG102 in this model, PG102 and positive control dexamethasone were administered either topically or orally to IMQ-treated mice. As shown in Fig. 9, vehicle group showed increase in skin thickness, erythema and scaling, as depicted in both photo and PASI score. Topical application of PG102 ameliorated symptoms of psoriasis, although it was not statistically significant while topical application of Dex significantly reduced PASI score. On the other hand, oral administration of both PG102 and Dex did not affect clinical symptoms of psoriasis. These results suggest that due to the acute and local inflammation induced by IMQ, oral administration of drug was not effective in ameliorating the clinical symptoms of disease while topical application of PG102 can relieve symptoms of psoriasis.

Next, in an attempt to find the most efficient vehicle for topically delivering PG102, PG102 was dissolved in three different types of solvents conventionally used for topical administration of drugs – triple distilled water (TDW), dimethyl sulfoxinate (DMSO) and mixture of polyethylene glycol and ethanol (PEG + EtOH). As shown in Fig. 10A, PG102 dissolved in PEG + EtOH showed precipitation while in DMSO, PG102 was completely dissolved. HPLC analysis of PG102 prepared in different solvents clearly showed that the constituents of PG102 were dissolved more effectively in DMSO compared to TDW or PEG + EtOH, as indicated by the area of each peak in chromatogram (Fig. 10B). Thus, DMSO was used as the vehicle to deliver PG102 for further experiments.

2.2.2. Establishment of optimal concentration for topical application of PG102

To determine the optimal concentration for topical application of PG102 in IPI model, three different concentrations – 100 mg/kg, 200 mg/kg, and 400 mg/kg – of PG102 were applied to mice and the mice were subjected to PASI scoring. As shown in

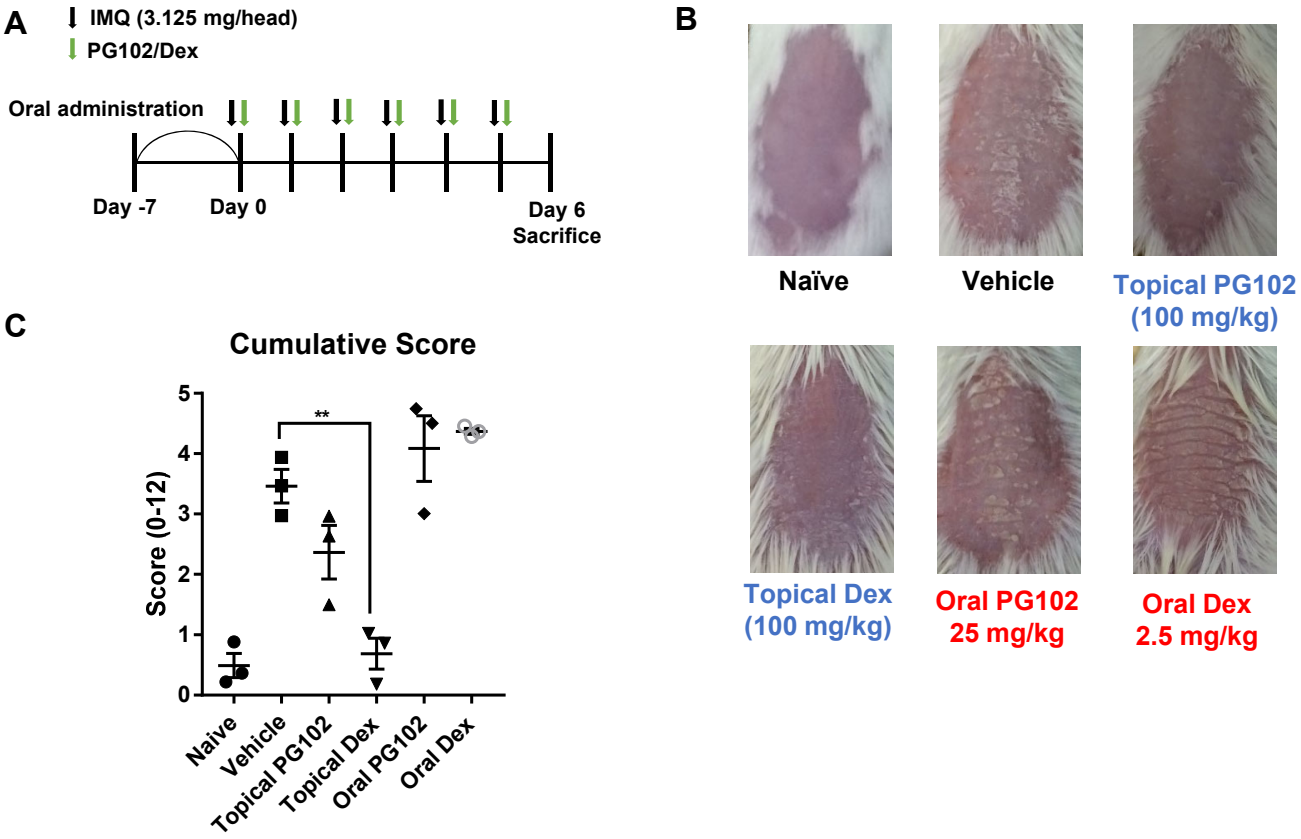


Fig. 9. Comparison of topical and oral administration of PG102 in IMQ-induced psoriasis-like skin inflammation (IPI) model. Mice were treated with IMQ and PG102 or Dex for 6 consecutive days (n=3). On day 6, mice were sacrificed and PASI score was calculated. Oral administration was initiated 7 days before the first IMQ treatment. (A) Experimental scheme (B) Photo of dorsal skin (C) Cumulative PASI score. ** $p<0.01$. The data are shown as the mean ± standard error mean (SEM).

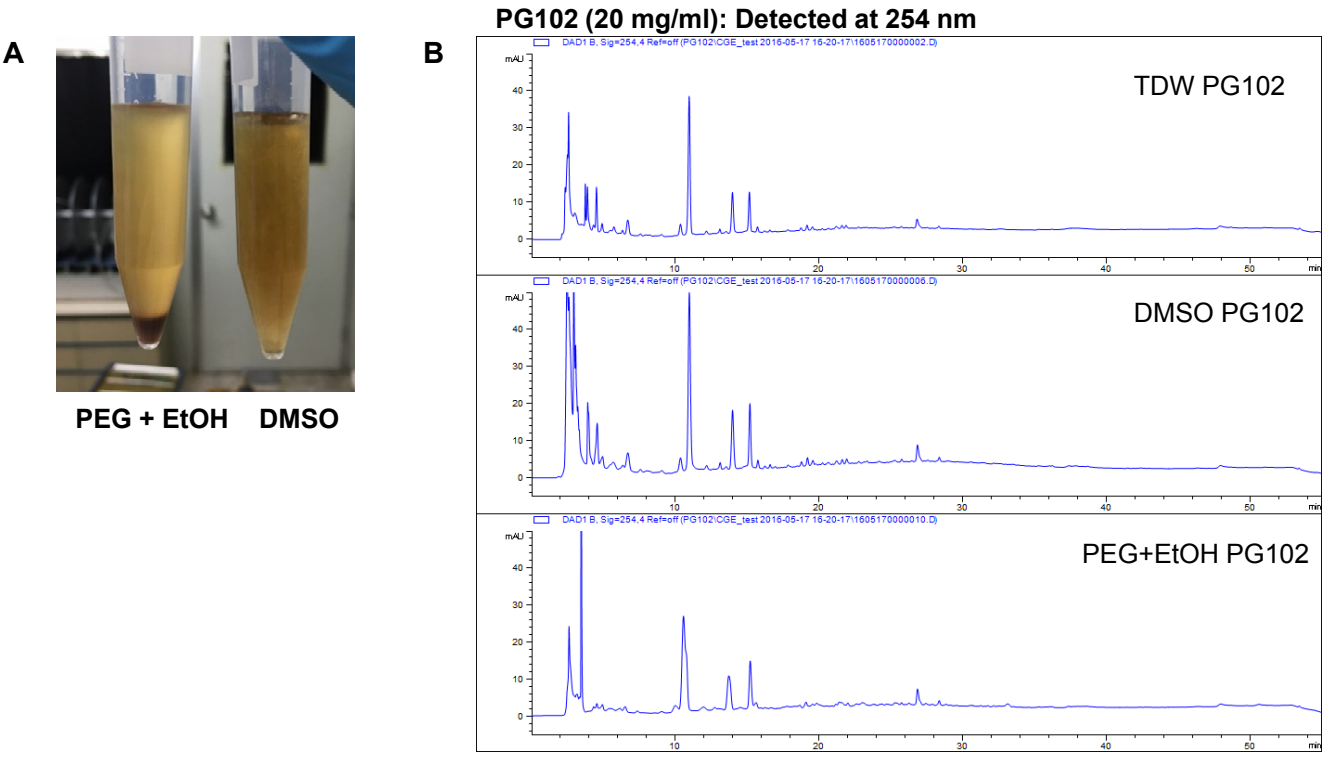


Fig. 10. Comparison of PG102 dissolved in different solvents. (A) Photo of PG102 dissolved in PEG+EtOH and DMSO. (B) HPLC fingerprint of PG102 dissolved in TDW, DMSO and PEG+EtOH.

Fig. 11, all concentrations of PG102 effectively alleviated symptoms of psoriasis, with PG102 at 400 mg/kg showing marked reduction in scaling. This was also seen in cumulative PASI score and only PG102 at 400 mg/kg showed statistically significant reduction in the PASI score when compared to the vehicle group (Fig. 11C). Yet, the differences between 100 mg/kg and 200 mg/kg or 200 mg/kg and 400 mg/kg were not significant, implying that increasing concentrations from 100 mg/kg might not show dose-dependency. Thus, in the next set of experiment, 25 mg/kg, a concentration lower than 100 mg/kg, was tested. As Fig. 12 shows, both 25 mg/kg and 100 mg/kg showed effective amelioration of symptoms of psoriasis. When the mice in each group were subjected to PASI scoring, 100 mg/kg group exhibited lower PASI scores than 25 mg/kg group, although the difference between these two groups was not statistically significant. From these data, it could be concluded that 100 mg/kg, among other concentrations, may be the optimal concentration for topical application of PG102 in IPI model.

2.2.3 Effects of PG102 on clinical parameters of IPI model

Effects of PG102 on IPI, other than PASI scoring, were assessed with the experimental scheme shown in Fig. 13A. Topical application of PG102 for six consecutive days improved clinical scores of psoriasis, mainly that of skin thickness (Fig. 13B and C). H&E staining of the back skin clearly shows reduced epidermal thickness compared to the vehicle group (Fig. 14). The levels of IL-17A from cells isolated from draining lymph nodes (dLN) and serum were decreased in PG102-treated mice (Fig. 15). Treatment with the positive control, dexamethasone, showed significant suppression of inflammatory responses but exerted severe adverse effects such as skin atrophy and weight loss (Fig. 13C and Fig. 16). These results suggest that topical treatment with PG102 could alleviate both local and systemic inflammation in IMQ-induced psoriasis-like inflammation of mice without causing adverse effects.

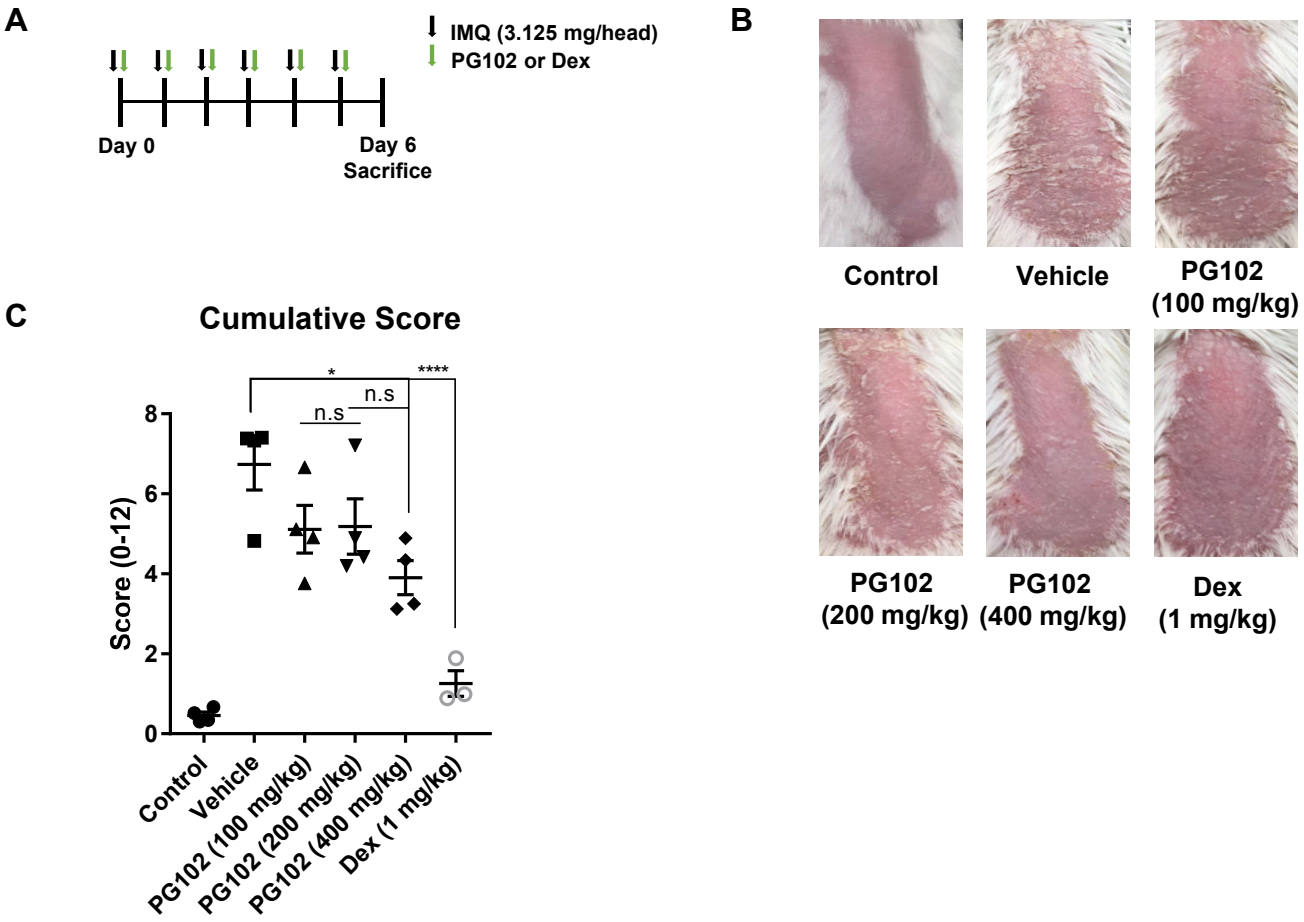


Fig. 11. Effects of different concentrations of PG102 on IPI model. Mice were treated with IMQ and PG102 or Dex for 6 consecutive days (n=4). On day 6, mice were sacrificed and PASI score was calculated. (A) Experimental scheme (B) Photo of dorsal skin (C) Cumulative PASI score. * $p<0.05$, **** $p<0.0001$. The data are shown as the mean ± standard error mean (SEM).

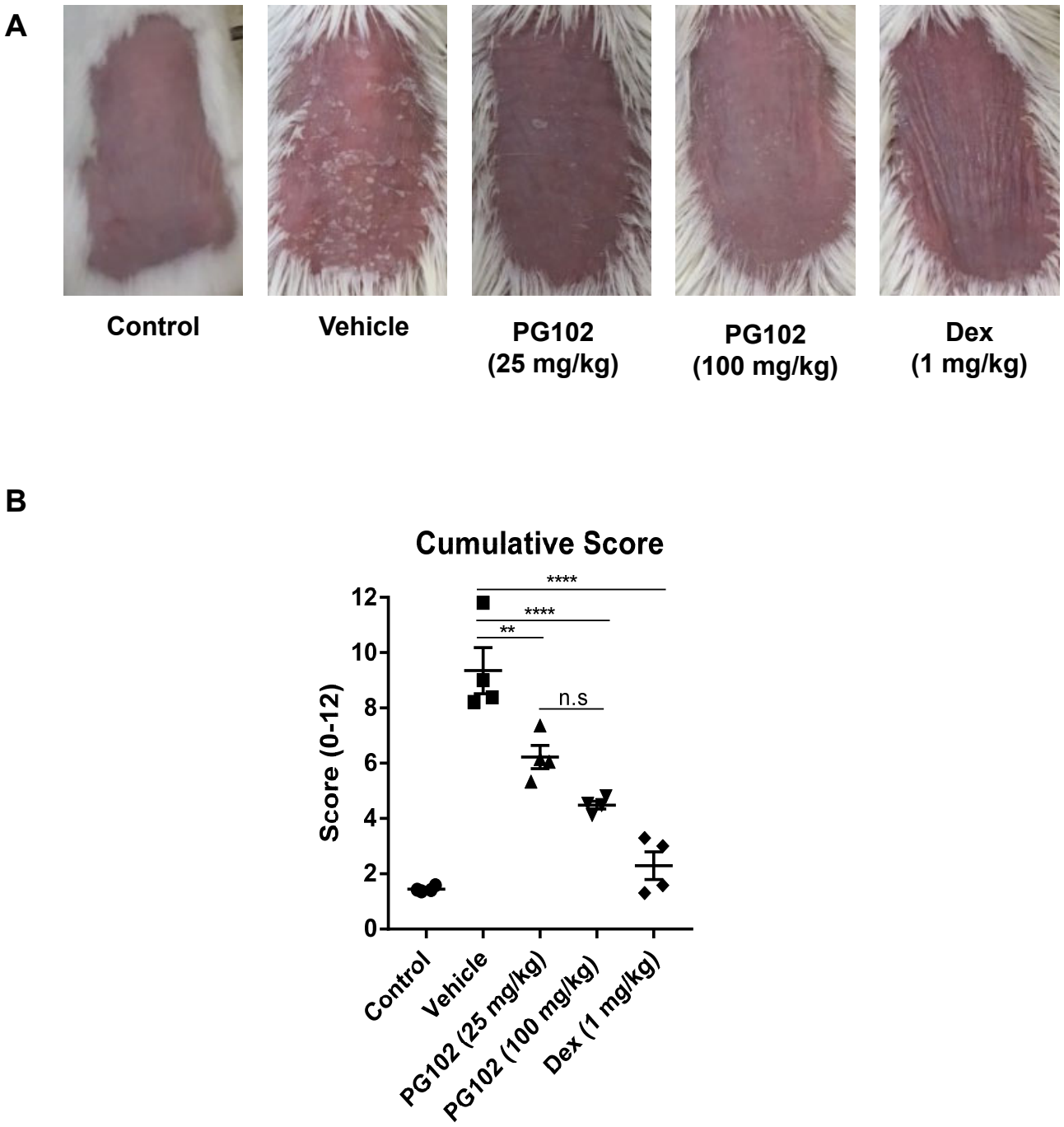


Fig. 12. Effects of two different concentrations of PG102 on IPI model. Mice were treated with IMQ and PG102 or Dex for 6 consecutive days (n=4). On day 6, mice were sacrificed and PASI score was calculated. (A) Photo of dorsal skin (B) Cumulative PASI score. ** $p<0.01$, **** $p<0.0001$. The data are shown as the mean ± standard error mean (SEM).

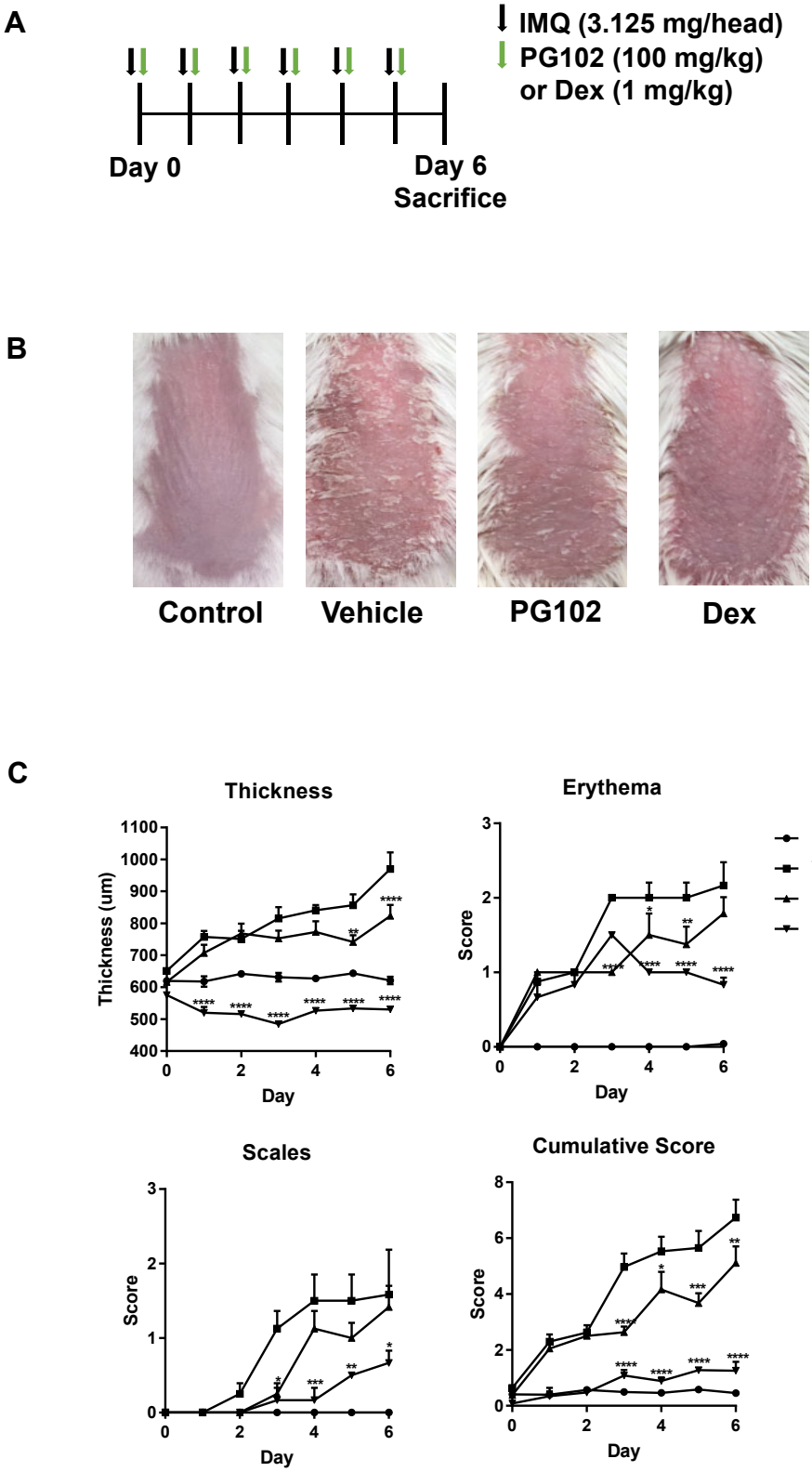


Fig. 13. Effects of PG102 on IPI model . Mice were treated with IMQ and PG102 or Dex for 6 consecutive days (n=4). On day 6, mice were sacrificed and PASI score was calculated.(A) Experimental scheme (B) Photo of dorsal skin (C) PASI score. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$ versus the Control group. The data are shown as the mean ± standard error mean (SEM).

