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Contents

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A New Design and Epitopes Analysis for Recombinant Vaccine against *Salmonella typhi* by *In silico* Analysis

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Running title: Epitope mapping of a new vaccine against *S.typhi*

ABSTRACT

Nowadays, foodborne diseases are one of the main problems of the world that infect humans due to consumption of contaminated water or food. Typhoid fever is one of the major causes of illness and death in the world caused by *Salmonella typhi*. Vaccination is one of the most effective approaches in order to reduction of the disease risk. The main goal of this study is designing and characterization of antigenic determinants of a fusion protein originated from *S.typhi* usable as an effective vaccine. In this study, the outer membrane proteins of salmonella have been considered as candidates conferring protection against typhoid. Considering the evidence, OmpA, OmpF and OmpC proteins of salmonella applied in a multivalent vaccine design. Conserved motives of these proteins were selected using the CLC software and then their extracellular regions of these peptides were identified with PRED-TMBB server. Appropriate motives were combined for design of final fusion protein. Finally epitops of designed protein with high antigenic properties were identified using BCPREDS, Ellipro, ABCpred, EpiJen, NetCTL-1.2, CTLpred, TAPpred, ProPred and VaxiJen servers. Predicted designed protein in this study reached a very high scores for antigenic indexes. Encoding Genetic construction of this fusion protein could be applied for production of the recombinant OmpA.OmpF.OmpC derived fusion protein with effective antigenic properties as a new vaccine against *S.typhi*. Laboratory experiments and animal challenging analyses is ongoing.

***Keywords*: *Salmonella typhi*; Recombinant Multiepitopic Vaccine**

1. Introduction

Foodborne diseases are dispersed in the world in humans due to consumption of contaminated water or food[1]. Typhoid fever is one of the major etiologic factors involved in illness and death in the world caused by *S.typhi* and it is the most common food poisoning[1,2]. Typhoid fever is a enteric bacterial infection[3], also *S. paratyphi* A and *S. paratyphi* B cause paratyphoid fever that is less common but clinically similar to the typhoid fever[4]. The disease is transmit and spread in a fecal-oral manner[3].

Nowadays in developed countries, salmonella is commonly associated with acute non-systemic gastroenteritis. But certain serovars of Salmonella, such as *S.typhi* and *S. paratyphi* are important as the causative agent of typhoid fever that is a common diseases in developing countries[5]. Typhoid fever is a public health problem that is endemic in many developed countries, such as Africa, Asia and south America regions[6,7]. The high incidence of the disease have been reported in South and Southeast Asia[8]. It is reported over 20 million cases of typhoid fever annually that led to the deaths of approximately 200,000 people[9,10]. Travelers to developing countries, civilians living in endemic areas, children and technicians in microbiology laboratories are at high risk of disease[4,6]. Typhoid fever is considered as a disease of school-aged children; and it is noteworthy economic and social impact on the communit[8,11].

Salmonella spp. are highly pathogenic members of the Enterobacteriaceae family in both humans and animals[12]. *S.typhi* is a human specific non-Sporulating gram-negative anaerobic pathogenic bacillus and facultative intracellular pathogen[13,14]. Salmonella is able to ad-

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apt with different conditions, such as low pH and high temperature[15]. The most important surface antigens of *Salmonella typhi* include lipopolysaccharide (O antigen), Flagella (H antigen) and capsular polysaccharide (VI antigen). More than 55% of patients who are infected with typhoid fever, ingest at least 10^5 organisms[4].

Although this disease is successfully treated with antibiotics but the increased emergence of the antimicrobial resistance phenotype in bacteria becomes a major problem that leads to difficulties in the treatment of the disease[4,13,16]. Also in endemic areas, early and accurate diagnosis of typhoid fever is difficult and important because, in addition typhoid fever, many other factors are involved in fever emergence[17]. On the other hand, the treatment of typhoid fever is significantly associated with direct and indirect costs which impose a burden on national health care facilities. Therefore, in order to reduce the impact of the disease, we are required to control the disease by preventive strategies, such as improved sanitation and application of effective vaccines[4]. Despite the improved quality of water and promoting health, vaccination is one of the most effective recommended ways by the World Health Organization for reduction of the disease risk[18]. Many studies have been done to develop the effective vaccines for protection against diseases caused by Salmonella species[16]. The first inactivated whole-cell vaccines was licensed in the 1970s in America and Europe. However due to the associated effects, it is not suitable for use [7]. The two typhoid vaccines that are currently available are the injectable VI polysaccharide vaccine and the oral live-attenuated Ty21a vaccine. Currently available vaccines are not sat- isfactory because of undesirable side effects or a lack of sustained effectiveness[19]. These vaccines have some limitations and cannot been used for children under two years of age and older age groups[16]. These reports show the need to development of new generation of vaccine against *S.typhi*.

Outer membrane of gram-negative bacteria has large number of proteins that are known as OMPs. A set of these proteins are called porins, which form channels in membrane to transport the small hydrophobic molecules[20]. Expression of OMPs is regulated according to the environmental conditions[21]. Porins are not only involved in a wide range of pathogenic process but also they are resistant to high temperatures and denaturant agent. OmpA, OmpF and OmpC are synthesized in salmonella. Osmolality and pH conditions affects the expression of porins. OmpF is expressed under low osmolality conditions, but OmpC is expressed under both low and high osmolality conditions[9,21,22]. Recent studies have shown the importance of the role of porins in bacte- rial pathogenesis and ability of them in promoting of humoral and cellular immune system in host[9]. Nowa- days there are many approaches to design vaccines, but we need new methods to access vaccines with better quality, less complications and more effectiveness. Due to the severity of the disease, relapse and spread through asymptomatic carriers, the incidence of drug resistance and use as a biological weapon, *Salmonella* has become a serious threat. Therefore in this study we try to design a new multiepitopic recombinant protein as a candidate model for new generation of vaccine against *Salmonella typhi*.

2. Material and methods

In this study salmonella enterica, Serovar typhi was selected as a model for epitope studies. Protein sequences of OmpF, OmpA and OmpC proteins (GeneBank accession number AAO69550.1, AAO69468.1 and AAO68302.1 respectively) were collected from NCBI genome database (www.ncbi.nlm.nih.gov.). The sequence of OmpF, OmpA and OmpC proteins belonging to *S. bongori, S. choleraesuis, S. enteritidis, S. gallinarum, S. typhimurium, S. sofia, S. Dublin* and *S, minessota species* collected from NCBI genome database, are involved in a homology study for identification of conserved motives. Using CLC main workbench 7.5.1 software[23], conserved blocks were identified for construction of recombinant fusion protein. In the next step, extracellular region of OmpF, OmpA and OmpC proteins sequences were considered by PRED-TMBB server (http:bioinformatics.biol.uoa.gr.PRED-TMBB)[24]. Finally some parts of three proteins that were conserved and belonged to the extracellular region were selected to design fusion protein.

Physical and chemical properties of the fusion peptide, such as number and percentage of constitutive amino acid residues were analyzed by ProtParam (web.expasy.org.protparam). Secondary structure of fusion protein was predicted by several different servers, such as Phyre2 (http:www.sbg.bio.ic.ac.uk.phyre2.html. page.cgi?id=index)[25], predict protein (https:www.pred-

ictprotein.org)[26] and PSIPRED (http:bioinf.cs.ucl.ac.uk. psipred)[27]. Tertiary structure of proposal fusion protein was predicted by Phyre2 and was analyzed by YASARA software[28].

Server	Address	Reference
EpiJen	http:www.ddg-pharmfac.net.epijen.EpiJen.EpiJen.htm	[32]
NetCTL-1.2	http:www.cbs.dtu.dk.services.NetCTL.	[33]
CTLpred	http:www.imtech.res.in.raghava.ctlpred.	[34]
TAPpred	http:www.imtech.res.in.raghava.tappre.	[35]
ProPred	http:www.imtech.res.in.raghava.propred.	[36]

Table 1. The List of some servers applied to the prediction of epitopes that intract with MHC and T cell receptors in this study

ictprotein.org)[26] and PSIPRED (http:bioinf.cs.ucl.ac.uk. psipred)[27]. Tertiary structure of proposal fusion protein was predicted by Phyre2 and was analyzed by YASARA software[28].

2.1 Prediction of antigenic properties

Antigen properties of OmpF, OmpA, OmpC proteins and derived fusion protein was calculated by Vaxi- Jen v2.1 (http:www.ddg-pharmfac.net.vaxijen. VaxiJen. VaxiJen.html). VaxiJen is a server developed to deter- mine antigenic probability based on the physicochemical properties of proteins without recourse to sequence alignment[29]. Accuracy of this predictions provided by VaxiJen is 70% to 89%.

2.2 Prediction of linear and conformational B cell epitopes

In order to the prediction of the linear B cell specific epitopes, the sequence of the proposed fusion protein was submitted to Ellipro (http: tools.immuneepitope.org.ellipro)[30] and ABCored servers (http:www.imtech.res.in.raghava.abcpred) seperately[31]. Conformational B cell epitopes were predicted by submission of PDB format of the proposed fusion protein to Ellipro server.

2.3 Prediction of MHC and T-cell related epitopes

Prediction of epitopes that interact with MHC-I and T-cells was carried out by some different servers as listed in **table 1**. Finally, concluded results were compared. MHC-II binding motives were predicted by ProPred server.

3. Results

Laboratory researches on this fusion vaccine presented in this paper is ongoing and the sequence of our proposed multiepitopic vaccine didn' t showed here, but it is available on request. Physical and chemical characterization showed that this fusion protein composed of 297 amino acid 3.2248 kD molecular weight. Based on ProtParam algorithms, iso- electric pH for this protein is 4.79 with hydrophobic property (GRAVY: -0.712). The secondary structure of this protein composes 3.4% a-helix, 49.8% β-sheet and 46.8 loop (**Figure 1**). Stability index (18.11) show that these protein is stable. Whole structure is very similar to structure of OMPs protein family.

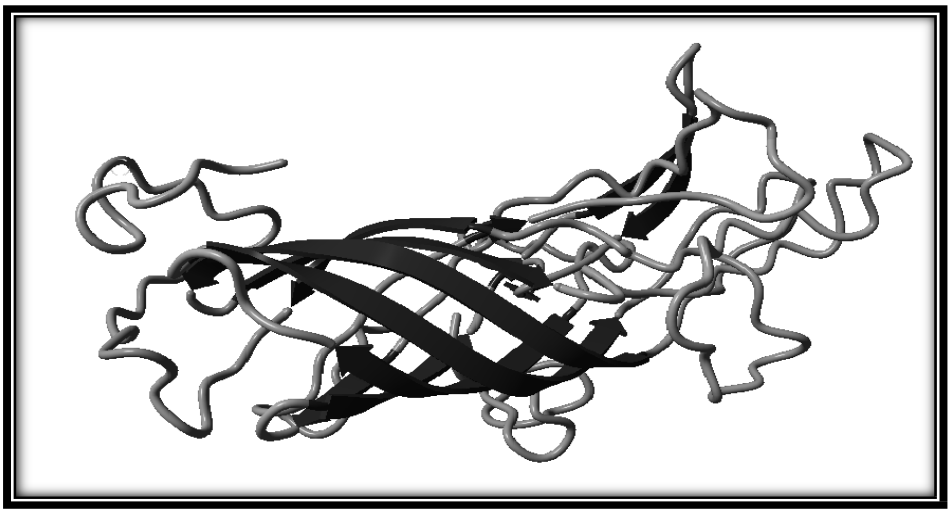


Figure 1; Tertiary structure of proposed protein. Beta-sheets were shown in dark ribbons. View by YASARA software**.**

3.1 Antigenic properties

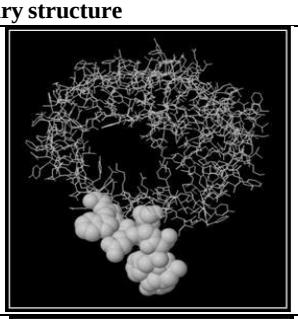
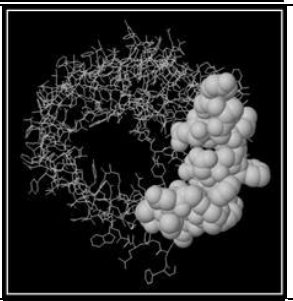
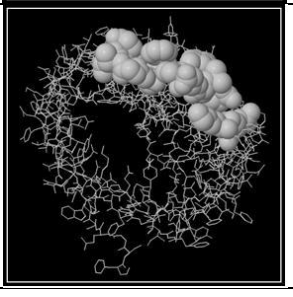
Linear B-cell Epitopes. Antigenic property probability of our proposed fusion protein was calculated 0.8896. The results from the different servers showed linear B-cell epitopes with nearby points. **Table 2** shows some epitope sequence extracted by two different servers.

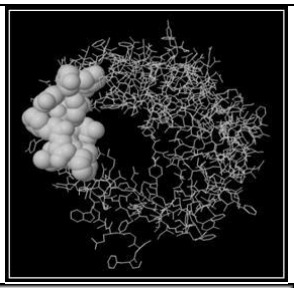
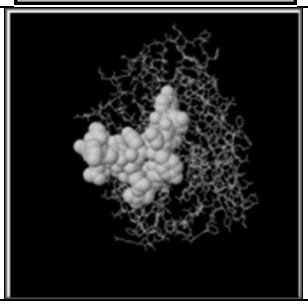
Conformational and discontinuous epitopes of proposed fusion protein were predicted by Ellipro. This server acts based on homology results and determining the tertiary structure of protein in order to identification of conformational epitopes (**Table 3**). Figures show conformational position of every epitope.

ABCpred server	**Start position**	**Score**	**Ellipro server**	**Start position**	**Score**
GDANTIGTRPDNGLLS	210	0.87	NNIGDA	207	0.828
NHSINSQNGDGVGYTM	55	0.81	GIQYQGKNQDNHSI	45	0.777
TSNGSNPSTSYGFANK	236	0.87	GSNPSTSYGFAN	239	0.726
VPGGASTKDHDTGVSP	168	0.86	WRADTKSNVPGGASTKD	160	0.691
GLRPSVAYLQSKGKDI	266	0.74	FEVVAQYQFDFGLRPSVAYLQSKGKDIS	255	0.661
PYKGDNTNGQGVQLTA	124	0.64	GDNTNGQGV	127	0.636
SSQTYNATRFGTSNGS	225	0.74	SQTYNAT	226	0.628
GKDISNGYGASYGDQD	278	0.81	SYGDQDIVKY	288	0.613
GETWGGAYTDNYMTSR	8	0.92	YTDNYMTSR	15	0.599
TNDQQDRDGNGDRAES	89	0.80			

*Start point relay on amino acid residues in proposed protein. Just motives with score more than 0.6 were listed. Reference score for ABCpred and Ellipro servers is 0/51 and 0/5.

Table 2. Linear B-cell Epitopes Predicted by ABCpred and Ellipro servers

Sequence	**Length**	**Score**	**Tertiary structure**
_:Y1, _:F2, _:S3, _:G4, _:E5, _:T6, _:W7, _:G8, _:F31	9	0.82	
_:F32, _:G33, _:L34, _:V35, _:D36, _:G37, _:L38, _:S39, _:F40, _:G41, _:I42, _:Y68, _:E69, _:F70, _:D71, _:G72, _:F73, _:G74, _:T76, _:Y119, _:G121, _:D122, _:N123, _:T124, _:N125, _:G126, _:A127, _:Y128	28	0.79	
_:K129, _:A130, _:Q131, _:D145, _:L147, _:D148, _:V149, _:Y150, _:F183, _:A184, _:G185, _:G186, _:I187, _:E188	14	0.754	

_:T226, _:Y227, _:Y259, _:Q260, _:F261, _:D262, _:F263, _:G264, _:L265, _:R266	10	0.739	
_:A14, _:Y15, _:T16, _:D17, _:N18, _:Y19, _:M20, _:T21, _:R23, _:G45, _:I46, _:Q47, _:Y48, _:Q49, _:G50, _:K51, _:N52, _:Q53, _:D54, _:N55, _:H56, _:S57, _:I58	23	0.704	

*Just conformational epitopes with score more than 0.7 is shown. Figures show conformational position of every epitope. Reference score for Ellipro server is 0/5.

Table 3. Conformational epitopes predicted by Ellipro server

T cell receptors and MHC-I related epitopes were predicted by different programs as listed in table 1. MHC-I binding motives were predicted by EpiJen, NetCTL, CTLpred and TAPpred servers (**Table 4**). MHC-II binding motives were predicted by ProPred server (**Table 5**)

.

Number	**sequence**	**Start point**	**score**	**Server**
1	SGETWGGAY	3	1.0776	NetCTL, EpiJen
2	YTDNYMTSR	11	1.9919	NetCTL, EpiJen
3	MTSRAGGLL	16	0.9978	NetCTL, EpiJen
4	ESWAVGAKY	99	0.8343	NetCTL, EpiJen
5	KGDNTNGAY	120	1.2016	NetCTL
6	VQLTAKLGY	133	0.7542	NetCTL ،TAPpred
7	ISNGYGASY	279	1.8013	NetCTL, EpiJen
8	YGDQDIVKY	287	1.0667	NetCTL, EpiJen
9	GTRPDNGLL	214	0.4527	EpiJen
10	PITDDLDVY	142	0.5682	EpiJen ،TAPpred
11	TAAYSNSKR	76	0.3841	EpiJen ،TAPpred
12	SRAGGLLTY	18	0.7454	EpiJen ،TAPpred
13	DGVGYTMAY	60	0.5260	EpiJen ،TAPpred
14	VGAKYDANY	103	0.5836	EpiJen ،CTLpred
15	NGLLSSQTY	219	0.5218	EpiJen ،TAPpred
16	DGLSFGIQY	36	0.5165	EpiJen
17	LTYRNSDFF	24	0.6201	EpiJen ،TAPpred
18	WRADTKSNV	158	0.3325	CTLpred ،TAPpred
19	IEYAITPEI	187	0.4093	CTLpred ،TAPpred

* Threshold for epitope identification in NetCTL is 0/75.

Table 4. MHC-I and T-cell receptors binding motives predicted by different servers. Mentioned Score in this table is based on predicted results in NetCTL server

number	sequence	Start point	score	allel
1	YKGDNTNGA	118	5.2	HLA-DRB1*0401
			2.8	HLA-DRB1*0405
2	LEYQWTNNI	198	2.9	HLA-DRB1*0401
			2.7	HLA-DRB1*0402
			2.4	HLA-DRB1*0405
3	LLSSQTYNA	220	1.7	HLA-DRB1*0401
			3.0	HLA-DRB1*0402
4	YKAQGVQLT	127	1.6	HLA-DRB1*0401
5	LRPSVAYLQ	264	1.6	HLA-DRB1*0401
			2.9	HLA-DRB1*0402
6	YQWTNNIGD	200	1.58	HLA-DRB1*0401
			2.6	HLA-DRB1*0405
7	MVWRADTKS	155	2.8	HLA-DRB1*0402

*Reference score is 1.

Table 5. MHC-II binding motives predicted by ProPred server

3.2 Discussion

Salmonella has become a serious threat in populations due to disease severity, the return and spreading manner of disease through the carriers without symptoms, the incidence of drug resistance and its application as a weapon in bioterrorism. Although the disease is rare in the developed industrial countries but is still considered as a serious problem in many developing countries, especially Southeast Asian countries, Latin America and Africa. In order to fight the disease and reduction of resulted mortality, many preventive methods are taken that the most important of them is vaccination[37]. There are two available vaccines against Salmonella, but these vaccines are not immunogenic in children under two years old. Due to some similar problems, design and development of new vaccines against Salmonella is recommended[38].

In this study, we selected three outer membrane proteins (OMP A, F and C) for designing of a proposal multiepitopic protein with effective antigenic properties as we carried out in a similar work previously [39]. It has focused on bacterial outer membrane proteins as diagnostic markers and effective candidate antigenic protein vaccine. These proteins have a potential for subunit vaccine development against Salmonella. These proteins, which are abundant on the surface of the cell have been reported to induce a significant increase in antibody titers as compared with other bacteria structure proteins[22,40]. An immune response to OMPs has been reported in people that were vaccinated with an attenuated live strain as well as in the patients with natural typhoid fever[16].

Studies on VaxiJen server showed that proposed fusion protein introduced in this study has high antigen probability compared with single candidate proteins. Antigenicity of proposed fusion protein was predicted 0.8896 which is a high value. Nowadays, the importance of in silico studies was emphasized in identifying the epitopes. B lymphocytes plays an important role in the formation of immune memory and remove pathogens by producing specific secretory antibodies. In Ellipro server, score higher than 0.5 and ABCpred, higher than 0.51 have potential in inducing humoral immunity. Ellipro and ABCpred servers were predicted 9 and 10 epitopes in designed protein with acceptable score, respectively. In Ellipro server, high score (0.828) belongs to NNIGDA and in ABCpred server, GETWGGAYTDNYMTSR had high score (0.92). However, score of NNIGDA is equivalent 0.87 in ABCpred. Also, conformational epitopes, _:Y1، _:F2، _:S3، _:G4، _:E5، _:T6، _:W7، _:G8، _:F31 had higher score that is 0.82; exactly 7 amino acid residues of this motif is located on an extracellular loop of Omp F derived domain.

Due to the important role of $CD4^+$ T cells in both innate and adaptive immune responses and involvement of $CD8^+$ cells in destruction of viral infected cells, identification of $CD4^+$ and $CD8^+$ T cells receptor related epitopes is essential for vaccine efficacy[41]. In this study, several servers were used to determine epitopes binding

to MHC-I and II which are necessary for successful presentation of antigen to $CD8^+$ and $CD4^+$ cells. NetCTL server identified eight effective epitopes that higher score (1.9919) belongs to YTDNYMTSR. This motif was predicted in EpiJen server too. In ProPred server, two motives (YKAQGVQLT and LRPSVAYLQ) were predicted as the most characteristic epitopes (score: 1.6). LEYQWTNNI motif was predicted in all three alleles, including HLA-DRB1*0401, HLA-DRB1*0402 and HLA-DRB1*0405 with a score of 2.9, 2.7 and 2.4 respectively and can act as a very effective epitope for MHC-II. Our results support proposed fusion protein presented in this study as an effective multiepitopic vaccine against salmonella. However laboratorial examination and animal challenge are necessary for a confident comment on this proposed protein.