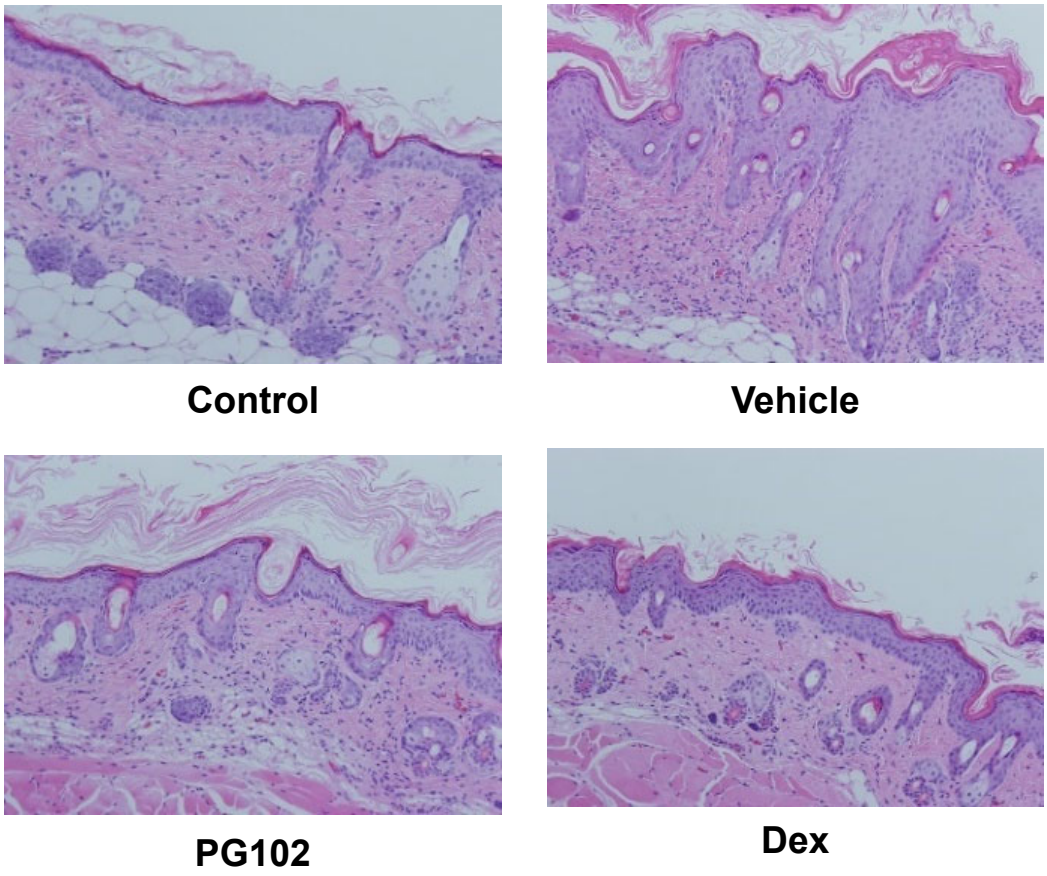


Fig. 14. H&E staining of dorsal skin. Dorsal skin of mice from each group was isolated and embedded in paraffin block and 3 μm-thick sections were subjected to H&E staining. Magnification: 200X.

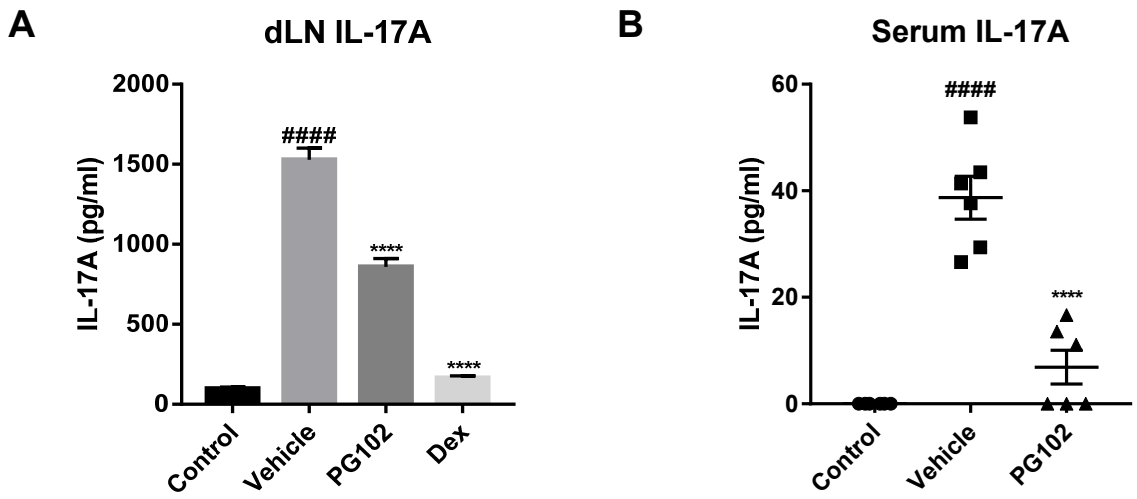


Fig. 15. Effects of PG102 on IL-17A levels. (A) Axillary and inguinal lymph nodes from mice of each group were isolated, pooled and restimulated with PMA/ionomycin. 24 hours later, IL-17A levels in the supernatant were measured by ELISA. (B) Serum from mice of each group were obtained and IL-17A levels were measured by ELISA. #### $p<0.0001$ versus the Control group; **** $p<0.0001$ versus the Vehicle group. The data are shown as the mean ± standard error mean (SEM).

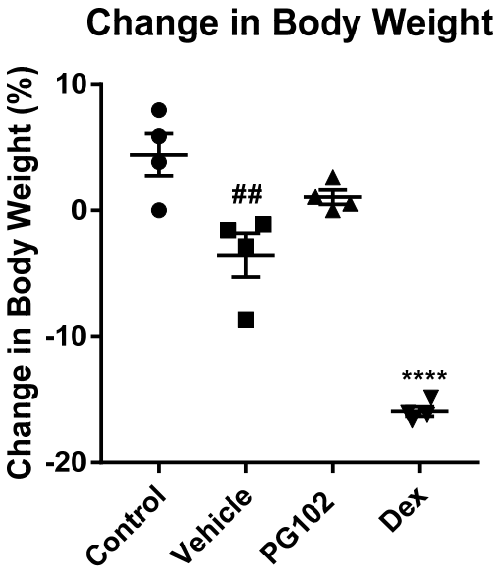


Fig. 16. Effects of PG102 body weight of mice. On day 6 of experiment, body weight of mice from each group was measured. ## $p<0.01$ versus Control group; **** $p<0.0001$ versus Vehicle group.

2.3. Molecular mechanism of anti-inflammatory effects of PG102

2.3.1 Effects of M5 stimulation on inflammatory mediators in HaCaT cells

As previous results implicated that topical, but not oral administration of PG102 was effective in ameliorating IPI model, it was speculated that PG102 exerted its effects mainly by acting on keratinocytes, the cell types present on the uppermost surface of skin. To further investigate molecular mechanism of anti-inflammatory effects of PG102, *in vitro* cell culture system using HaCaT keratinocyte cell line was employed. In the study presented by Guillteou et al. (2010), primary human keratinocytes were stimulated with M5 to mimic conditions of psoriasis *in vitro* but since HaCaT cells were used in this study, it was necessary to find out time kinetics of different inflammatory mediators upon stimulation with M5. Hence, HaCaT cells were treated with M5 (10 ng/ml) for 0, 6, 12 and 24 hours, followed by total RNA extraction and RT-qPCR. As shown in Fig. 17A, the mRNA levels of inflammatory chemokines CXCL1, CXCL3 and CXCL8 peaked at 6 hours and steadily decreased while that of CXCL5 peaked at 12 hours and decreased at 24 hours after M5 stimulation. On the other hand, mRNA levels of antimicrobial peptides (AMPs) β-Defensin 2 (hBD-2), β-Defensin 3 (hBD-3), Cathelicidin (hCAP-18) and S100A7 gradually increased until 24 hours after M5 stimulation (Fig. 17B). These results showed that the two distinct groups of inflammatory mediators involved in psoriasis – chemokines and AMPs – were induced at maximal levels 6 hours and 24 hours, respectively, after stimulation with M5 and these time points were chosen for analysis in further experiments.

2.3.2 Effects of PG102 on inflammatory mediators in M5-stimulated HaCaT cells

In psoriatic epidermis, keratinocytes actively participate in the immune responses by releasing pro-inflammatory cytokines, chemokines and AMPs to recruit various immune cells [10]. Thus, blocking expression of these inflammatory mediators

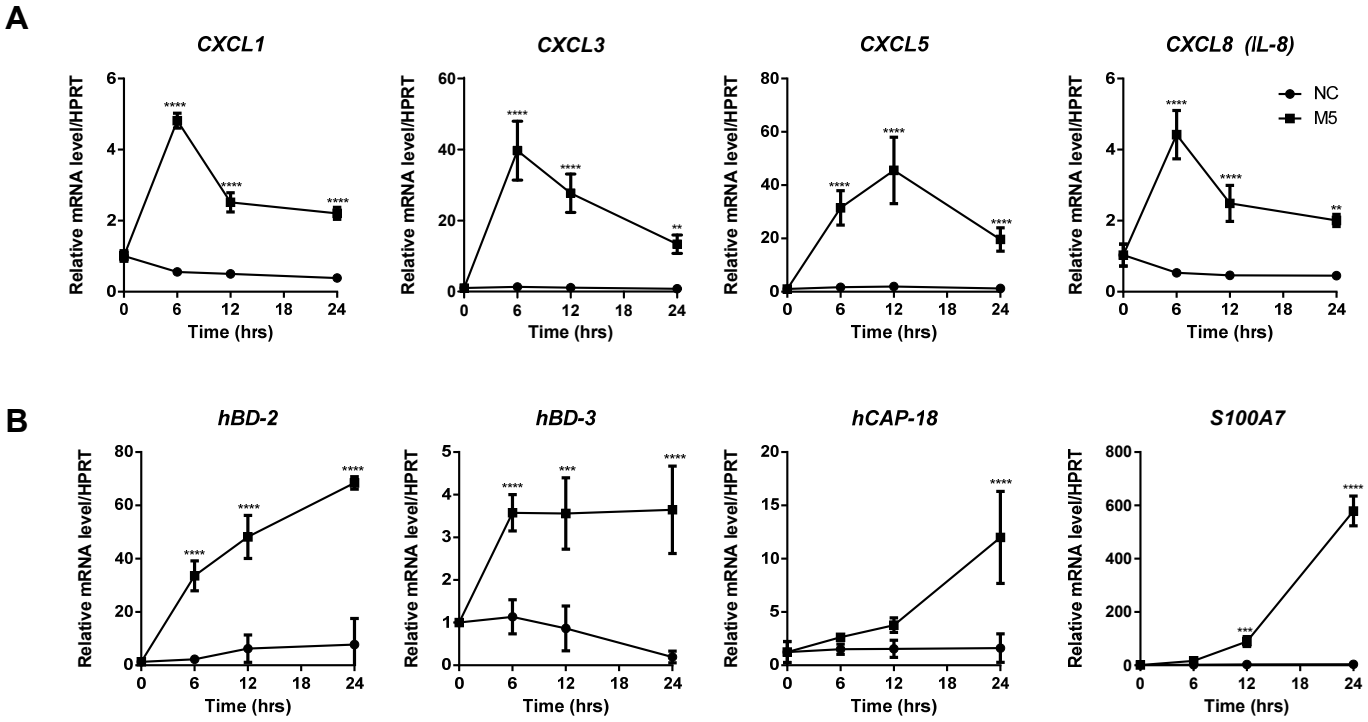


Fig. 17. Expression pattern of inflammatory mediators in HaCaT cells stimulated with M5. Cells were treated with mixture of 5 cytokines (M5) for 0, 6, 12, and 24 hours and isolated RNA were subjected to RT-qPCR. (A) mRNA levels of chemokines (B) mRNA levels of antimicrobial peptides (AMPs). * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$ versus 0 hr. The data are shown as the mean ± standard deviation (SD).

has beneficial roles in regulating psoriasis. CXCL1, CXCL5 and IL-8 are chemokines released by keratinocytes and recruit neutrophils through interactions with CXCR2 and CXCR1, respectively [70]. In M5-stimulated HaCaT cells, their mRNA and protein levels are dramatically increased but treatment with PG102 effectively downregulated expressions of these chemokines in a concentration-dependent manner (Fig. 18). These data showed that PG102 might exert its biological activities through the negative regulation of chemokines.

Next, it was assessed whether PG102 could suppress the expression of AMPs. S100A8 and S100A9 are antimicrobial peptides that form a heterodimer called calprotectin which interacts with Toll-like receptor 4 (TLR-4) and exacerbates psoriasis by activating complement factor C3 and recruiting neutrophils [18, 71]. hBD-2, a serum biomarker for psoriasis, is another antimicrobial peptide that is known to induce chemotaxis of immune cells through interaction with CC chemokine receptor 2 (CCR2) [17]. In the M5-treated group, the mRNA levels of S100A8, S100A9, cathelicidin and hBD-2 were highly increased, but treatment with PG102 reduced their levels in a dose-dependent manner without cytotoxic effects (Fig. 19). Similar observations were made when protein levels of S100A8/A9 heterodimer and hBD-2 were measured, suggesting that PG102 might regulate the expression of antimicrobial peptides at both mRNA and protein levels (Fig. 19).

2.3.3 Effects of PG102 on cell proliferation in HaCaT cells

Since hyperproliferation of keratinocytes is one of the main manifestations of psoriatic lesions, anti-proliferative effects of PG102 were assessed. To evaluate effects of PG102 on proliferation of keratinocytes, HaCaT cells were treated with M5 and PG102 for 24 and 48 hours. The M5-stimulated group showed significant increase in proliferation at 48 hrs, while treatment with PG102 suppressed the proliferation (Fig. 20).

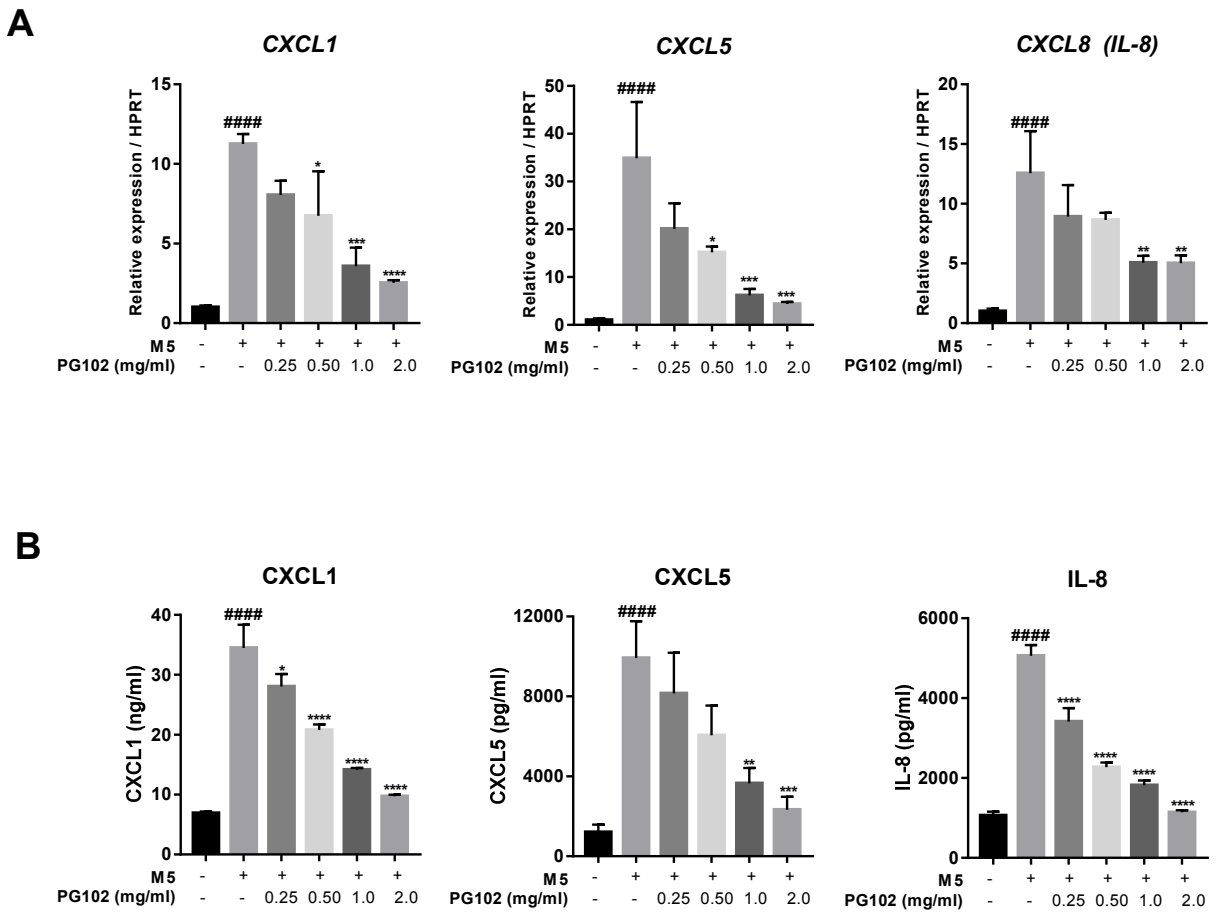


Fig. 18. Effects of PG102 on chemokine expressions in M5-stmulated HaCaT cells. (A) Cells were treated with M5 and PG102 for 6 hours and isolated RNA were subjected to RT-qPCR. (B) Cells were treated with M5 and PG102 for 24 hours and the culture supernatants were subjected to ELISA. #### $p<0.0001$ versus negative control group; * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$ versus M5-only treated group.

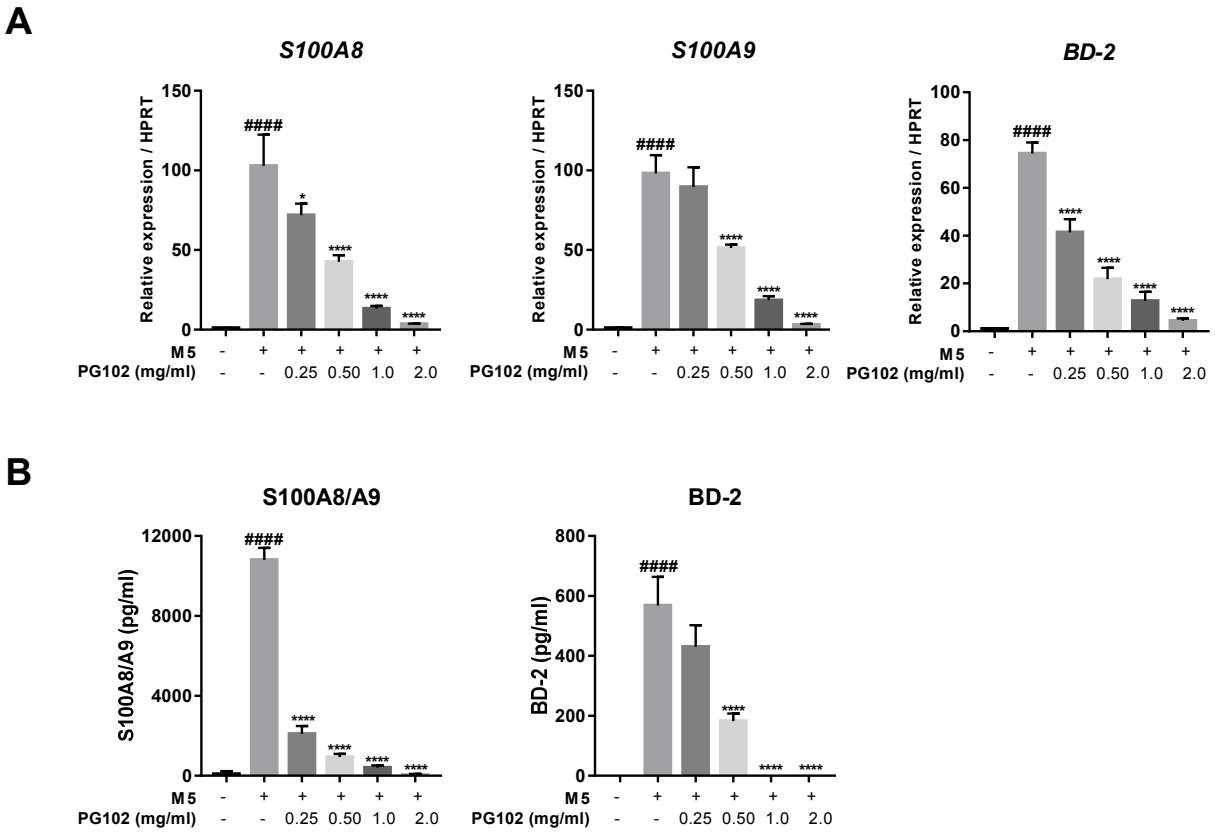


Fig. 19. Effects of PG102 on AMP expressions in M5-stmulated HaCaT cells. (A) Cells were treated with M5 and PG102 for 24 hours and isolated RNA were subjected to RT-qPCR. (B) Cells were treated with M5 and PG102 for 48 hours and the culture supernatants were subjected to ELISA. #### $p<0.0001$ versus negative control group; * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$ versus M5-only treated group.

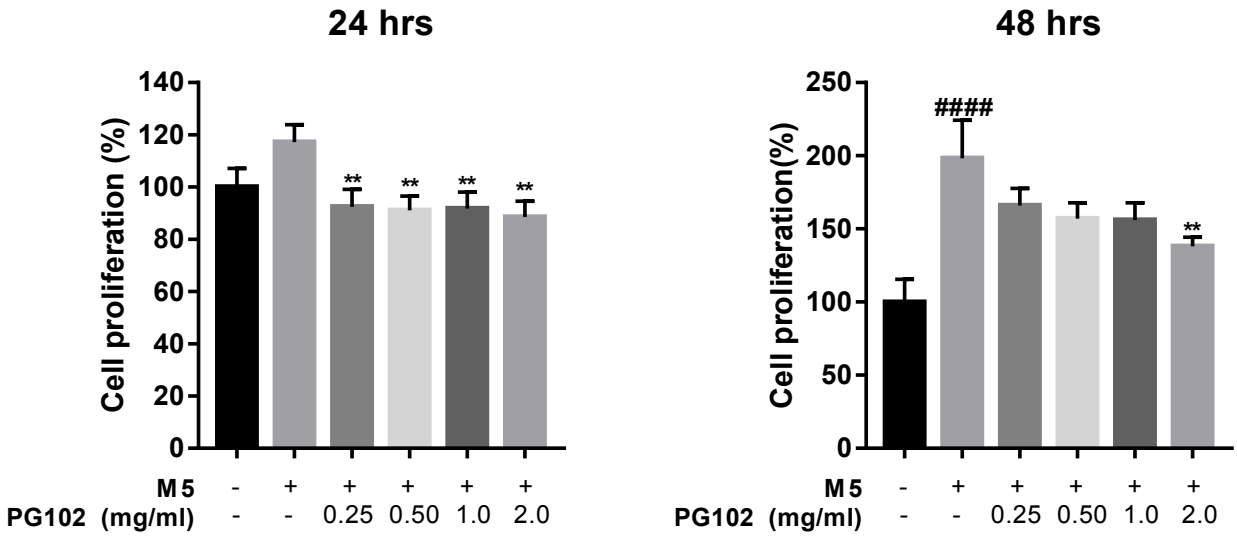


Fig. 20. Effects of PG102 on hyperproliferation of HaCaT cells. Cells were treated with M5 and PG102 for 24 hours and 48 hours. Cell proliferation was measured by WST-1 assay. #### $p<0.0001$ versus negative control group; ** $p<0.01$ versus M5-only treated group.

Next, we corroborated the effects of PG102 on STAT3 signaling whose activation has been known to induce proliferation of keratinocytes [72]. Consistent with previous reports, M5 treatment increased phosphorylation of STAT3 at both tyrosine 705 and serine 727 residues while PG102 effectively reduced their expression levels (Fig. 21). These results indicated that the effect on epidermal thickness is, in part, due to suppression of STAT3 phosphorylation and hyperproliferation of keratinocytes (Fig. 14).

2.3.4 Effects of PG102 on NF-kB and STAT1 signaling pathway in HaCaT cells

Transcription of chemokines and antimicrobial peptides involved in psoriasis has been reported to depend largely on STAT and NF-κB signalings [73, 74]. In an effort to understand molecular mechanisms underlying the anti-inflammatory activities of PG102, HaCaT cells were co-treated with M5 and PG102 for 30 minutes, and the levels of various signaling proteins were measured by Western blot. M5 stimulation increased the level of phosphorylated STAT1 while co-treatment with PG102 lowered its amount in a concentration-dependent manner. M5 also induced degradation of IκB-α and subsequent phosphorylation of p65, while PG102 treatment prevented degradation of IκB-α and phosphorylation of p65 (Fig. 22). These results demonstrate that PG102 exerts anti-inflammatory activities through inhibition of phosphorylation of STAT1, NF-κB p65 and degradation of IκB-α.

2.3.5. Effects of PG102 on neutrophil chemotaxis

One of the hallmarks of psoriatic lesions is the marked infiltration of neutrophils, among many other immune cells [11]. We hypothesized that PG102 might exert its anti-psoriatic effects by regulating the expression of chemokines and antimicrobial peptides and inhibiting subsequent neutrophil infiltration. To test this hypothesis, HL-60, a neutrophil-like cell line, was subjected to chemotaxis assay.

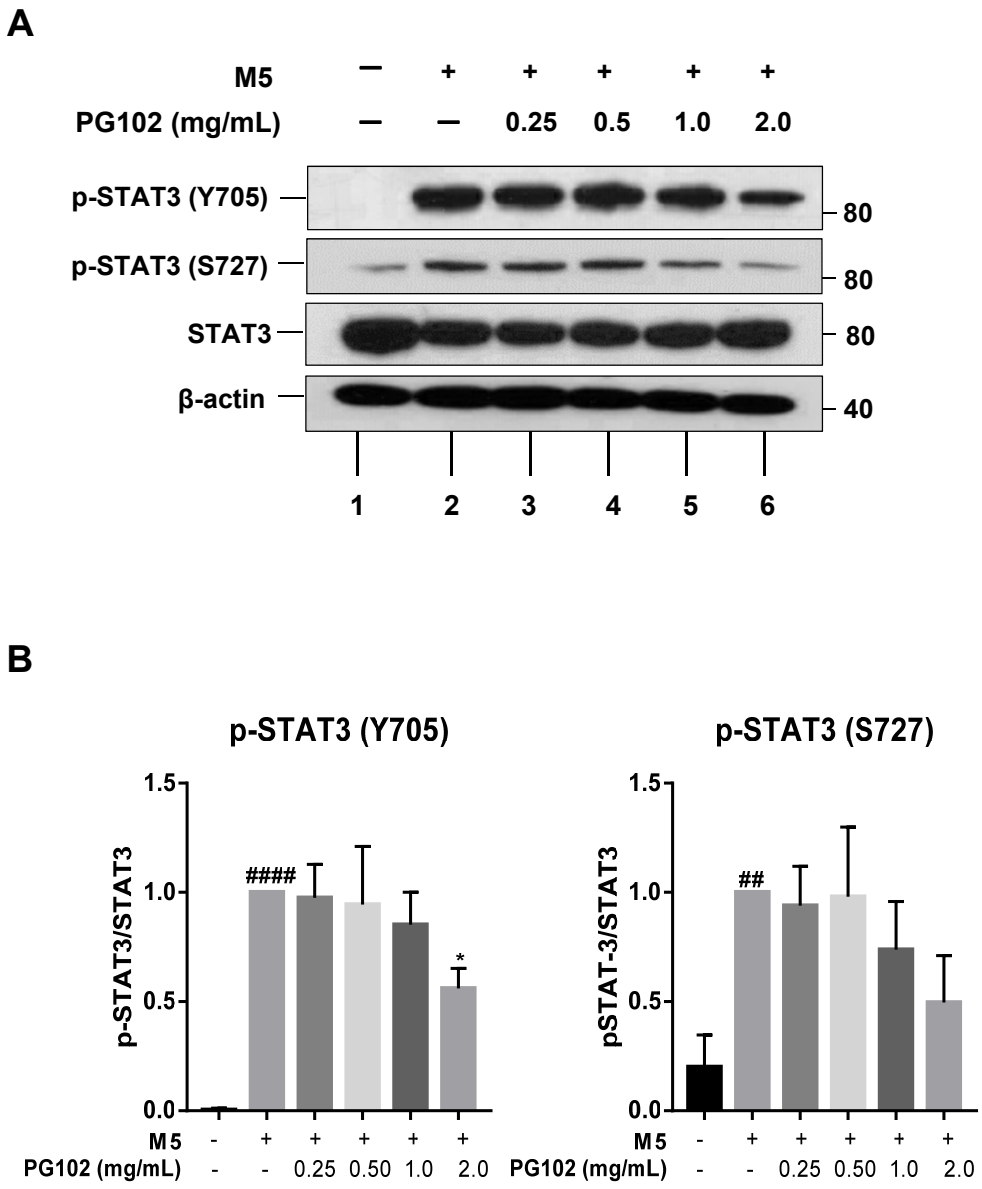


Fig. 21. Effects of PG102 on phosphorylation of STAT3. (A) Cells were treated with M5 and PG102 for 30 minutes and the cell lysates were subjected to Western blot analysis. (B) Densitometry results of Western blot. The means of three independent experiments are shown. ## $p<0.01$, #### $p<0.0001$ versus negative control group; * $p<0.05$ versus M5-only treated group.

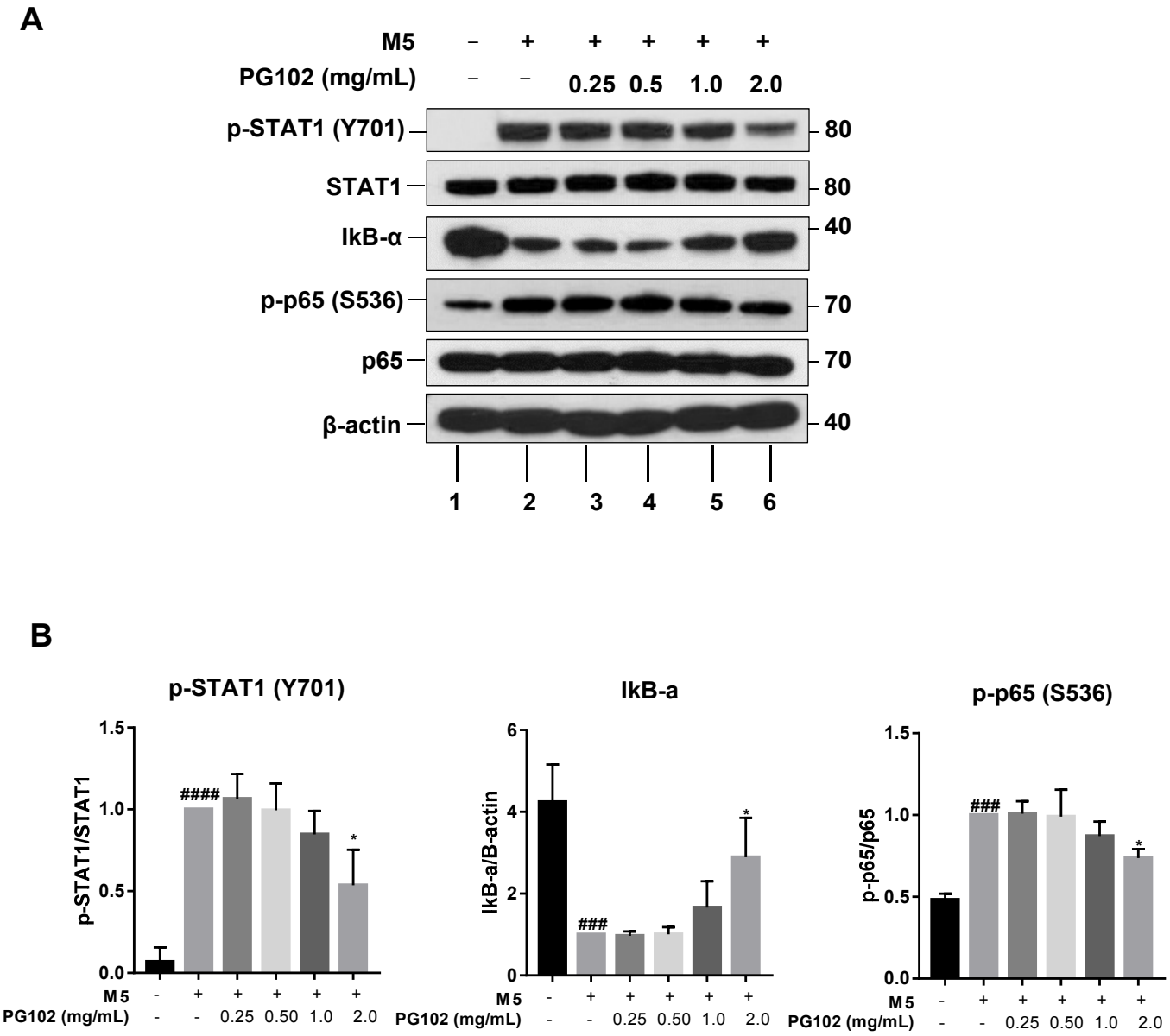


Fig. 22. Effects of PG102 on NF-κB and STAT signaling. (A) Cells were treated with M5 and PG102 for 30 minutes and the cell lysates were subjected to Western blot analysis. (B) Densitometry results of Western blot. The means of three independent experiments are shown. #### $p<0.0001$ versus negative control group; * $p<0.05$ versus M5-only treated group.

Differentiated HL-60 cells (dHL-60) were placed in the upper well of the Boyden chamber, in the presence of cell culture supernatants from HaCaT cells treated with M5 and PG102 for 48 hours in the bottom well. Medium containing 20% FBS served as the positive control. The number of HL-60 cells migrated to the bottom well were measured by fluorescence and PG102-treated supernatant resulted in a lower number of migrated HL-60 cells in a concentration-dependent manner (Fig. 23).

The extent of neutrophil infiltration to dorsal skin in IMQ-treated mice was measured by immunostaining of the lymphocyte antigen 6 complex locus G (Ly6G) protein. In the vehicle-treated group, the number of $Ly6G^+$ cells were greatly increased in the dermal layer of the skin (Fig. 24). However, treatment with PG102 or dexamethasone suppressed neutrophil infiltration to the skin, along with reduced thickness of the epidermis. The RNA levels of CXCL1, MIP-2, S100A8 and S100A9 were reduced in the PG102-treated group (Fig. 25). Taken together, these data suggested that PG102 might ameliorate IMQ-induced psoriasis-like skin inflammation by suppressing neutrophil chemotaxis induced by stimulated keratinocytes.

3. Discussion

PG102 is a botanical extract derived from an edible portion of Actidinia arguta. It has previously been shown to contain potent anti-inflammatory and anti-oxidative activities both *in vitro* and *in vivo* [55-60]. Based on these reports, we investigated the effect of PG102 on the murine IMQ-induced psoriasis-like skin inflammation model. Because PG102 is a mixture of compounds, we initially established two quality control methods to minimize batch-to-batch variations in two marker compounds and the ability to suppress IL-8 in HaCaT cells. Only batches which satisfied the standards for both assays were used. The quality of PG102 was found to be remarkably consistent in different batches. In this study, we demonstrate that topical application of PG102 ameliorates clinical symptoms of psoriasis by inhibition of STAT3-mediated

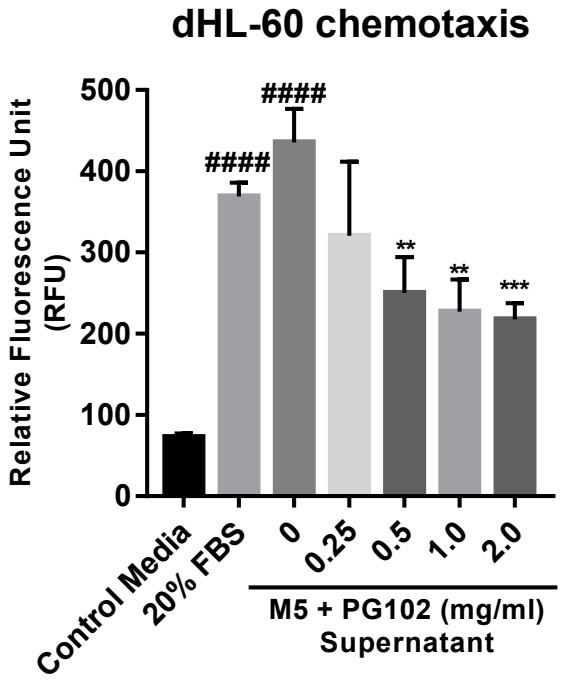


Fig. 23. Effects of PG102 on dHL-60 chemotaxis. Differentiated HL-60 (dHL-60) neutrophil-like cell line was seeded on the upper well of the transwell and cell culture supernatants from HaCaT cells treated with M5 and PG102 for 48 hours were placed on the bottom well. The number of migrated cells were quantified by fluorescence. Media containing 20% FBS served as the positive control. #### $p<0.0001$ versus Control Media group; ** $p<0.01$, *** $p<0.001$ versus M5-only treated group.

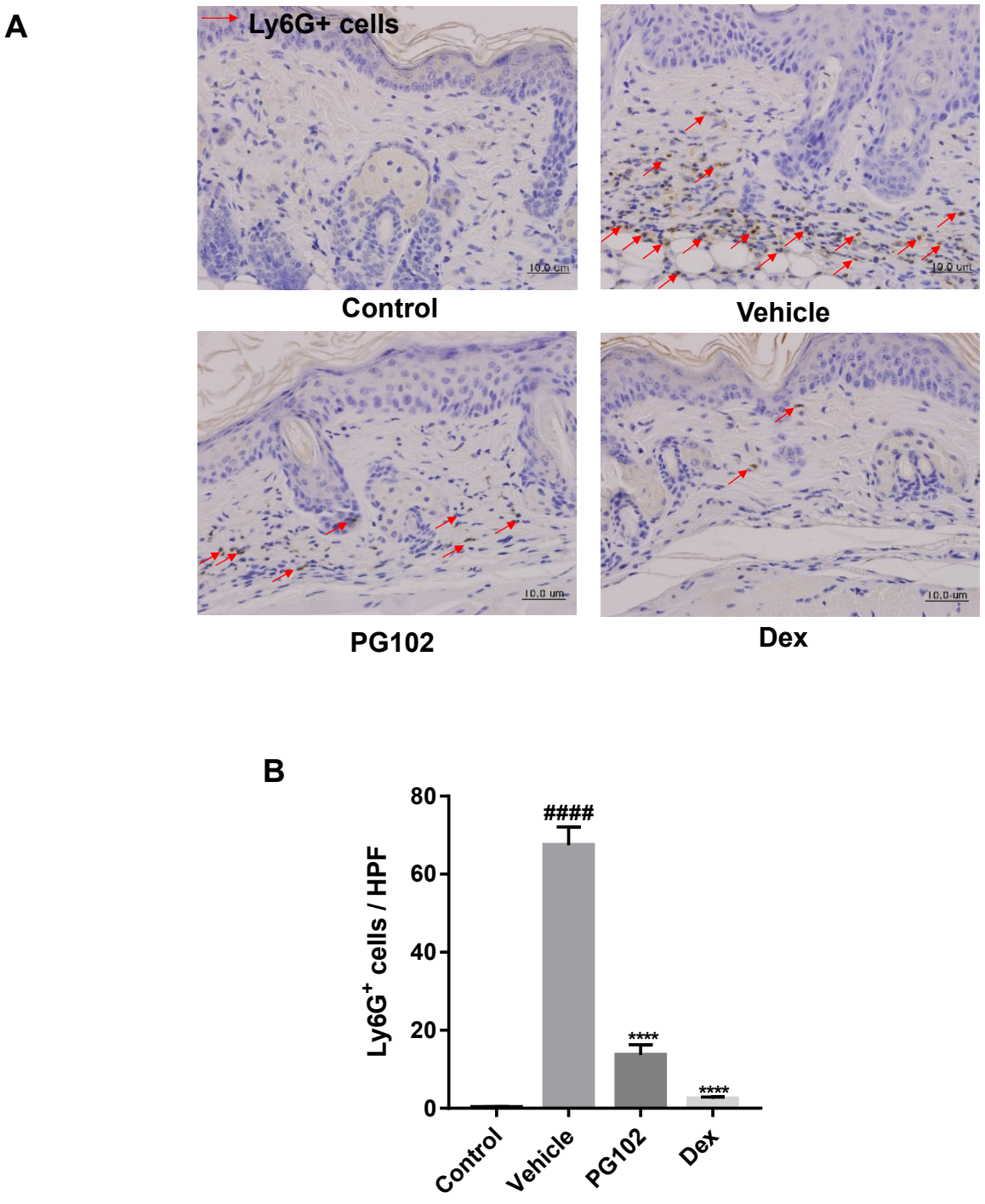


Fig. 24. Effects of PG102 on neutrophil chemotaxis to skin. (A) IHC staining for Ly6G in dorsal skin of control, vehicle, PG102 (100 mg/kg) and Dex (1 mg/ml) treated mice (400X). (B) Quantification of Ly6G positive cells per five random high power fields (HPF) (n=3~4). The data are shown as the mean ± SEM. #### $p<0.0001$ versus Control group; **** $p<0.0001$ versus Vehicle group.

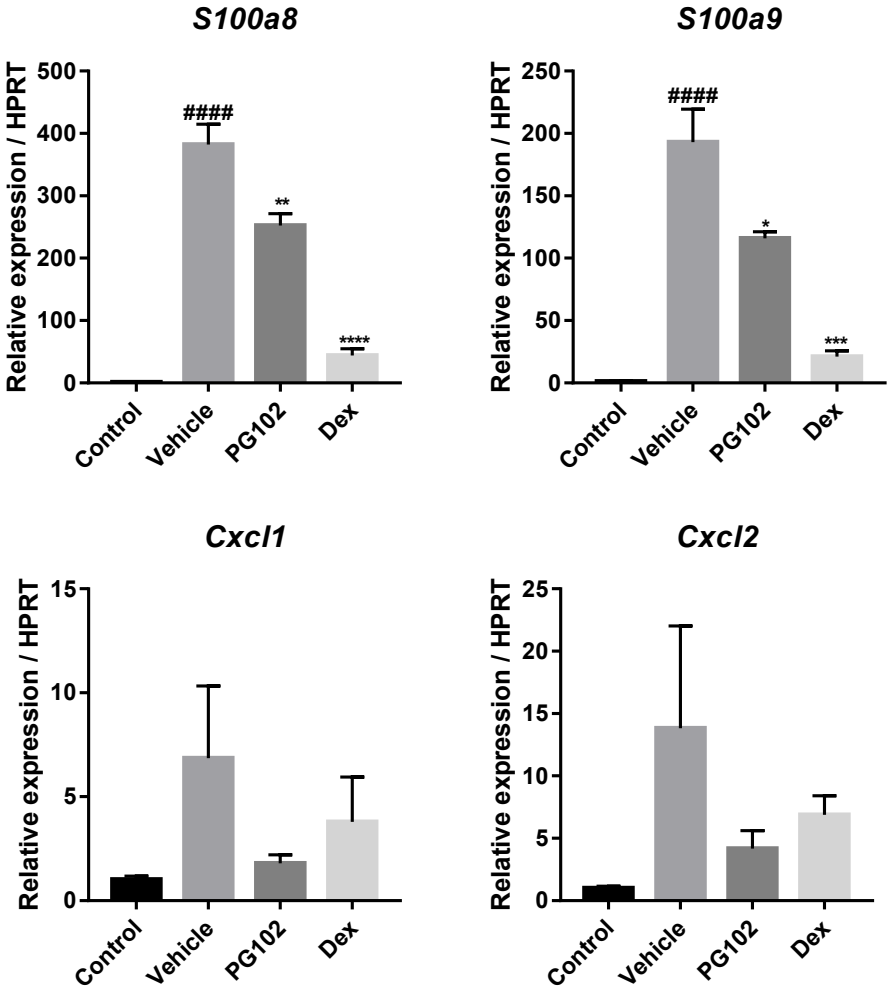


Fig. 25. Effects of PG102 on expressions of AMPs and chemokines. mRNA expression levels of antimicrobial peptides and chemokines in dorsal skin of mice were analyzed by RT-qPCR. The data are shown as the mean ± SEM. #### $p<0.0001$ versus Control group; **** $p<0.0001$ versus Vehicle group.

proliferation of keratinocytes and neutrophil infiltration to skin.

One of the hallmark features of psoriasis is epidermal thickening caused by hyperproliferation of keratinocytes along with abnormal differentiation of keratinocytes, which ultimately leads to dysfunctional skin barrier [75]. IL-22, produced by Th17 and Th22 cells, is the key cytokine responsible for hyperproliferation of keratinocytes and disruption of terminal differentiation by activating STAT3 [72]. Thus, STAT3 has been aroused as an ideal therapeutic target for psoriasis. Indeed, a recent study has shown that calcipotriol, which has been used widely for the treatment of psoriasis, inhibits proliferation of keratinocytes by downregulating phosphorylation of STAT3 [76]. Our data showed a decrease in epidermal thickness in IMQ-treated mice by topical application of PG102 as well as suppression of proliferation and phosphorylation of tyrosine 705 and serine 727 residues of STAT3 in HaCaT cells. Besides, we have observed an increase in late differentiation markers, such as filaggrin and involucrin, by treatment of PG102 in HaCaT cells, suggesting possible therapeutic roles of PG102 on differentiation of keratinocytes.

Another hallmark of psoriasis is the marked infiltration of neutrophils into epidermis that form microabscesses and neutrophil extracellular traps (NETs) composed of DNA and AMPs [77]. Although many of the drugs developed for psoriasis primarily focus on the IL-23/Th17 axis of the disease, the roles of neutrophils should not be overlooked, as neutrophils are one of the most predominant cell types in psoriatic skin [78]. Indeed, it has been shown that depletion of neutrophils rapidly improved the symptoms of psoriasis while recovery of neutrophils reversed this effect [79]. Pathological significance of neutrophils has been confirmed by the observation that RAR-related orphan receptor gamma t (RORγt), the key transcriptional regulator of IL-17A, is expressed in neutrophils present in psoriatic lesions [80]. Moreover, neutrophils were identified as the numerically largest source of IL-17A in psoriasis along with Th17 cells [78]. Thus, targeting neutrophil trafficking appears to be a viable approach to

relieve psoriasis. Our results clearly showed that topical application of PG102 can reduce both neutrophil infiltration to skin and IL-17A production from the cells present in the draining lymph node yet in this study, we have not identified the IL-17A-producing cells affected by PG102. Reduction in IL-17A level could be due to direct suppression of IL-17A-producing cells or due to upregulation of anti-inflammatory mediators. The latter may be a possible explanation based on the previous report describing an increase in regulatory T cell generation by PG102 [57]. It would be worth further investigating the cellular target of PG102 in psoriasis-like skin inflammation.

In the current study, we also observed dramatic downregulation of neutrophil-chemotactic chemokines and antimicrobial peptides at both RNA and protein levels by treatment with PG102. It is well documented that the canonical NF-κB signaling, which initiates by degradation of IκB-α and subsequent phosphorylation and translocation of NF-κB p65 subunit, is involved in the transcription of numerous chemokines, including CXCL1, CXCL5 and IL-8 [81]. Moreover, promoters of S100A8 and S100A9 include binding sites for NF-κB and STAT3, while that of β-defensin 2 contains binding sites for NF-κB and STAT1 [74, 82]. To understand the molecular mechanism of PG102, we analyzed the effects of PG102 on STAT1 and NF-κB p65 phosphorylation in M5-stimulated HaCaT cells. Our data suggested that treatment with PG102 effectively inhibited phosphorylation of these signaling molecules, in accordance to downregulation of chemokines and antimicrobial peptides. In addition, activation of mitogen-activated protein kinases (MAPKs) are also involved in transcription of these inflammatory factors. Indeed, treatment with M5 increased phosphorylation of ERK, p38 and JNK MAPKs. Yet, co-treatment with PG102 did not affect phosphorylation of the MAPKs, suggesting that anti-inflammatory effects of PG102 were mediated mainly through suppression of NF-κB and STAT signalings.

Identification of active compounds and additional cellular targets of PG102 remain to be elucidated. The effects of PG102 on NF-κB, STAT signaling pathway

could be due to simultaneous actions of multiple components of PG102 or selective targeting of upstream regulators. Given the high degrees of safety and efficacy of PG102 in the psoriasis-like model, further investigation is warranted to identify and isolate active compounds responsible for its biological activities.

Chapter IV

Regulation of IL-37 expression by PG102 through ERK, p38 and Smad3 pathways

1. Background

Interleukin 37 (IL-37) is a member of the IL-1 cytokine family whose function as a fundamental inhibitor of innate immunity was first discovered in 2010 [38]. Unlike other IL-1 family members such as IL-1β, IL-18, and IL-36, which are pro-inflammatory, IL-37 exhibits anti-inflammatory properties in various cell types [38, 49, 83]. Although the mouse ortholog of IL-37 has not been discovered, delivery of the human IL-37 gene to mice showed dampened inflammatory responses in animal models of lipopolysaccharide (LPS)-induced shock, inflammatory bowel disease, asthma and insulin resistance [38, 45, 47, 48].

IL-37 can be induced by pro-inflammatory mediators such as IL-1β, tumor necrosis factor (TNF)-α, toll like receptor (TLR) agonists such as LPS, and growth factors such as epidermal growth factor (EGF) and transforming growth factor beta (TGF-β) [38, 43]. Its expression and activation require cleavage by caspase-1, which induces both secretion and nuclear translocation of the IL-37 protein [44]. When acting intracellularly, IL-37 forms a functional complex with phosphorylated Smad3 and translocates into the nucleus [38, 84]. On the other hand, inhibition of Smad3 suppresses not only the expression of IL-37 but also reverses the inhibition of pro-inflammatory cytokines in macrophage and lung epithelial cell lines [38]. Thus, Smad3 activities are essential for IL-37 to exert its anti-inflammatory effects.