4. Conclusion

We have introduced a vaccine here which at the same time is capable of producing an immune response against some important structural proteins of *S.typhi*. In this paper, in addition to designing the structure and the exact identification of the relevant epitopes, the response rate of various immune cells is also measured. Laboratory studies and immunization of this vaccine are also underway in the laboratory and will be presented in the future.

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Conflict of interest

The authors declare that there is no conflict of interest regarding the publication of this manuscript.

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Peroxisome Proliferator-Activated Receptors (PPARs) Activation as Therapeutic Targets in Skin Inflammation

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ABSTRACT

Peroxisome proliferator-activated receptors (PPARs) are fatty acid activated transcription factors that belong to the nuclear hormone receptor family. They are initially known as transcriptional regulators of lipid and glucose metabolism, although further evidence has also been accumulated for other functions. Due to the nature of all PPAR isotypes which are expressed and exert effects by regulating the functions of cell types residing and infiltrating in the skin, PPARs represent a major research target for the understanding and treatment of many skin diseases. Atopic dermatitis (AD) is a chronic and relapsing disease characterized by skin barrier dysfunction and immune dysregulation. Skin barrier disturbance is one of the exacerbation factors of AD, due to facile penetration of molecules such as antigens. From the aspect of immune dysregulation, innate and acquired immunity including cell proliferation, cell differentiation, and cytokine network are involved in the pathogenesis. In this review, the role of PPAR in AD and the possibility of its agonist for the treatment of AD are discussed.

Keywords: Peroxisome Proliferator-activated Receptors; Skin; Inflammation; Atopic Dermatitis

1. Introduction

PPARs are classified three different isoforms termed PPARα, PPARβ/δ and PPARγ[1,2]. Initial studies demonstrated that PPARs are pivotal participants in the regulation of energy homeostasis by modulating glucose and lipid metabolism and transportation[3], and then subsequent studies have shown that PPARs regulate in other cellular functions such as cell proliferation, cell differentiation, apoptosis and inflammation. Because all PPAR isotypes are expressed[4] and exert effects by regulating the functions of cell types residing and infiltrating in skin, PPARs represent a major research target for the understanding and treatment of many skin diseases. Atopic dermatitis (AD) is a chronic and relapsing disease. AD is characterized skin barrier dysfunction and immune dysregulation. A typical characteristic of AD is xerosis which affects lesional and non-lesional skin areas, due to increased transepidermal water loss. This skin barrier disturbance exacerbates AD, due to facile penetration of high molecules such as antigens[5]. Thus, the application of emollients is one of the basic treatments to support skin barrier function and allow hydration of the skin as conservative treatments [6]. Immune dysregulation occurs in both innate immunity and acquired immunity. The innate immunity is presented in the epidermis as the front line defense against infection. Antimicrobial peptides (AMPs), directly kill a broad spectrum of microbes, are secreted from keratinocytes and activated to respond immediately after microbial invasion. Although it is supposed that AD patients have a higher prevalence of infection with bacteria, fungi, and viruses due to skin barrier disruption, the defects of innate immune system are demonstrated previously [7]. Regarding dysregulation of acquired immunity, AD is originally regarded as a Th2-mediated disease because of the systemic elevation of Th2 cytokines with increased IgE levels and eosinophilia in the acute phase[6,8]. However, Th1cytokines are detected in chronic AD, suggesting that Th1 cytokines are involved in the maintenance of chroic

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AD skin[8,9]. Additionally, a study has reported that number of Th17 cells is significantly increased in AD patients[10]. Thus these alternative concepts in AD should be addressed. Tacrolimus mainly acts on both Th1 and Th2 cells and then IFN-γ, IL-2, IL-4 and IL-5 are potently inhibited by tacrolimus[11]. Moreover, treatments with several monoclonal antibodies for AD are clinically applied or the clinical trials are underway[12-14]. In this review, the role of PPAR in AD and the possibility of its agonist for the treatment of AD are discussed.

2. Peroxisome proliferator-activated receptors

PPARs are fatty acid activated transcription factors that belong to the nuclear hormone receptor family. They are initially known as transcriptional regulators of lipid and glucose metabolism, although further evidence has also accumulated for their other functions. Three PPAR isotypes, PPAR-α, PPAR-β/δ and PPAR-γ, encoded by separate genes, have been identified in vertebrates. The expression of each isotype exhibits distinct tissue distribution reflecting their functions[15]. The highest expression of PPAR-α is found in liver, and preferentially expressed in metabolically active tissues including kidney, heart, skeletal muscle and brown fat[15-17]. PPAR-β/δ is expressed in a wide range of tissues such as brain, kidney, heart and skin[18,19]. PPAR-γ is expressed in heart, skeletal muscle, colon, intestines, kidney, pancreas and spleen. In human skin, all PPAR isotypes are expressed[4]. In skin, PPAR isotypes show the different expression pattern. PPAR-β/δ is ubiquitously present throughout the epidermis while the expression of PPAR-α and -γ increase along with the differentiation of keratinocytes[20]. Ligands of PPARs comprise long chain polyunsaturated fatty acid (**Table 1**). For example, α-linoleic acid, docosahexaenoic acid, arachidonic acid metabolites, and leukotrienes are the well-known endogenous ligands for PPARs. Many synthetic ligands for PPARs have been developed. Of them, fabric acid derivatives, dual-selective agonists for PPAR-α and -γ, and thiazolidinedione and derivatives, single-selective agonists for PPAR-γ, are successfully used in treatments of cardiovascular diseases and diabetes mellitus type 2[21,22]. However, any ligand for PPARs has not been clinically applied for the treatments of skin diseases. Previous studies have demonstrated underlying mechanisms in PPARs actions[23]. Once PPARs bind to their ligands, they form heterodimers with the retinoid X receptor (RXR), followed by direct binding to DNA response element, termed PPAR response elements (PPREs), located in the promotor regions of target genes[24-26]. Binding of ligand leads to the recruitment of coactivator complexes which modify chromatin structure and facilitate assembly of the general transcriptional machinery to the promoter[27]. This transactivation induces the expression of target genes, involved in PPARs functions (**Figure 1**).

	Single-selective				Dual-selective		
Ligand	PPAR-α	PPAR-β/δ	PPAR-γ	Ligand	PPAR-α	PPAR-β/δ	PPAR-γ
15-deoxy-D-12,14-PGJ_2			+	9-HODE	+		+
Leukotrien B_4	+			13-HODE	+		+
PGA_1		+		15-HETE	+		+
PGA_2		+		Linoleic acid		+	+
PGD_2		+		Palmitic acis	+	+	
PGI_2		+		Eicosapentanoic acid		+	+
8(S)-HETE	+			Clofibrate	+		+
Oleic acid	+			Fenofibrate	+		+
Oleoylethanolamide	+			WY14643	+		+
Thiazolidinediones			+	Ibuprofen	+		+
Fmoc-leucine			+	Indomethacin	+		+
Sulindac			+	Fenoprofen	+		+
GW0742		+		Farglitazar	+		+
GW1929			+	GW2331	+		+
GW2570			+	GW2433	+	+	
GW7845			+	GW409544	+		+
GW9578	+						
GW501516		+					

HETE: hydroxyeicosatetraenoic acid, HODE: hydroxyoctadecadienoic acid, PG: prostaglandis

Table 1. Endogenous and synthetic ligands of PPARs

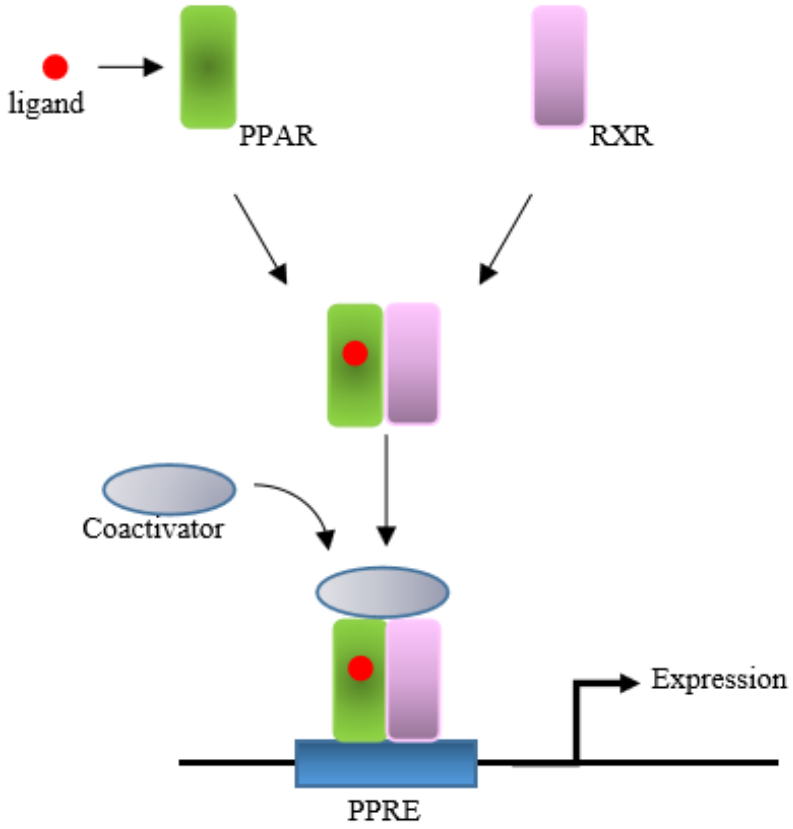

Figure 1; Mechanism of gene expression by PPAR activation. Specific ligands-activated PPARs form hetrodimers with retinoid X receptors (RXRs) and recruit cofactors. The complexes then modulate DNA transcription by binding to peroxisome proliferator response element (PPRE) in the promoter region of target genes.

3. Roles of PPARs in inflammation

Inflammation evoked by detrimental stimuli is a protective response in order to maintain homeostasis. Because innate immunity is considered as the first line of host defense against onset of harmful stimuli, immune cells such as macrophages, dendritic cells, mast cells, lymphocytes and neutrophils play crucial roles in complicated inflammation response. Apart from immune cells, non-immune cells such as keratinocytes, fibroblasts, epithelial cells and endothelial cells contribute the response as well[28,29]. In skin, once inflammatory stimuli are recognized by pattern-recognition receptors on the plasma membrane, inflammatory cytokines (e. g. TNF-α, IL-1β, IL-6) released from keratinocytes, fibroblasts and dendritic cells induce mediators during autocrine and paracrine signaling, followed by progression of the sophisticated inflammation process. Leukocyte adhesion, extravasation and migration to the inflammatory site are important events in leukocyte recruitment. Vascular cell adhesion molecule 1 (VCAM-1) and intercellular adhesion molecule 1 (ICAM-1) play pivotal roles in leukocyte adhesion and their expression is the consequence of stimulation by TNF-α and IL-1, while they are not present on quiescent endothelial cells[30]. On the other hand, IL-8, which is induced by TNF-α, leads leukocyte, especially neutrophil, to migrate along a chemotactic gradient to the inflammatory site[31]. The initial demonstration of a regulatory function of PPAR-α in inflammation signaling was obtained in PPAR-α-deficient mice that display an exacerbated response to inflammatory stimuli[32]. Consequently, intensive studies on the effects of PPAR activators on inflammatory responses have been revealed that all of PPAR isotypes exert distinct and overlapping anti-inflammatory effects[33-40]. The effects of PPARs activation on inflammatory molecules are listed in **Table 2**[39,41]. Previous studies reported that a crosstalk between PPARs and transcription factors mediating inflammatory signaling including C/EBP, STAT, AP-1 and NF- B and proposed that five mechanisms of PPAR-mediated transrepression; i) direct interaction, ii) induction of IκBα, iii) regulation of kinase activity, iv) coactivator competition and v) co-repressor interaction[27,39,42]. The

reduction of IL-1-stimulated IL-6 production from human smooth muscle cells by fenofibrate is caused by the repression of c-Jun, a component of AP-1, and NF- B-induced transcription of the human IL-6 promotor. This transcription interference occurs independent to the promotor context. Furthermore, in vitro protein-protein interaction assay showed fibrate-activate PPAR-α binds directly to c-Jun and NF-κB (**Figure 2a**)[43]. Another study demonstrated a distinct mechanism that fenofibrate induces the expression of IκB, which inhibits NF-κB by masking the nuclear localization signals of NF-κB proteins and keeping them sequestered in an inactive state in the cytoplasm, in human aortic smooth muscle cells and hepatocytes, accompanied with a decrease in NF-κB DNA binding activity[44]. This suggests that PPAR activation inhibits NF-κB DNA binding by IκB induced by PPAR activation (**Figure 2b**). In mice colon inflammation, troglitazone reduces TNF-α and IL-1β mRNA levels, accompanied with reduction of NF-κB DNA binding activity, c-Jun NH2-terminal kinase (JNK), and p38 activities[45]. Oxidative stress-induced production of TNF-α and IL-1β is reduced in PPAR-γ overexpressing Ad/PPARγ C2C12 cells, compared to Ad/LacZ C2C12 cells. At the same time, phosphorylation of ERK1/2 and p38 is inhibited in Ad/PPARγ C2C12 cells, concomitant with inhibition of NF-κB translocation from cytosol to nucleus[46]. Likewise, Shi and the colleagues demonstrated that alline, a potent PPAR-γ activator, ameliorates LPS-induced production of iNOS, IL-1β, IL-6 and TNF-α from RAW264.7 cells through the reduced phosphorylation of ERK1/2, JNK and p38, suggesting that PPAR-γ activation regulates MAPKs activity[47]. These suggest PPAR activation attenuates inflammatory response through regulation of protein kinase activity (**Figure 2c**). Several members of the nuclear receptor family including PPARs and RXR require coactivator such as CREB-binding protein (CBP)/p300 to exert their functions. Similarly, AP-1 also requires CBP/p300 to regulate the target gene expression. Thus, PPARs and AP-1 scramble competitively for limiting pool of overlapping sets of coactivator in cells[48]. Li *et al*. have demonstrated that transrepression by PPAR-γ is achieved by targeting CBP through direct interaction with its N-terminal domain and via SRC-1-like bridge factors[49]. This is the fourth mechanism of transrepression (**Figure 2d**). Lee and the colleagues proposed a ligand-dependent transcriptional pathway in which PPAR-β/δ controls an inflammatory switch through its association and disassociation with BCL-6[50]. PPAR-β/δ-BCL-6 complex possesses pro-inflammatory effect when PPAR-β/δ is unliganded. Once PPAR-β/δ activated by the ligand, BCL-6 is released from the complex and then suppresses the production of cytokines and chemokines (**Figure 2e**). Pascual *et al* proposed another corepressor-dependent model that PPAR-γ mediates transrepression of a subset of inflammatory response genes in macrophages by preventing the signal-dependent clearance of corepressor complexes on inflammatory promoters downstream of LPS signaling[51]. Based on its anti-inflammatory activities as described above, PPARs are expected to be therapeutic targets for treatment of different inflammatory skin diseases[52].

Up-regulation	Down-regulation
IL-4, IL-5, sIL-1ra	IL-1β, IL-6, IL-12, IL-23, IL-27
	CCL2 (MCP-1), CCL4 (MIP), CXCL8 (IL-8)
	IFN-γ, TNF-α
	ICAM-1, VCAM-1
	ET-1
	COX-2, iNOS

Table 2. Influence of PPAR activation on inflammatory molecule expression

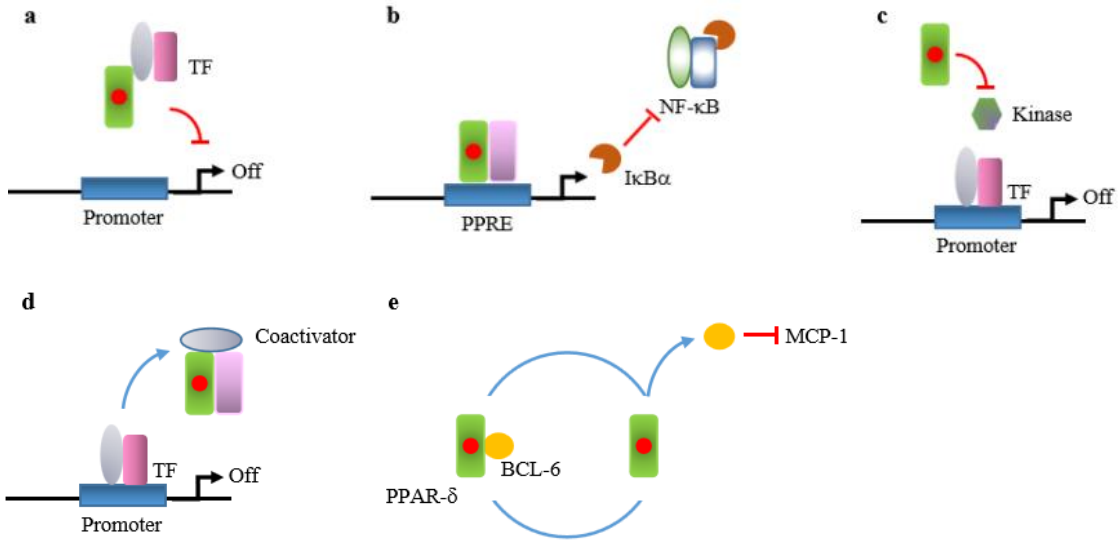

Figure 2; Mechanisms of liganded PPAR-mediated transsppression. a) Direct interaction with transcription factor (TF), b) Induction of IκBα, c) Kinase inhibition, d) Competitive scramble for coactivator, e) Association and disassociation with BCL-6**.**

4. Skin barrier disruption in atopic dermatitis

Atopic dermatitis (AD) is a chronic and relapsing disease. Its increasing prevalence to be estimated up to 20% in children and 10% in adults represents a major public-health problem. AD is characterized skin barrier dysfunction and immune dysregulation. From the aspect of skin barrier dysfunction, a typical characteristic of AD is xerosis which affects lesional and non-lesional skin areas, due to increased transepidermal water loss. Previous studies have proposed two major causes of increased transepidermal water loss: (i) decreased ceramide content in strarum corneum[53], (ii) filaggrin gene mutation[54]. This skin barrier disturbance exacerbates AD, due to facile penetration of molecules such as antigens[5]. Thus, the application of emollients such as urea and heparinoid is one of the basic treatments to support skin barrier function and allow hydration of the skin as conservative treatments[6]. On the other hand, it is supposed that hyperproliferation and hypodifferentiation of keratinocyte are the factors for skin barrier dysfunction in AD, other than gene mutation[55,56]. A previous report demonstrated that PPARβ/δ plays crucial roles in keratinocyte proliferation, maintenance of cutaneous barrier homeostasis and regulation of inflammation in PPARβ/δ deficient mice[57]. To establish a mature skin barrier function mechanically, sequential and orchestrated cross-linking of filaggrin, involucrin, loricrin and ceramides by transglutaminase 1 along with keratinocyte differentiation is required. Hanley *et al*. reported that clofibrate, a PPARα agonist, decelerates keratinocyte proliferation and accelerate differentiation with enhancement of mRNA expression of involucrin and transglutaminase 1[58]. Other studies demonstrated that caffeic acid induces keratinocyte differentiation via PPAR-α activation[59], and that GW0742, a PPAR-β/δ selective activator, induces keratinocyte differentiation and inhibits proliferation[60]. Addition to the regulation of keratinocyte differentiation, intracellular lipid accumulation and lamellar body secretion are crucial for the construction of intercellular lipid alignment to contribute skin barrier function. Schmuth *et al*. provided crucial evidence on relation between PPAR-β/δ activation and skin barrier homeostasis: i) PPAR-β/δ activator, GW1514, stimulates the recovery of acute and chronic skin perturbation in hairless mice, ii) GW1514 stimulates an increase in the expression of the differentiation markers, loricrin and filaggrin, iii) GW1514 increases accumulation of triglyceride[61]. A consecutive study from the same group demonstrated that application of activators of PPARα (WY14643), PPARβ/δ (GW1514) and PPARγ (ciglitazone) to hairless mice enhances synthesis of cholesterol, fatty acid and ceramides, and consequently that the activators accelerate the recovery from acute disruption of skin barrier function[62]. These results suggest that PPAR activators are expected to improve cutaneous barrier homeostasis by control of keratinocyte differentiation. Further, other studies showed that activation of PPAR-α by WY14643 improves skin barrier

with normalization of the molar ratio of the main skin barrier lipids to 1:1:1 (free fatty acids:ceramides:cholesterol) and upregulation of filaggrin expression[63], and that oat lipid extract, which demonstrates robust dual agonism for PPAR-α and PPAR-β/δ, enhances keratinocyte differentiation and ceramide synthesis[64]. These results suggest that PPAR activators are expected to be alternative treatments to support skin barrier function.

5. Attenuation of innate immunity in AD

On the other hand, immune dysregulation in both innate and acquired immunity is another important aspect in AD. Especially, cytokines in innate and acquired immunity contribute to establish the pathology of AD[65]. The innate immunity presents in epidermis as the front line defense against infection. Antimicrobial peptides (AMPs) such as cathelidin (LL37) and β-defensins, directly kill a broad spectrum of microbes, including Gram-positive and Gram-negative bacteria as well as fungi and certain viruses, are secreted from keratinocyte and activated to respond immediately after microbial invasion. Although it is supposed that AD patients have a higher prevalence of infection with bacteria, fungi, and viruses due to skin barrier disruption, the defects of innate immune system are demonstrated previously[7]. Ong *et al*. reported that the expression of LL37 and human β-defensin 2 (HBD-2) was suppressed in AD patients[8]. As the expression of AMPs arises during keratinocyte differentiation, the disturbance of keratinocyte differentiation is a considerable reason why suppression of LL37 and HBD-2 occurs in AD patients. Because PPARs activators induce keratinocyte differentiation[59,61,66,67], PPAR activation may improve AMPs production in AD. Furthermore, a previous study reported that apoptosis signal-regulating kinase-1 (ASK1), an intracellular regulator of keratinocyte differentiation, enhances the expression of LL37 and HBD2 via p38 cascade[68]. Since PPAR/p38 pathway is one of the signal cascade to exert the functions, similar to ASK1[69,70], PPAR activation is expected to induce AMP expression via p38. In fact, Dai *et al*. showed that PPARγ regulates the 1α, 25-dihydroxyvitamin D_3-induced production of HBD-3 and LL37, whose gene is a direct target of the vitamin D receptor, in keratinocytes through the regulation of AP-1 and p38 activity[71-74].

6. Dysregulation of acquired immunity in AD

Regarding dysregulation of acquired immunity, AD is originally considered as a Th2-mediated disease because of the systemic elevation of Th2 cytokines with increased IgE levels and eosinophilia in the acute phase[6,8]. Once keratinocytes, locating the outmost of the body, are activated by diverse stimuli including chemicals, allergens, microbes and scratching, they release thymic stromal lymphopoetin (TSLP), IL-25 and IL-33. TSLP is produced from keratinocytes by tape stripping-induced skin barrier disruption and by *Staphylococcus aureus*, as well as antigen-activated mast cells (MC)[75-77]. Additionally, the high expression of TSLP in keratinocytes from patients with AD implies involvement of TSLP in AD[78]. Previous studies demonstrated that IL-25 expression is found in Th2 cells, allergen-activated MCs, eosinophils, basophils, dendritic cells (DC) and human skin of AD patients[79-82]. IL-33 is expressed by a wide variety of cell types, including residing and infiltrating cells in skin[83]. These cytokines share the properties which induce IL-4, IL-5 and IL-13 production to lead skewing and augmenting Th2 response in AD[79,81]. Interestingly, a previous study demonstrated that TNF-α-induced HBD-2 production from HaCaT cells is significantly decreased in the presence of IL-4 or IL-13[7], suggesting that IL-4 and IL-13 affect the innate immune system in AD. In addition, IL-25 is suggested to participate in barrier dysfunction in AD because IL-25 reduces filaggrin expression in keratinocyte[81]. Additionally, IL-33 stimulates MCs to produce IL-5, IL-6, IL-10, IL-13, TNF-α and GM-CSF[84]. Of them, TNF-α stimulates keratinocytes to produce TSLP[65]. Following the production of TSLP, IL-25 and IL-33 to target Th2 cells, Th2-cytokines including IL-4, IL-5 and IL-13 are released. Their functions in acquire immune response are; i) IL-4 induces immunoglobulin class switch form IgM to IgE, and upregulates IgE receptors on monocytes, as well as promotion of Th2 skewing, ii) IL-5 induces the production of IL-25 from eosinophils and stimulates maturation and also activation of eosinophis, iii) the effects of IL-13 are similar to those of IL-4. The patients with AD are divided into extrinsic AD (EAD) and intrinsic AD (IAD). In EAD, increased total serum IgE and a higher

expression of IgE receptors on monocytes are found, compared with IAD. On the other hand, higher expression of IL-5 and IL-13 are detected in EAD than IAD. However, the expression of Th2 cytokines including IL-4, IL-5 and IL-13 in skin lesions of both group is elevated, compared with normal control skin[85-87]. IL-31, belonging to an IL-6 family in terms of its structure and receptor complex, is expressed by Th2 cells[88]. Raap *et al*. reported a correlation between serum levels of IL-31 and the severity of AD[89]. A role of IL-31 is to induce the release of pro-inflammatory cytokines including IL-1β and IL-6, and AD-related chemokines including CXCL1, CXCL8, CCL2 and CCL18 from eosinophils whose infiltration in skin lesions is a predominant pathological feature of AD[90]. In addition, IL-31 is focused as a major pruritogen associated with AD[91]. Because scratching behavior due to pruritus is an exacerbation factor to influence the quality of life, the control of pruritus is important. In NC/Nga mice developing spontaneously AD-like skin lesions, long-lasting scratching behavior and IL-31 expression is enhanced, while both of them is unchanged in TNCB-induced contact dermatitis and this scratching behavior is ameliorated by administration of anti-IL-31 antibody[92,93]. These suggest the importance of Th2 cytokines in the pathogenesis of acute phase in AD. It is well known that cytokine profile in AD shifts from Th2 dominant in acute phase to Th1 dominant in chronic phase, as it is called "Th1/Th2 paradigm"[94-97]. Indeed, increased levels of IL-12 and IFN-γ, which represent Th1 cytokines, are detected in chronic AD lesions, compared with normal skin[8]. Previously, Aral *et al*. demonstrated that serum level of IL-18 is found significantly higher in AD patients than in controls and that a statistically significant relationship between the severity of AD, and serum levels of IL-18 and IL-12/p40 is determined, suggesting the involvement of IL-18 in AD[9]. IL-18, derived from dendritic cells, induces Th1 cells to produce Th1 cytokines[66, 98]. Moreover, other studies suggest the roles of IL-18 in the pathogenesis of AD[99-101]. However, because the conflict results in relationship between IL-18 and atopic dermatitis-like inflammation[102], the role of IL-18 in atopic dermatitis should be further addressed. IL-21, a member of the type I cytokine family, is produced by lymphoid cells such as activated $CD4^+$ T cells and exerts its pleiotropic function by binding to IL-21 receptor (IL-21R). Upregulation of IL-21 and IL-21R in skin lesions from AD patients and elevated levels of IL-21 in serum form AD patients are reported[103, 104]. In mice, skin barrier disruption, a surrogate for scratching, enhances the expression of IL-21 and IL-21R, as well as IL-6[103]. Further, IL-21 enhances CCR7 expression, migration to local lymphnode and antigen presentation of DCs[105]. In addition to Th1/Th2 paradigm, Th17 cells and Th22 cells emerged as new participants in the pathogenesis of AD. The cell number of intracellular IL-17 positive circulating lymphocyte, mRNA expression of IL-17 in peripheral blood mononuclear cells and IL-17 concentration in serum are upregulated in the patient with AD, correlated with the severity of AD[106,107]. IL-17 directly enhances IgE production, but not IgG, IgM or IgA, in human by triggering rapid degradation of IκBa and subsequent translocation of NF-κB into the B-cell nucleus[108]. Th22 cells were identified as CD4+ T cell producing IL-22 and lacking production of IL-17 and are distinct from Th1, Th2 and Th17 cells[109-111]. IL-22 induces the expression of S100A7, S100A8 and S100A9, a group of proinflammatory molecules, in human keratinocyte, as well as matrix metalloproteinase 3 and CXCL5. In addition, IL-22 induces keratinocyte migration in an in vitro injury model and downregulates the expression of keratinocyte differentiation markers including involucrin, loricrin, heat shock protein 27, calmodulin-related protein and heme oxygenase 1. Further, in reconstituted human epidermis, IL-22 induces strongly hyperplasia[112]. The number of Th2 and Th22 cells are significantly elevated in AD, whereas psoriatic skin has significantly increased frequency of Th1 and Th17 cell. The levels of IL-22 is upregulated in AD lesions, associated with the severity of AD symptoms[113]. These findings suggested that IL-22 affects to maintenance of inflammation and epidermal hyperplasia in AD. Taken together, AD is a Th2/Th22 skewed disease, with additional contributions from Th1 cytokines occurring in the chronic stage. Overlooking this complicated pathogenesis of AD, a simple question, whether this complicated cytokine network in AD can be regulated by the activation of PPARs, is raised. To simplify the cytokine network, the intracellular signaling pathway activated by these cytokines are focused. As shown in **Table 3**, JAK/STAT or NF-κB is involved in all

signaling pathways activated by cytokines in AD. PPAR-α interacts with NF-κB and AP-1 and PPAR-γ interacts with STAT, NF-κB, AP-1 and NF-AT[42]. Consequently, the gene expression in the down-stream involving by these transcription factors is reduced. Likewise, we examined the effects of PPAR-δ activation by GW501516 on IL-6 and IL-8 production from HaCaT cells, an immortalized keratinocyte derived from human epidermis. Expectedly, LPS-induced IL-6 production and TNF-α-induced IL-8 production are reduced with GW501516 treatment (**Figure 3**). Following the results from in vitro experiments, in vivo experiments in animal models, including gene-modified animals and classical (traditional) animal, are required to elucidate the effect of PPAR activation on AD. Actually, Kim *et al*. demonstrated that ursolic acid, a potential PPAR-γ agonist, suppresses ovalbumin-induced airway inflammation with the downregulation of IL-5, IL-13 and IL-17[114]. In dermatological field, two groups reported the effect of PPAR activators on oxazolone-induced contact dermatitis in mice, as an atopic dermatitis model[115,116]. NC/Nga mouse is known as an animal model for AD. NC/Nga mice are originated from Japanese fancy mice (Nishiki-Nezumi) and were established as a inbred strain in 1955. The most important characteristic in NC/Nga mice is that spontaneous AD-like dermatitis appears in the mice raised under ambient laboratory conditions, while no skin lesion is detected clinically in the mice raised under specific pathogen-free condition. Additionally, previous studies have revealed the other features, including the skin barrier dysfunction with the reduction of ceramide contents, IgE hyperproduction, cytokine profiles and long-lasting scratching behavior, corresponding to human AD[117-120, 92]. Therefore NC/Nga mice are widely used for evaluation of the therapeutic effect for AD. Chiba *et al*. showed that topical application (transdermal) of PPAR-α suppresses atopic dermatitis in NC/Nga mice[121]. Recently, a study showed that tannic acid ameliorates clinical severity in house dust mite extract-induced AD-like dermatitis in NC/Nga mice, with pathologically inhibition of hyperkeratosis, parakeratosis, acanthosis and infiltration of inflammatory cell[122]. To follow the antecedent studies on the effect of PPAR activation on skin barrier dysfunction in AD, further studies should be performed to elucidate the effects of PPAR activation on immune-modulation in AD.

Cytokine	Kinase/Transcription factor
IL-4	JAK3/STAT6
IL-5	RAS/MAPK, JAK/STAT, PI3K
IL-13	JAK3/STAT6
IL-22	JAK1/STAT3, Tyk2/STAT3, MAPK/STAT3
IL-25	NF-κB
IL-31	JAK1/STAT3, JAK1/STAT5, JAK2/STAT3, JAK2/STAT5, PI3K/AKT
IL-33	NF-κB
TSLP	JAK2/STAT3, JAK2/STAT5

Table 3. Kinases/Tanscription factors in signaling pathways of AD cytokines

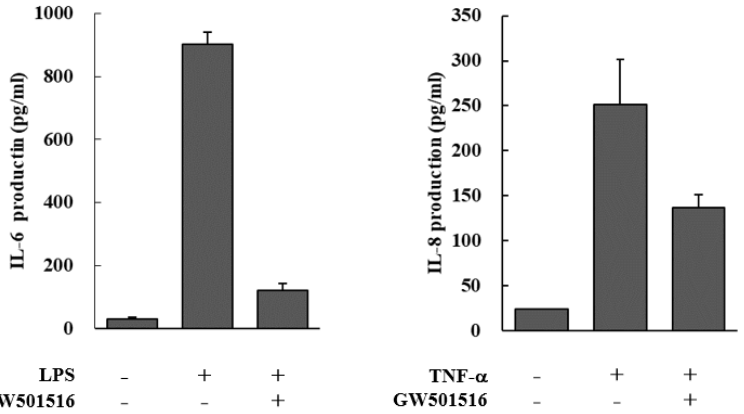

Figure 3; Suppression of inflammatory cytokine production by PPAR-δ agonist (GW501516). Treatment with GW501516 suppresses LPS-induced IL-6 production and TNF-α-induced IL-8 production from HaCaT cells**.**

7. Conclusion

Depending to pleiotropic function of PPARs, therapeutic applications of PPAR activators have been expected. Actually, some of agonists for PPAR-γ have already used in diabetes therapy. It is easily hypothesized that PPAR activators, which possess suppressive effects on transcription factors, may improve skin inflammation, including AD. Indeed, numerous numbers of in vitro experiments have been performed and provided useful information. Regarding to AD, although previous studies suggest that PPAR activation may be useful for improvement of skin barrier dysfunction and that PPAR activation suppresses the inflammatory molecules via inhibition of transcriptional pathways, the usefulness of PPAR activation for immune dysregulation is still unclear, due to its complicated cytokine network. However, some in vivo studies put the beacons to resolve the underlying issues. Thus, PPAR activation is expected to be one of the immune-modulating therapy for AD.

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Eucalyptus Citriodora Extract Regulates Cutaneous Homeostasis Including Immune Dysregulation and Skin Barrier Dysfunction Via the Modulation of Peroxisome Proliferator-Activated Receptor-β/δ (PPAR-β/δ) Pathway

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ABSTRACT

Perturbation of cutaneous homeostasis including immune dysregulation and skin barrier dysfunction evokes skin disorders. In this study, we examined the effect of *Eucalyptus citriodora* (Euc-c) extract on cytokine production, cell proliferation and cell differentiation in HaCaT cells to elucidate its influence on cutaneous homeostasis. Euc-c suppressed significantly LPS-induced IL-6 and TNF-α-induced IL-8 production from HaCaT cells. Conversely IL-1ra production was significantly enhanced by Euc-c. The expressions of IVL, CERS3 and CERS4, keratinocyte differentiation markers, were upregulated to 3.1, 2.8 and 2.7-fold respectively by Euc-c treatment, compared to the control, while the proliferation was downregulated. The lipid contents in Euc-c-treated cells tended to increase, compared with non-treated cells. To explore the underlying mechanism of these effects, we next performed siRNA experiments against PPAR-β/δ. Euc-c enhanced PPAR-β/δ mRNA expression to 3.25-fold, while PPAR-β/δ mRNA expression in transfected cells was suppressed. The expressions of IVL, CERS3 and CERS4 in transfected cells were suppressed to 1.48, 0.82 and 0.72-fold respectively, concomitant with suppression of PPAR-β/δ mRNA expression. These results indicated that Euc-c exerts anti-inflammatory effects and regulates keratinocyte differentiation via the modulation of PPAR-β/δ pathway. Therefore, the application of Euc-c is expected to exert beneficial effect on skin disorders evoked by perturbation of skin homeostasis.

Keywords: *Eucalyptus citriodora;* PPAR-β/δ, Inflammation; Barrier Function; Cutaneous Homeostasis

1. Introduction

The primary functions of the epidermis, especially stratum corneum which is comprised terminally differentiated cornified cells, are to serve permeability barrier function and water holding property. As reviewed by Menon, physical and/or structural features of stratum corneum have attracted researchers since 19th century[1]. Then the subjects of studies passed into biological and/or biochemical characteristics of keratinocytes with increasing the evidence that keratinocytes play crucial roles in cutaneous biology and immune system in 1980s[2]. To concoct these functions maturely, inter- and intracellular events undergo in sophisticated systems of different proteins and lipids during keratinocyte differentiation[3-6]. Therefore, it is important to regulate keratinocyte differentiation which is affected by skin environment such as dryness and inflammation[7-9]. Peroxisome proliferator-activated receptors (PPARs), which have been cloned as a member of the steroid hormone receptor superfamily in mouse liver, are classified three different isoforms termed PPAR-α, PPAR-β/δ and PPAR-γ sharing considerable sequence and structural homologies[10,11]. Activated-PPARs regulate the target gene expression by binding to a PPAR response element (PPRE) in the promotors of target genes as heterodimers with retinoid X receptors[12]. Initial studies demonstrated

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that PPARs are pivotal participants in the regulation of energy homeostasis by modulating glucose and lipid metabolism and transport[13],furthermore subsequent studies have shown that PPARs regulate in other cellular functions such as cell proliferation, cell differentiation, apoptosis and inflammation. The expressions of all subtypes of PPAR are identified in normal human epidermal keratinocytes. Of all the subtypes, PPAR-β/δ is expressed dominantly and consistently in keratinocytes, whereas PPAR-α and -γ are expressed lower quantities[14]. As previously reviewed the role of PPARs in skin diseases including psoriasis, atopic dermatitis and skin cancer[15-17], PPARs are involved in keratinocyte differentiation, epidermal hyperplasia, inflammation and permeability barrier function. Therefore the activation of PPAR-β/δ by exogenous ligands is expected to maintain the skin homeostasis. *Eucalyptus citriodora* (Euc-c) is an ever green tall tree, originated from temperate and tropical northeastern Australia. Their essential oil is used in perfumery and insect repellents, and applied as an alternative medicine against respiratory problems[18]. Gbenou and colleagues demonstrated that the essential oil exhibited anti-inflammation and analgesic properties in rats[19]. Further in vitro experiments showed that the essential oil and its major monoterpenes exhibited moderate to strong antioxidant activity[20], in which the constituents of the essential oil inhibited nitric oxide and prostaglandin E_2 production with altered expression of inducible nitric oxide synthase and cyclooxygenase-2[21] and that the resin inhibited the proliferation of B16F10 cells via apoptosis[22]. In this study, we demonstrated the effect of Euc-c extract on cytokine production, cell proliferation and cell differentiation in HaCaT cells, a spontaneously immortalized human keratinocyte cell line.

2. Materials and Methods

2.1 Reagents

Lipopolysaccharide (LPS) and Tumor necrosis factor-α (TNF-α) were purchased from Merk (St. Louis, MI). Ca^{2+}-free Dulbecco's modified Eagle medium (DMEM) without pyruvate and glutamine, 200mM L-glutamine, 100mM sodium pyruvate and fetal bovine serum (FBS) were purchased from Thermo Fisher Scientific (Waltham, MA).

2.2 Preparation of Euc-c extract

Leaves of Euc-c were kindly provided from Lone Pine Koala Sanctuary (Brisbane, QLD, Australia). The leaves were dried at room temperature and pulverized, followed by extraction procedure according to previous studies[23,24]. Briefly, the powder (250mg) was applied onto solid phase extraction tube and extracted with 5ml of dichloromethane and 11ml of methanol. After evaporation, the residue was dissolved in 1ml of dimethyl sulfoxide. The concentration of the solution was expressed 250mg equivalent (mge)/ml.

2.3 Cell culture

HaCaT cells, a spontaneously immortalized human keratinocyte cell line, were kindly gifted from Professor Michael Roberts at University of Queensland (Brisbane, QLD, Australia). To maintain HaCaT cells in the distinct stage of differentiation, the cells were cultured according to the method previously reported[25]. Calcium in FBS was depleted by incubation with Chelex 100 resin (Bio-Rad, Hercules, CA) for 1hr at 4°C. The resin was removed with 0.22μm filter. HaCaT cells were maintained in Ca^{2+}-free DMEM supplemented with 4mM L-glutamine. 1mM sodium pyruvate, 5% Ca^{2+}-depleted FBS, and 0.05 (LC) or 1.25 (HC) mM $CaCl_2$.

2.4 Cell proliferation assay

LC- and HC-HaCaT cells were seeded into a 96-well plate at the cell density of 2.0 x 10^3 and then were maintained in at 37°C in humidified atmosphere containing 5% CO_2 for 24hr. BrdU incorporation assay kit (Cell Signaling Technology, Danvers, MA) was employed for cell proliferation assay, according to the manufacturer's instruction.

2.5 Cytotoxicity

LC-HaCaT cells were seeded into a 96-well plate at the cell density of 2 x 10^3 cells/well and maintained in a humidified atmosphere of 5% CO_2 at 37°C for 24hr. The cells were treated with 50, 100 and 250μge/ml of Euc-c for 24hr and then subjected to neutral red assay

2.6 Cytokine production

LC-HaCaT cells were seeded into a 96-well plate at the cell density of 5 x 10^3 cells/well (for IL-6 and IL-8) or 1 x 10^4 cells/well (for IL-1ra) and maintained in a humidified atmosphere of 5% CO_2 at 37°C for 24hr. To estimate IL-6 production, the cells were treated with 100ng/ml LPS and 250μge/ml Euc-c for 24hr. For IL-8 production assay, the cells were treated with 10ng/ml TNF-α and 250μge/ml Euc-c for 24hr. The productions of IL-6 and IL-8 was measured by ELISA kit (R&D sys-

tems, Minneapolis, MN), according to the manufacturer's instruction. To measure the production of IL-1ra, the cells were treated with 250μge/ml Euc-c for 24hr. After discarding the medium, the cells were washed with PBS twice and lysed by freeze-thaw cycle three times in 200μl of PBS. After removing cell debris from the lysates by centrifugation x10,000g for 10 min, the supernatant were subjected to IL-1ra ELISA kit (R&D systems, Minneapolis, MN), according to the manufacturer's instruction.

2.7 Cell differentiation

To evaluate differentiation stage in LC- and HC-HaCaT cells, the cells were seeded into a ϕ6cm dish at 2.5 x 10^5/dish and maintained in LC-DMEM or HC-DMEM in a humidified atmosphere of 5% CO_2 at 37°C till >80% confluent, followed by real-time PCR as described below. To evaluate the effect of Euc-c on LC-HaCaT differentiation, LC-HaCaT cells maintained in LC-DMEM in a humidified atmosphere of 5% CO_2 at 37°C till >80% confluent. The cell were treated with 250μge/ml Euc-c for further 24hr and then subjected to real-time PCR.

2.8 Small interfering RNA (siRNA) Transfection

LC-HaCaT cells were seeded into a ϕ6cm dish at 2.5 x 10^5/dish and maintained in a humidified atmosphere of 5% CO_2 at 37°C for 24hr. Predesigned PPARD siRNA (NM_006238) obtained from Merk (St. Louis, MI) was transfected with Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA) according to manufacturer's instruction and maintained in a humidified atmosphere of 5% CO_2 at 37°C for 24hr, followed by Euc-c treatment for further 24hr. Then the harvested cells were subjected to total RNA extraction, reverse transcription and real-time PCR to assess the expression of PPAR-β/δ, IVL, CERS3 and CERS4.