Psoriasis is an inflammatory skin disease characterized by the abnormal differentiation and hyperproliferation of keratinocytes [10]. Keratinocytes in psoriatic epidermis are constitutively stimulated by cytokines like IL-17A and IL-22, and they in turn secrete various antimicrobial peptides (AMPs) that are known to amplify the disease [85]. While these factors are overexpressed in psoriatic lesions, transcriptome analysis of psoriatic lesional skin has revealed that IL37 was one of the most downregulated genes compared to healthy skin [52]. In addition, the overexpression of IL-37 in HaCaT keratinocytes suppressed the production of pro-inflammatory

cytokines, and the delivery of plasmid encoding IL-37 into Keratin 14-VEGF transgenic mice ameliorated symptoms of psoriasis [86]. Thus, the upregulation of IL-37 in skin may be an effective therapeutic approach to alleviating inflammatory skin diseases.

IL-37 exhibited bioactivities similar to PG102 such as amelioration of asthma and psoriasis symptoms and promotion of regulatory T cell differentiation *in vitro* [87]. This has led us to speculate that IL-37 might be involved in the biological activities of PG102. To date, there is no published results reporting an agent that can upregulate IL-37 in keratinocytes, and the role of IL-37 in keratinocytes has remained elusive. In this chapter, we demonstrated that silencing IL-37 leads to increased inflammatory responses in HaCaT cells and that PG102 upregulated IL-37 levels through extracellular signal-related kinases (ERK), mothers against decapentaplegic homolog 3 (Smad3) and p38 while promoting the colocalization of IL-37 and phospho-Smad3. These results suggest potential anti-inflammatory roles of PG102 through the regulation of IL-37 expression and possible application of PG102 against inflammatory skin diseases.

2. Results

2.1. Silencing IL-37 increases expression of antimicrobial peptides in HaCaT cells

IL-37 is a suppressor of pro-inflammatory responses induced by inflammatory insults such as LPS or cytokines. The effects of silencing endogenous IL-37 have been reported in human peripheral blood mononuclear cells (PBMCs), aortic valve interstitial cells and renal tubular epithelial cells, but have never been tested in the context of keratinocytes [44, 87-89]. We initially tested the effects of IL37 knockdown by siRNA in human keratinocyte cell line, HaCaT cells (Fig. 26A). After silencing IL37, cells were stimulated with a mixture of five cytokines designated as M5 (IL-1α, IL-17A, IL-22, Oncostatin M and TNF-α) to mimic the microenvironment of keratinocytes in psoriasis [68]. In cells transfected with control siRNA, treatment with M5 resulted in increased expressions of various AMPs including DEFB4A (hBD-2), DEFB103 (hBD-

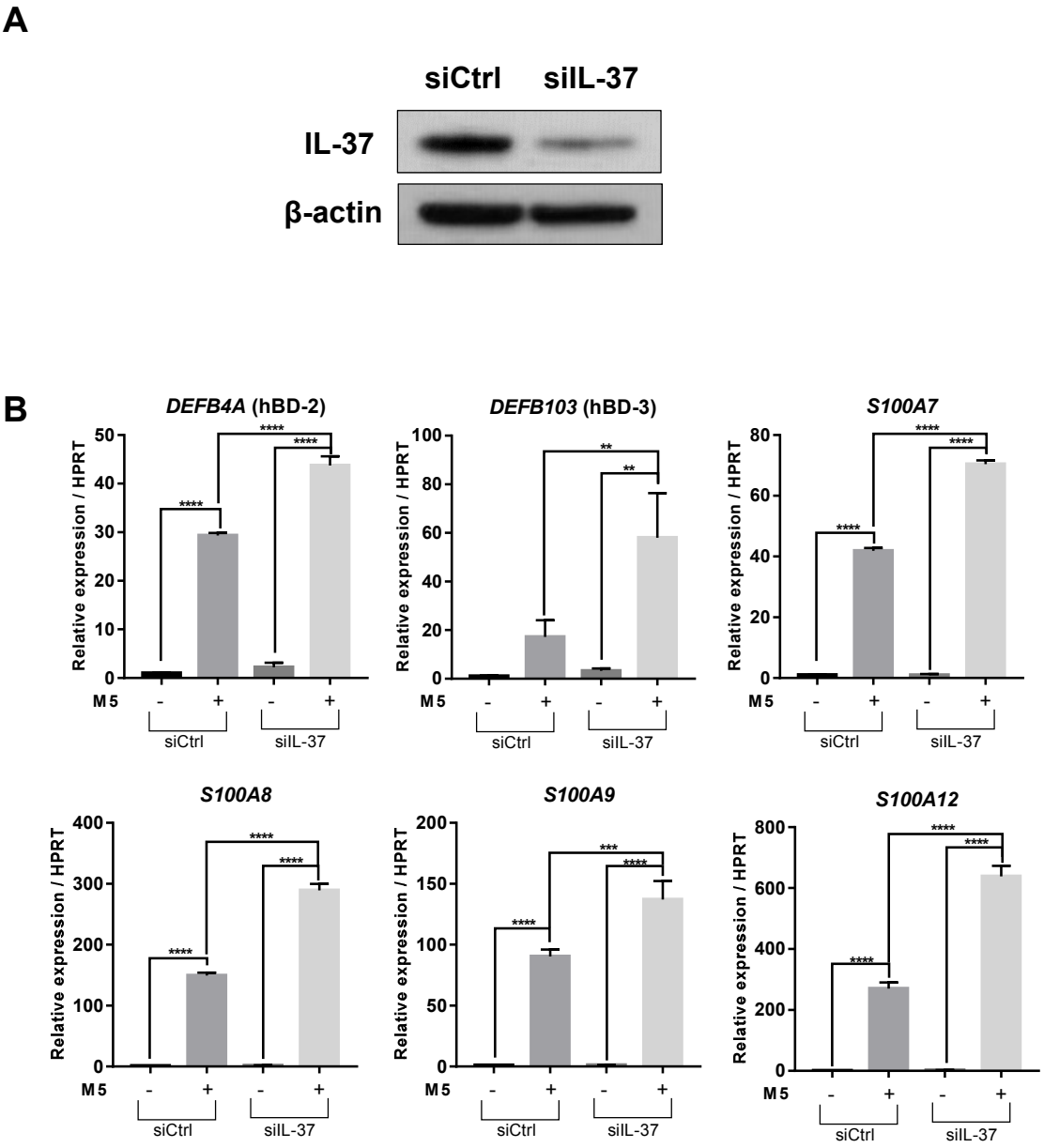


Fig. 26. Effects of silencing *IL37* in HaCaT cells. (A) siRNA-mediated knockdown of IL-37 was confirmed by Western blot analysis. (B) HaCaT cells were transfected with control and IL-37 siRNAs and treated with M5 for 24 hours, followed by RT-qPCR analysis. Representative results from three independent experiments are shown. Each point represents mean ± SD. ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

3), S100A7, S100A8, S100A9 and S100A12 (Fig. 26B). These effects were further augmented by transfection of IL37 siRNA, suggesting that IL-37 acts as a natural inhibitor of inflammation in HaCaT cells, as in the case of other cell types. Based on this result, it was hypothesized that upregulating IL-37 may be an effective approach to regulating excessive inflammation in keratinocytes.

2.2. PG102 upregulates IL-37 mRNA and protein levels in HaCaT cells

We have previously shown that PG102 repressed the expression of AMPs such as hBD-2 and S100A8/A9, which are highly increased upon exposure to inflammatory cytokines in HaCaT cells [90]. Based on the anti-inflammatory properties of PG102, it was tested whether PG102 might also enhance the expression of IL-37. When HaCaT cells were treated with PG102, the mRNA level of IL37 started to increase from 24 hours after treatment and continued to the last time point, 48 hours (Fig. 27A). Treatment with PG102 at different concentrations induced IL37 expression in a dose-dependent manner (Fig. 27B). At 2 mg/ml of PG102, the mRNA level of IL37 increased more than 10-fold compared to the control group. The protein expression of IL-37 was also increased by PG102 (Fig. 28). These results suggest that PG102 upregulates IL-37 expression at both mRNA and protein levels in HaCaT cells.

2.3. Activation of Smad3 is essential for the induction of IL-37 by PG102 in HaCaT cells

It has previously been shown that activation of Smad3 is essential for the expression of IL-37 [38]. Therefore, it was tested whether Smad3 phosphorylation was involved in the PG102-mediated induction of IL-37. As shown in Fig. 29A, HaCaT cells treated with PG102 showed an increase in the phosphorylation of Smad3 in a concentration-dependent manner. Thus, it was predicted that PG102 could enhance IL-37 expression by activating Smad3. To confirm this, SIS3—a specific inhibitor of

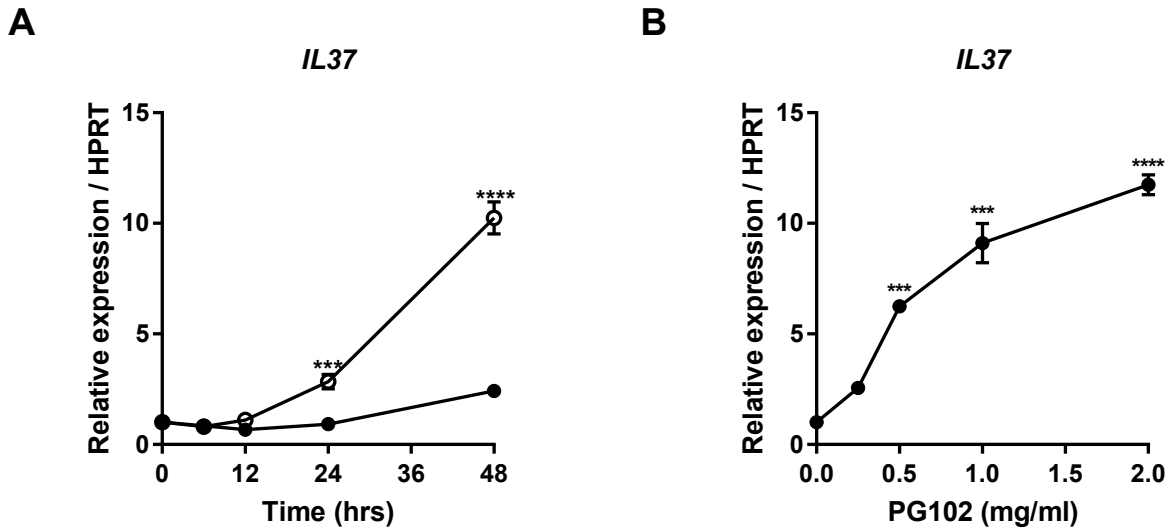


Fig. 27. Effects of PG102 on IL-37 expression in HaCaT cells. (A) PG102 (1 mg/ml) was treated to HaCaT cells for different time points, followed by RNA extraction and RT-qPCR analysis. Closed circle: control; Open circle: PG102 (1 mg/ml) (B) HaCaT cells were treated with PG102 at different concentrations for 24 hours, followed by RT-qPCR. *** $p < 0.001$, **** $p < 0.0001$ compared control cells.

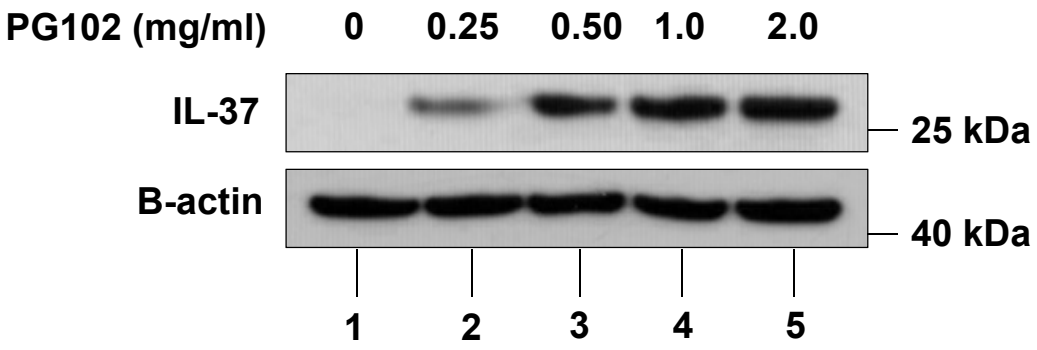


Fig. 28. Effects of PG102 on protein expression of IL-37 in HaCaT cells. HaCaT cells were treated with PG102 at different concentrations for 48 hours, followed by Western blot analysis.

Smad3—was used to block the activation of Smad3. Cells were pretreated with SIS3 for two hours followed by a treatment with PG102. As shown in Fig. 29B, PG102-mediated expression of IL-37 was effectively suppressed by pretreatment with SIS3. These results show that Smad3 is necessary for the induction of IL-37 by PG102.

3.4. PG102 increases IL-37 expression through phosphorylation of ERK and p38 MAPKs in HaCaT cells

Activation of mitogen-activated protein kinases (MAPKs) leads to phosphorylation of a variety of their downstream targets including Smad3, and promotes transcription of various genes [91]. Since MAPKs have been shown to be required for the induction of IL-37, we investigated the role of MAPKs in the PG102-mediated upregulation of IL-37. Treatment with PG102 enhanced phosphorylation of p38 and ERK in a concentration-dependent manner (Fig. 30A and 31A).

Next, it was tested whether inhibiting p38 and ERK activities reverses the effects of PG102 on Smad3 phosphorylation and IL-37 expression. Cells were incubated in the presence of SB203580 and U0126—specific inhibitors of p38 and ERK respectively—for 1 hour prior to treatment with PG102. As shown in Fig. 30B, inhibition of p38 reduced the expression of IL-37 and phosphorylated Smad3. In addition, pretreatment with U0126 diminished both phosphorylated and total forms of Smad3, as well as expression of IL-37, suggesting that both p38 and ERK are the upstream kinases responsible for phosphorylating Smad3 and inducing IL-37 (Fig. 31B). Taken together, these results show that both p38, ERK and Smad3 pathways are involved in PG102-mediated induction of IL-37.

3.5. PG102 promotes colocalization of IL-37 and Smad3 in perinuclear regions in HaCaT cells

IL-37 can bind to intracellular Smad3 to form a functional complex in the

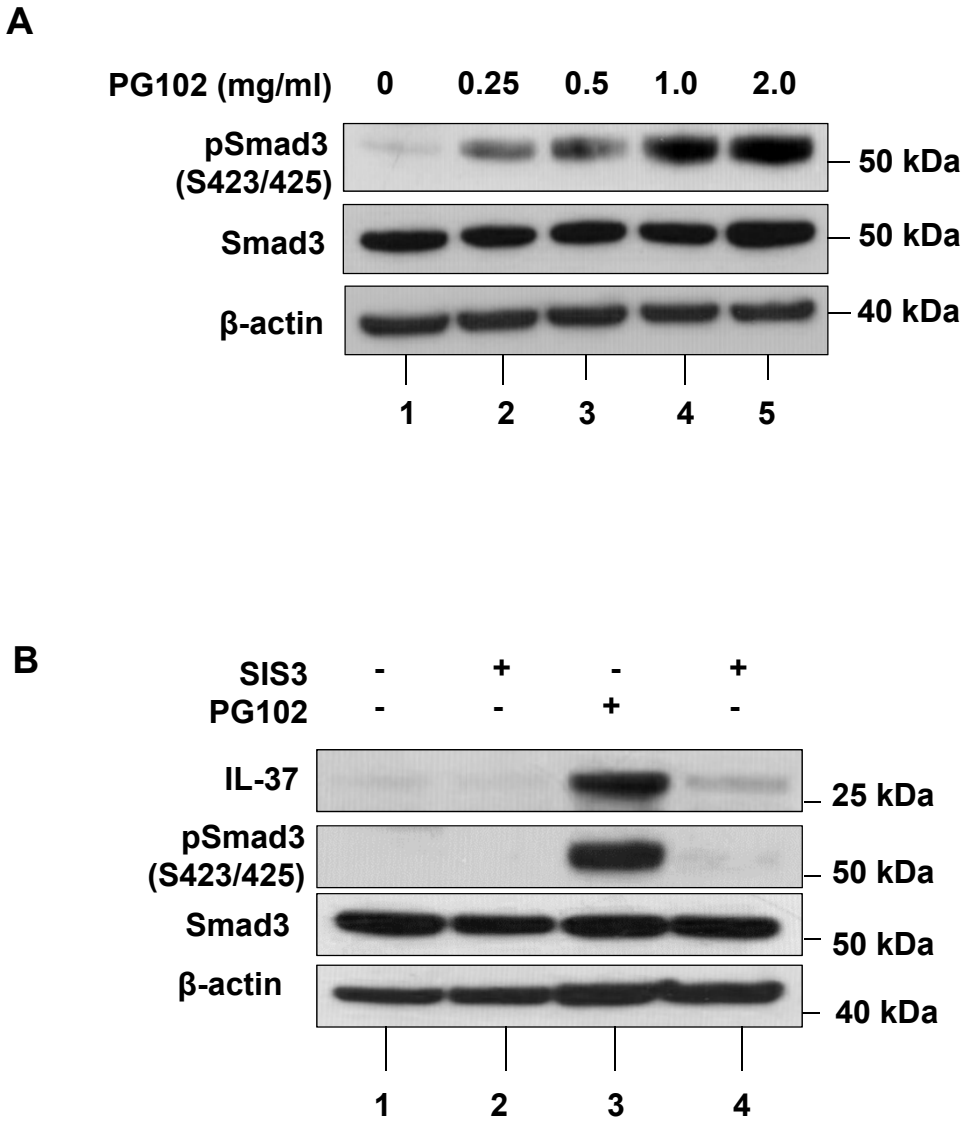


Fig. 29. IL-37 induction by PG102 is dependent on activation of Smad3. (A) HaCaT cells were incubated with PG102 for 48 hours and the cell lysates were subjected to Western blot analysis using antibodies against phospho-Smad3, Smad3 and β-actin. (B) HaCaT cells were pretreated with 10 μM SIS3 for 1 hour, followed by incubation with or without PG102 (1 mg/ml) for 48 hours and Western blot analysis. Representative results from three independent experiments are shown.

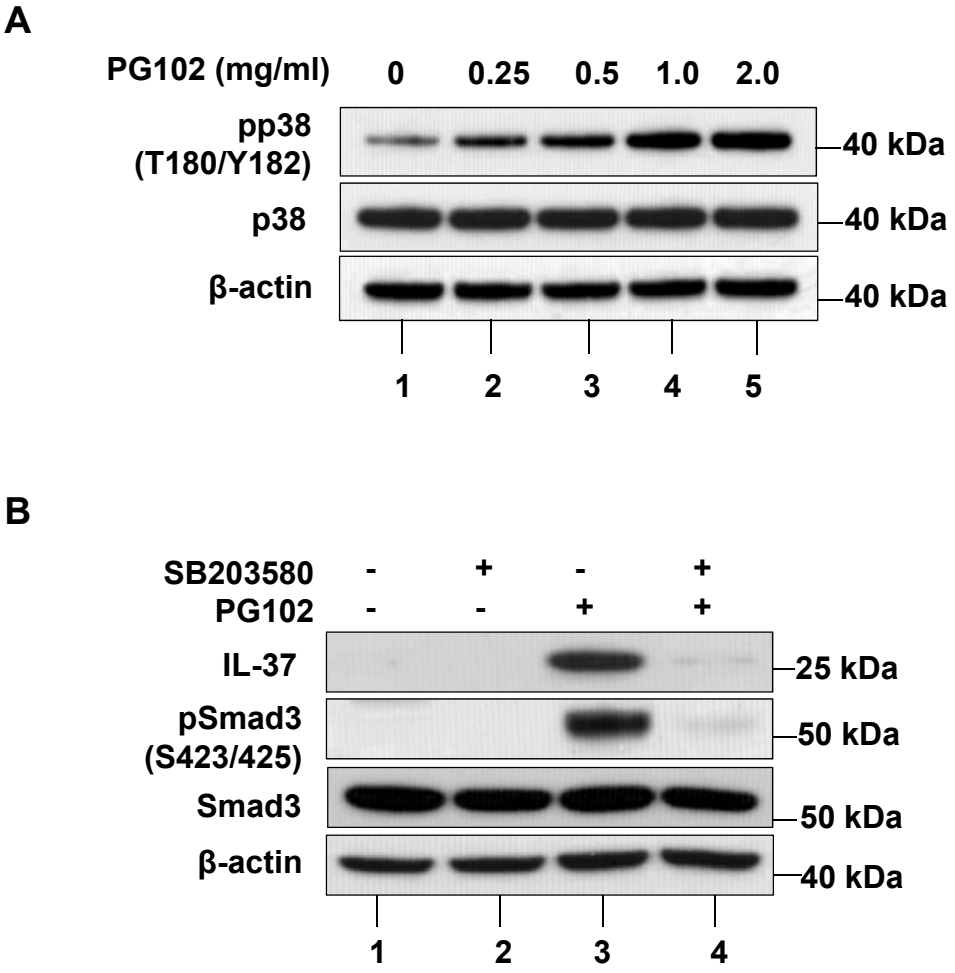


Fig. 30. PG102 increases IL-37 expression through p38 pathway. (A) HaCaT cells were treated with PG102 at different concentrations for 30 minutes and the protein levels of phospho-p38 and p38 were analyzed by Western blot analysis. (B) Cells were pretreated with SB203580 (20 μM) for 1 hour, followed by treatment with PG102 for 48 hours. The protein levels of IL-37, phospho-Smad3 and Smad3 were analyzed by Western blot analysis. Representative results from three independent experiments are shown.

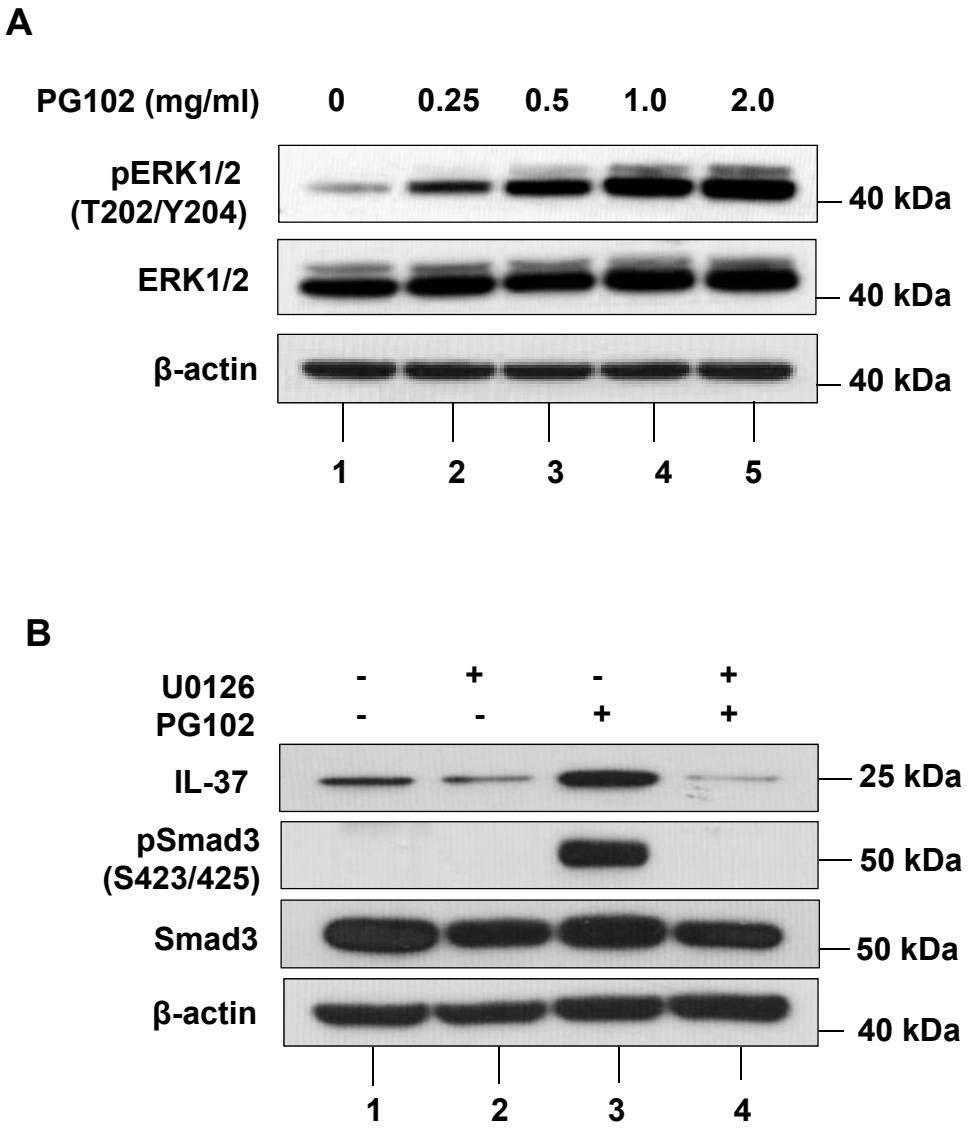


Fig. 31. PG102 increases IL-37 expression through ERK pathway. (A) Cells were treated with PG102 at different concentrations for 30 minutes and the protein levels of phospho-ERK1/2 and ERK1/2 were analyzed by Western blot analysis. (B) Cells were pretreated with U0126 (5 μM) for 1 hour, followed by treatment with PG102 for 48 hours. The protein levels of IL-37, phospho-Smad3 and Smad3 were analyzed by Western blot analysis. Representative results from three independent experiments are shown.

perinuclear regions, ultimately translocating to the nucleus and controlling transcription of a wide variety of inflammatory genes [38]. Using immunofluorescence, we assessed whether PG102 could promote colocalization of IL-37 and Smad3. As observed in Fig. 32, IL-37 and phospho-Smad3 were induced by PG102 and they co-stained in perinuclear regions. These results suggest that PG102 not only increases the expression of IL-37, but also induces the formation of a functional complex of IL-37 and Smad3.

4. Discussion

IL-37 is a potent immunomodulatory cytokine that exerts a broad range of biological functions. Silencing IL-37 results in excessive inflammatory responses to external stimuli in various cell types, but little is known about the role of IL-37 in keratinocytes [92]. Here, we demonstrated that knockdown of IL37 augmented the expression of AMPs in cytokine-stimulated HaCaT keratinocytes. Based on this result, we speculated that upregulation of IL-37 in HaCaT cells might be an effective approach to suppressing excessive inflammation. Since PG102, a standardized water-soluble extract from *Actinidia arguta*, has previously been shown to inhibit expression of various AMPs in HaCaT cells, it was investigated whether IL-37 was involved in the biological effects of this botanical extract. In this study, we showed that PG102 could significantly increase IL-37 levels through the control of p38 and ERK/Smad3 pathways.

In the cytokine milieu of psoriatic skin, keratinocytes release a variety of AMPs such as S100 proteins and beta defensins. Although AMPs were initially known as simple peptides with antimicrobial functions, it is now widely accepted that they actively participate in immune responses [93]. For instance, S100A8 and S100A9 form a complex called calprotectin that aggravates psoriasis by regulating expression of the C3 complement factor, while hBD-2 promotes chemotaxis of various leukocytes in a C-C chemokine receptor (CCR) 2- and CCR6-dependent manner [17, 71]. Consistent with

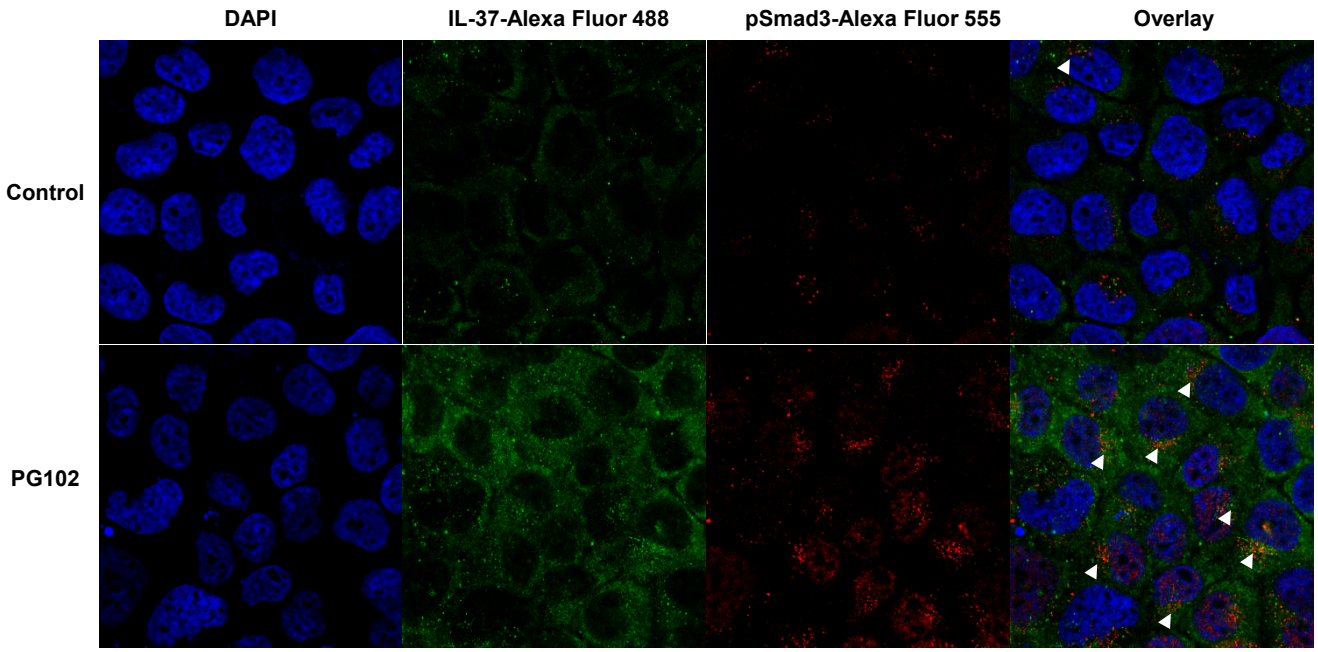


Fig. 32. PG102 promotes colocalization of IL-37 and phospho-Smad3. Confocal microscopy of HaCaT cells treated with PG102 (1 mg/ml) for 24 hours. Localization of nuclei (DAPI), IL-37 (Alexa Fluor 488) and pSmad3 (Alexa Fluor 555) are shown. Colocalized proteins are shown in white arrowheads. Scale bar: 10 μm.

these results, transcriptome analyses of psoriatic lesions have identified AMPs as one of the most upregulated group of genes [94, 95]. On the other hand, IL37 was shown to be one of the most downregulated gene in psoriatic lesions [52]. These results raise the possibility that the chronic inflammation in psoriasis may be due, in part, to the reduced expression of anti-inflammatory mediators like IL-37. This is supported by another study that has shown that overexpression of IL-37 in HaCaT cells effectively mitigated production of IL-6, IL-8 and S100A7 [86]. Therefore, induction of IL-37 in keratinocytes by PG102 may be a novel approach to alleviating psoriasis.

To study the molecular mechanism underlying PG102-mediated upregulation of IL-37, we investigated the effects of PG102 on Smad3 and MAPKs. One study has indicated that hBD-3 increases the expression of IL-37 in human keratinocytes through the use of such molecules as Smad3, ERK1/2, c-Jun N-terminal kinases (JNK), and nuclear factor- κB (NF-κB) [43]. PG102 upregulated IL-37 by activating Smad3, ERK1/2, and p38, but not involving JNK. In Chapter III, it was shown that PG102 suppresses NF-κB signaling in HaCaT cells. Hence, it is anticipated that the signaling pathways involved in the expression of IL-37 may vary depending on the cell type and stimulants used. Whatever the case is, Smad3 seems to be the key molecule orchestrating IL-37 across different cell types.

IL-37 has dual functions in that it can act both extracellularly and intracellularly [40, 44]. Similar to other IL-1 family cytokines, IL-37 is secreted outside cells after cleavage by caspase-1 and binds to either IL-18 receptor α (IL-18Rα) or single Ig IL-1–related receptor (SIGIRR) to exert its effects [40, 96]. Inside cells, however, it forms a functional complex with Smad3, which is required for its anti-inflammatory effects [38]. It has been reported that silencing Smad3 diminishes the anti-inflammatory effects of IL-37 in various cell types and IL-37tg mice [38, 97]. These observations are consistent with the well-known function of Smad3, which translocates into the nucleus after being phosphorylated and binds to the Smad-binding element (SBE) present in the

promoter of respective genes [98]. For instance, it antagonizes the activation of signal transducer and activator of transcription 3 (STAT3) and the subsequent production of IL-6, which is a major inflammatory molecule involved in psoriasis [99]. Our results suggested that PG102 might control the intracellular function of IL-37 in HaCaT cells as we could not detect IL-37 in cell culture supernatants using ELISA. However, it may also be possible that HaCaT cells may be defective in the machinery involved in the processing of IL-37. It has been reported that HaCaT cells express a relatively low level of caspase-1 compared to primary keratinocytes or THP-1 cells [100]. Thus, to elucidate whether PG102 affects secretion of IL-37, different cells such as primary keratinocytes should be employed.

To date, 5 proteins are known to interact with IL-37: Caspase-1, Caspase-4, IL-18 receptor 1 (IL-18R1), IL-18BP and Smad3 [101]. Caspase-1 and Caspase-4 are known to cleave their substrates in the cytosol and IL-37 is known to bind to IL-18R1 and IL-18BP in the extracellular space [96, 102]. Thus, Smad3 is the only protein that is reported to interact and form functional complex with IL-37 in the perinuclear regions. Our results showed colocalization of IL-37 and pSmad3 in the perinuclear regions of HaCaT cells which implies formation of functional complex of these two proteins. Yet, because IL-37 was prevalent in HaCaT cells after treatment with PG102, there is a possibility that these two proteins were co-stained by chance. Thus, using methods such as co-immunoprecipitation will support the IFA results showing interaction between IL-37 and pSmad3.

It is not yet clear which compounds in PG102 upregulate the expression of IL-37. PG102 contains numerous compounds with anti-inflammatory properties [56]. One particular group of molecules of interest is the glycosides, as these molecules isolated from *Tripterygium wilfordii* Hook F have been shown to possess strong anti-inflammatory properties and upregulate IL-37 in THP-1 human macrophage cell line [103, 104]. Glycosides of various compounds are abundant in PG102 as well, so they

can be obvious candidate molecules responsible for the upregulation of IL-37 [54, 105]. Further investigations are underway to identify active compounds from PG102.

In conclusion, our findings demonstrate that silencing IL37 intensifies the expression of AMPs in response to cytokine stimulation in HaCaT cells. PG102 significantly increased IL-37 expression at both mRNA and protein levels, and these effects were mediated through ERK/Smad3 and p38 pathways. Furthermore, PG102 promoted colocalization of IL-37 and phosphorylated Smad3 in perinuclear regions, which is essential for the anti-inflammatory activities of IL-37. Currently, there is no report on agents that can induce the expression of IL-37 in keratinocytes. Our results suggest that PG102 might be used as a basis for developing therapeutics for inflammatory skin diseases such as psoriasis.

Chapter V

Fractionation of PG102

1. Background

Previous reports have shown anti-inflammatory and anti-oxidative effects of PG102 in various animal models and *in vitro* systems. For instance, PG102 was shown to regulate balance between Th1 and Th2 cytokines and decrease production of IgE in OVA-sensitized mice [60]. It also promoted expansion of regulatory T cells (Tregs) in atopic dermatitis model and alleviated clinical symptoms [57]. The multifaceted biological activities of PG102 may be due to actions of multiple compounds included in the extract, working in either additive or synergistic effects. Indeed, numerous herbal medicines exert their therapeutic effects through combinatorial effects of multiple compounds on multiple targets [106].

One of the most conventional methods to identify active components in a mixture is to fractionate it using organic solvents of different polarities [107]. A group of compounds with similar polarities and chemical properties are separated and enriched into each solvent. By testing bioactivity of each fraction, it would be possible to narrow down the fraction with the highest activity and that fraction might be further analyzed to isolate active compounds.

Actinidia arguta is known to contain several aromatic terpenoids and esters, which are responsible for its characteristic odor, along with organic acids, sugars and glycosides, which contribute to the taste of *Actinidia arguta* [54, 105, 108]. Although components of *Actinidia arguta* have been studied by various groups, its active compounds haven't been identified yet. Kim et al. (2013) has previously shown that anti-allergic effects of *Actinidia arguta* could be partially reconstituted by combining 6 compounds present in *Actinidia arguta* at appropriate ratio [56]. This implies that the holistic effect of PG102 is the result of more than at least 6 compounds present in the mixture and to fully recapitulate the effects of PG102, additional compounds needs be identified.

Because the compounds involved in anti-allergic effects and anti-psoriatic

effects of PG102 might be different, bioactivities of PG102 fractions were studied with a similar approach. In this chapter, PG102 was fractionated with three organic solvents and biological activity of each fraction was assessed both *in vitro* and *in vivo*, in an attempt to identify fraction with the highest anti-psoriatic effects. Originally, it was speculated that this approach would ultimately facilitate identification of active compound(s) present in PG102 and contribute to standardization of PG102 and possibly, other herbal medicines.

2. Results

2.1. Fractionation of PG102

In previous study, total PG102 (named PG102T) was fractionated using four different organic solvents [chloroform, ethyl acetate (EA), butanol and water, named PG102C, PG102E, PG102B and PG102W, respectively] and efficacy of each fraction was tested in IL-4-based bioassay and murine atopic dermatitis model. It was shown that PG102E showed around 9 times higher IL-4-suppressing activity than total PG102 [60]. In this study, PG102T was fractionated with the same organic solvents with slight modifications (Fig. 33). Because PG102 is a water-soluble extract from fruits of *Actinidia arguta*, it contains high proportion of sugars and glycosides which are soluble in water [105]. Therefore, PG102T dissolved in water was initially fractionated with butanol to exclude sugars and the butanol fraction was further fractionated using chloroform and EA.

As shown in HPLC chromatograms in Fig. 34, PG102C, PG102E and PG102B each exhibits different chemical profiles. Due to the complex nature of water fraction, which contains plethora of saccharides and glycosides, however, PG102W could not be analyzed by HPLC. It could be inferred from the different chemical profile of each PG102 fraction that each fraction possibly possesses different biological activities. Therefore, the anti-inflammatory effects of each fraction were tested both *in vitro* and *in*

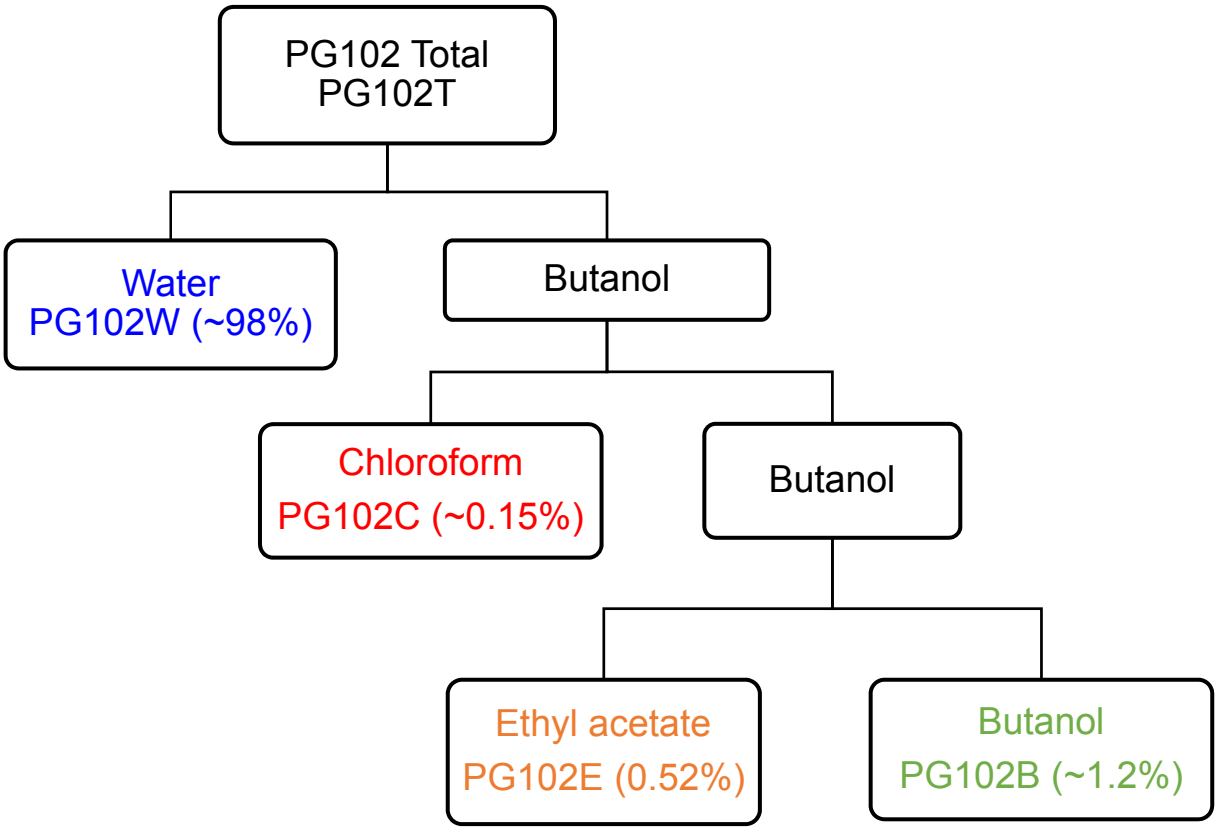


Fig. 33. Fractionation scheme of PG102. Total PG102 (PG102T) was initially dissolved in distilled water and fractionated using water-saturated butanol. The water layer was collected and was designated PG102W. The butanol layer was further fractionated with chloroform and ethyl acetate and these fractions were designated as PG102C and PG102E, respectively. The remaining butanol layer was designated as PG102B. All fractions were concentrated using rotary evaporator and stock solution were prepared at 400 mg/ml in DMSO.

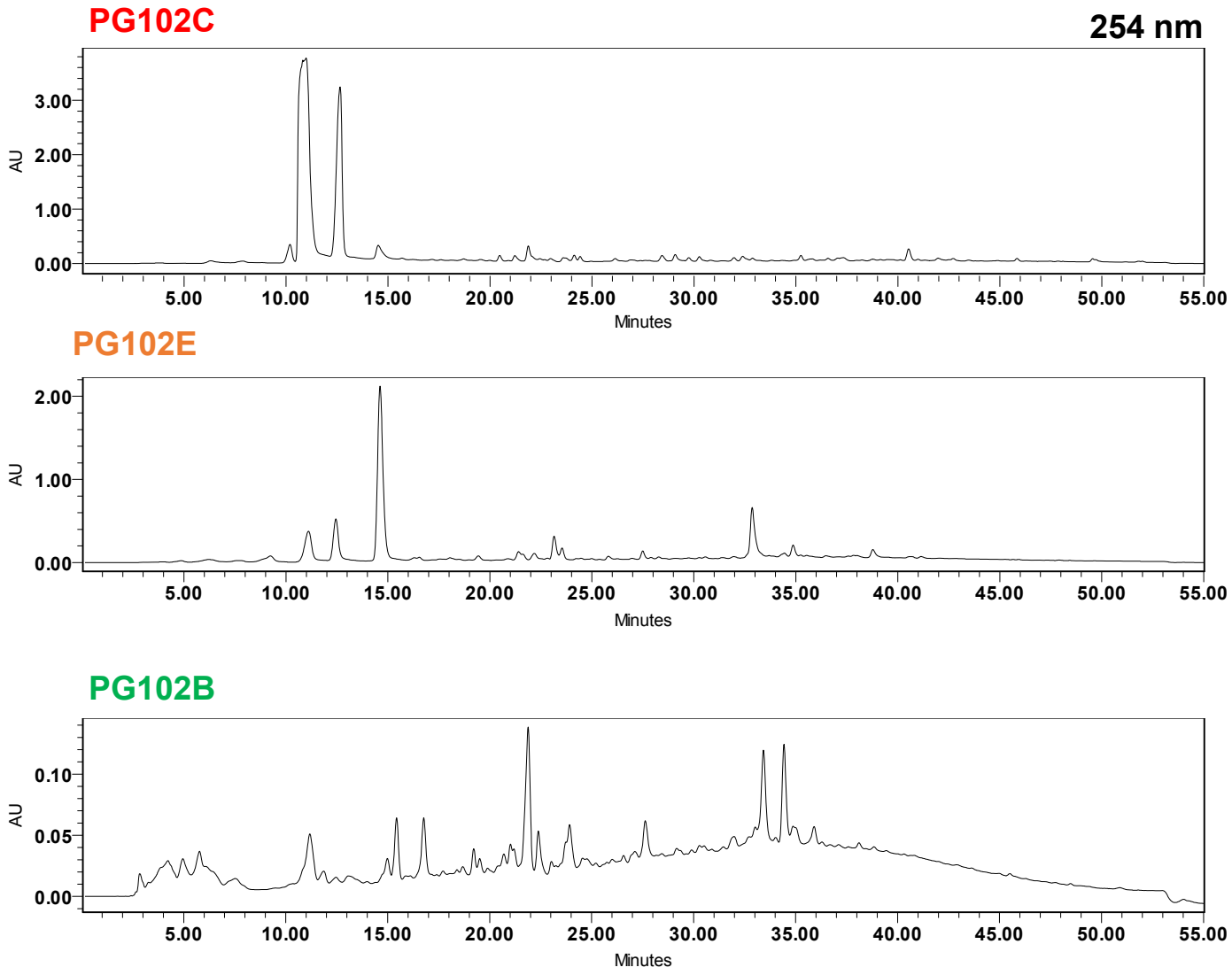


Fig. 34. HPLC fingerprints of PG102C, PG102E and PG102B. Chemical compositions of PG102C, PG102E and PG102B prepared at 20 mg/ml were analyzed by HPLC at 254 nm.

vivo.

2.2 Effects of chemokine secretion by HaCaT cells

Anti-inflammatory effects of each fraction was initially tested *in vitro* by IL-8-based bioassay system described in Chapter X. HaCaT cells were treated with M5 (10 ng/ml) and various concentrations of PG102 (100, 200 and 400 μg/ml) for 24 hours. Cytotoxicity of each fraction was measured by WST-1 assay, cell culture supernatant was collected and IL-8 level was measured by ELISA. As shown in Fig. 35, PG102T, PG102E, PG102B and PG102W did not show any cytotoxicity in the range of 100 ~ 400 ug/ml but PG102C fraction showed high cytotoxicity at 400 ug/ml. Due to the high cytotoxicity exhibited by PG102C, it was not used for further experiments. When IL-8 level was measured in the supernatant, PG102T and PG102W showed comparable IC50 values (150.8 and 142.8 ug/ml) while PG102C, PG102B and PG102E showed lower IL-8-suppressing activities (Fig. 36).

To further compare bioactivities of PG102T and PG102W, HaCaT cells were treated with M5 and both fractions at higher concentrations (up to 800 μg/ml) for 24 hours and CXCL1 and IL-8 levels in the cell culture supernatants were measured by ELISA. As shown in Fig. 37, both PG102T and PG102W did not show any cytotoxicity up to the concentration of 800 μg/ml. CXCL1 and IL-8 levels were highly increased by M5 treatment but both fractions of PG102 lowered them in a dose-dependent manner (Fig. 38). Although PG102T showed slightly lower IC50 values, it was not significantly different from that of PG102W. Taken together, these data indicated that bioactivity of PG102 was divided upon fractionation and that PG102W possesses bioactivity closest to the PG102T, in terms of its suppressive effects on chemokines such as CXCL1 and IL-8.