2.9 Real-time PCR

Total RNA was extracted from HaCaT cells with SV RNA isolation kit (Promega, Madison, WI), according to the manufacturer's instruction and then performed reverse transcription reaction (42°C 15min, 95°C 5min) with RT system (Promega, Madison, WI). PCR amplification and detection were performed on Rotar-Gene Q (Corbett) using the initial denaturation condition of 95°C for 5 min, followed by 40 cycles at 94°C for 10sec, 62°C for 15sec and 72°C for 20sec each with primers as described in **Table 1**. Expression of target mRNA was quantified using the comparative threshold cycle (Ct) method for relative quantification ($2^{-\delta\delta Ct}$), normalized to the geometric mean of reference genes β-actin.

Name	Sequence
β-actin	forward: GATGAGATTGGCATGGCTTT reverse: CACCTTCACCGTTCCAGTTT
IVL	forward: GGCCCTCAGATCGTCTCATA reverse: CACCCTCACCCCATTAAAGA
CERS3	forward: ACATTCCACAAGGCAACCATTG reverse: CTCTTGATTCCGCCGACTCC
CERS4	forward: GGAGGCCTGTAAGATGGTCA reverse: GAGGACCAGTCGGGTGTAGA
PPARD	forward: ACTGAGTTCGCCAAGAGCAT reverse: TGCACGCCATACTTGAGAAG

Table 1. Primer sequences

2.10 Lipid accumulation in HaCaT cells

LC- and HC-HaCaT cells were seeded into a flasks (75cm^2) at 1.0 x 10^6/flask and maintained in LC-DMEM or HC-DMEM in a humidified atmosphere of 5% CO_2 at 37°C. After reaching >80% confluent, LC-HaCaT cells were treated with 250μge/ml Euc-c for 24hr, harvested by trypsinization and the cell number was adjusted to 2 x 10^6 cells/tube. After washing three times with 1ml of PBS, the cells were lyzed with 700μl of $CHCl_3$:methanol (2:1) to extract lipids followed by addition of 400μl H_2O and centrifugation at 5000g for 5min. The lower layer was transferred to glass tube and dried under vacuum.

The residue was dissolved in 20μl of $CHCl_3$:methanol (2:1). Ten μl of lipid solution was applied on a thin-layer chromatography plate and developed with $CHCl_3$:methanol:CH_3CO_2H (190:9:1) twice. After drying, the plated was charred at 180°C for 10min and subjected to dencitmetric analysis.

2.11 Statistical analysis

Data are expressed as means±SD. Statistical comparison between experimental groups and controls was performed using an unpaired Student's t-tests. P values less than 0.05 were considered significant.

3. Results

3.1 Difference in differentiation stage between HaCaT cells maintained in different concentration of Ca^{2+}

To evaluate the effect of Ca^{2+} concentration, morphological chages in LC-HaCaT cells and HC-HaCaT cells are observed. LC-HaCaT cells showed less compacted and spindle shape with absence of cell to cell tight junction (**Figure 1a**). On the other hand, HC-HaCaT cells showed a more spread-out squamous shape with tight junction among the cells (**Figure 1b**). Next, to evaluate the effect of Ca^{2+} concentration on cell proliferation, BrdU up-take assay was performed. The cell proliferation ratio was remained in HC-HaCaT cells, compared to LC-HaCaT cells (**Figure 1c**). To estimate differentiation stage, the expressions of involucrin (IVL), ceramide synthase 3 (CERS3) and ceramide synthase 4 (CERS4) in LC- and HC-HaCaT cells were evaluated. The expressions of IVL was enhanced 3.6-fold with significance in HC-HaCaT cells, compared with LC-HaCaT cells. Likely, CERS3 and CERS4, which are dominantly expressed in differentiated keratinocytes, were expressed significantly in higher levels in HC-HaCaT cells, compared with LC-HaCaT cells (**Figure 1d**).

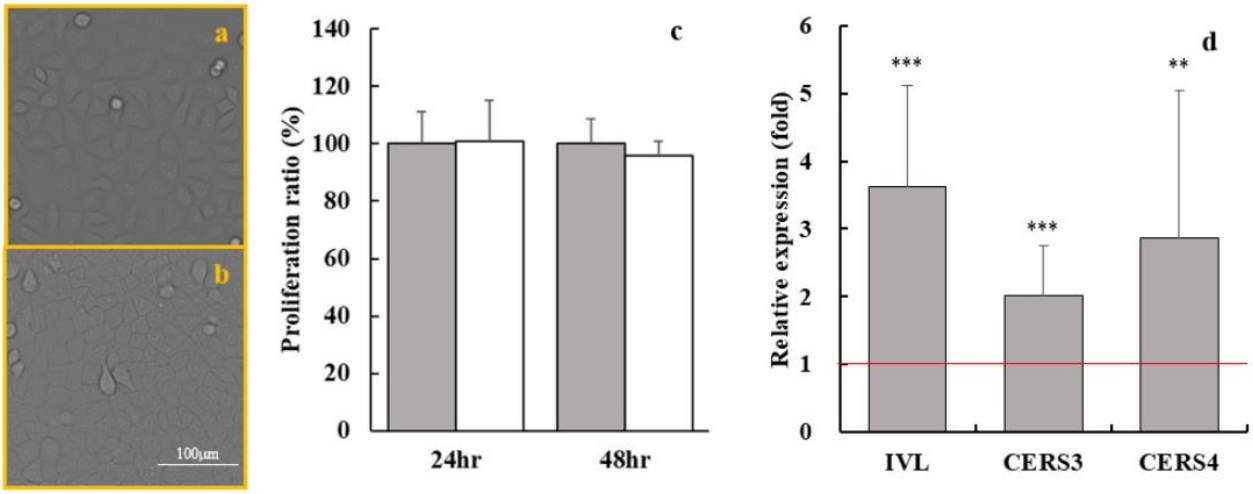

Figure 1; Differences on morphology, cell proliferation and differentiation maker expression between LC-HaCaT and HC-HaCaT. Less compacted and spidle shape with absence of tight junction was observed in LC-HaCaT (a), while HC-HaCaT cells showed spread-out squamous shape with tight junction (b). There was no significant difference in cell proliferation ratio (c) between LC-HaCaT (closed column) and HC-HaCaT cells (open column). Significant enhancement of differentiation marker expression was detected in HC-HaCaT cells, compared with LC-HaCaT cells (d). Each value represents the mean ± SD from three independent experiments (**p<0.01, ***p<0.001 compared with LC-HaCaT).

3.2 Cytotoxicity of Euc-c

The cytotoxicity of Euc-c on LC-HaCaT cells was evaluated with neutral red assay to determine the concentration of Euc-c for further experiments. Significant cytotoxicity was not detected within the range of concentration applied in this experiment (**Figure 2**). Therefore, the cells were treated with 250μge/ml in the following experiments.

3.3 Anti-inflammatory effect of Euc-c

To evaluate the anti-inflammatory effect of Euc-c, the production of IL-6, IL-8 and IL-1ra from LC-HaCaT cells was measured with ELISA. LPS-stimulation induced 904±38pg/ml of IL-6 secretion while unstimulated LC-HaCaT cells secreted 31.1±4.0pg/ml. The enhanced production of IL-6 was significantly reduced to 653±50pg/ml in the cells treated with Euc-c (**Figure 3a**).

IL-8 production was enhanced to 251±51pg/ml with stimulation of TNF-α, while the production in untreated LC-HaCaT cells was 23.8±0.5pg/ml. The enhanced production of IL-8 was significantly suppressed to 80.5±6.6pg/ml in the cells treated with Euc-c (**Figure 3b**). IL-1ra production in Euc-c-treated cells was significantly enhanced to 1371±93pg/ml, compared with the untreated cells (**Figure 3c**).

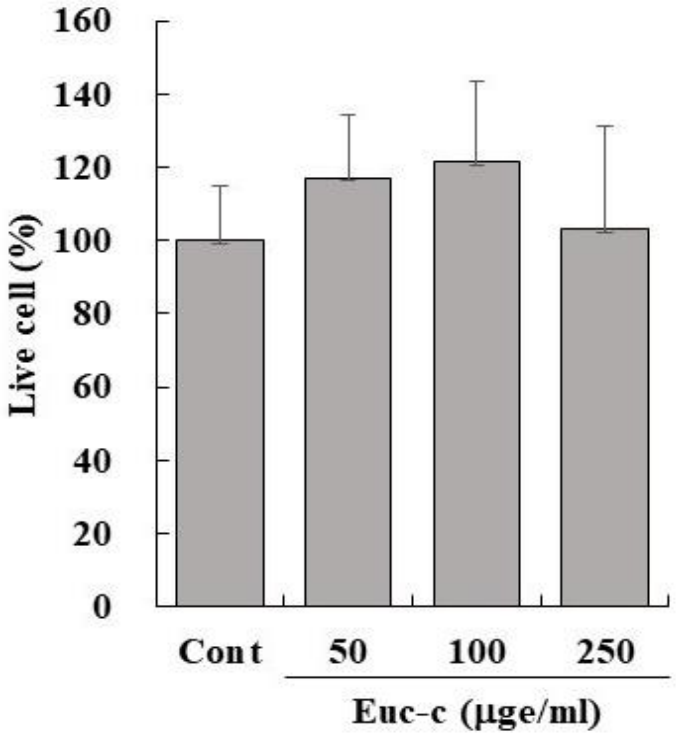

Figure 2; Cytotoxicity of Euc-c. Significant cytotoxicity was not detected at range of concentration applied in this experiment. Each value represents the mean ± SD from three independent experiments.

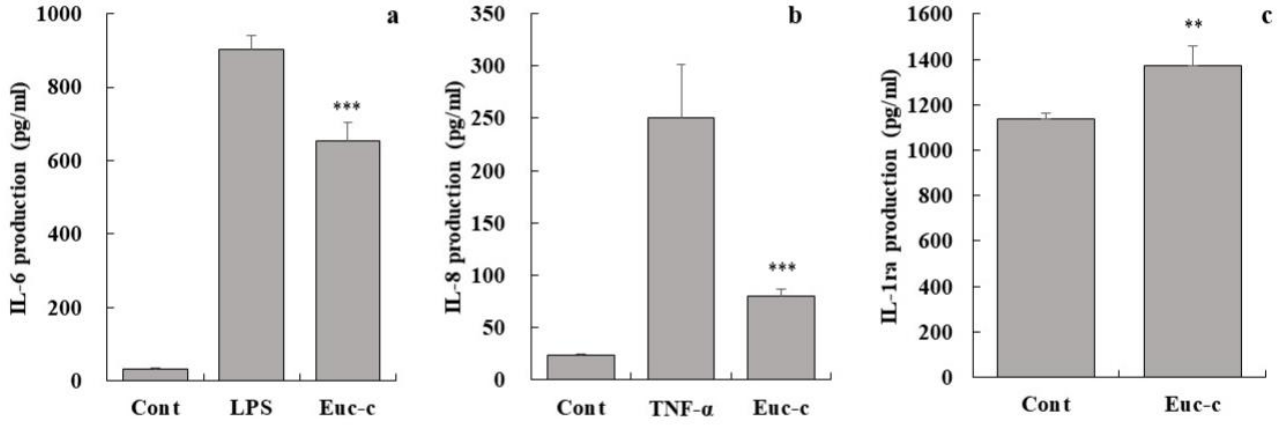

Figure 3; Anti-inflammatory effect of Euc-c. To evaluate the anti-inflammatory effect of Euc-c, the production of IL-6 (a), IL-8 (b) and IL-1ra (c) from LC-HaCaT cells was measured. LPS-induced IL-6 production was significantly reduced (a). TNF-α-induced IL-8 was also reduced significantly (b). IL-1ra production in Euc-c-treated cells was significantly enhanced (c). Each value represents the mean ± SD from three independent experiments (**p<0.01, ***p<0.001 compared with the production from stimulated cells)

3.4 Euc-c suppresses proliferation of LC-HaCaT

BrdU up-take assay was employed to estimate whether Euc-c has effects on LC-HaCaT proliferation ratio. The relative proliferation of LC-HaCaT cells treated with Euc-c was decelerated to 33.0±2.4% of the

control with significance (**Figure 4**).

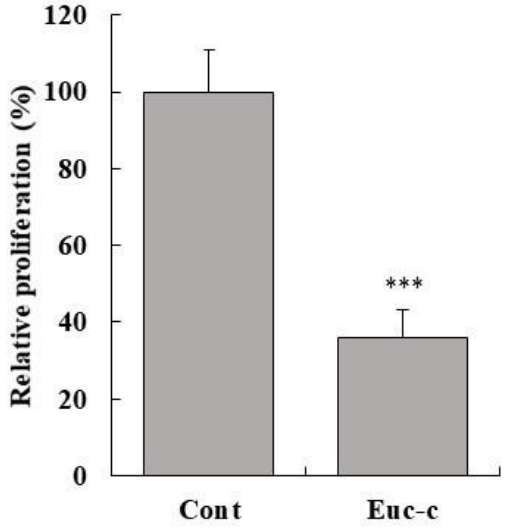

Figure 4; Euc-c suppresses proliferation ratio of LC-HaCaT. The relative proliferation of LC-HaCaT cells treated with Euc-c was significantly decelerated. Each value represents the mean ± SD from three independent experiments (***p<0.001 compared with the control)**.**

3.5 Euc-c promotes LC-HaCaT differentiation

To examin the effect of Euc-c on LC-HaCaT differentiation, the relative expressions of IVL, CERS3 and CERS4 were assessed. As shown in **Figure 5**, every expression of IVL, CERS3 and CERS4 was significantly accelerated to 3.1, 2.8 and 2.7-fold respectively, compared to the control.

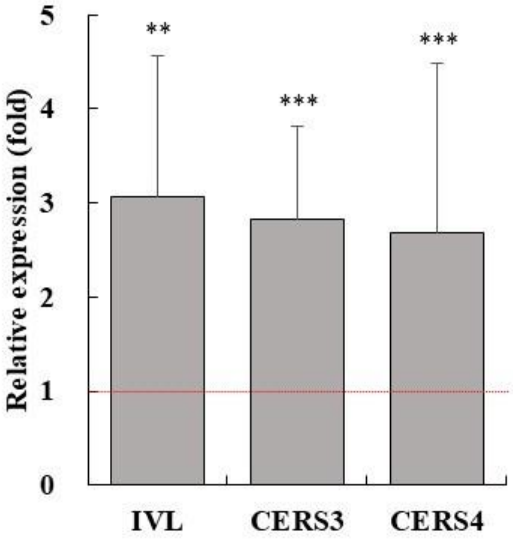

Figure 5; Euc-c promotes LC-HaCaT differentiation. The expressions of IVL, CERS3 and CERS4 was significantly accelerated. Each value represents the mean ± SD from three independent experiments (**p<0.01, ***p<0.001 compared with the control)**.**

3.6 Euc-c enhances lipid accumulation in HaCaT cells

Because lipid accumulation in keratinocytes is important to exert skin barrier function, we examined whether Euc-c is involved in lipid accumulation in LC-HaCaT cells. The upper spots were detected in all lipid extract from LC-HaCaT cells, Euc-c-treated LC-HaCaT cells and HC-HaCaT cells (**Figure 6**). The results in dencitmetric analysis showed an increase in the intensity of the upper spot in Euc-c-treated LC-HaCaT cells, compared to LC-HaCaT cells and HC-HaCaT cells (**Table 2**). On the other hand, the lower spot was detected in Euc-c-treated LC-HaCaT cells, while the spot was not detected in LC-HaCaT cells and HC-HaCaT cells

(**Figure 6**).

	Upper spot	Lower spot
LC-HaCaT	1539000	ND
Euc-c	1664000	791400
HC-HaCaT	1543000	ND

Table 2. Dencitmetric analysis of lipid spots

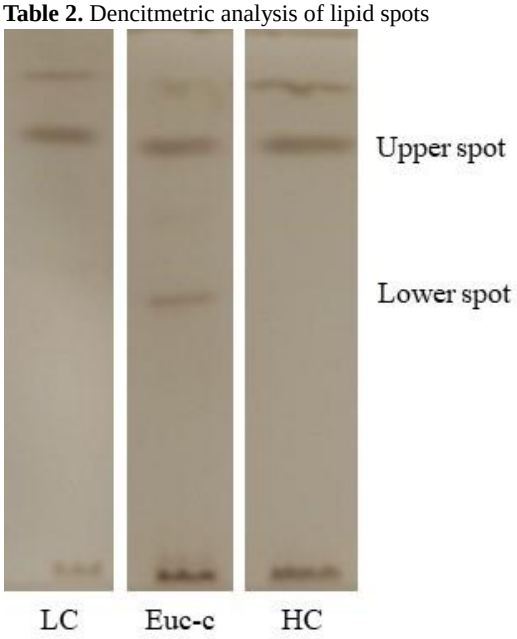

Figure 6; Euc-c enhances lipid accumulation in HaCaT cells. The upper spots were detected in all lipid extract from LC-HaCaT cells, Euc-c-treated LC-HaCaT cells and HC-HaCaT cells. The lower spot emerged only in Euc-c-treated LC-HaCaT cells**.**

3.7 PPAR-β/δ is involved in the effects induced by Euc-c

Since the results described above strongly suggested that Euc-c exerts the effects on LC-HaCaT cells through PPAR-β/δ activation, we performed a siRNA-mediated PPAR-β/δ knockdown in LC-HaCaT cells to examine whether PPAR-β/δ might be activated by Euc-c. PPAR-β/δ siRNA reduced the PPAR-β/δ mRNA level in Euc-c-treated LC-HaCaT cells by 0.56 fold compared with the control, while Euc-c treatment enhanced PPAR-β/δ expression to 3.25-fold in siRNA-untransfected LC-HaCaT cells (**Figure 7a**). The mRNA expression levels of IVL, CERS3 and CERS4 in siRNA-transfected LC-HaCaT cells treated with Euc-c were significantly reduced to 1.48, 0.82 and 0.72-fold respectively, whereas Euc-c treatment enhanced the expression levels in wild-type LC-HaCaT cells (**Figure 7b**).

4. Discussion

The primary function of the epidermis, which is located in the outmost of the body, is to serve a permeability barrier between the external environment and the host. In order to provide the mature permeability barrier, the orchestrated inter- and intracellular events undergo in the epidermis. Due to the disturbance of skin micro-environment including inflammation, cell proliferation and cell differentiation impairs the permeability barrier function[7-9], it is important to regulate complex biological events in the epidermis. Since PPARs are initially identified as transcriptional regulators of lipid and glucose metabolism[10,13], further evidence has also

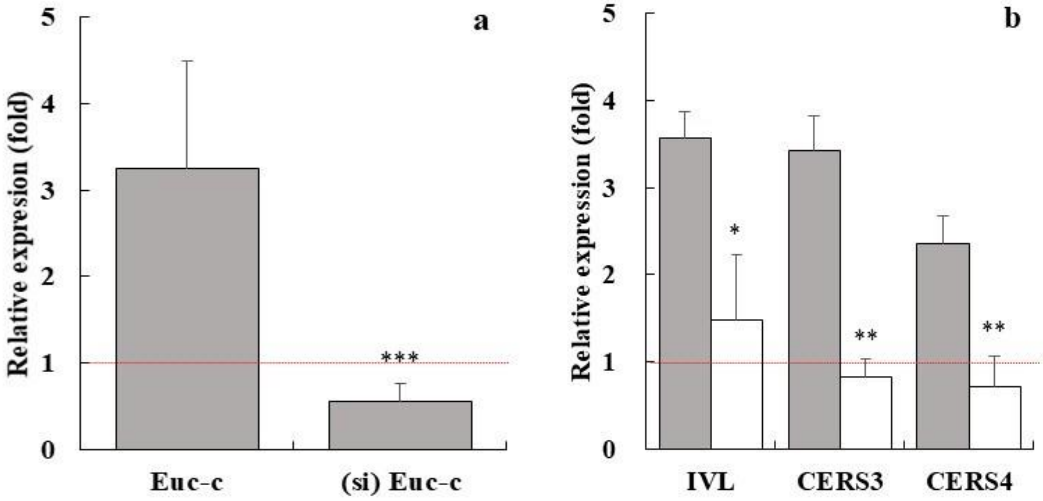

Figure 7; PPAR-β/δ is involved in the effects induced by Euc-c. PPAR-β/δ siRNA reduced the PPAR-β/δ mRNA level in Euc-c-treated LC-HaCaT cells compared with Euc-c-treatet cells (a). The mRNA expression levels of IVL, CERS3 and CERS4 in siRNA-transfected LC-HaCaT cells treated with Euc-c (open column) were reduced significantly (b). Each value represents the mean ± SD from three independent experiments (*p<0.05, **p<0.01, ***p<0.001 compared with the untransfected cells).

accumulated for their tissue distribution and other functions. Regarding their distribution in human skin, previous studies showed that all PPAR isotypes are expressed in human skin and that PPAR-β/δ is ubiquitously expressed throughout the epidermis while the expressions of PPAR-α and -γ increase along with the keratinocyte differentiation[14,26]. In addition, other studies on their function in the skin demonstrated that PPARs were involved in keratinocyte differentiation, epidermal hyperplasia, inflammation and permeability barrier function[15-17]. Euc-c is an ever green tall tree, originated from temperate and tropical northeastern Australia. The diverse biological activities of Euc-c extract, especially as an essential oil, have been reported previously[18-22] and its safety has been proved by long history of traditional usage. In this study, we demonstrated the effect of Euc-c extract on cytokine production, cell proliferation and cell differentiation in HaCaT cells. To elucidate the diverse events involved in inflammation, cell proliferation and differentiation in epidermis, normal human epidermal keratinocytes (NHEKs), which are supplied commercially, provide an ideal in vitro experimental system. However their usage is limited due to the complexities involved in cultivation and a limited number of passages. HaCaT cells, which have been established as spontaneously immortalized human keratinocytes maintaining full epidermal differentiation capacity[27], are widely employed as a model for the study of keratinocyte functions to exclude the problems arose in the system using NHEKs. Similar to NHEKs, Ca^{2+} addition induces morphological changes, concomitant with expressions of differentiation makers such as keratin 1, keratin 10 and involucrin in HaCaT cells[28]. Interestingly, Deyrieux *et al*. reported that HaCaT cells exhibited reversible differentiation by switching the Ca^{2+} concentration from 2.8mM to 0.03mM[25]. First, we elucidated the differences in morphology, proliferation rate and differentiation maker expression between LC- and HC-HaCaT cells. As showed in **Figure 1,** LC-HaCaT cells exhibited less compacted spindle shape with less mRNA expression of IVL, CES3 and CERS4, whereas HC-HaCaT cells exhibited a more spread-out squamous shape with enhanced mRNA expressions of the markers and maintained proliferation rate. These results suggested that HaCaT cells cultured in low Ca^{2+} condition maintained a basal-like state both morphologically and biochemically, and that the differentiated-state was induced and maintained, once the cells were cultured in high Ca^{2+} condition. We employed LC-HaCaT cells for further experiments, while HC-HaCaT cell were used as the differentiated-state control. The evidences for anti-inflammation effects of PPARs activators have been examined previously[29], therefore, the effect of Euc-c on cytokine production at a concentration of 250μge/ml, which was the highest no-cytotoxic concentration, was examined. Euc-c treatment down-regulated LPS-induced

IL-6 production and TNF-α-induced IL-8 production (**Figure 3a** and **b**). The initial demonstration of a regulatory function of PPAR-α in inflammation signaling was obtained in PPAR-α-deficient mice that displayed an exacerbated response to inflammatory stimuli[30]. Consequently, intensive studies on the effects of PPAR activators on inflammatory responses showed that all of PPAR isotypes exerted distinct and overlapping anti-inflammatory effects, as listed in **Tables 3**[31-39]. Regarding the underlying mechanism of the inhibition of pro-inflammatory cytokines, Ricote and Glass proposed five diversity mechanisms of PPAR-mediated transrepression; i) direct interaction, ii) induction of IκBα, iii) regulation of kinase activity, iv) coactivator competition and v) co-repressor interaction[40].

Up-regulation	Down-regulation
IL-4, IL-5, sIL-1ra	IL-1β, IL-6, IL-12, IL-23, IL-27
	CCL2 (MCP-1), CCL4 (MIP), CXCL8 (IL-8)
	IFN-γ, TNF-α
	ICAM-1, VCAM-1
	ET-1
	COX-2, iNOS

Table 3. Influence of PPAR activation on inflammatory molecule expression

Since NF-κB is a crucial transcriptional factor in both LPS and TNF-α signaling cascade, NF-κB may be involved in the underlying mechanism of the down-regulation of IL-6 and IL-8 by Euc-c. In contrast, IL-1ra production was up-regulated by Euc-c (**Figure 3c**). Previous reports showed that WY14643, a PPAR-α agonist, and GW501516, a PPAR-β/δ agonist, enhanced IL-1ra production and that IL-1ra gene was a direct target of PPARs[41,42]. Our results, which are consistent with previous studies, suggest that Euc-c exerts an anti-inflammatory effect by both down-regulating expression of pro-inflammatory genes and direct up-regulation of anti-inflammatory gene through PPAR activation. The function of PPAR-β/δ in keratinocyte proliferation still remains controversial. Some studies showed that PPAR-β/δ accelerates keratinocyte proliferation in psoriasis[43] and that PPAR-β/δ played a vital role in EGF-stimulated proliferation of HaCaT cells[44]. Conversely, other reports demonstrated that activation of PPAR-β/δ by GW0742 inhibited proliferation and enhanced terminal differentiation in keratinocyte[45], and that GW0742 inhibited cell growth of human N/TERT-1 keratinocytes[46]. We also obtained the contradictory results on cell proliferation by the different methods, neutral red assay and BrdU cell proliferation assay. The result in neutral red assay showed that Euc-c treatment did not affect cell viability whereas the proliferation of HaCaT cells treated with Euc-c was significantly reduced in BrdU cell proliferation assay, as well as GW501516, a selective PPAR-β/δ activator (data not shown). Because BrdU cell proliferation assay reflects BrdU incorporation during DNA synthesis, our results suggest that the ratio of cells in S phase was decreased by Euc-c treatment. However, further experiments are required to elucidate the effect of Euc-c on cell cycle. Next, we evaluated the expression of keratinocyte differentiation markers in Euc-c-treated LC-HaCaT cells. The mRNA expression of IVL, a well-known keratinocyte differentiation marker, was accelerated in the cells treated with Euc-c. Concurrently, the mRNA expressions of CERS3 and CERS4 were enhanced by Euc-c treatment (**Figure 5**). The enzymes are dominantly expressed in keratinocyte[47] and their expressions are upregulated upon keratinocyte differentiation[48]. Previous studies demonstrated that the activation of PPARs induced keratinocyte differentiation[49,50]. Westergaard and the colleagues showed that L165041, a selective PPAR-β/δ activator, was the most potent for keratinocyte differentiation, compared with PPAR-α and PPAR-γ activators[26]. The results we obtained also suggest that Euc-c treatment induced keratinocyte differentiation. Lipids including ceramide, cholesterol and free fatty acid are required to establish a mature permeability barrier. The deficiency of essential fatty acids, which are components of ceramide, results in abnormalities of permeability barrier[51]. Moreover, previous studies showed that PPAR-β/δ activators improved

epidermal barrier homeostasis through stimulation of lipid synthesis[52,53]. We showed here that the lipid extract from Euc-c-treated LC-HaCaT cells demonstrated two lipid spots (the upper and the lower spot) whereas the extract from LC-HaCaT and HC-HaCaT cells exhibited only the upper spot (**Figure 6**). Dencitmetric analysis revealed that the treatment of Euc-c tended to increase the intensity of the upper spot, compared to LC- and HC-HaCaT cells (**Table 2**). Scince the lower spot was detected in the extract of LC-HaCaT cells treated with GW501516 as well (data not shown), the spot was not a result of Euc-c extract. Thus the emergence of the lower spot suggests that the treatment with Euc-c induced synthesis of another kind of lipid. Because the upregulation of CERS3 and CERS4 mRNA expression was found, and Chon *et al*. demonstrated that an oat lipid extract enhanced ceramide synthesis via PPAR pathways[54], the enhancement of ceramide synthesis can be presumed although ceramide spots could not be detected in our experimental conditions. Finally, we performed siRNA experiments for PPAR-β/δ to clarify the effects of PPAR-β/δ on the above effects of Euc-c. According to results described above, we at first hypothesized that Euc-c activated PPAR-β/δ pathway. However, the treatment with Euc-c accelerated the expression of PPAR-β/δ mRNA expression which was suppressed by siRNA transfection (**Figure 7a**). Concomitantly, the mRNA expressions of IVL, CERS3 and CERS4, which were upregulated by Euc-c, were suppressed by siRNA transfection (**Figure 7b**). It is still unclear whether Euc-c activates PPAR-β/δ, however we confirmed here that Euc-c upregulated the mRNA expression of PPAR-β/δ, accompanied by enhancement of the accumulation of fatty acids which can be endogenous ligands for PPAR-β/δ. Taken together, these results suggest that Euc-c affected keratinocyte functions via modulation of PPAR-β/δ pathway. Atopic dermatitis is a chronic and relapsing disease characterized skin barrier dysfunction and immune dysregulation. The application of Euc-c is expected to exert beneficial effect on atopic dermatitis by improving both skin barrier dysfunction and immune dysregulation, as well as other PPAR-β/δ activators (Panduratin A and GW0742) previously reported[55,56]. On the other hand, because previous studies demonstrated that the modulation of PPAR-β/δ pathway exhibited therapeutic effects on wound healing[57], melanoma[58, 59] and UV-induced extrinsic skin aging[60], further experiments evaluating the effects of Euc-c on other skin disorders should be explore to develop the application of Euc-c for a wide range of skin disorders.

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Conflict of interest

The authors declare that there is no conflict of interest regarding the publication of this manuscript

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Epidermal Growth Factor Inhibitor-Induced Cutaneous Toxicity Improves with Moisturizers

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ABSTRACT

Although epidermal growth factor receptor (EGFR) inhibitors are one of the most effective treatment options for lung cancer, they frequently cause cutaneous toxicity that can lead to treatment discontinuation. Dryness, which is a common form of cutaneous toxicity, is usually treated using medical moisturizing agents. We aimed to investigate the treatment of cutaneous toxicity caused by EGFR inhibitors by comparing patients who used a cosmetic moisturizer with those who used conventional medical moisturizers. This study included 12 patients with lung cancer, who were receiving EGFR inhibitors and using topical medical moisturizers. The participants were assigned to a group that continued using medical moisturizers or a group that began using NOV® skin cream D. The study's findings showed that like conventional medical moisturizers, NOV® skin cream D improved the cutaneous dryness caused by EGFR inhibitors and that it might additionally improve patients' quality of life. Also, we obtained novel findings that NOV® skin cream D normalized keratinization, which is a component of normal skin cell differentiation impeded by EGFR inhibitors. Hence, the cosmetic moisturizer may help to prevent the discontinuation of EGFR inhibitors, thereby ensuring their continuous therapeutic effects.

Keywords: Cutaneous Toxicity; Epidermal Growth Factor Receptor Inhibitors; Moisturizer; Quality of Life; Stratum Corneum Water Content

1. Introduction

Epidermal growth factor receptor (EGFR) inhibitors, which target *EGFR* mutations, are used to treat lung cancer[1]. However, cutaneous toxicities, including dry skin, acne-like eruptions, and rashes, occur frequently[2], and the quality of life (QOL) of the patients declines. Therefore, administering EGFR inhibitors continuously is difficult[3]. Given that side-effects indicate cancer cell sensitivity[4] and they correlate with the therapeutic effect, it is important to continue the administration of EGFR inhibitors while managing their side-effects.

Of the cutaneous toxicities, which are a major side-effect of EGFR inhibitors, dryness can be alleviated or prevented by using moisturizers. Medical moisturizers, including preparations that contain heparin-like compounds, are widely used[5-7], but many patients complain about the sticky feeling associated with using these medical moisturizers, and if the use of moisturizers is to continue, moisturizers that match patients' preferences are required. This study aimed to investigate the treatment of cutaneous toxicities caused by EGFR inhibitors by comparing patients who used a cosmetic moisturizer, namely, NOV® skin cream D (Noevir Co., Ltd., Kobe, Japan), with those who used conventional medical moisturizers.

2. Materials and methods

2.1 Participants

The participants were patients with *EGFR* mutation-positive lung cancer who visited Takatsuki Red Cross Hospital Department of Dermatology between January and October 2018. These patients were adminis-

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tered afatinib (Giotrif®), gefitinib (Iressa®), or osimertinib (Tagrisso®), and they applied moisturizers, including NOV® skin cream D, Hirudoid® soft ointment (0.3%) (Maruho Co., Ltd., Osaka, Japan), or Besoften® Lotion (0.3%) (Teikoku Seiyaku Co. Ltd., Kagawa, Japan), externally to dry skin to counter the drugs' side-effects.

2.2 Moisturizer application

The patients were randomly assigned to two groups, of which, one continued using the medical moisturizers (Medical moisturizer group) and the other started using the cosmetic moisturizing cream (Moisturizing cream group). The participants applied the moisturizers (1–2 mg/cm^2) systematically at home in the morning and evening, or after bathing for 8 weeks. The patients did not apply the moisturizers to the upper back region, which comprised the control region.

2.3 Observation and assessments

The patients' inner forearms, which received the moisturizers (application area), and their upper backs (control area) were evaluated.

2.3.1 Evaluation of skin condition

The levels of dryness, itchiness, and erythema on the left and right inner forearms and upper back were scored using a 5-grade scale, as follows: none: 0; slight: 1; mild: 2; moderate: 3; or severe: 4, and the total scores were calculated.

2.3.2 Stratum corneum analysis

The stratum corneum water content was evaluated using a 3.5-MHz high-frequency conductance instrument (Skicon®-200EX; Yayoi Co., Ltd., Tokyo, Japan). Areas (24 mm × 50 mm) of the stratum corneum on the right inner forearm and upper back were sampled using tape stripping (Cellotape®; Nichiban Co., Ltd., Tokyo, Japan). The interleukin (IL)-1 receptor antagonist (IL-1ra) and IL-1α levels were quantified using an enzyme-linked immunosorbent assay[8], and the IL-1ra to IL-1α ratios were calculated. The stratum corneum samples were stained immunohistologically for thymic stromal lymphopoietin (TSLP)[9], and the fluorescence intensities per cell area were calculated. After staining the samples with gentian violet and brilliant green, the captured images were binarized, and the multilayer exfoliated cell area to the total cell area ratios were calculated to determine the degree of multilayer exfoliation. The levels of trypsin activity were then measured[10].

2.3.3 Patient surveys

The patients' QOL was evaluated using the Skindex-16 questionnaire. Furthermore, a questionnaire was administered to the patients to assess the ease of application and the moist feelings associated with the using of the moisturizers.

2.4 Statistical analyses

The Wilcoxon signed-rank test was used to analyze the skin condition's scores, the water content of the stratum corneum, IL-1ra to IL-1α ratios, TSLP levels, degrees of multilayer exfoliation, trypsin activity, and the QOL scores on weeks 0–8. The Mann-Whitney U test was used to compare data from the inner forearm (application area) and the upper back (control area) regions, and the Medical moisturizer and the Moisturizing cream groups. The groups' questionnaires were analyzed using the χ^2-test. The statistical analyses were performed using SPSS® 21.0 (SPSS Inc., Chicago, IL, USA), and a value of $p < 0.1$ was considered significant.

2.5 Ethics statement

The Takatsuki Red Cross Hospital Ethics Committee approved this study (H29-20). All study participants provided informed consent.

3. Results

3.1 Background of participants

The participants comprised one man and 11 women whose mean age was 72.8±8.3 years (58–85 years). The groups did not differ regarding sex and age. All of the patients used the moisturizers continuously without any adverse events. The skin symptoms did not worsen in either group.

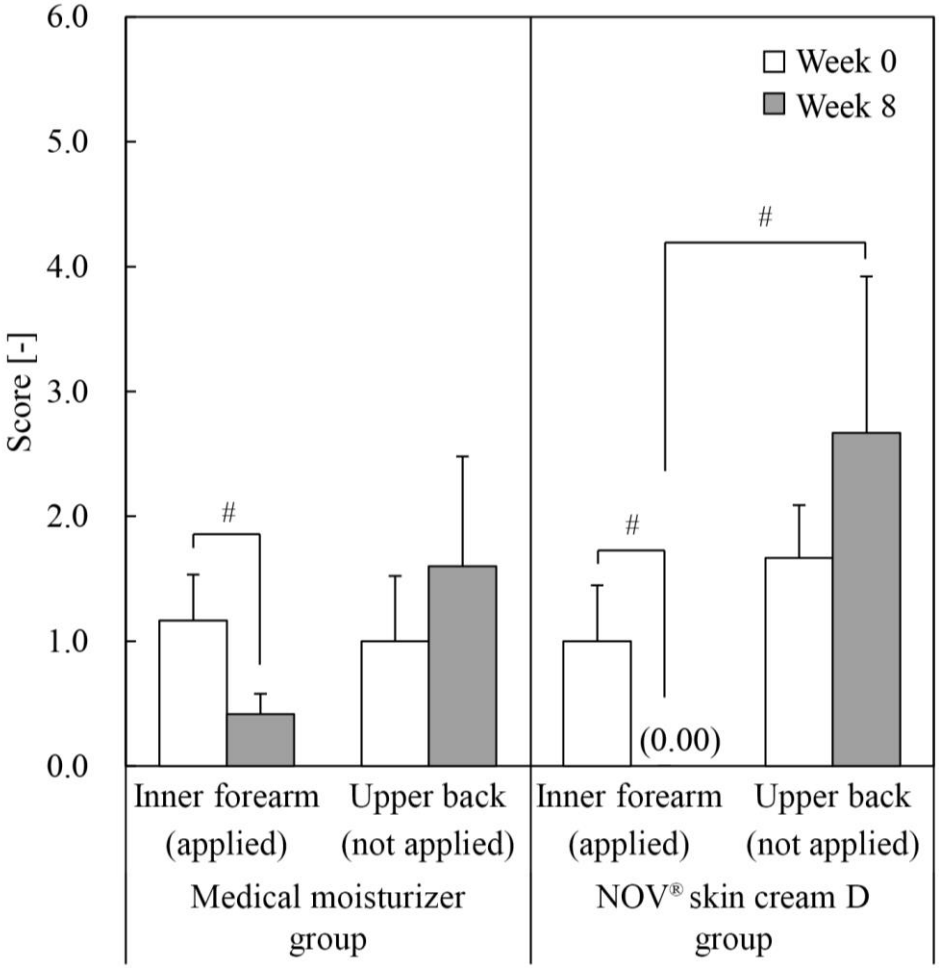
3.2 Evaluation of skin condition

Figure 1 shows the total dryness, itching, and erythema scores from the patients' inner forearms and upper backs. In both groups, the inner forearms' total scores had decreased significantly by week 8 compared with those at week 0. At week 8, the inner forearm's total score was significantly lower than the upper back's total score in the Moisturizing cream group.

3.3 Stratum corneum analyses

Table 1 shows the analyses of the stratum corneum. Compared with week 0, the water content of the upper back's stratum corneum was significantly lower at week 8 in the Medical moisturizer group. In both groups,

the TSLP levels in the stratum corneum of the inner forearm and upper back were significantly higher at week 8 compared with those at week 0. In the Moisturizing cream group, the degree of multilayer exfoliation of the inner forearm was significantly lower at week 8 compared with that at week 0. In the Moisturizing cream group, the degree of multilayer exfoliation of the inner forearm was significantly lower compared with that of the upper back at week 8. In the Moisturizing cream group, the degree of multilayer exfoliation of the inner forearm was significantly lower than that in the Medical moisturizing group at week 8.



Skin finding scores: Total score for dryness, itching, and erythema

Mean ± S.E., medical moisturizer group (inner forearm, n = 6; upper back, n = 5), NOV® skin cream D group (n = 6), Week 0 vs. Week 8: Wilcoxon signed-rank test inner forearm (applied) vs. upper back (not applied) and medical moisturizer group vs. NOV® skin cream D group: Mann-Whitney U test, # : $p < 0.1$

Figure 1. Skin finding scores.

	Medical moisturizer group				NOV® skin cream D group				*p*-value
			p-value				*p*-value		Medical moisturizer group vs NOV® skin cream D group (Week 8)
	n	Ratio to Week 0	Week 0 vs. Week 8	Inner forearm vs. upper back (Week 8)	n	Ratio to Week 0	Week 0 vs. Week 8	Inner forearm vs. upper back (Week 8)	
Stratum corneum water content									
Inner forearm (applied)	6	1.51±0.40	0.173	0.093	6	1.84±0.49	0.173	0.180	0.699
Upper back (not applied)	6	0.74±0.11	0.028 *		6	1.07±0.17	0.753		0.132
IL-1ra / IL-1α									
Inner forearm (applied)	6	1.64 ± 1.24	0.345	0.699	6	1.03 ± 0.54	1.000	0.589	0.394
Upper back (not applied)	5	3.06 ± 3.05	0.249		6	0.85 ± 0.64	0.463		0.240
TSLP									
Inner forearm (applied)	6	1.90 ± 1.25	0.028 *	0.589	6	1.82 ± 0.92	0.028 *	0.699	1.000
Upper back (not applied)	6	2.62 ± 1.54	0.046 *		6	1.51 ± 0.42	0.028 *		0.240
Degree of multilayer exfoliation									
Inner forearm (applied)	6	1.40 ± 0.93	0.345	0.699	6	0.30 ± 0.26	0.028 *	0.004**	0.009 **
Upper back (not applied)	6	0.93 ± 0.86	0.600		6	1.36 ± 1.20	0.753		0.699
Trypsin activity									
Inner forearm (applied)	6	1.06 ± 0.60	0.600	0.818	6	1.36 ± 0.83	0.345	0.699	0.818
Upper back (not applied)	6	1.84 ± 2.60	0.917		6	1.20 ± 1.13	0.753		1.000

Table 1. Stratum corneum analysis

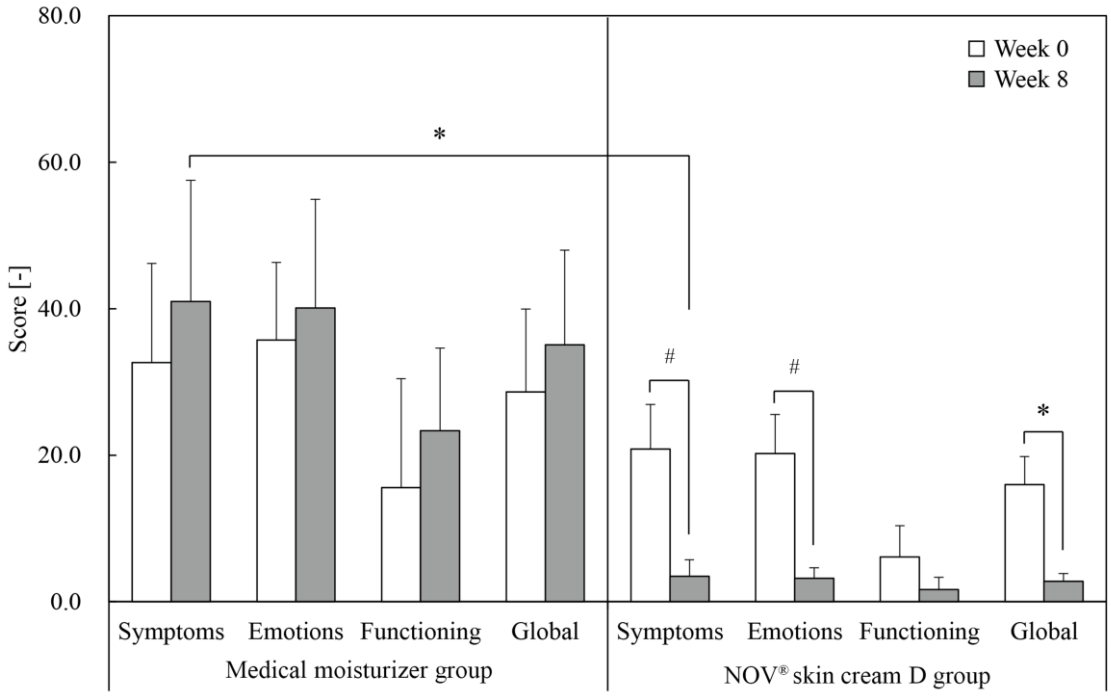
Ratio to Week 0: Ratio in Week 8 compared with Week 0 as 1, values represented as mean ± S.D., Week 0 vs. Week 8: Wilcoxon signed-rank test inner forearm (applied) vs. upper back (not applied) and medical moisturizer group vs. NOV® skin cream D group: Mann-Whitney U test, * : $p < 0.05$, ** : $p < 0.01$

TSLP: thymic stromal lymphopoietin

3.4 Patient survey findings

Figure 2 shows the results from the QOL evaluations using the Skindex-16 questionnaire. In the Moisturizing cream group, the patients' symptoms, emotions, and global scores were significantly lower at week 8 than those at week 0. The symptom score was significantly lower in the Moisturizing cream group than that in the Medical moisturizing group at week 8.