2.3 Effects of PG102 fractions on antimicrobial peptide expression in HaCaT cells

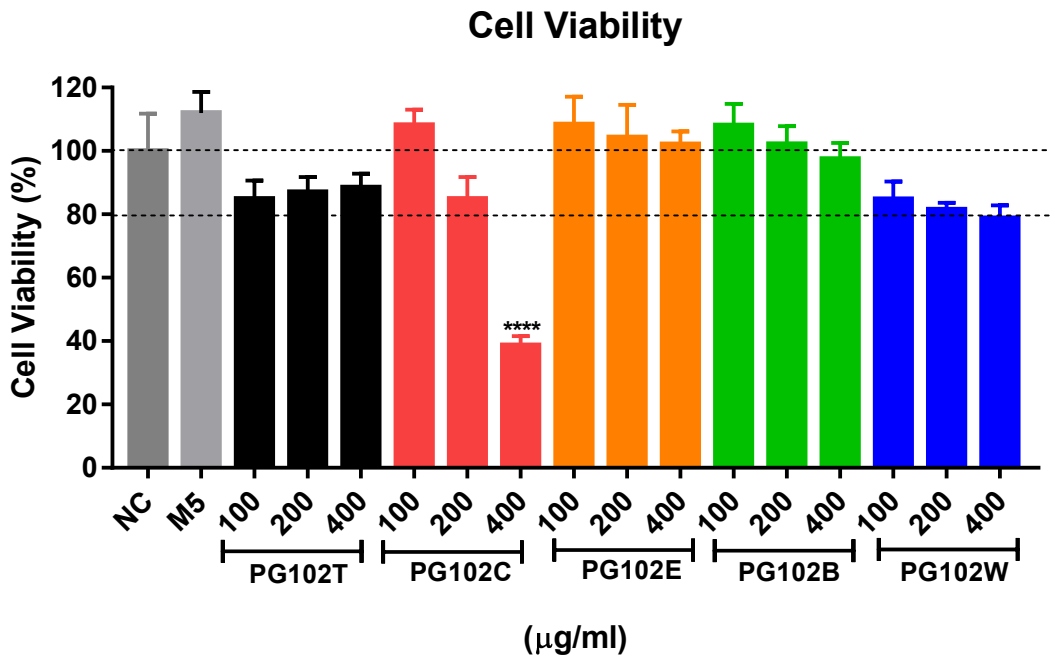
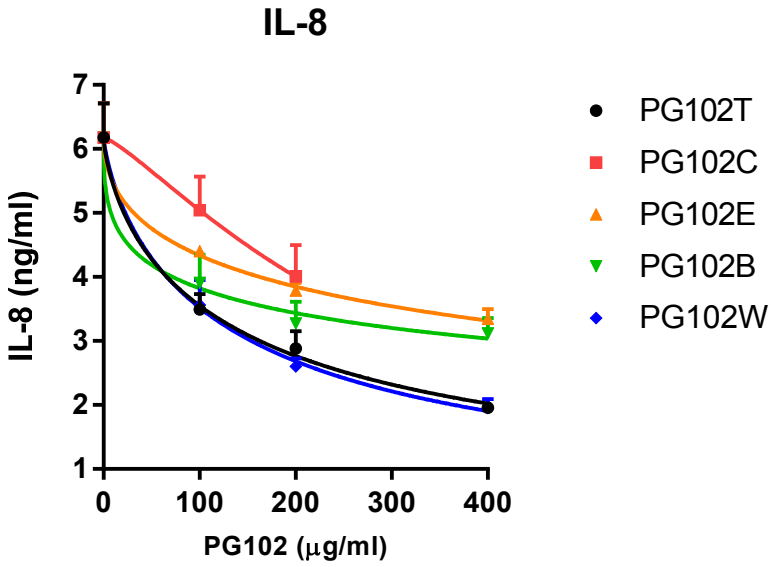


Fig. 35. Effects of PG102 fractions on cell viability of HaCaT cells. HaCaT cells were treated with M5 and four PG102 fractions at different concentrations. After 24 hours, cells were subjected to WST-1 assay to measure cell viability. **** $p<0.0001$ versus M5-treated group.



Fraction	IC_{50} (µg/ml)
PG102T	150.8
PG102C	324.1
PG102E	509.3
PG102B	365.7
PG102W	142.8

Fig. 36. IL-8 bioassay of PG102 fractions. HaCaT cells were treated with PG102 fractions at different concentrations for 24 hours. Cell supernatants were collected and the level of IL-8 was measured by ELISA.

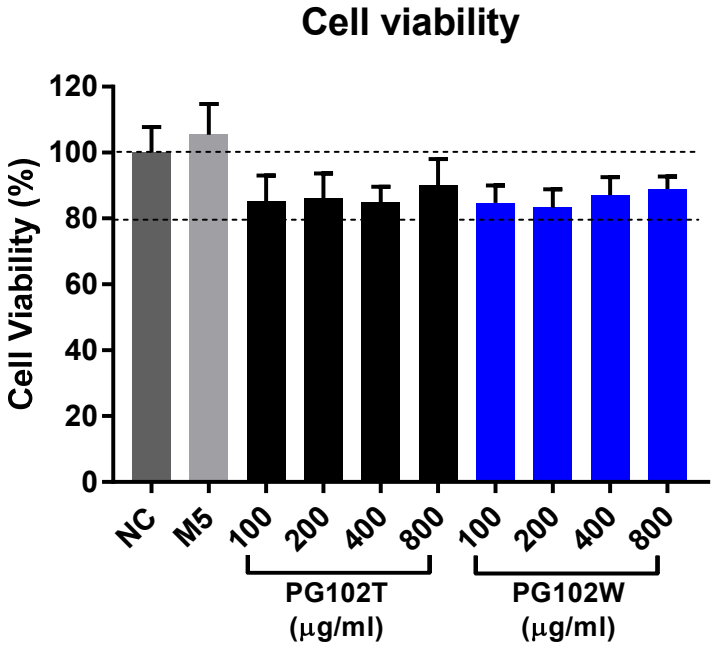
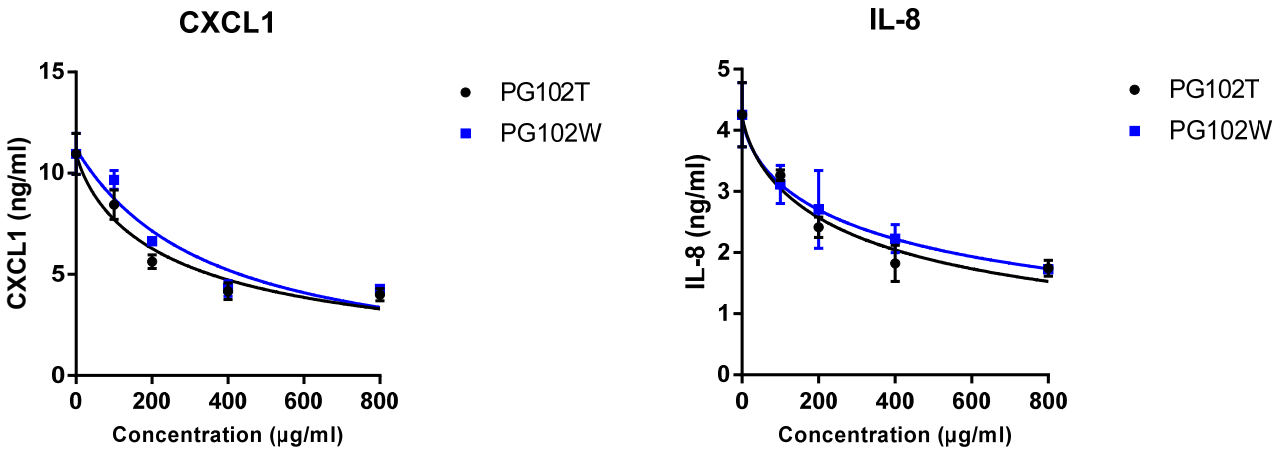


Fig. 37. Effects of PG102T and PG102W on cell viability of HaCaT cells. Cells were treated with M5, PG102T and PG102W at various concentrations for 24 hours and cell viability was measured by WST-1 assay.



Fraction	IC_{50} (µg/ml)
PG102T	279.6
PG102W	350.3

Fraction	IC_{50} (µg/ml)
PG102T	351.6
PG102W	456.7

Fig. 38. Effects of PG102T and PG102W on chemokine secretion in HaCaT cells. Cells were treated with M5, PG102T and PG102W at different concentrations for 24 hours. Cell culture supernatants were collected and were subjected to CXCL1 and IL-8 ELISA.

As described in Chapter 3, PG102 could effectively suppressed expression of antimicrobial peptides (AMPs). Therefore, effects of PG102 fractions on AMP expression were investigated. HaCaT cells were treated with M5 (10 ng/ml) and PG102T, PG102E, PG102B and PG102E at 400 ug/ml for 24 hours, followed by total RNA extraction and RT-qPCR. As shown in Fig. 39, total PG102 decreased expression of AMPs BD2, S100A7, S100A8, S100A9 and S100A12 most potently. Among PG102 fractions, PG102E showed the highest bioactivity, followed by PG102W and PG102B. These data suggest that of AMP-inhibiting activity of PG102 was again divided upon fractionation and PG102E possesses bioactivity closest to the PG102T, while PG102B shows minor effects on AMP expression.

2.4 Effects of PG102 fractions on anti-inflammatory mediators in HaCaT cells

In Chapter 3, I showed that PG102 could increase the expression levels of two anti-inflammatory mediators BD3 and IL-37. To test which fraction could increase these mediators, HaCaT cells were treated with M5 (10 ng/ml) and each fraction at 400 ug/ml for 24 hours, followed by total RNA extraction and RT-qPCR. As shown in Fig. 40, PG102W could upregulate the expression levels of BD3 and IL-37 at the level comparable to total PG102. Although PG102E increased IL-37 mRNA level more than PG102T and PG102W, the difference between PG102E and PG102T group was not statistically significant. Taken together, PG102W was most effective in increasing BD3 expression while PG102E was most effective in increasing IL-37 expression. Previous report has shown that BD3 increases expression of IL-37 in keratinocytes [43]. These results imply that the increase in IL-37 expression is not due to the increased expression of BD3 but through a different pathway.

2.5 Comparison of anti-psoriatic effects of PG102T, PG102C, PG102E and PG102W

The above data suggested that PG102B might not have anti-psoriatic

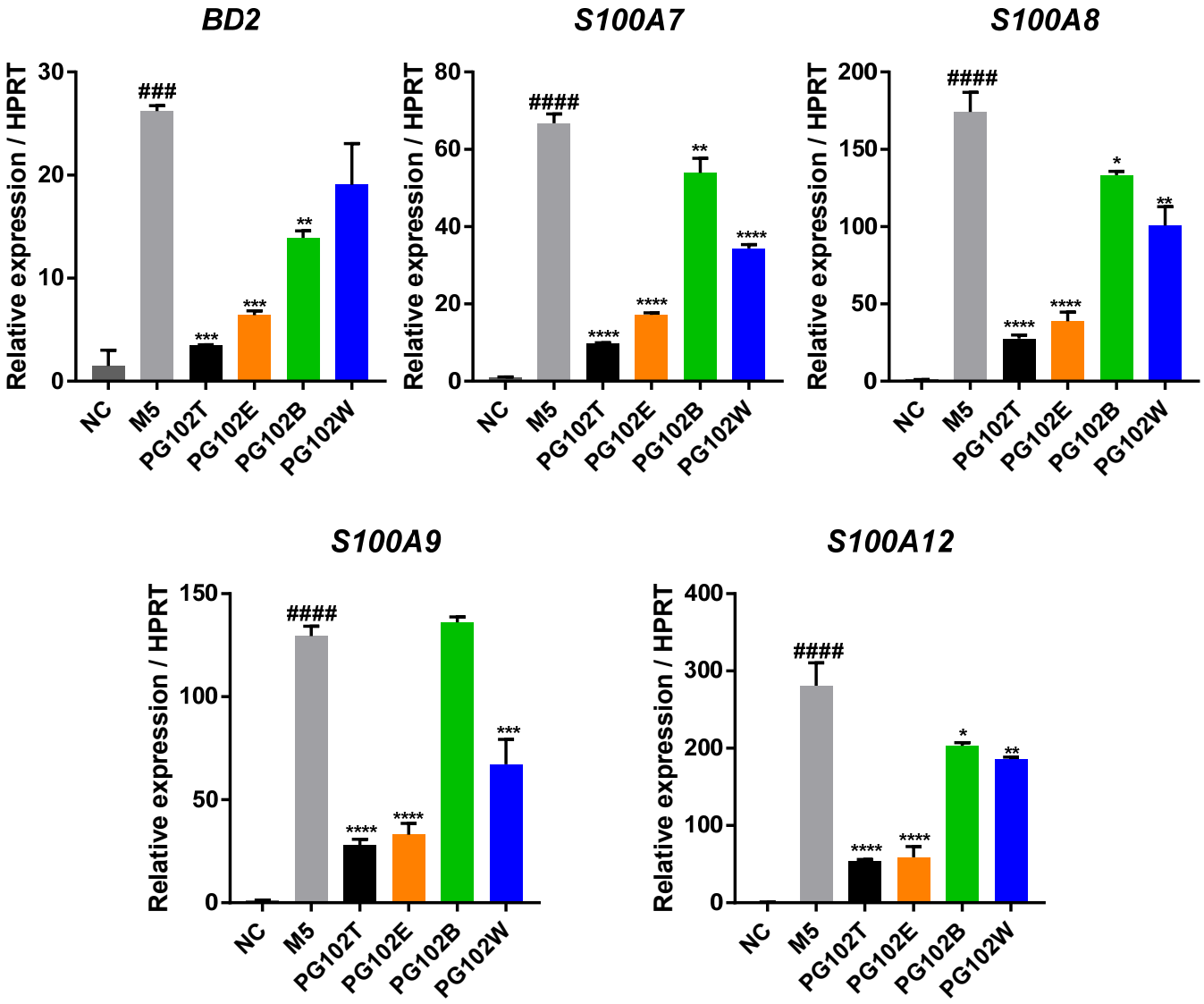


Fig. 39. Effects of PG102 fractions on expression of AMPs. Cells were treated with M5 and PG102 fractions (400 µg/ml) for 24 hours. Total RNA was isolated and relative mRNA levels were quantified by RT-qPCR analysis. #### $p<0.0001$ versus NC; **** $p<0.0001$, *** $p<0.001$, ** $p<0.01$, * $p<0.05$ versus M5-treated group.

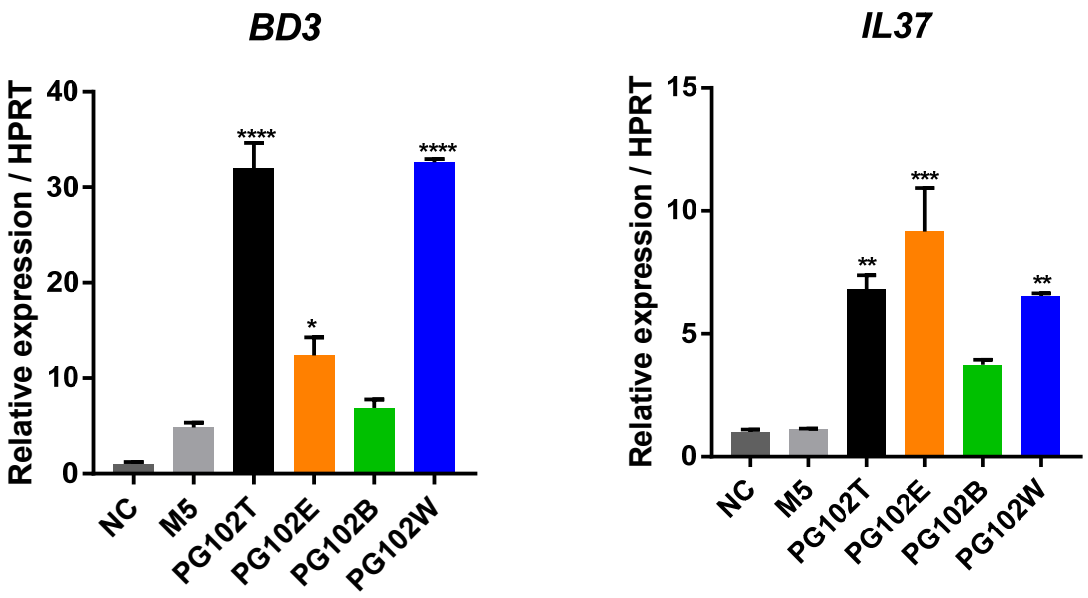


Fig. 40. Effects of PG102 fractions on expression of anti-inflammatory mediators. Cells were treated with M5 and PG102 fractions (400 µg/ml) for 24 hours. Total RNA was isolated and relative mRNA levels were quantified by RT-qPCR analysis. #### $p<0.0001$ versus NC; **** $p<0.0001$, *** $p<0.001$, ** $p<0.01$, * $p<0.05$ versus M5-treated group.

bioactivities, so this fraction was excluded from further *in vivo* experiments. Even though PG102C exerted substantial cytotoxicity, it did show IL-8-suppressing activities at low concentration (200 ug/ml), so its efficacy was initially tested in IMQ-induced psoriasis model, along with PG102T, PG102E and PG102W. As shown in Fig. 41, vehicle group showed significant increase in thickness, scales, erythema and cumulative PASI scoring while topical application of PG102T ameliorated each of the clinical parameters. PG102E and PG102W each showed slight ameliorating effects on PASI scoring. Of note, PG102W significantly reduced serum IL-17A level and exhibited higher bioactivities compared to PG102E (Fig. 42). Next, RNA levels of AMPs and inflammatory cytokine/chemokines were measured. PG102T suppressed expression levels of S100A9, CXCL1 and IL-17F while only CXCL1 and IL-17F were suppressed by PG102W (Fig. 43). On the other hand, PG102C had no effects on clinical scorings, serum IL-17A level and mRNA levels of AMPs and inflammatory cytokine/chemokines. These results suggest that PG102C not only causes cytotoxicity in HaCaT cells but also does not show any beneficial effects on IMQ-induced psoriasis model. In addition, similar to the results obtained from *in vitro* experiments, none of the PG102 fractions could achieve bioactivity comparable to that of PG102T.

2.6 Comparison of anti-psoriatic effects of PG102T, PG102E, PG102W and mixture of PG102E and PG102W

It was speculated that the holistic effects of PG102T could be achieved by mixing PG102E and PG102W as PG102W effectively downregulated expression of chemokines while PG102E effectively downregulated expression of AMPs in HaCaT cells (Fig. 38 and 39). To test this hypothesis, PG102T, PG102E, PG102W and PG102E + PG102W mixed at 1:1 ratio were topically applied on dorsal skin of mice along with IMQ. Due to the hair grown during the experiment, it was not possible to perform PASI scoring (Fig. 44). The epidermal thickness was greatly increased in vehicle group, as

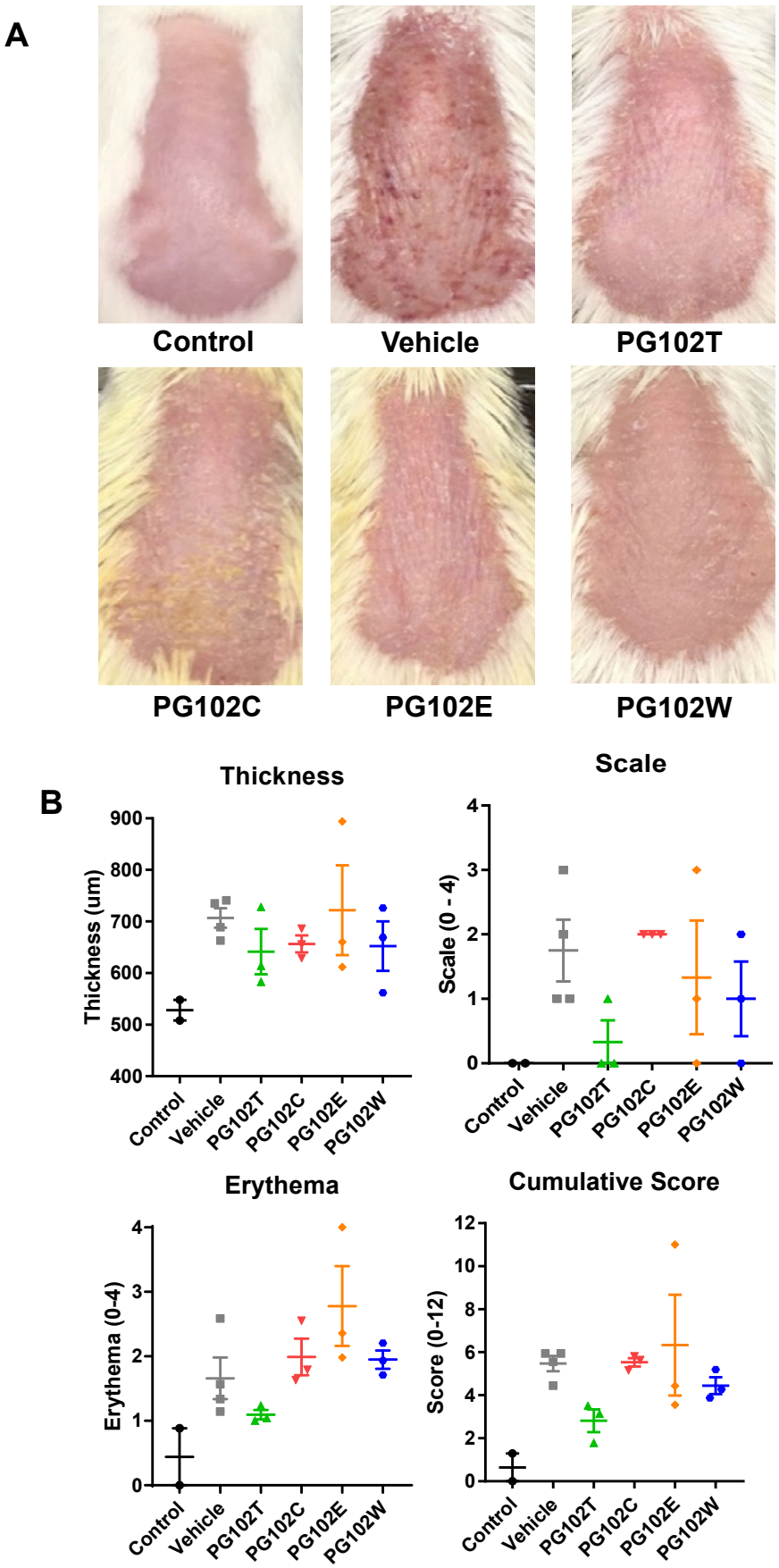


Fig. 41. Effects of PG102 fractions on IPI model. Mice were topically administered with IMQ and four different PG102 fractions (100 mg/kg) for 6 consecutive days (n=4). On day 6, mice were sacrificed and analyzed. (A) Photo of dorsal skin. (B) PASI scores.

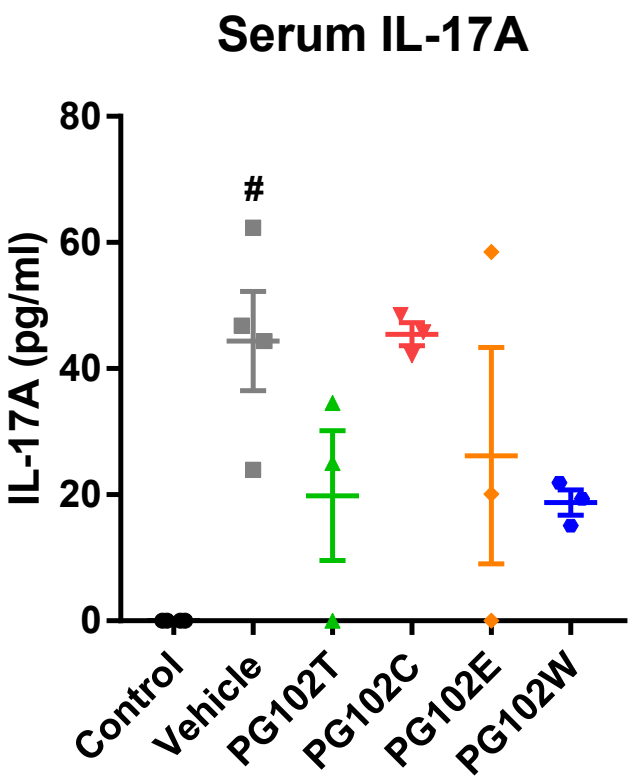


Fig. 42. Effects of PG102 fractions on serum IL-17A level in IPI model. Serum from mice of each group were collected on day 6 and IL-17A levels were measured by ELISA. # $p<0.05$ versus Control group.