Regarding "ease of application", 50% (3/6) of the patients in the Medical moisturizer group and 67% (4/6) of the patients in the Moisturizing cream group indicated that the moisturizers were easy to apply. Regarding "moist feeling", 33% (2/6) of the patients in the Medical moisturizer group and 67% (4/6) of the patients in the Moisturizing cream group indicated that the moisturizers were moisturizing.



Mean ± S.E., medical moisturizer group (n = 6), NOV® skin cream D group (n = 6), Week 0 vs. Week 8: Wilcoxon signed-rank test, medical moisturizer group vs. NOV® skin cream D group: Mann-Whitney U test, # : $p < 0.1$, * : $p < 0.05$

Figure 2. Quality of life assessment based on Skindex-16.

4. Discussion

In this study, patients with lung cancer who were administered EGFR inhibitors were assigned to the Medical moisturizer group or the Moisturizing cream group, and the effectiveness of medical moisturizers and a moisturizing cream was compared for 8 weeks. None of the moisturizers were associated with any side-effects. After 8 weeks, the Moisturizing cream group had significantly lower degrees of multilayer exfoliation and lower symptom scores than the Medical moisturizing group. There was almost no difference in the other parameters investigated.

The results from the evaluation of the multilayer exfoliation suggested that the moisturizing creams normalized keratinization, which is a component of normal skin cell differentiation that is impeded by EGFR inhibitors[11]. Patients with atopic dermatitis have high TSLP levels in the stratum corneum[12]. The current study's results showed that TSLP production was similarly enhanced during the administration of EGFR inhibitors. EGFR inhibitors increase the levels of different chemokines[13], but this is the first study of TSLP expression in the stratum corneum. The IL-1ra/IL-1α ratio is an index of inflammation[8], and trypsin is involved in stratum corneum exfoliation[10]. This is the first analysis of physiologically active substances in the stratum corneum following the administration of EGFR inhibitors. However, because of the small number of cases in this study, detailed analysis is limited. To elucidate the relationships among the physiologically active substances in the stratum corneum, the administration period, and skin symptoms, we intend to accrue a large number of patients. Compared with week 0, the symptom, emotion, and global scores were significantly lower in the Moisturizing cream group only at week 8. Reducing a patient's QOL may hinder their on-going treatment with EGFR inhibitors[3], and moisturizing creams may alleviate patients' psychological burdens.

5. Conclusions

The results from this study suggest that compared with conventional medical moisturizers, the moisturizing cream was superior at improving skin dryness associated with EGFR inhibitor administration. Furthermore, it may improve the QOL of patients. Regarding lung cancer treatment, applying the moisturizing cream should facilitate the continued administration of EGFR inhibitors and, thus, their therapeutic effects.

Conflicts of interest

Tokiwa Pharmaceutical Co., Ltd. funded this clinical trial and the test products.

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CAR-T Therapy for Solid Tumors: Development of New Strategies

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ABSTRACT

The recent approval of two CAR-T therapies by US Food and Drug Administration (FDA) marks a very significant development in cell-based cancer immunotherapy. This milestone was demonstrated by the effectiveness of eradicating hematologic cancers using CD19-specific CARs. The success spurred development of immune cell therapies for other cancers, especially solid tumors. The generation of novel CAR constructs for these cancer types represents a major challenge in bringing the technology 'from-bench-to-bedside'.In this review, we outline some new technologies we have developed to equip CAR-T cells to enhance efficiency while decreasing toxicity of CAR-T therapies in solid tumors.

***Keywords*:** Cancer Immunotherapy; Chimeric Antigen Receptor T cell Therapy; Lymphoma; Solid Tumors; Cancer Molecular Profiling

1. Introduction

In the past, surgery, radiation and chemotherapy were at the forefront of recommended and accepted treatments for different cancer types. However, the efficacy of these therapies were limited due to (*a*) high recurrence rate[1,2,3], (*b*) hard-to-detect residual metastasis[4,5], (*c*) frequent late stage diagnosis[6,7], (*d*) elevated refractory cases from resistant cancers[8,9] and (*e*) invasiveness and toxicity to patients. In response to addressing these drawbacks, a new method entered the treatment group – *immunotherapy*. This kind of therapy utilizes the body's immune function to detect cancer antigens and to mount an attack against cancer cells. Due to the remarkable positive clinical outcome brought by immunotherapy, it is now becoming as the first line of treatment in some cancer types[10]. The technology involves transfusions with (autologous or allogeneic) T cells that are engineered to recognize cancer cells (**Figure 1a**), known as chimeric antigen receptor T (CAR-T) cell therapy[11,12].

CAR-Ts are engineered T cells expressing scFv (single chain variable fragment) domain of antigen-specific antibody linked to a TCR (T cell receptor)-associated intracellular signaling domain such as CD3 zeta[13] (**Figure 1b**). The scFv redirects CAR-T cells to recognize cancer cells in an HLA (human leukocyte antigen)-independent manner and the TCR intracellular domain induces T-cell dependent cancer killing[14,15]. After the remarkable demonstration of efficiency by the first engineered T cells pioneered by Eshhar and coworkers[16], variability in T cell functionalities have emerged. The 1st generation CAR expresses the CD3 zeta domain alone[17] while the 2nd generation is made by tandem with CD28[18] and the 3rd generation has an added domain from either CD137, CD134, ICOS or CD27[19-22]. The emerging 4th generation has an added inducible IL2 or IL12 cytokine secretion[23] for more potent immune activity (**Figure 1c**).

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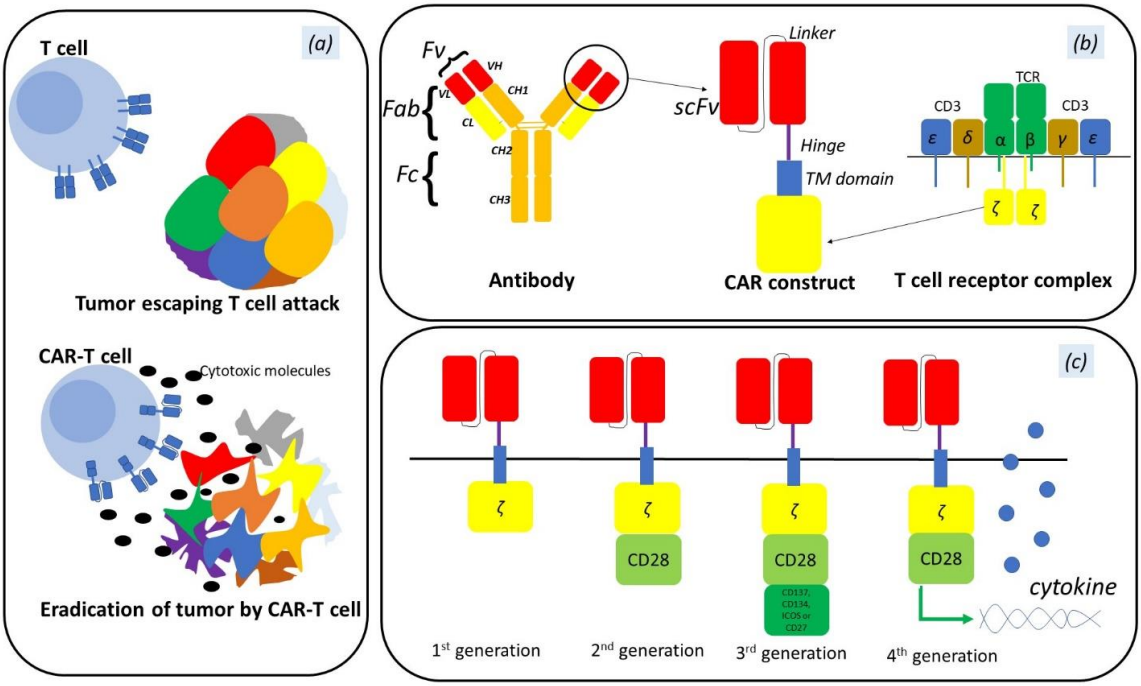

Figure 1; Schematic representation of chimeric antigen receptor (CAR) T cell therapy against cancer. Engineered T cells harboring CARs are more efficient in eradicating cancer cells compared to their un-engineered counterparts (a). This enhance anti-cancer activity is made possible by expression of an extracellular CAR domain with tumor associated antigen (TAA)-binding moiety, usually a single chain variable fragment (scFv) which was cloned from antibody gene with specificity to the desired TAA. A hinge (or spacer) region is placed after the scFv for flexibility followed by a transmembrane (TM) domain and one or more signaling domains involved in T cell activation (b). Functionality of CARs were enhanced by modifying the number and type of intracellular signaling domains of CAR. The first-generation CAR is equipped with the stimulatory domain of the T cell receptor complex zeta (ζ) chain. The second-generation CAR has the addition of CD28 co-stimulatory domain to ensure full activation of T cell response. The third-generation CAR is generated by adding a third co-stimulatory domain (CD137, CD134, ICOS or CD27) in tandem with CD28/zeta chain to potentiate maximally the immune response against cancer. The lastly, the fourth-generation CAR includes an inducible cytokine such as IL2 or IL12 to deliver enhanced anti-tumor effect and prevent down-modulation of CAR-T cytotoxic activity (c).

Over the past two decades since the description of first CAR-T trials, there have been more than 200 CAR-T cell therapies being evaluated in clinical trials globally (based on database search in Clinicaltrials.gov); and yet, there were only two CAR-T cell therapies approved by US FDA (Food and Drug Administration) for treatment of hematologic cancers[24]. With these approvals, more CAR-Ts are projected to emerge for evaluation in clinical studies. Several of these CAR-T constructs are directed to solid tumors (**Table 1**). In recent years, the developments in molecular genetics, molecular immunology and precision medicine directed to solid tumors have opened exciting opportunities for engineering immune cells directed to the many different human solid tumors and for customizing treatments based on the molecular characteristics of each patient's tumor.

Target Antigen	**Cancer Type**	**Initial Posting**	**Strategy**	**References**
EGFR (Epidermal Growth Factor Receptor)	Lung cancer and other EGFR+ solid tumors	Jun-13	EGFR-specific	NCT01869166
	Advanced solid tumor	Jun-17	CTLA-4 and PD1 antibodies expressing	NCT03182816

			CAR-T cells	
	Colorectal cancer	May-18	IL-12 inducible	NCT03542799
EGFRvIII	Malignant glioblastoma	Oct-11	EGFRvIII-specific	NCT01454596
	Residual glioblastoma	Aug-14	EGFRvIII-specific	NCT02209376
MUC1	Malignant glioblastoma, colorectal and gastric cancers	Nov-15	MUC1-specific	NCT02617134
	Advanced solid tumor	Jun-17	CTLA-4 and PD1 antibodies expressing CAR-T cells	NCT03179007
IL13Rα2	Malignant glioblastoma	Aug-14	IL13Rα2-specific	NCT02208362
	Brain tumors	Aug-08	Containing Hy/TK suicide gene	NCT00730613
Mesothelin	Cervical cancer and other mesothelin-positive solid cancers	Apr-12	Mesothelin-specific	NCT01583686
	Solid tumors	Jan-17	PD-1 antibody expressing	NCT03030001
CD70	Pancreatic and other CD70-expressing tumors	Jul-17	CD70-specific	NCT02830724
CD171	Neuroblastoma and ganglioneuroblastoma	Dec-14	CD171-specific	NCT02311621
CEA (carcinembryonic antigen)	Lung, colorectal, gastric, breast and pancreatic	Jan-15	CEA-specific	NCT02349724
	Liver metastasis	Aug-16	Regional delivery of CAR-T cells	NCT02850536
EpCAM	Nasopharyngeal carcinoma and breast cancer	Sep-16	EpCAM-specific	NCT02915445
	Colon, esophageal, pancreatic, prostate, gastric and hepatic cancer	Jan-17	EpCAM-specific	NCT03013712
Her2	Her-2 positive solid tumors	Sep-13	Her2-specific	NCT01935843
	Central nervous system tumor	Apr-18	Tumoral delivery	NCT03500991
FAP (fibroblast activation protein)	Malignant Pleural Mesothelioma	Nov-12	FAP-specific	NCT01722149
EphA2	Malignant glioma	Oct-15	EphA2-specific	NCT02575261
GD2	Sarcoma, osteosarcoma, neuroblastoma and melanoma	Apr-14	caspase-9 inducible	NCT02107963
	Sarcomas	Oct-13	caspase-9 inducible and VZV vaccine activation	NCT01953900
	Solid tumors	Dec-16	caspase-9 and cytokine inducible	NCT02992210
	Neuroblastoma	Apr-13	caspase-9 inducible	NCT01822652
	Cervical cancer	Nov-17	Multi-antigen target-	NCT03356795

			ing	
	Glioma	Aug-17	GD2-specific	NCT03252171
CD133	Liver cancer and other CD131 positive tumors	Sep-15	CD133-specific	NCT02541370
GPC3	Hepatocellular carcinoma	Mar-16	GPC3-specific	NCT02723942
	Hepatocellular carcinoma and squamous lung carcinoma	Jun-17	GPC3-specific	NCT03198546
	Hepatocellular carcinoma	May-17	GPC3-specific	NCT03146234
MG7	Liver metastases	Aug-16	MG7-specific	NCT02862704
PSCA	Pancreatic cancer	Apr-16	PSCA-specific	NCT02744287
ErbB	Head and neck cancer	Mar-13	Intratumoral delivery	NCT01818323

Table 1. Target tumor-associated antigens in solid cancers, clinical trial duration and type of CAR-T strategy (Data from Clinicaltrials.gov).

2. Discussion

2.1 Challenges in CAR-T Therapy for solid tumors and how to overcome them:

The success of CD19-targetted CAR-T cells against hematological cancers is aided by the ability to recognize and bind to cancer cells readily upon CAR-T infusion[18,12]. In solid cancers, particularly in bulky tumors, there are multiple factors that complicate efficient targeting of cancer cells, including penetrability of the tumor and specificity of scFv to antigens present in the cancer but not, or much less, in normal cells[25]. Several potential targets in solid tumors have been identified and some of them are being evaluated for clinical efficiency (Table 1)[26,27]. Numerous factors contribute to the complexity in targeting solid tumors, including mechanisms that hinder access of these CAR-T cells to the site of tumor, cell trafficking, homing and extravasation, tumor infiltration, circumventing the tumor microenvironment, CAR affinity, CAR-T toxicity and other characteristics of cancer cells such as tumor heterogeneity, genomic instability, immune-checkpoint regulation and target down regulation (**Figure 2**)

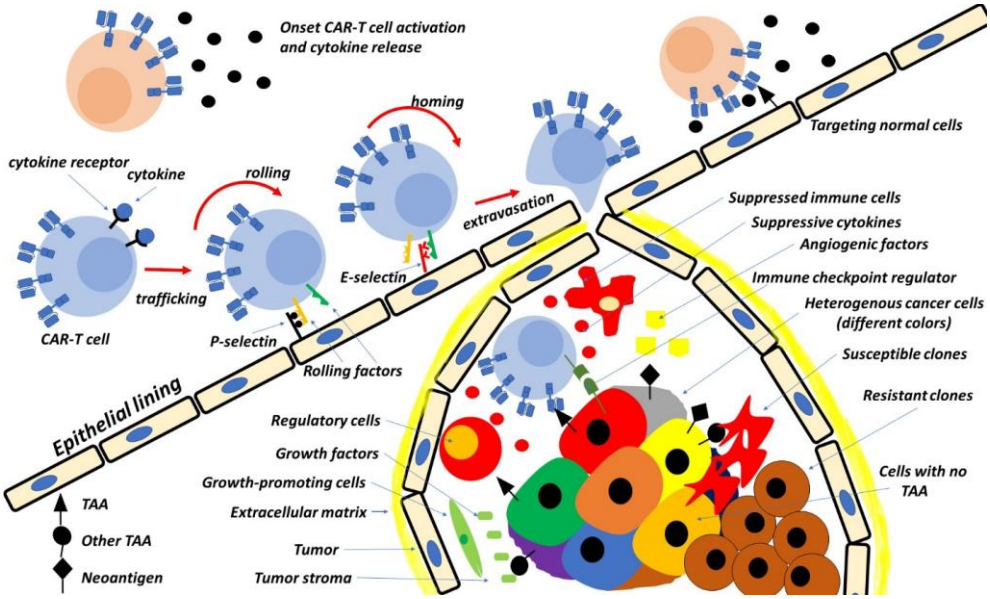

Figure 2; Challenges and point-of-improvements in CAR-T for solid tumors. This schematic representation shows the major hindrances encountered by CAR-Ts in delivering cytotoxic effect against solid cancers. These hindrances limit the efficiency of CAR-T therapy which could be focus for potential improvement**.**

2.2 Enhancing CAR-T cell efficiency:

A. CAR-T cell trafficking. Inefficient migration at the tumor site essentially limits the efficiency of CAR-T therapy against tumor cells[26]. This restrictive impact might be due to chemokine mismatch released by cancer cells with the chemokine receptors expressed by CAR-T cells[28,29]. Previous study demonstrated that activated $CD8^{+}$ $CXCR3^{high}$ tumor-infiltrating lymphocytes render inefficient for recruitment due to lack of receptor expression for related chemokine ligands such as CXCL9 and CXCL10 produced by cancer cells[30]. In other reports, arming CAR-T cells with receptors for cancer-specific chemokines such as CCR2B (CCL2 receptor)[31], CXCR2 (CXCL1 receptor) [32], CCR4 (CCL17 receptor)[33] and modifying the expression of immune activation pathway molecules such as protein kinase A [34] to increase baseline expression of chemokine receptor improved trafficking and cancer eradication.

The workflow of re-engineering T cells for added receptor is customarily difficult as cancers from different patients produce different chemokine profiles. In an attempt to circumvent this, local instillation approach has emerged in clinical trials. This site-specific CAR-T administration bypasses the drawbacks of inefficient trafficking; however, this may not prove to be beneficial for those with multiple and residual metastasis or tumors that are concealed within multiple organs of the body. Hence, technical administration is somehow challenging. Nonetheless, preclinical results of regional and intratumoral delivery of CAR-T cells provide promising results against glioblastoma [35], liver cancer [36], and in some types of head and neck cancers[37].

Another effort to increase CAR-T trafficking emerged from the use of oncolytic vaccinia virus strain[38]. While some CAR-Ts are engineered to harbor receptors for chemokines, these oncolytic viruses can serve as tumor-specific delivery of chemokine genes so cancer cells release matching chemokines that are efficiently recognized by CAR-T cells; thus, enhancing recruitment of tumor infiltrating effector T cells. Preclinical reports on oncolytic virus-mediated transgenic delivery of CXCL-11[39], CCL-5[40], CCL-19[41] resulted in elevated expression of granzyme B and INF-y in tumor site with enhanced tumor mass reduction.

B. Homing and extravasation of CAR-T cells to tumor site. The interaction of chemokine receptors with their ligands induces expression of T cell rolling-associated proteins [E- and P- selectin ligands[42,43] including related homing and adhesion molecules in T cells such as LFA-1 and VLA-4 integrins to track the gradient of chemokines released through the blood stream[44]. However, the efficiency of extravasation and homing into the tumor site remained challenging. Escape from neo-vessel epithelium by extracellular-matrix (ECM) degradation hinders CAR-T cells from reaching the target site[45]. Histopathological features of solid tumors display high concentration of blood vessels and the extracellular lining of epithelium is composed of protective barriers that need to break down. In vitro and mouse model studies on transduction of heparanase (HPSE) gene in CAR-T cells enhanced heparan sulfate proteoglycan degradation in the ECM resulting in more efficient targeting of neuroblastoma cancer cells[46]. Other ECM-targeted approach such as anti-fibulin 3 CAR-T cells was found to efficiently eradicate glioblastoma cells in mouse models evidenced by increased expression of IFN-gamma, IL-2, perforin and granzymes in the site of tumor[47]. While some are targeting ECM-components, some CAR-Ts are modified to target VEGFR (vascular endothelial growth factor receptor) [48] to specifically direct T-cell effect as guide from tumoral activities that hijacks vasculature formation.

Advances in oncolytic virus strategy have pronounced other modes of assisting CAR-T cell extravasation. Some studies demonstrated the enhancement of CAR-T cell infiltration following administration of oncolytic viruses that express different ECM-degrading enzymes such as collagenase[49], hyaluronidase[50] and matrix metalloproteinase 9 (MMP-9)[51]. Other modes of oncolytic virus-assisted CAR-T extravasation includes the arming of these viruses with anti-VEGF[52,53] or anti-VEGFR[54] or other inhibitors of these molecules to inhibit vasculature growth.

Cancer cells also secrete angiogenic factors such as Ang-1 or angiopoietin[55,56] that downregulates expression of T-cell adhesion factors ligands such as ICAM-1, VCAM-1 and other T-cell rolling molecules such as E-selectins. Theoretically, blocking or targeting these angiogenic factors and upregulation of these adhesion

molecules and T cell rolling factors may result in enhanced trafficking of CAR-T cells into tumor site.