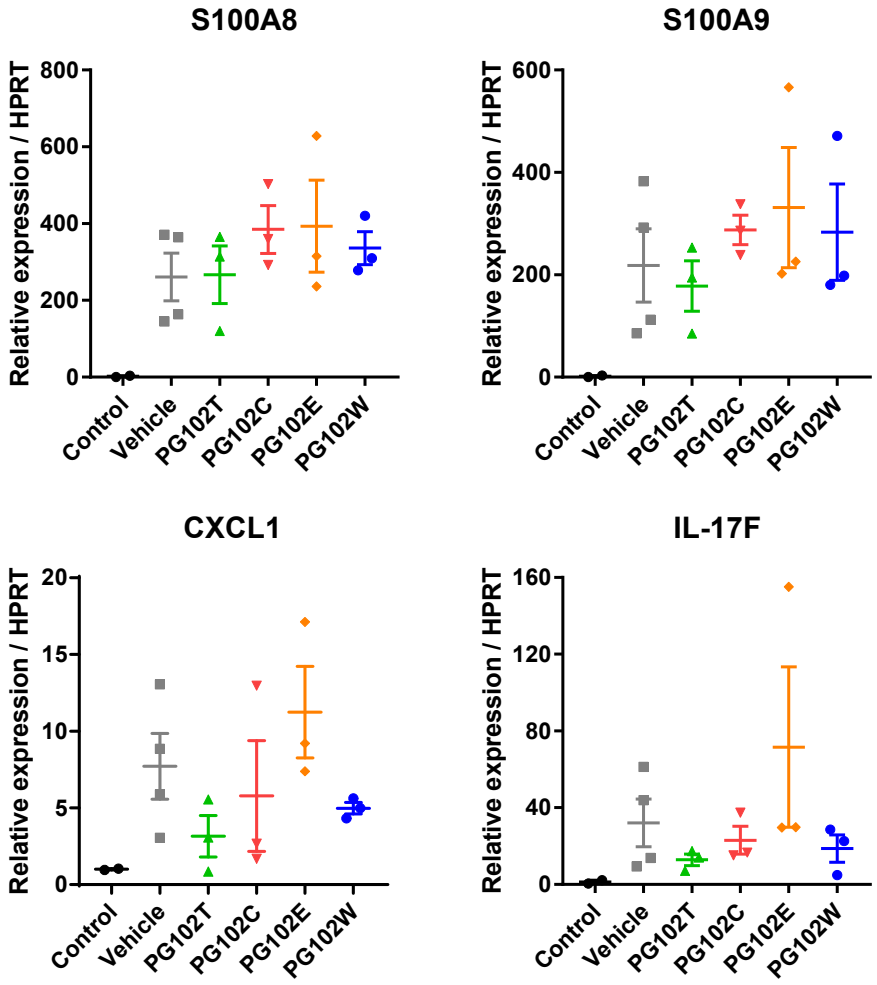


Fig. 43. Effects of PG102 fractions on expression of inflammatory mediators in skin. Dorsal skin from each mice was collected and total RNA was isolated from each sample (n=3~4). Relative expression levels of each gene were measured by RT-qPCR analysis.

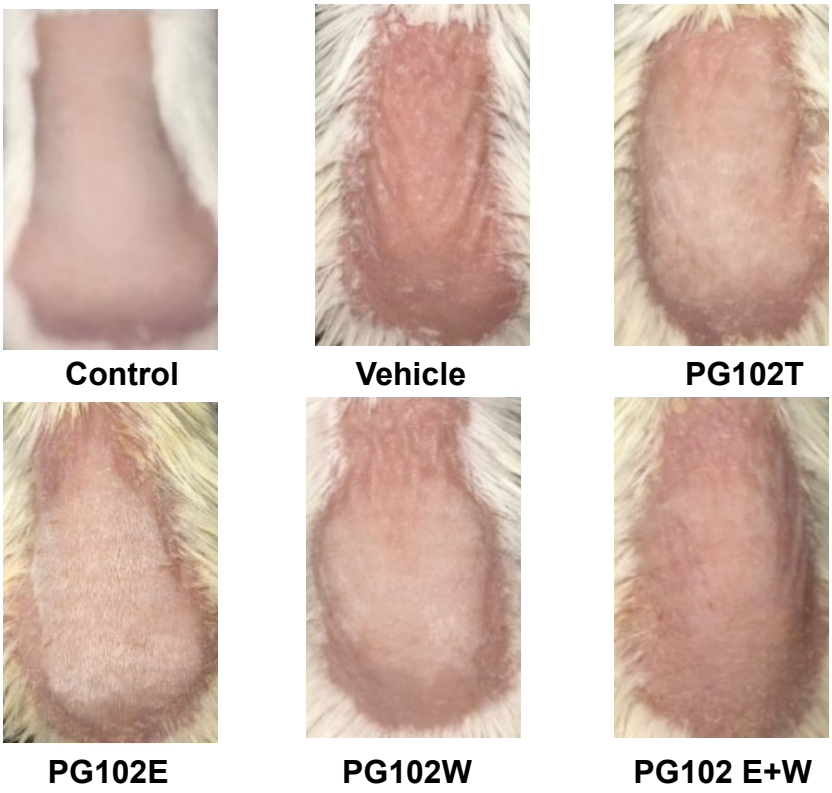


Fig. 44. Effects of PG102 fractions on IPI model. Mice were treated with IMQ and different fractions of PG102 (100 mg/kg) for 6 consecutive days (n=3~4). Photo of dorsal skin was taken on day 6, before sacrifice.

shown in H&E staining of dorsal skin (stained in dark purple), showing characteristic rete ridges (Fig. 45A). On the other hand, PG102T group did not show rete ridges and showed clearly thinner epidermis compared to the vehicle group. PG102E and PG102E + W group showed a number of rete ridges while PG102W showed fewer rete ridges compared to the PG102E group (Fig. 45A). When skin thickness was measured by micrometer, PG102T and PG102W showed reduction in thickness (Fig. 45B). Furthermore, IL-17A levels in serum and dLN were lower in PG102W group compared to PG102E group (Fig. 46). Although serum IL-17A level was comparatively higher in PG102E + W group, IL-17A was not detected in dLN of PG102E + W group.

Effects of PG102 fractions on mRNA levels of pro-inflammatory mediators S100A8, S100A9 and IL-17F were also measured by RT-qPCR. As shown in Fig. 15, PG102E group showed expression levels of these mediators higher than the vehicle group, showing that in contrast to the *in vitro* results, PG102E could not suppress expression of AMPs *in vivo*. PG102E + W group reduced mRNA levels of S100A8 and S100A9 at similar degree to PG102W but it did not reduce IL-17F level (Fig. 47).

Considering various clinical parameters of psoriasis, PG102W showed effects that was the most comparable to PG102T. Mixture of PG102E and PG102W showed neither additive nor synergistic effects. These results suggest that *in vivo*, PG102W is more effective in ameliorating symptoms of psoriasis than PG102E and mixture of PG102E and PG102W at 1:1 ratio was not sufficient to achieve the holistic efficacy of PG102T.

3. Discussion

One of the main hurdles in the development of botanical drugs is the identification of active compounds. Characterization of active compounds would not only aid discovery of mechanism of action but also facilitate quality control of the drug. For decades, researches have focused on isolating single active compound(s)

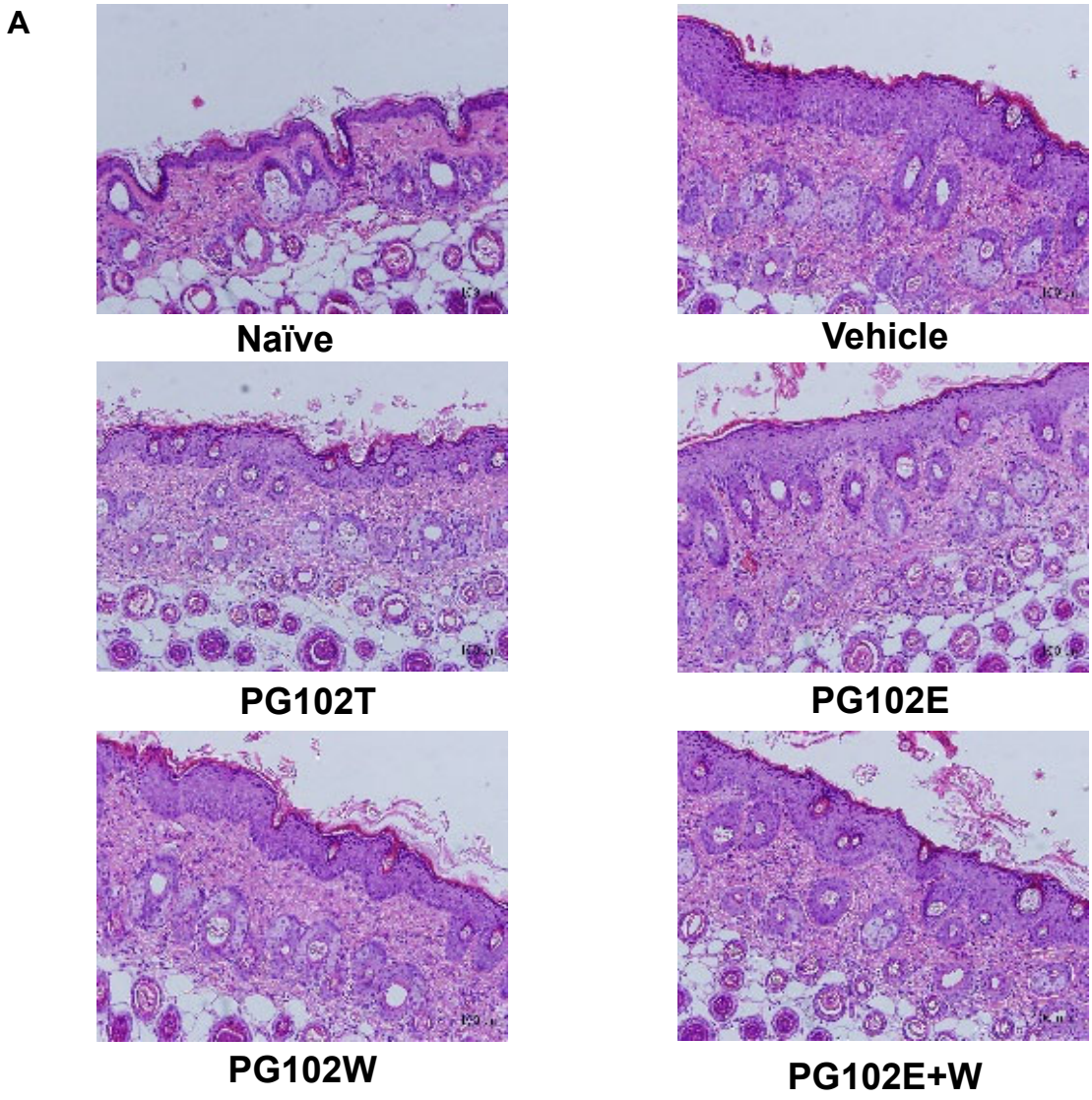


Fig. 45. Effects of PG102 fractions on skin thickness in IPI model. Mice were treated with IMQ and different fractions of PG102 (100 mg/kg) for 6 consecutive days (n=3~4). (A) H&E staining of dorsal skin on day 6. (B) Thickness of dorsal skin measured by micrometer. #### $p<0.0001$ versus Control group.

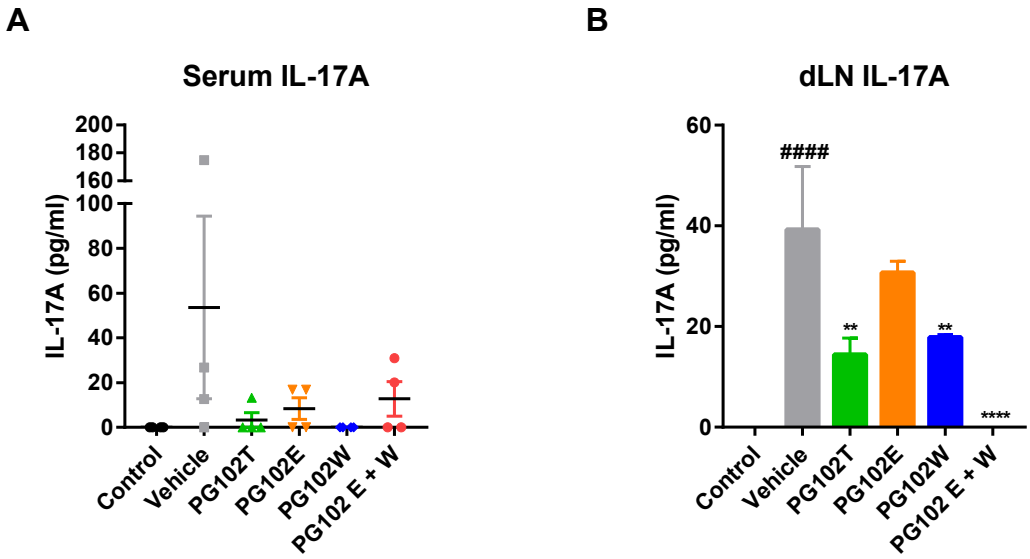


Fig. 46. Effects of PG102 fractions on IL-17A levels in serum and dLN. (A) Serum from mice of each group were obtained and IL-17A levels were measured by ELISA. (B) Axillary and inguinal lymph nodes from mice of each group were isolated, pooled and restimulated with PMA/ionomycin. 24 hours later, IL-17A levels in the supernatant were measured by ELISA. #### $p<0.0001$ versus the Control group; ** $p<0.01$, **** $p<0.0001$ versus the Vehicle group. The data are shown as the mean ± standard error mean (SEM).

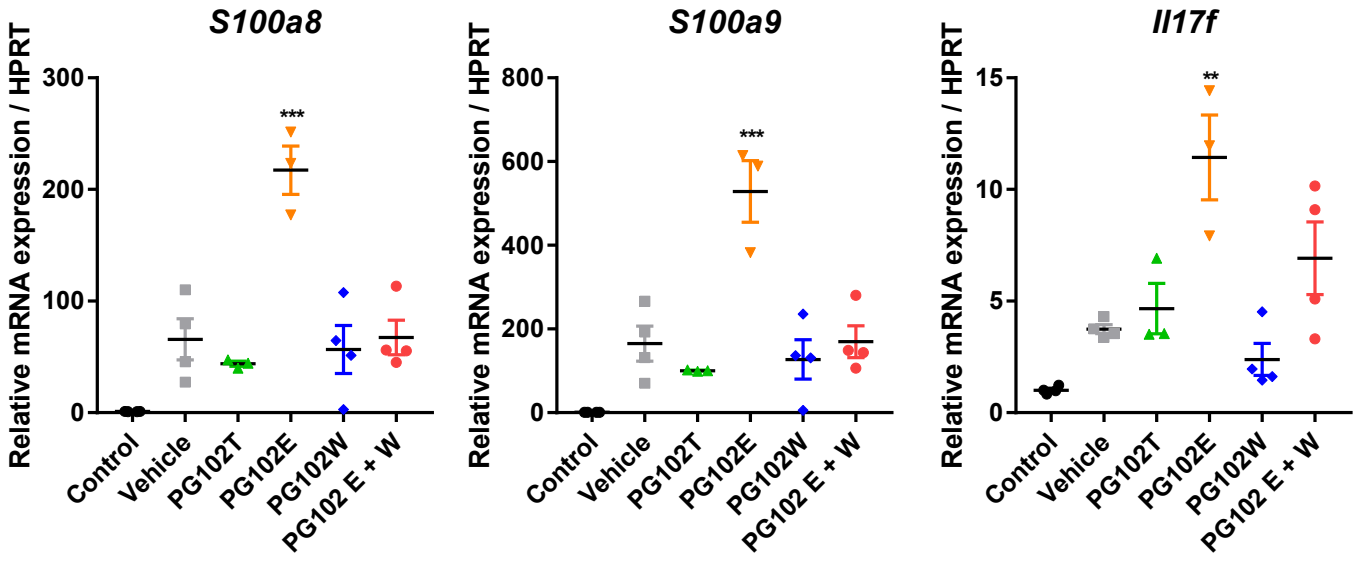


Fig. 47. Effects of PG102 fractions on expression of inflammatory mediators in skin. Dorsal skin from each mice was collected on day 6 and total RNA was isolated from each sample (n=3~4). Relative expression levels of each gene were measured by RT-qPCR analysis. *** $p<0.001$ versus Vehicle group.

responsible for respective biological activities from plant sources. However, most of these attempts have not been successful as they disregarded the combinatorial roles of numerous different compounds in the whole extract. Rather than screening for a single effective constituent, isolating a fraction with concentrated activity may be a more plausible approach to characterize the herbal medicine.

In this chapter, PG102 was fractionated with chloroform, EA, butanol and water and anti-psoriatic effects of each fraction was tested in both *in vitro* and *in vivo* systems. In HaCaT cells, effects of each fractions on chemokine expression and AMP expression were assessed. Previous studies have shown PG102E possesses 12 times higher IL-4 suppressing activity in RBL-2H3 cells compared to PG102T (unpublished results). On the other hand, none of the PG102 fractions exerted IL-8 suppressing activity higher than that of PG102T in HaCaT cells, although each fraction suppressed IL-8 secretion from HaCaT cells to different degrees. Thus, it can be speculated that the bioactivity of PG102T is split upon fractionation. Of note, PG102W showed comparable CXCL1 and IL-8 suppressing activity to PG102T, implying PG102W is the main fraction accounting for the chemokine-suppressing activity of PG102. In fact, the yield of PG102W accounts for more than 80% of PG102T so it can be expected to contain the majority of bioactivities of PG102T. When the effects of PG102 fractions on AMP expression were tested, PG102E was shown to be more effective in downregulating them than PG102B or PG102W. Yet, it was not more effective than PG102T which indicates that PG102E is not the main concentrated fraction responsible for regulation of AMP expression (Table V).

Based on previous reports, it could be hypothesized that a number of compounds in PG102W and PG102E possess immunomodulatory functions [109-114]. For instance, *Actinidia arguta* is rich in sugars such as sucrose, glucose, fructose, mannose with myo-inositol being the major sugar and it can be predicted that these sugars are fractionated into PG102W [115]. Myo-inositol and its phosphorylated form

Fraction	Cytotoxicity (100 ~ 400 μg/ml)	*in vitro*		*in vivo*	
		Effects on chemokines	Effects on AMPs	Effects on PASI score	Effects on biochemical parameters
PG102T	X	↓↓↓↓	↓↓↓↓	↓↓↓↓	↓↓↓
PG102C	O	↓↓	N/A*	–	–
PG102E	X	↓	↓↓↓	↓	↓
PG102B	X	↓↓	↓	N/A	N/A
PG102W	X	↓↓↓	↓↓	↓↓	↓↓

* N/A: Not assessed

Table V. Summary of anti-psoriatic effects of various PG102 fractions. AMP, antimicrobial peptides; PASI, psoriasis area severity index.

myo-inositol hexaphosphate were shown to downregulate IL-8 secretion from colonic epithelial cells [116]. In addition, it has recently been reported that D-mannose induces generation of Tregs in mouse models of autoimmune diabetes [117]. Consistent with this report, PG102 treatment was shown to induce Tregs both *in vivo* mouse atopic dermatitis model and ex vivo cell culture system [57]. Thus, these sugars may be the compounds responsible for immunomodulatory effects of PG102W. A number of compounds in PG102E have also been identified and they are known to exert anti-inflammatory effects in various cells. For instance, gallic acid is known to downregulate expression of IL-6 and TNF-α in LPS-stimulated Raw264.7 cells while caffeic acid suppressed iNOS and COX-2 expression in HaCaT cells [118-120]. Because these compounds exist in minute amounts in PG102, the holistic effects of PG102 may be the result of additive or synergistic effects of these compounds (Table VI).

As many chronic diseases result from multiple factors, single compound-single target strategy in drug development is facing limitations. On the other hand, therapeutic potential of botanical drugs that can target multiple mediators of the diseases is increasingly emphasized [121]. However, conventional fractionation method using solvents of different polarities has not been always successful in deciphering active compounds since the bioactivity of the whole extract may be separated upon fractionation, as in the case of PG102. Therefore, a novel approach to screen active compounds in herbal extracts is needed to expedite the development of botanical drugs. One such method is chemical knock-out strategy, proposed by Liu et al (2014) and Song et al (2016). Similar to the idea of knocking out genes to study their function in cells, this strategy selectively removes each component in the herbal medicine using preparative HPLC [122, 123]. For instance, at the first round of screening, the selection threshold was set at 10% of the total peak area of the whole HPLC chromatogram and all compounds accounting for more than 10% of the whole extract are collected, mixed and were subjected to respective bioassays. In the next rounds of screening, the

Compound	Role(s)	Reference(s)
Ascorbic acid	Promotes epidermal differentiation and formation of epidermal barrier *in vitro*	[107]
Caffeic acid	Induces differentiation of keratinocytes *in vitro*; anti-pruritic effects *in vivo*	[117]
Citric acid	Reduces inflammation in LPS-treated mice	[108]
Gallic acid	Anti-oxidative effects in UVB-treated HaCaT cells	[116]
5-Hydroxymethyl-2-furaldehyde	Suppresses ICAM-1 and production of ROS in HUVEC cells	[109]
Isoquercitrin	Suppresses NF-kB signaling and histamine production in basophil cell line	[111]
Linoleic acid	Downregulates IL-4 production in RBL-2H3 cells	[110]
Malic acid	Inhibits proliferation of keratinocytes *in vitro*	[112]
Mannose	Induces differentiation of regulatory T cells *in vivo*	[115]
myo-Inositol	Downregulates IL-8 production from colonic epithelial cells	[114]
Quinic acid	Inhibits NF-κB signaling in A549 lung epithelial cells	[118]

Table VI. List of major components of *Actinidia arguta* and their anti-inflammatory bioactivities.

selection threshold sequentially reduced at 1% and 0.1% of the total peak area and they were subjected to bioassays. These mixture of compounds, coined as bioactive equivalent combinatorial components (BECCs), showed efficacy that was comparable to the whole extract. This method could be utilized to a wide range of herbal medicines such as PG102, in which bioactivity is separated by conventional fractionation method.

In summary, PG102 was fractionated with chloroform, EA, butanol and water and each fraction suppressed expression of chemokines and AMPs to different extents. PG102W showed bioactivity closest to PG102T in terms of chemokine-suppression, while PG102E showed bioactivity closest to PG102T, in the context of AMP-suppression in HaCaT cells. On the other hand, mixture of PG102E and PG102W did not show any additive or synergistic effects and PG102W was the most effective fraction in ameliorating symptoms of psoriasis *in vivo*. Although PG102E showed concentrated bioactivities in two different allergy models and RBL-2H3 cells, there was no single fraction showing dramatically increased bioactivities in psoriasis model and HaCaT cells. These results suggest that the active compounds of PG102 involved in suppression of Th2-mediated responses of allergy and Th1/Th17-mediated responses of psoriasis are different and thus, an alternative approach, other than fractionation, may have to be employed to identify active compounds with anti-psoriatic effects.

Chapter VI

Concluding Remarks

PG102, first reported in the year 2005 in our laboratory, has been studied over a decade, as reflected in extensive list of publications (Table IV). PG102 was indeed an intriguing material because it contains potent anti-inflammatory activities not only *in vitro* but also in various disease models. Furthermore, being prepared from an edible part of *Actinidia arguta*, PG102 has been shown to be safe in mouse, dogs and human. Until I started my thesis work, most of the studies of PG102 focused on Th2-mediated allergic diseases. Based on previous findings, I hypothesized that the essence of PG102 is its properties of maintaining immune homeostasis; its therapeutic effects are not limited to specific diseases but may be context-dependent. For instance, in the Th2-skewed microenvironment, it promoted Th1 responses to maintain balance between Th1 and Th2 cells and alleviated allergic symptoms. In another study, PG102 increased the frequency of Tregs to maintain immune homeostasis. In my thesis work, I showed that PG102 could also suppress Th17-mediated inflammation and therefore, studies on PG102 might be expanded and applied to other Th17-mediated diseases, such as rosacea (Fig. 48).

In this study, I have shown that topical application, but not oral administration, of PG102 can alleviate psoriasis-like skin inflammation of mice. In an attempt to identify the solvent that can best deliver the components of PG102 topically, DMSO was chosen in this thesis work. Even though PG102 is a water-soluble extract of *Actinidia arguta*, it includes a mixture of both high-polarity and low-polarity components and considering lipid-rich structure of the stratum corneum, the outermost layer of the skin, DMSO was more efficient than distilled water or PEG plus EtOH in delivering PG102. For PG102 to be developed as a topical formulation for human psoriasis, a safer vehicle that can enhance skin penetration and maximize bioavailability should be employed.

My results showed that topical application of PG102 effectively suppressed the increase in thickness and IL-17A levels in the dLN and serum. Treatment with PG102

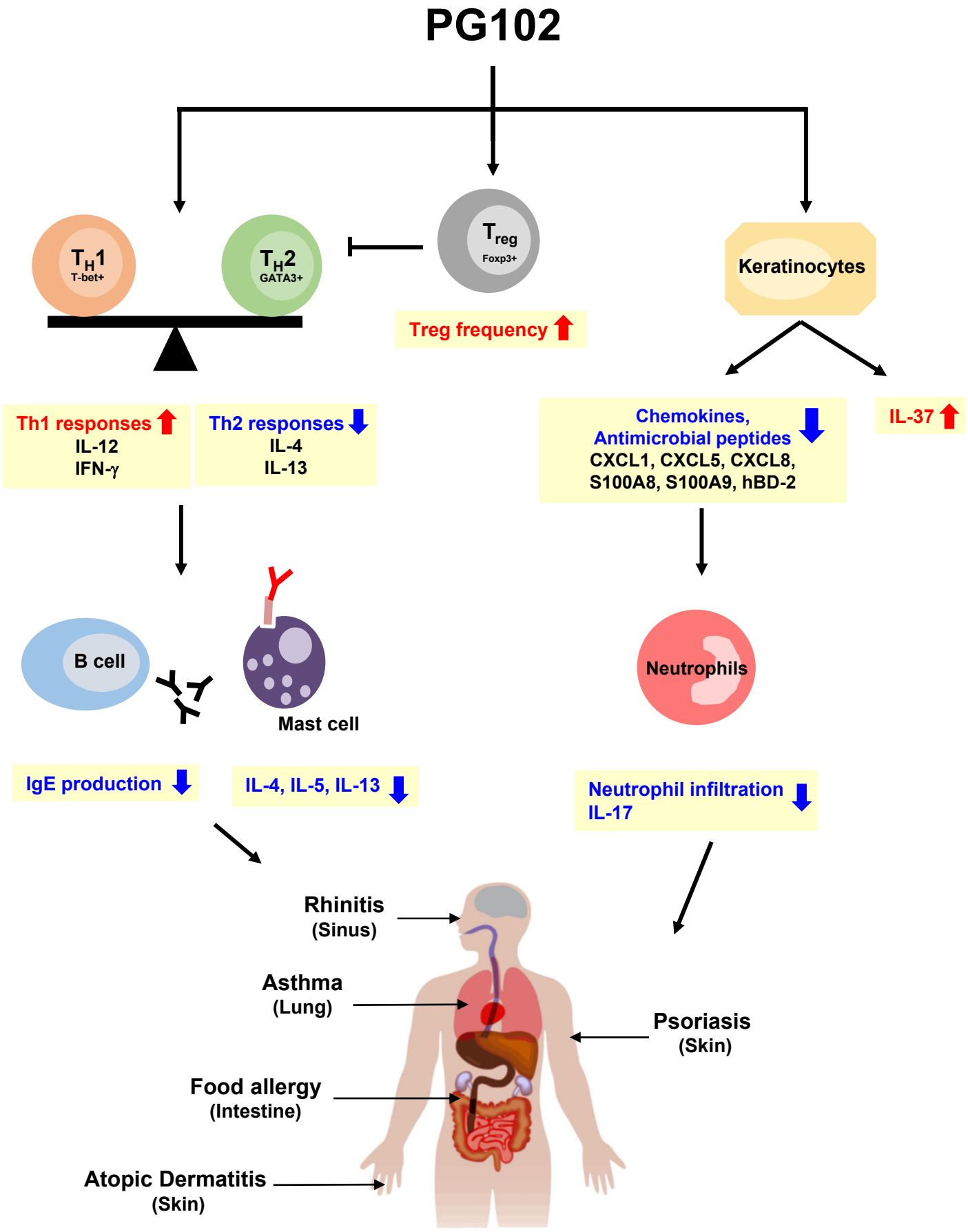


Figure 48. Summary of immunomodulatory effects of PG102. The diagram was created based on all published articles of PG102 from year 2005 to 2016 and this study.

suppressed STAT3-mediated cell proliferation of HaCaT keratinocytes, indicating that the inhibition of skin thickening might be a direct effect of PG102. However, this effect could also be the result of reduction in overall inflammation as levels of other inflammatory mediators and immune cell infiltration was reduced in PG102-treated mice. Results from *in vitro* experiments showed that HaCaT cell could downregulate expression of various chemokines and AMPs, which are responsible for infiltration of neutrophil the skin. Since neutrophils are the main cell types, aside from Th17 cells, present in the psoriatic lesions, reduction in neutrophil chemotaxis may have beneficial roles in treating psoriasis. Inhibition of neutrophil infiltration by PG102 was observed both *in vitro* and *in vivo* – when chemotaxis assays with dHL-60 cells were performed using cell culture supernatants from HaCaT cells stimulated with M5 and PG102, the degree of chemotaxis was reduced in the supernatant with PG102, in a dose-dependent manner. In IPI model, the frequency of $Ly6G^+$ cells were reduced in the skin. Consistently, improvements were observed in the PASI scoring and serum IL-17A level which are the indicators of systemic inflammation (Fig. 49).

As described in Chapter I, the antibody targeting IL-23, which is under phase III clinical trial, was shown to be superior to the antibody targeting IL-17A. It can be speculated that this is because IL-23 is the upstream molecule that induces Th17/IL-17A. As shown in Figure 3, it is the immune cascade beginning from Th17 differentiation that makes psoriasis a complex and multifaceted disease. Blocking IL-23 can essentially inhibit initiation of Th17-mediated immune responses. However, IL-23 is not the molecule located at the uppermost part of the pathogenesis of psoriasis. It is self-DNA/AMP complex that induces IL-23 secretion by DCs by binding to TLR of pDCs. Thus, in theory, inhibiting production of AMPs from keratinocytes might be a more effective approach to blocking initiation of psoriasis pathology. PG102 was shown to suppress expression of various AMPs, including β-defensin 2, S100A8/A9 and LL-37. Among these AMPs, LL-37 was identified as the self-antigen of psoriasis while β-

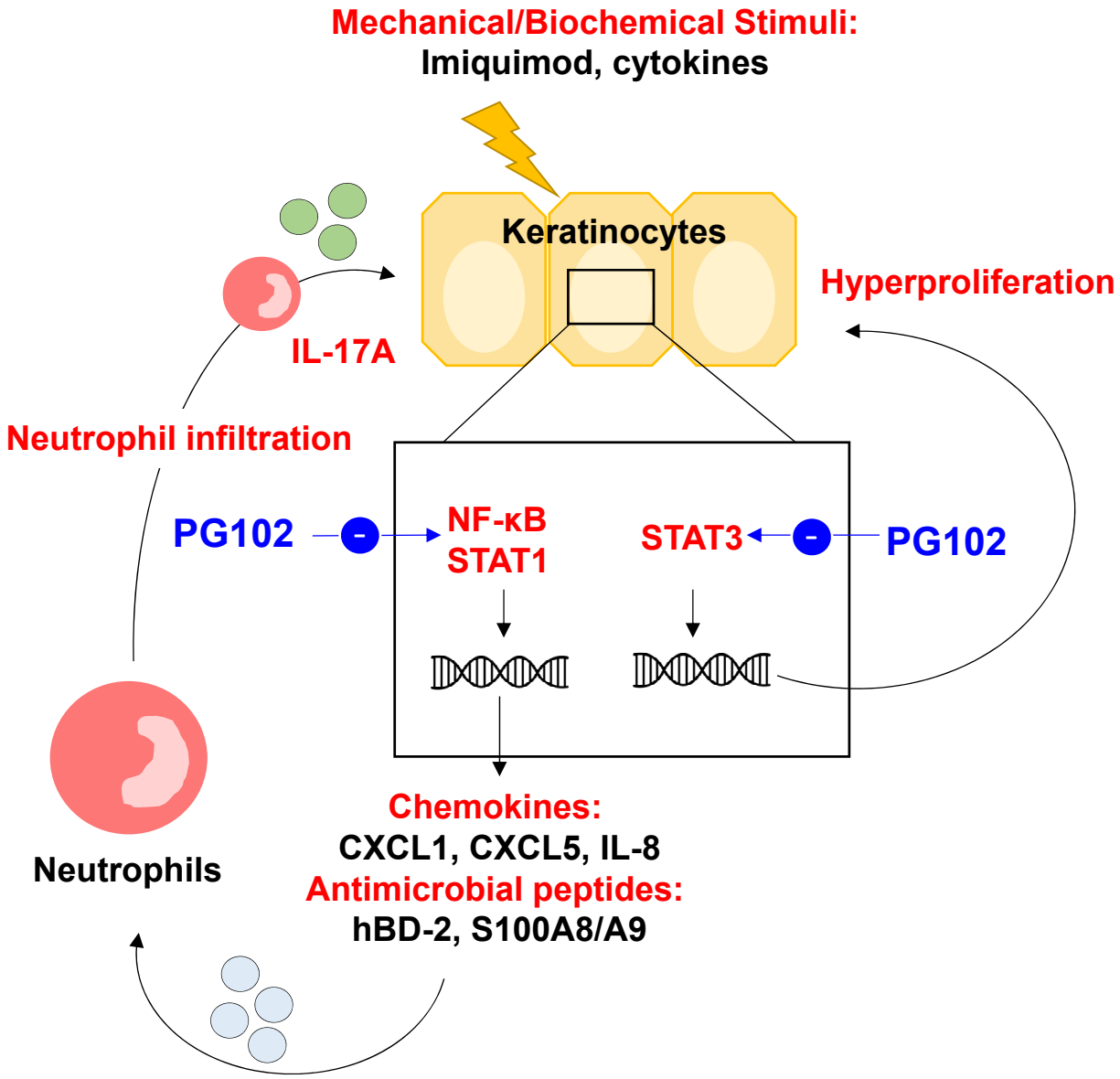


Figure 49. Schematic diagram of mechanism(s) underlying anti-psoriatic effects of PG102.

defensin 2 can also bind to self-DNA to form a complex that acts as TLR9 ligand. Nevertheless, it is not clear whether AMP expression is suppressed by PG102 in the initiation stage or in the later stage of the disease. Also it has to be elucidated whether ameliorating effects of PG102 is solely due to the inhibition of AMP production. It would be worth addressing these questions in future studies.

Above results indicated that the therapeutic effects of PG102 was mediated by direct suppression of inflammatory mediators. Many drugs exert their therapeutic effects indirectly by upregulating anti-inflammatory molecules as well. The broad immunomodulatory effects of PG102 shown in previous studies and in my thesis research raised a possibility that PG102 might upregulate an anti-inflammatory molecule. Among many anti-inflammatory molecules screened, IL-37 gained particular attention because expression of IL-37 has recently been shown to exert beneficial effects in psoriasis and its expression was dramatically increased by treatment with PG102. In this thesis research, the specific role of IL-37 in keratinocytes was first investigated and it was shown that silencing endogenous IL-37 resulted in augmented expression of AMPs upon stimulation with M5. Consistent with results from other cell types, my data indicated that endogenous IL-37 acts as a natural inhibitor of inflammation in HaCaT cells.

It was further investigated whether IL-37 was involved in the biological activities of PG102. Treatment with PG102 increased both RNA and protein levels of IL-37 in a dose-dependent manner. Activations of Smad3 and MAPK are essential for induction of IL-37 so the effects of PG102 on these signaling molecules were also studied. PG102 increased phosphorylation of Smad3, ERK and p38 in a concentration-dependent manner as well and these effects were reversed by pretreatment with specific inhibitors of these proteins. PG102 not only increased the expression of IL-37 but also promoted formation of functional phospho-Smad3/IL-37 complex in the perinuclear regions. These results suggested that PG102 might exert its AMP-suppressing effects, in

part, by upregulating IL-37 (Fig. 50).

It remains to be elucidated whether IL-37 induced by PG102 directly contributed to its AMP-suppressing effects. If PG102 suppressed AMPs solely by increasing expression and activities of IL-37, treatment with PG102 would have not exerted any effects on AMPs in IL-37-silenced cells. However, treatment with PG102 still downregulated expression of AMPs in IL-37-silenced cells. This may be because PG102 contains numerous compounds with anti-inflammatory properties that can suppress expression of AMPs via different mechanisms. It may also be possible that IL-37 induced in HaCaT cells is not functional if HaCaT cells have defective machineries through which IL-37 is processed. To address the question of whether IL-37 increased by PG102 actually suppresses AMPs, one may need to isolate compounds that only affects IL-37 expression and also use different cell types which possess functional processing machineries.

Isolating active compounds from PG102 has always been a major hurdle in studying PG102 as (1) there was no one compound standing out in terms of both quantity and strong bioactivity and (2) the sugar-rich characteristic of PG102 makes it difficult to analyze the bioactive components, though sugar molecules themselves might be the real bioactive compounds of PG102. Total PG102 is already an effective immunomodulatory reagent but when it is considered for botanical drug, identification of active compounds (or at least a fraction) may be the crucial step in facilitating studies of molecular mechanisms, standardization and developing PG102 with enriched bioactivities.

Previous studies employing atopic dermatitis model and RBL-2H3 cells did show that PG102E exhibited concentrated bioactivities in terms of suppressing Th2 cytokines, such as IL-4 and IL-13. However, in IPI model or M5-stimulated HaCaT cells, there was no one fraction that showed concentrated bioactivities of total PG102. Initially, it was speculated that this was due to the fact that the fractionation scheme

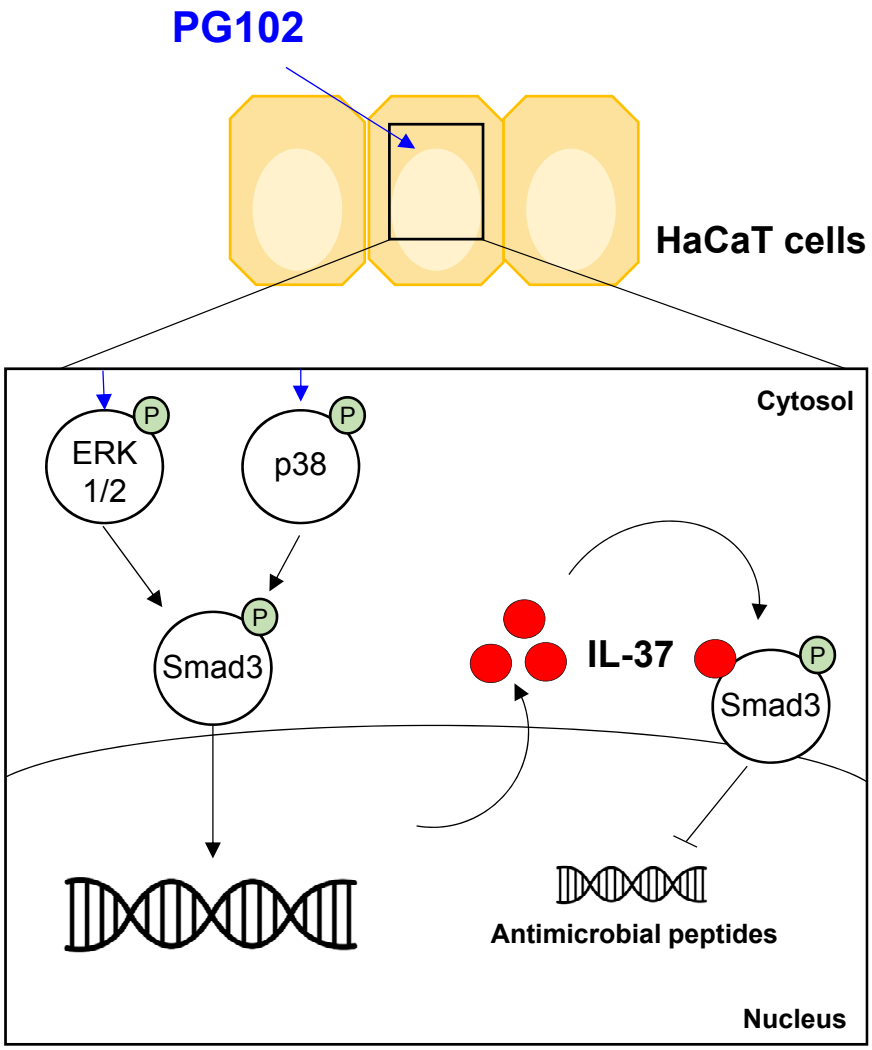


Figure 50. Schematic diagram of mechanism(s) underlying upregulation of IL-37 by PG102.

used in this study was different from the one used in the past studies. However, when I tested its IL-4-suppressing effects using the same total, chloroform, EA and butanol fractions in A23187-stimulated RBL-2H3 cells, the results from the past studies were reproduced. PG102E used in this thesis work also showed around 3-times higher IL-4-suppressing activity compared to PG102T. Thus, it could be concluded that the anti-allergic effects of PG102 is concentrated in the EA fraction but the anti-psoriatic effects of PG102 are separated into different fractions when fractionated with chloroform, EA, butanol and water. It was shown that water fraction contained comparable bioactivity to the total PG102 in its effects on chemokines such as CXCL1 and IL-8, while EA fraction possessed relatively higher AMP-suppressing effects, although other fractions also showed bioactivities to different extents.

As mentioned above, there was no one compound standing out in terms of both quantity and anti-inflammatory effects. HPLC analysis of PG102 showed one compound with the largest peak area at retention time of 9 minutes, which was identified as 5-hydroxymethyl-2-furaldehyde. Initially, it was speculated that this compound might be the main active compound of PG102, but it did not possess any bioactivity and it was merely a compound produced in the process of dehydration of sugars during boiling of *Actinidia arguta*. The effects of PG102, observed in my work as well as previous researches, may be due to the synergistic actions of both major and minor compounds present in PG102. It is now widely accepted that many botanical drugs exert their effects through actions of multiple compounds rather than just one or two strong bioactive compounds. Analyses on the chemical composition of *Actinidia arguta* revealed many potential immunomodulatory compounds and the effects of PG102 may be attributed to interactions of these compounds. Therefore, I have concluded that conventional fractionation method is not suitable for dissecting bioactivities of PG102 and a different analysis method, such as creating bioactive equivalent combinatorial components, may have to be employed for further studies.

Studies on molecular mechanisms underlying anti-inflammatory bioactivities of PG102 have been conducted in RBL-2H3 mast cell line, primary T cells and HaCaT cells. Although different stimulants and cell types were used in these studies, it was observed that three common factors were affected by PG102 across these systems: Phosphorylation of STAT1 and STAT3 were repressed, differentiation of regulatory T cells and phosphorylation of Smad3 was induced independently of TGF-β, and ERK was activated, among other MAPKs. Due to the nature of botanical extracts which possess various compounds, it is not possible to deduce one main target molecule. Yet it can be hypothesized that activation of ERK is one of the central axes in the observed bioactivities of PG102 across different cell types, as ERK is reported to control STAT and Smad3 signaling pathways. Further studies are warranted to investigate whether PG102 regulates molecules upstream of ERK as ERK might be merely one of the effector molecules affected by PG102.

In summary, my thesis work showed anti-psoriatic effects of PG102 in IPI model and M5-stimulated HaCaT cells. PG102 effectively downregulated expression of various chemokines and AMPs and suppressed neutrophil infiltration to skin. These effects were collectively manifested as reduction in skin thickness, erythema, scales and IL-17A level. At molecular level, these effects were mediated by directly affecting phosphorylation of NF-kB and STAT signaling pathways. These results suggested possible therapeutic application of PG102 in Th17-mediated diseases or other skin diseases. At the same time, I have shown that PG102 could upregulate the recently-discovered anti-inflammatory cytokine IL-37, which is known as the fundamental inhibitor of innate immunity. This effect was mediated by ERK/Smad3 and p38 signaling – inhibition of these pathways dampened the increase in IL-37 expression. To date, there are only two published papers reporting agents that can upregulate IL-37 expression. My thesis work raises possibility that many other botanical products may also increase expression of IL-37. Finally, PG102 was fractionated into four different

fractions and their anti-psoriatic effects were tested in an attempt to find the fraction with the highest bioactivity. My results showed that anti-psoriatic activities of PG102 were spread over all four fractions and it was not possible to isolate a specific fraction with concentrated bioactivity. These results suggest that conventional fractionation methods may not be an effective way of isolating biologically active compounds, and a completely different approach may have to be devised. Taken together with previous findings, PG102 may be developed as a safe and effective agent for the treatment of psoriasis and other inflammatory skin diseases.

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국문초록

다래 (*Actinidia arguta*)로부터 열수 추출한 PG102는 다양한 세포 및 동물 질병 모델에서 강력한 항염증 및 항산화 활성을 보였다. PG102의 보고된 효과들을 바탕으로 본 연구에서는 염증성 피부질환의 한 종류인 건선에 대한 PG102의 면역조절 효과를 연구했다. 이 때 이미퀴모드로 유도한 마우스 건선 모델과 피부각질세포주 HaCaT을 이용해 PG102의 활성을 분자생물학적으로 이해하고, 나아가 PG102의 분획을 통해 가장 높은 활성을 가진 분획물을 분리하기 위한 실험을 수행하였다.

PG102의 활성을 먼저 이미퀴모드로 유도한 건선 모델에서 관찰했다. PG102의 국소도포는 피부 두께 증가를 억제하고 배수림프절 및 혈청에서 IL-17A 분비를 감소 시켰다. 또한 염증성 사이토카인으로 자극한 HaCaT 피부 각질세포주에 PG102 처리 시 CXCL1, IL-8, S100A8/9, hBD-2 등의 키모카인 및 항균펩타이드들의 발현을 RNA 및 단백질 수준에서 감소 시켰다. 이러한 효과는 NF-kB 및 STAT 신호전달경로를 조절함으로써 작용하는 것을 웨스턴 블롯 분석을 통해 관찰했다. 이 인자들은 건선 증상을 악화시키고 호중구 화학주성 (chemotaxis)을 유도한다고 알려져 있는데 migration assay를 통해 PG102가 in vitro에서 호중구 세포주의 이동을 억제하고 마우스 건선 모델에서 피부로 침투한 호중구 세포의 수를 유의미하게 감소 시키는 현상을 관찰했다. 이 결과들은 PG102가 호중구 세포의 침투를 억제 시킴으로써 건선 완화 효과를 나타낼 수 있다는 것을 의미한다.

다음으로 항염증 사이토카인 IL-37이 각질세포에서 하는 역할과 PG102가 이의 발현에 미치는 영향을 밝히는 연구를 수행하였다. HaCaT 세포에서 siRNA를 이용해 IL-37를 억제한 후 사이토카인으로 자극 시 항균펩타이드의 발현이 대조군에 비해 유의미하게 증가함을 발견했다. 이는 각질세포에서 IL-37을 증가 시키는 것이 건선 완화에 도움을 줄 수 있음을 시사한다. 본 연구에서는 PG102가 HaCaT 세포에서 IL-37 발현을 RNA 및 단백질 수준에서 유의미하게 증가시킴을 관찰했다. PG102 처리 시 ERK, p38 MAPK와 Smad3의 인산화가 농도의존적으로 증가했다.반면 이 kinase 들의 억제제를 처리하면 IL-37 발현이 증가하지 않는 것을 관찰했다. 이는 PG102가 ERK, p38 MAPK 및 Smad3 신호전달경로 활성화를 통해 IL-37의 발현을 조절한다는 사실을 의미한다. 또한

IL-37은 phospho-Smad3와 결합하여 항염증 효과를 나타내는데 PG102는 이 둘의 결합을 촉진했다. 이 결과들은 PG102의 효과가 IL-37의 발현 및 기능을 조절함으로써 작용할 수 있다는 것을 의미한다.

마지막으로 PG102의 항건선 활성을 가진 물질, 또는 분획물을 분리하기 위해 클로로포름, 에틸아세테이트, 부탄올, 물을 이용해 PG102를 분획했다. 이후 각 분획물의 활성을 이미퀴모드로 유도한 마우스 건선 모델 및 HaCaT 세포에서 관찰했다. 그 결과 PG102의 총 활성은 어느 한 분획물로도 농축되지 않고 각 분획으로 분리 되었다. 그 중 에틸아세테이트 분획물은 상대적으로 높은 항균펩타이드 발현 억제능을 보였고 물 분획물은 상대적으로 높은 키모카인 발현 억제능을 HaCaT 세포에서 보였다. 하지만 각 분획물들은 동일한 농도에서 PG102 보다 낮은 활성을 보였다. 이는 각 분획물들에서 항건선 활성을 가진 특정 성분들이 복합적으로 작용하여 PG102의 전체적인 효과를 나타낼 가능성을 시사한다.

이 연구를 통해 PG102의 국소도포는 마우스 건선모델에서 치료효과를 나타낸다는 것을 밝혔다. PG102는 직접적으로 다양한 염증성 인자, 신호전달경로 및 호중구의 침투를 조절을 막고 항염증 사이토카인의 발현을 증가 시킴으로써 간접적으로 각질세포의 염증 반응을 조절한다는 것을 관찰했다. 선행 및 본 논문 연구의 결과들을 종합해 볼 때, PG102는 다양한 염증성 피부질환들에 안전하고 효율적인 치료제로 사용될 수 있는 가능성을 제시하였다.

핵심어: 다래, 천연물 의약품, 분획, PG102, 건선, 각질세포, IL-37

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