C. CAR-T cells infiltrating the tumor microenvironment (TME). Physically, the extracellular-matrix (ECM) serves as scaffold for all cells in the tumor microenvironment (TME). However, attacking the ECM itself is not a guarantee to successfully kill cancer cells. While ECM serves as barrier, the TME provides another level of comfort for cancer cells to evade immune destruction by CAR-T cells[57]. As a cancer-made habitat, TME is dominated by tumor-induced interactions favoring cancer growth and suppressing immune functions including promoting metastasis, nurturing mutational accumulation, resisting apoptotic signals and concentrating proliferative factors. All these events are orchestrated by the ability of cancer cells to turn all other related cells as traitors to the body's natural processes. Key players in building TME involves an interplay of different cells and complex factors.

i. Tumor growth-inducing cells. Myofibroblasts or fibroblastic cells are specialized cells that develop in response to injury[58]. In TME, myofibroblasts are called CAFs (cancer associated fibroblasts). These cells play a very significant role in promoting tumor growth as they secrete growth factors such as insulin-like growth factor 1 (IGF-1) and fibroblast growth factor (FGF)[59]. CAFs also secrete anti-inflammatory cytokines such as TGF-B (transforming growth factor beta)[60] which suppresses immune attack while contributing to metastatic potentials of cancer cells. Chemokine CXCL12 derived from fibroblast of TME can serve as chemoattractant for recruitment of other TME-associated cells [61] which will be discussed below. Other key factors in tumorigenesis is the added support provided to tumor vasculature and blood vessel formation by other cellular components such as stromal endothelial cells[62], vascular endothelial cells [63] , and pericytes [64] which are all known to create dynamic interplay in providing the overall TME region a suitable habitat for cancer growth.

Recent advancement in CAR-T therapy found that CARs directed against fibroblast activation protein (FAP) have better anti-tumor effects[65]. Other CARs as previously described above targeting receptors for growth factors such as VEGFR can circumvent this active involvement of CAFs and other tumor growth-inducing cells in tumorigenesis. It is projected that the combinatorial use of ECM degrading and TME-associated stromal cells such as HSPE and FAP-targeted CARs may efficiently increase the chance of CAR-T cells to reach the site of tumor.

ii. Immune cells and associated immune-suppressing cells. Surprisingly, the body's immune cells reside within TME, but their functions have been deactivated and altered. A subclass of T cell population, $CD4^{+}Foxp3^{+}CD25^{+}$ regulatory T cells (Tregs), and B cell subclass, $CD5^{+}CD1d^{high}$ regulatory B cells (Breg or B10) are concentrated within the tumor site[66,67] which secrete IL-10, TGF-B (transforming growth factor beta) and activates immune-checkpoint receptors in most pro-inflammatory T cell population leading to their deactivation. Other groups of immune cells found in TME are natural killer (NK) and natural killer T (NKT) cells while their functions in tumor stroma are unknown, a number of studies demonstrated their anergic phenotype and might have the potential to secrete anti-inflammatory cytokines[68]. Phagocytes such as dendritic cells (DCs) and macrophages are also associated with TME. With their ability to engulf and present antigens via HLA pathway, these two types of immune cells are supposed to cascade immunologic events leading to cancer eradication. However, DCs are found to have defective antigen processing and presentation of tumor-associated antigens (TAAs) due to strong immunosuppressive effects of anti-inflammatory cytokines secreted by other cells of TME[69]. Macrophages on the other hand are discovered to have converted from cancer-killing (M1) to cancer-promoting phenotype (M2) or also called tumor-associated macrophages (TAM)[70]. M2 cells residing in TME converts from producing IL-12 cytokines, which is essential for activating immune function, in to IL-10 which favors immune suppression. In fact, clinical data suggest that high TAM correlates to poor cancer prognosis[71].

Several other key players of TEM such as myeloid-derived suppressor cells deactivate CD8+ T cells and cooperatively converts M1 to TAM phenotype[72]. Tumor-associated neutrophils (TAN) are found to enhance angiogenesis and cancer metastasis[73]. Adipocytes[74] and neuroendocrine cells[75] produce and stimulate hormone-like factors that induce hormone-depended

cell growth and downregulate immune response along with anti-inflammatory cytokines released by themselves and all other cells.

With the continuous development of CARs, the 4th generation CAR-T cells (also called TRUCK) can be equipped with inducible pro-inflammatory cytokine such as IL-2, IL-18 and IL-12 to circumvent the saturation of anti-inflammatory cytokines present in tumor stroma. Recent report showed superior antitumor activity of 4th generation CAR-T cells where T-bet expression have increased in T cell population accompanied with reduction of TAMs and Tregs in tumor site with promising clinical correlation of increased conversion to CD8+ and CD4+ subtypes[23]. The induction of immune-activating cytokines can also elicit NK and NKT cells activation in solid tumors leading to more efficient cancer eradication. Consecutively, administration of oncolytic virus which is armed to express inflammatory cytokines following CAR-T administration can theoretically circumvent the immunosuppressive nature of TME.

iii. Non-cellular components of TME. The creation of sub-habitual location of tumor inside the body creates a condition where some of the normal cellular functions are impossible to carry out. Generally, TME and the tumor itself is packed with rapidly dividing cells where oxygen (hypoxia) and nutrients are usually limiting, leading to an environment that is more acidic and lower in glucose concentration(76]. This acidic environment and lack of nutrients in tumor stroma generate a stress response leading to T cell anergy or apoptosis or conversion into Treg alongside with immune suppressing activities of anti-inflammatory cytokines[77]. Hypoxia was found to have big impact in tumor initiation and progression by activating hypoxia inducible factor (HIF)[78] which target and transcribe multiple genes associated with survival of cells and also cooperatively induces pro-inflammatory environment that initially recruits immune cells in TME and are coaxed to become traitors of immune activity by releasing cytokines to antagonize inflammatory reactions and later on impart in conversion of T- and B- cell population into regulatory subclasses.

Hostile environment resulting to hypoxia was found to decrease cytotoxic tumor infiltrating T cells and even when reactivated with IL-2, their viability has decreased[79]. These unfavorable condition for T cells has proposed requirement as limiting factor for CAR-T therapy in solid tumors where tumor size should be minimal, otherwise, any other therapies may fail. Despite of challenges in delivering and striving CAR-T cells in hypoxic environment, there has been pioneering study aimed to armor CAR-T cells with sensor to sense hypoxic environment[80]. The team added the oxygen-sensing domain of HIF gene to the intracellular domain of CAR construct. They found that CAR-T functionalities in killing cancer is not hindered in a low oxygen environment. Therefore, retaining immunologic function despite hostile environment.

D. Potentiating tumor targeting by fine-tuning scFv affinity of CAR. We discussed previously the role of scFv (single chain variable fragment) domain of CAR in locating target cells. Antigen-recognition is vital in directing CAR-T cell effect against cancer. Other key factors that relates to the quality of scFv includes binding affinity which determines the efficiency of antigen recognition. In immunotherapy, scFv affinity is a dual edged functionality where too high interaction results in poor tissue penetration and distribution which may pose risk of side-effects due to potential concentration of immune effect in normal tissues. On the other hand, too low interaction may result in poor targeting of the desired antigen, hence causing low efficiency[81].

CARs are derived from antibody scFv with unknown binding affinity or may have affinity that is altered after recombinant fusion with the intracellular domain. Study presented by Park *et al*[82] demonstrated the use of enhanced anti-ICAM1 CAR-T cells targeting solid tumors in mouse. Increased molar affinity of ICAM-1 resulted in better distribution and eradicated preferentially tumor cells while keeping normal cells unharmed. In another study, anti-ErbB2 CAR harboring scFv with lower affinity has comparative anti-tumor activity against solid tumors with high-affinity CARs[83]. These two contradicting affinity features of CAR indicate the necessity of fine-tuning binding affinities based on complex factors such as cancer type, antigen density or may either be traced back to identifying suitable hybridoma or phage clones during antibody scFv development.

As discussed above, most of target antigens in solid cancer are also expressed in normal tissues. These studies on scFv fine-tuning are promising platform to teach

CAR-T cells in discriminating normal from the cancer cells. It is important to note that fine-tuning scFV to harbor high or low binding affinity is an important avenue for improving CAR-T cells in providing safer and better anti-tumor effect. Various protein engineering approaches such as directed evolution[84], domain exchange[85], coupled with high-throughput analysis using phage display[88] can assist in scFv fine tuning.

E. Neutralizing CAR-T toxicity. Currently, most of the targets in solid tumors are molecules found relatively in normal cells. CAR construct is designed to recognize specific antigen but CAR-T cells cannot distinguish between normal and cancer cells. Severity of this "on-target/off-tumor" toxicity range from cell linage depletion or aplasia with some reports describing severe toxicity leading to death. This problem is demonstrated by CD19 and carcinoembryonic antigen-directed CAR-T cells where normal cells are also recognized[87,88]. In some reports this type of toxicity have been addressed by fine-tuning scFv[89] or identifying other targets that are more specific to cancer cells such as neoantigens[90].

The most prevalent side effect of CAR-T therapy is the early or onset immune activation known as cytokine release syndrome (CRS)[91,27]. Even prior to encountering cancer antigen, CAR-T cells may start releasing cytotoxic molecules which orchestrate severe noncancer-specific inflammatory processes inside the body, targeting different organs and tissues. There are plenty of reasons why CRS occurs which include complexity of generating chimeric T cell functionalities and the generation of fine-tuned scFv as described above. Enhancing the anti-tumor activities of CAR-T cells is a "double-edged sword" that may either enhance tumor eradication or may escalate patients to life threatening situation[34]. Current CAR-T platforms developed to address cytotoxicity include developing switchable CAR (sCAR) equipped with switch-on mechanism to prevent early activation of CAR-T cells[92,93]. These sCARs utilize an anti-PNE (peptide neo-epitope) CAR with scFv domain that is specific to PNE epitope which is not found in human proteome. The sCAR is activated once it encounters a PNE-coupled antigen-specific scFv. Report on anti-CD19 sCAR-T cells showed a dose-dependent response without unwanted immune activation[92].

Other modes of CAR-T variants being developed to bypass onset activation includes platforms that contain 'safety switch' such as iCAR and caspase-9-inducible CAR. The antigen-specific inhibitory chimeric antigen receptors or simply iCAR is another advancement in CAR-T therapy dampening T cell activation when scFv of iCAR recognizes normal cell antigen. This platform contains tumor-specific CAR that allows cancer-specific killing and an inhibitory iCAR that suppresses immune attack on normal tissue. Sadelain and coworkers[94] demonstrated the use of PD-1 and CTLA-4 inhibitory domains to offset immune activation of cytotoxic CAR when CAR-T cells are trafficked in non-tumoral region. They showed that CD19-CAR/PSMA-iCAR T cells killed CD19+/PMSA- cells but not CD19+/PMSA+ cells. This iCAR is a promising approach to preventing CAR-T cells from eliciting immune attack when not needed. Same cytotoxicity management is employed in caspase-9-inducible CAR proposed by the team of Diaconu *et al*[95]. However, the CAR construct does not employ the use of immune checkpoint proteins but has an added caspase-9 intracellular domain activated by induced-dimerization of FK506-binding protein in the presence of pharmacological drug known as AP1903. This drug-induced dimerization to activate caspase-9 affords to manage toxicity by terminating the effects of CAR-T cells by apoptosis.

2.3 Identifying weak spots in cancer cells:

A. Targeting heterogeneous population of cancer cells in the tumor site. Cancer develops from accumulated mutations that initiates a malignant phenotype. As cancer cells continue to replicate, other clones harbor different genetic and epigenetic anomalies as they are exposed to different microenvironmental pressures[96] such as deprivation of nutrients and oxygen as described above. Cancer cells in tumors are highly heterogeneous[97,98] and CAR-T cells targeting only one specific antigen might not be sufficient enough to eradicate all targets, especially in metastatic cases where associated antigens are different from succeeding tumors. The big challenge in addressing heterogeneous cancer antigens in solid tumors is that most of the targets currently being employed for therapeutic evaluation are also expressed by normal cells[87]. However, some CAR-T cells would preferentially target tumor sites as most of these

antigens are upregulated in tumor population however the greatest challenge is to execute T cell killing immediately on cancer cells before any harmful side effects have been made to normal cells and considering all other factors in directing CAR-T cells to the site of tumor (as described above).

In lieu of constructing CARs that express scFv against single targets, there have been reports that utilized dual or multiple targeting of tumor-associated antigens to bypass drawbacks in tumor heterogeneity[99,100]. Some innovative CAR designs employed the added inhibitory signals where one scFv recognize a tumor antigen while the other recognizes a protein expressed by normal tissue to make CAR-T therapy safer for use in cancer immunotherapy. Dual TAA-targeting has been also shown to enhance tumor eradication.

B. Evading complexity of cancer genomic instability. From the view point of mutations and epigenetic changes described recently, accumulated genomic changes may result in various genetic anomalies such as indels (insertion/deletion) or rearrangements leading to fused, altered or truncated proteins[101]. As clinical consequence, cancer cells might become less aggressive and patient respond easily to available treatment or they might become refractory or more aggressive leading to more serious conditions. Depending on kind of aberration, increased mutational load and high neoantigen frequency might be beneficial to some tumors such as high microsatellite instability (MSI^{high}) in colorectal cancer[102,103]. However, some genomic aberrations result in poor prognosis such as high mutational burden in TP53 and RET in pancreatic cancer and APOBEC family of genes in multiple myeloma[104].

In a bright note, these mutations and aberrant changes in chromosomal arrangements creates an altered protein product known as neoantigen. Cancer neoantigens are gene products with altered sequence or structure that may present immunogenic epitope for immunologic response [90]. Current approach in neoantigen treatment includes vaccine design which requires cloning and expression of the neoantigens which are induced to be immunogenic and later on triggers cancer killing[105].

Neoantigens expressed on the surface of cancer cells could be potential targets and because they are only present in cancer cells, they offer a safer and more specific tumor-associated targeting for CAR-T therapy. Roughly around 28 neoantigen clinical trials are being evaluated (based on Clinicaltrials.gov database search query) and one of them includes redirecting CAR-T cells to target neoantigens in solid tumors such as metastatic glioblastoma, lung cancer, ovarian, breast and gastrointestinal tumors[106]. However, the clinical efficacy and safety of this CAR-T platform is yet to be evaluated.

C. Bypassing immune checkpoint inhibition. As quality check process, T cells are regulated in two stages, central and peripheral tolerance. These T cell regulations are important to prevent auto-reactive T cells from attacking the normal tissues. Unlike developing T cells, CAR-T cells and their unmodified counterparts, are controlled at the periphery (peripheral tolerance) which is orchestrated by different immune checkpoint proteins. The PD1/PDL-1 axis is one of the very well-known immune checkpoint proteins associated with T cell suppression[107]. The interaction of PD-1 (programmed cell death 1-receptor) on T cells to the ligand (PDL-1 or PDL-2) on normal tissues prevents autoimmunity[108]. However, this T cell suppression mechanism is also used by cancer where expression of these ligands (PDL1 and PDL2) are very high, correlating to poor prognosis in some cancer types[109,110]. Clinical results on mune-checkpoint blockade using antibodies produced encouraging remission outcomes and increased patient survival in many types of solid cancers[111]. Some immune checkpoint blockade targets include PD-1, PDL-1, CTLA-4, TIM-3, LAG-3, and A2AR[112,108,113], all are clinically evaluated for efficiency in eradicating cancer cells by antibody-mediated cancer killing.

CAR-T cells are not exempted from this immune checkpoint suppression as they also express these inhibitory molecules owing to their innate T cell nature. So far, we discussed the importance of overcoming the presence of immune suppressing cytokines and other soluble factors that saturate the tumor stroma. In order to bypass this suppressing environment, additional inducible cytokine has to be equipped with CAR-T to potentiate immune attack. However, the presence of immune checkpoint proteins on cancer cells present another danger that might render CAR-T cells inefficient even equipped with various inflammatory cytokines.

In various CAR-T clinical trials, some patients were

found to have increased PD-1 expression after few weeks of infusion[114]. In fact, some participant showed higher PD-1 expression in CAR-T cells compared to endogenous T cells[115]. These problems have led to combinatorial treatment of anti-PD1 or anti-PDL1 antibody following CAR-T treatment. Preclinical research and experimental animal models showed better tumor killing and enhanced CAR-T cell survival in the presence of PD-1 pathway blockade. Pioneering study by group of John *et al*. [116] confirmed that anti-Her2 CAR-T cells can undergo T cell exhaustion after continuous stimulation with PDL-1+ Her-2^+ tumor cells. In an in vitro set-up, they showed that treatment of anti-PD1 antibody in combination CAR-T cells enhanced T cell activation and proliferation. In their transgenic model, their anti-Her2 CAR-T cells strikingly produced better anti-tumor effect in the presence of anti-PD1 antibody.

Despite positive results of this combinatorial dosing, the separate cost and the independent side effects of each therapy could hinder access to this treatment. New platforms of CAR-T cells are now developed and currently under clinical trials to evaluate efficiency and safety of co-engineering CAR-T cells with antibody genes targeting these inhibitory molecules such as PD-1, CTLA-4 and PDL-1. These antibody-expressing CAR-T cells provide a "built-in" therapy that will no longer require co-administration of antibodies. Targets in solid tumors include MUC1[117], EGFR families[118] and mesothelin[119]; however, their clinical efficiency are still being evaluated in clinical trials.

Other modes of engineering T cell in bypassing PD-1-mediated T cell suppression was demonstrated by chimerizing the PD-1 intracellular domain with the CD28 signaling region [120]. In this report, the scientists demonstrated increased T cell activation and proliferation accompanied with increased cytokine secretion and granzyme B release in their experimental model. This promising approach to turning inhibitory molecules to activate T cells by engineering the intracellular domain is a promising approach.

In some other reports, CAR-T cells can be co-engineered using CRISPR (clustered regularly interspaced short palindromic repeats)-Cas9[121] or RNAi (RNA interference) technology[122] to harbor dominant-negative inhibitory receptor or could be knocked out, knocked down or removed from the loci to render the PD-1/PDL-1 axis inefficient to suppress T cell activity.

D. Circumventing down-regulation of antigen targets. As a well-established fundamental physiology by which cancer escape cell death or evade immune destruction is through downregulating tumor-associated antigens (TAAs) among all other things. Factors leading to reduced or loss of expression of these TAAs may attribute to genetic malfunctions already discussed above or may have been a physiological feedback or sporadic response which reduce the efficiency of any cancer therapy[123]. As exemplified by some clinical reports on breast cancer, endocrine therapy using tamoxifen was shown to induce loss of expression of target receptors such as ER (estrogen receptor) and progesterone receptor (PR) with clinical significance of resistance reaching to almost 20%[124]. It is now being recognized that the loss of presentation of internal TAAs or even neoantigens by MHC (major histocompatibility complex) class I hide cancer cells from being detected by immune cells, be it endogenous or the engineered counterpart[125-127]. Downregulation of latent membrane protein (LMP) 2, LMP7[128,129], transporter associated with antigen processing (TAP) 1 and TAP2[130-132] are some of the genes scientists are now looking in response to finding key features of developing immunotherapy-resistant cancer cells.

Similarly, in case of anti-CD19 CAR-T therapy targeting melanoma, scientists found that an isoform of CD19 with skipped exon 2 (CD19-e2) was upregulated leading to downregulation of the full-length CD19 target[133]. This downregulation of the whole CD19 protein led to loss of cognate epitope necessary for CAR-T recognition. It is now being recognized that loss of antigen expression on tumor cells presents a very dramatic problem in CAR-T therapy and immunotherapy in general.

Current approach in CAR-T therapy using dual targeting CAR-T cells for two different TAAs might overcome this antigen downregulation by cancer cells. Preclinical and clinical studies using this approach produced a very promising result. Hedge *et al*. demonstrated that the combinatorial targeting of HER2 and IL-13Rα2 by CAR-T offset antigen escape and enhanced anti-tumor

activity in vitro and in xenogeneic mouse model[134].

3. Molecular profiling of solid tumors

Molecular changes that underlie tumorigenesis have been widely elucidated. In fact, a cancer cell from one patient or in a certain cancer group differs in molecular background[135-137]. This heterogeneity of cancer pathophysiology may provide answers why only a portion of the treated population respond to immunotherapy and why some tumors develop resistance to the treatment[138,139].

Molecular profiling has proven to be effective in providing adequate information to conclude prognosis and diagnosis of some diseases including providing clinical decisions for treatment and disease management[140,141]. Tumor profiling provides information on the molecular characteristics of cancer cells[142]. The elucidation of these 'characteristics' provides better understanding about the cancer cells and in translation may give clue to identifying appropriate therapy for patients. For example, 4th generation caspase-9-inducible anti-CD19 CAR-T was found to rescue patient with chemo-refractory acute lymphoblastic leukemia carrying Bcr-Abl cytogenetic fusion and C275Y TP53 mutation[143] while some leukemia with different profiles might be refractory to the therapy. This finding correlates the importance of identifying biomarkers that might involve in sensitizing cancer cells for CAR-T therapy. Thus, tumor profiling allows tailor-fitting the specific CAR-T platforms needed by certain stratified tumor profile.

4. Conclusion

In this review, we outlined the major challenges of CAR-T therapy in solid tumors. The success of CAR-T therapy is affected by two factors: 1) the strategic efficiency of CAR-T cells; 2) and the susceptibility of cancer cells to immunotherapy. While it is important to note factors affecting CAR-T delivery and toxicity it is also very important to identify the extent of cancer heterogeneity and treatment sensitivity.

Recently, tumor profiling test has been ed by US FDA (Food and Drug Administration) for cancer diagnosis and prognosis[144,145]. This molecular profiling of tumors allows analyzing multiple genes that are associated with tumorigenesis that might aid in screening novel biomarkers for use in CAR-T therapy. The success of CAR-T treatment will be greatly influenced by the identification of these target antigens that are unique for each patient's solid tumor. This strategy will not be amenable to a mass-produced general CAR-T construct that can be recommended, for example, for all patients with lung cancer, breast cancer or pancreatic cancer. Considering the heterogeneity and variability of antigen expression of each patient's cancer, a personalized molecular-genetic approach will be needed for effective targeting of each patient's cancer, beyond the general organ-related categories in use currently.

Conflict of Interest Statement

The authors do not identify conflicts of interest in writing this review.

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Novel Disease-Modifying Drugs against Skin Fibrosis of Systemic Sclerosis

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ABSTRACT

Systemic sclerosis (SSc) or scleroderma is an autoimmune disorder characterized by tissue fibrosis of the skin and internal organs. The etiology of the skin fibrosis is thought to be thickened dermis due to uncontrolled excessive deposition of various extracellular matrix, mainly type I collagen.Systemic treatments with anti-inflammatory and cytotoxic immunosuppressive properties, such as corticosteroids and immunosuppressants, are usually considered for skin sclerosis of patients with SSc. However, their approach must be initiated at the early stage, before the fibrosis is completed, and the effects of the corticosteroids and immunosuppressants are known to be reduced in the late stages of the sclerosis. Furthermore, various significant adverse effects of these treatments must be considered.This paper discusses the present day understanding of therapeutic options using disease-modifying drugs against skin sclerosis of SSc patients and the possible mechanisms.

***Keywords*:** Collagen; Scleroderma; Steroid

1. Introduction

Systemic sclerosis (SSc) or scleroderma is an autoimmune disorder characterized by tissue fibrosis of the skin and internal organs (e.g. lung or esophagus). The etiology of the skin fibrosis is thought to be thickened dermis due to uncontrolled excessive deposition of various extracellular matrix (ECM), mainly type I collagen. However, the mechanisms responsible for the pathologic increase of ECM in SSc skin have not been fully clarified, despite recent advances in understanding of the regulation of ECM gene expression. The current hypothesis is that inflammation, autoimmunity, and vascular abnormality may lead to activation of dermal fibroblasts, which result in the overproduction of ECM (1, 2).

SSc patients are clinically sub grouped according to the classification system proposed by LeRoy *et al.* (3) either diffuse cutaneous SSc (dcSSc) or limited cutaneous SSc (lSSc). The definition of dcSSc is progressive skin sclerosis involving proximal extremities and the trunk. Systemic treatments with anti-inflammatory and cytotoxic immunosuppressive properties, such as corticosteroids and immunosuppressants, are usually considered for skin sclerosis of dcSSc patients. However, their approach must be initiated at the early stage of dcSSc (‘early diffuse’ SSc), before the fibrosis is completed, and the effects of the corticosteroids and immunosuppressants are known to be reduced in the late stages of the sclerosis. Furthermore, various significant adverse effects of these treatments must be considered.

This paper discusses the present day understanding of therapeutic options using disease-modifying drugs against skin sclerosis of SSc patients and the possible mechanisms.

2. Review

2.1 Corticosteroid

Corticosteroid treatment for skin sclerosis of patients with SSc remains controversial because a retrospective case–control study showed that high-dose corticosteroid is a risk factor of life-threating renal crisis (4, 5). On the other hand, corticosteroid treatment is more commonly used in Japan, especially for patients in early stages of dcSSc.

Japanese SSc patients have lower susceptibility to renal crisis than other ethnic SSc patients. However, no

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controlled clinical trials have fully proven the efficacy of corticosteroids for treatment of skin thickening. Sharada *et al*. reported a randomized placebo-controlled, double-blind study on 35 patients with dcSSc in India (6). Significant improvement of total skin score (28.5 to 25.8) was seen in 17 patients received monthly intravenous dexamethasone pulse therapy (100 mg dexamethasone in 250 ml 5% dextrose) for six courses, but not in 18 control patients (30.6 to 34.7). Contrary to concerns, adverse effects of corticosteroid therapy were limited to an increase in minor infections. On the other hand, Takehara conducted an uncontrolled prospective study of 23 Japanese patients with dcSSc, and described that low-dose oral corticosteroid (initial dose of prednisolone 20 mg/day) significantly ($P<0.001$) reduced the mean skin score (20.3 to 12.8) after one year of treatment, and to 8.7 at final evaluation (7). Based on the result, the report recommended low-dose oral corticosteroid treatment for patients with two or more of following three conditions: (1) early onset; (2) edematous changes, and (3) rapid progression.

Although the mechanism of the efficacy of corticosteroid is unknown, it may involve immunosuppressive effects by inhibition of cellular activity, and by reduction of antibody production. In addition, inhibition of collagen transcription, inhibition of collagen mRNA stability, and the increase of matrix metalloproteinase (MMP) expression may also be involved in the mechanism (8, 9).

2.2 Immunosuppressants

The few controlled clinical studies of immunosuppressants to date have not always shown efficacy. Nonetheless, various immunosuppressants are widely used for the treatment for skin sclerosis of patients with dcSSc.

1) Cyclophosphamide

Cyclophosphamide is an alkylating agent that affects growth of normal and malignant cells. Cyclophosphamide is sometimes used to improve interstitial lung disease (ILD) in patients with SSc, although the mechanisms underlying the effect of cyclophosphamide on tissue fibrosis are still to be clarified. The efficacy on skin sclerosis has also been determined. In a multicenter double-blind study, Tashkin *et al*. reported that oral cyclophosphamide (initially 1 mg/kg/day) improved skin sclerosis as well as ILD after 12 months of treatment (10). The skin score significantly ($P<0.05$) improved from 15.5 to 11.9 in 54 patients treated with cyclophosphamide. To note, the significant difference in improvement of the skin score in the dcSSc group disappeared after an additional year (11).

On the other hand, cyclophosphamide may cause adverse events, such as cytopenia and immunosuppression, and the risk of malignancies will be increased by the accumulating dose. Thus, to avoid side effects and to reduce the total dose, intravenous cyclophosphamide pulse therapy (IVCY) is often considered. The combination therapy of corticosteroid and IVCY for ILD of SSc has already been evaluated. One study demonstrated that prednisone (10 mg/day) and IVCY (750 mg/m^2 month for one year) were effective for the suppression of active alveolitis in SSc patients (12). However, other randomized control trials could not show significant effects (13), and the effect of IVCY for ILD of SSc patients remains controversial.

Previous literature has also indicated the significant efficacy of IVCY on skin sclerosis, but is made up of mainly case reports or case series (14, 15). Randomized control trial studies are therefore required.

2) Cyclosporine A

In spite of small number of patients (only 20 subjects in a single facility), a double-blind study has indicated that treatment with oral cyclosporine (2.5 mg/kg/day) and monthly iloprost for one year significantly improves skin sclerosis ($P=0.008$) as well as microvascular abnormalities and esophageal involvement compared to monthly dose of iloprost alone (16). Suppression of serum IL-6 concentration was suggested to be the mechanism of the effects of cyclosporine. Further larger studies are required to confirm treatment efficacy. Furthermore, the possibility that cyclosporine induced a renal crisis in SSc patients should be considered.

3) Methotrexate

Methotrexate is a dihydrofolate reductase inhibitor, which interferes with DNA synthesis and cell production, and has been used for many human disorders. Two randomized controlled trials (29 patients with 15-25 mg/week in 1996 and 71 patients with 10-17.5 mg/week in 2001) showed that methotrexate improves the skin score in early dcSSc, albeit in a statistically insignificant rate at 24 weeks ($P=0.06$ and <0.17, respectively)(17,

18). Additional analyses were performed, showing significant effects of the drug on the skin score. Patients treated with methotrexate should be carefully observed for occurrence or worsening of ILD, especially Japanese SSc patients.

4) Mycophenolate mofetil

Mycophenolate mofetil inhibit inosine monophosphate dehydrogenase, and reduce proliferation of T and B cells, which resulted in immunosuppressive effects. The therapeutic values against lung, muscle, and joint involvements of SSc have been reported. Several reports have also evaluated the effect of mycophenolate mofetil for treating skin thickening in small open-label studies with small number of patients. For example, in 15 dcSSc patients, the skin score significantly improved (22.5 to 8.4) in patients who tolerated the treatment (starting from 1,000 mg/day, increased to 2-3,000 mg/day) for >3 months ($P < 0.0001$) (19).

However, Nihtyanova *et al*. retrospectively compared the effect of mycophenolate mofetil (~2000 mg/day) in 109 SSc patients and that of other immunosuppressants including azathioprine, anti-thymocyte globulin, D-penicillamine, intravenous cyclophosphamide, oral cyclophosphamide, α-interferon and methotrexate in 63 patients, which showed no significant difference in the change of skin score between the two groups (20): 26 to 11 in mycophenolate mofetil group and 26 to 15 in control group. Accordingly, the efficacy of the drug on skin sclerosis remains controversial.

5) Tacrolimus

Morton *et al*. reported that oral tacrolimus (mean 0.07 mg/kg/day) improved skin sclerosis in four of eight patients in a small open-label study (21). However, the risk of renal crisis is also carefully considered in SSc patients.

6) Azathioprine

A previous randomized study showed skin score was significantly improved (14.7 to 5.2, $P<0.001$) in 30 patients treated with cyclophosphamide (2 mg/kg/day for 12 months, followed by 1 mg/kg/day for 6 months), but not (14.3 to 14.5) in those with azathioprine (2.5 mg/kg/day for 12 months, followed by 2 mg/kg/day for 6 months) (22). Accordingly, this report indicated that azathioprine did not clearly affect skin sclerosis.

2.3 Anti-thymocyte globulin

Anti-thymocyte globulins have been used as polyclonal antibodies for the depletion of T lymphocytes to prevent graft-versus-host disease in organ transplantation. Several older case reports have indicated its efficacy for SSc, but in studies of 10 cases, the effect of anti-thymocyte globulins (10 mg/kg over 4 hours, on five days) was not proven (only two patients showed improvement in the skin, whereas five patients were worse and three were stable after 12 months). Moreover, various side effects, including allergic reaction appeared (23). One recent paper described the significant ($P<0.01$) efficacy of anti-thymocyte globulin plus mycophenolate mofetil on skin score (24). Furthermore, the drug is commonly used at the time of autologous stem cell plantation in SSc patients.

2.4 D-penicillamine

D-penicillamine was believed to have anti-fibrotic effect, to stabilize or even to improve ILD, and to have a beneficial effect on patient survival (25, 26). Jayson *et al*. studied 22 SSc patients and concluded that D-penicillamine (~1250 mg/day) showed ous benefit in 15 patients, but due to either side effects or relapse, overall good results occurred in five patients (27). On the other hand, other investigators question whether D-penicillamine does indeed have a positive effect in SSc patients. For example, Clements *et al*. compared the effects of high-dose D-penicillamine (750~1,000 mg/day) on improvement of skin score with that of low-dose D-penicillamine (125 mg/, every two days) in early-stage dcSSc patients, but there was no statistically significant difference (-4.9 vs -6.7, $P=0.38$) (28). After this double-blind study, the frequency of usage of this drug is decreased, in spite of a recent retrospective randomized cohort study showing efficacy of D-penicillamine (750 mg/day) on skin sclerosis ($P<0.01$) (29). Side effects include cytopenia, anorexia, dysgeusia, vomiting, and diarrhea.

2.5 Tocilizumab

Interleukin (IL)-6 is a multifunctional proinflammatory cytokine produced by various cell types, including lymphocytes and fibroblasts. In SSc, serum levels of IL-6 is significantly elevated, and serum levels of IL-6 positively correlated with the skin score (30), suggesting the involvement of IL-6 in the pathogenesis of skin fi-

brosis. IL-6 has already been shown to stimulate collagen production in cultured human dermal fibroblasts (31), and the possible effect of tocilizumab, a monoclonal antibody against the IL-6 receptor, on SSc skin fibrosis may be via the blockade of inflammatory response and the profibrotic effect of IL-6 on dermal fibroblasts.

The efficacy of tocilizumab on skin sclerosis has been demonstrated by case reports and case series. However, Phase 2 Safety and Efficacy of Subcutaneous Tocilizumab in Adults with Systemic Sclerosis (faSScinate) randomized controlled trial including 87 patients (43 treated with tocilizumab and 44 with placebo) showed no significant reduction in skin score by tocilizumab (P=0.058, subcutaneous 162 mg weekly). The difference of skin score, however, was greater in the tocilizumab group (-6.3) than in the placebo group (-2.8) at 48 weeks (32). On the other hand, Khanna *et al*. described an additional report of patients with SSc who were treated for 48 weeks in an open-label extension phase of the faSScinate study with weekly 162 mg subcutaneous tocilizumab (33). In the report, 24 out of the 44 SSc patients treated with placebo-tocilizumab, and 27 out of the 43 SSc patients treated with continuous-tocilizumab patients completed 96-week treatments. Mean change of skin score from the baseline was -3.1 for patients with placebo and -5.6 for those with tocilizumab at week 48, and -9.4 for placebo-tocilizumab and -9.1 for continuous-tocilizumab at week 96. Accordingly, improvements to skin score were observed in placebo-treated patients who crossed over to tocilizumab and were maintained in the open-label period.

A Phase II/III, multicenter, randomized, double-blind, placebo-controlled trial is currently being performed to prove the efficacy of tocilizumab on SSc cutaneous sclerosis. In the study, patients will be randomized to receive either tocilizumab 162 mg subcutaneously or a placebo each week for 48 weeks. Between weeks 48 and 96, all patients will receive open-label tocilizumab 162 mg/week.

2.6 Ritsuximab

B cells are known to play various roles in the human immune system, including antibody generation to specific antigens, which are able to not only neutralize pathogens, but are also able to enhance their elimination by activating phagocytosis or complement proteins (34). Furthermore, independent from such antibody producing function, B cells also exert important regulatory roles, including efficient antigen presentation to the T cells, cytokine secretion, and immune cell differentiation.

Rituximab is a chimeric monoclonal antibody for CD20 expressed on mature B cells. Since its first use in human lymphoma in the 1990s, this biologic drug has been available for various diseases. Among autoimmune diseases, ritsuximab was firstly tested in patients with rheumatoid arthritis. In non-responders to other therapies, ritsuximab was demonstrated to be effective in preventing progression of articular erosion. Since then, off-label use of ritsuximab has been reported for intractable cases of various autoimmune diseases, including systemic lupus erythematosus (SLE), autoimmune hemolytic anemia, Evans syndrome, granulomatosis with polyangiitis, pemphigus, pemphigoid, type 1 diabetes mellitus, Sjogren's syndrome and autoimmune pancreatitis.

In SLE patients, although phase II trials resulted in good tolerance and had preferable therapeutic effects, phase III double-blind placebo-controlled studies could not show significant effects in this disease. Nonetheless, many experts have regarded B cell depletion with rituximab as promising treatment for refractory lupus. Rare, but fatal side effects, such as progressive multifocal leukoencephalopathy (PML) infection, should be considered, however.

B cell abnormalities in the pathogenesis of SSc may include polyclonal B cell activation and autoantibody production. Various abnormal immune activations including the production of disease-specific autoantibodies are observed in SSc. Although such autoantibodies may not have functional roles, they are strongly correlated with unique clinical manifestations. B cell activation in SSc was also characterized by chronic hyper-reactivity of memory B cells. In addition, the percentage of IL-10-producing regulatory B cells, which negatively control immune response, was significantly lower in SSc patients than in healthy controls (35). In addition, levels of regulatory B cell in SSc patients negatively correlated with the titer of anti-topoisomerase I antibody and anti-centromere antibody. Based on the above notion, B cell depleting therapy with rituximab may also be utilized as a promising treatment for SSc (34). In tight skin

mice, anti-mouse CD20 monoclonal antibody could significantly suppresses the skin fibrosis as well as prevents hypergammaglobulinemia and autoantibody production. Down-regulation of profibrogenic cytokines including TGF-β or normalization of Th1/Th2 cytokine balance in the skin was also described (8, 36).

To date, many recent reports have indicated that rituximab improved skin sclerosis of dcSSc: for example, as one of the first reports, 15 patients were recruited to receive intravenous doses of rituximab (1000 mg) twice (37). The modest B cell infiltrates that were seen at baseline were completely depleted in the skin and blood of most patients after six months. On the other hand, autoantibody titers were only slightly decreased after the treatment. Furthermore, the mean change in the skin score after six months was not statistically significant (20.6 to 20.2, P=0.83). Rituximab treatment was concluded to be safe and well tolerated, and effectively depletes both circulating B cells and dermal B cells. However, the drug had little effect on the levels of SSc-associated autoantibodies or skin sclerosis. Nonetheless, a lot of reports of case series have indicated the efficacy for skin sclerosis. In addition, in a EUSTAR group observational study, improvement of skin scores were significantly larger (P=0.03) in the rituximab group (n=63, -24.0%) than matched controls (n=25; -7.7%) (38). Moreover, in patients treated with rituximab, the mean skin score was also significantly reduced compared with baseline (26.6 vs 20.3, P=0.0001).

Taken together, additional controlled trials are needed to evaluate both the effects and the side effects. Several phase III studies versus placebo are currently being performed.

2.7 Intravenous immune globulin

Intravenous immune globulin (IVIG) is firstly used as treatment of agammaglobulinemia. Subsequently, IVIG was found to be effective for idiopathic thrombocytopenic purpura in the 1980s. The drug has become available for various human autoimmune diseases. Furthermore, the efficacy of IVIG has been reported in many skin diseases including pemphigus, dermatomyositis/polymyositis, or Stevens-Johnson syndrome/epidermal necrolysis because of its more effective therapeutic values and fewer side effects in comparison with conventional treatments.

Although the detailed mechanisms by which IVIG improves autoimmune diseases remain unknown, several hypotheses have been suggested (39). For example, IVIG is thought to exert the effects via the Fc region of IgG. Fc portion competes with autoantibodies in binding to Fc receptors on the surface of B-cells and macrophages, resulting in the elimination of autoantibodies. Alternatively, the IgG polymer generated by IVIG may compete with immune complexes induced by autoantibodies. IVIG may also modulate lymphocyte functions, cytokine synthesis, or complement activation.

Moreover, IVIG treatment is thought to suppress the expression of chemokines including monocyte chemoattractant protein (MCP)-1, macrophage colony-stimulating factor (M-CSF) and granulocyte–macrophage colony stimulating factor (GM-CSF) in fibrotic diseases (5, 40). We also found the serum levels of Th1 cytokines (IFN-γ and IL-12), not Th2 cytokines, were recovered by the IVIG treatment in SSc patients (41). Blank *et al*. reported that IVIG administration on tight-skin mice significantly reduced collagen mRNA levels and cutaneous collagen deposition (42). In addition, in mice splenocytes, IVIG treatment reduced the secretion of profibrotic cytokines (TGF-β1 and IL-4), but not interferon-γ.

There have been case series and a small number of open-label, uncontrolled studies suggesting the efficacy of IVIG for SSc skin (43, 44), indicating that IVIG may be a promising option. For example, IVIG (400 mg/kg daily for five consecutive days) dramatically reduced histological skin fibrosis, and significantly improved the skin score (P<0.01) (43). A large randomized controlled study has been completed in Japan to further validate the efficacy, but is currently unpublished.

2.8 Interferon

Several reports have shown that interferon-α suppresses the expression of type I collagen in SSc dermal fibroblasts in vitro (45). This notion prompted a randomized, double-blind, placebo-controlled trial. However, interferon-α treatment ($13.5x10^6$ units/week) for one year did not improve skin sclerosis in patients with dcSSc (mean skin score change=-4.7) compared to placebo (-7.5, P=0.36) (46). Furthermore, interferon-α exacerbated ILD. These results are consistent with cases treated with interferon-α and -β for the treatment of

chronic hepatitis C and multiple sclerosis, who developed SSc and SSc-like disorders (5). Taken together, these clinical and laboratory observations suggest that interferon-α is positively associated with the fibrotic process of SSc, and blockade of interferon-α, not interferon supplementation, may be effective (5).

2.9 Anti-tumor necrosis factor (TNF)-α therapy

TNF-α is thought to inhibit the production of profibrotic factors and stimulate proteolytic activation, such as MMP expression. There have also been reports describing immune activation by anti-TNF-α therapy in autoimmune diseases, such as lupus- or psoriasis-like symptoms. Furthermore, rheumatoid arthritis-related pulmonary fibrosis can be exacerbated by the therapy (47-49). On the other hand, anti-TNF-α therapies have been beneficial in certain inflammatory bowel diseases that are characterized by inflammation and fibrosis (49).

In the report by Lam *et al*, 18 female SSc patients with inflammatory joint involvement were treated with etanercept 50 mg/week for 2 to 66 months (mean: 30 months) (50). Eight patients were positive for rheumatoid factors and three had positive anti-cyclic citullinated peptides (CCP) antibodies. In the retrospective cohort study, 15 out of the 18 patients (83%) were considered as responders with a decrease in inflammation of joints. On the other hand, the skin score decreased from 6.6 to 3.9, but the change was not significant (P=0.12). In another open-label study of 16 dcSSc patients with progressive skin sclerosis, infliximab at a dose of 5 mg/kg at five courses was tested, but the effect on skin score was only slight at 26 weeks (from 26 to 22) (49, 51). Serum levels of propeptide of collagen III and collagen I were significantly reduced after the treatment compared with the baseline. The expression of TGF-β in lesional skin biopsies was not be affected by the treatment (51). Bosello *et al*. reported that infliximab (3 mg/kg) at four times together with methotrexate, followed by etanercept (25 mg) improved skin score in four patients with SSc (35 to 16, 12 to 7, 16 to 7, and 8 to 3), albeit insignificantly (52). Accordingly, there is still not enough evidence to show the effect of anti-TNF therapy against skin involvement. The EUSTAR group do not recommend use of this drug (49, 53). Treatment-related death was rarely reported (47), and long-term tolerance of anti-TNF-α in SSc patients should be examined in the future.

2.10 Imatinib

A protein tyrosine kinase inhibitor, imatinib mesylate, blocks c-kit and PDGF receptor. Imatinib also inhibits non-canonical TGF-β signaling through c-Abl, which is one of the important downstream molecules of the TGF-β pathway. Imatinib is currently used for the treatment of chronic myeloid leukemia and gastrointestinal stromal tumors.

Imatinib mesylate is given attention for its possible application as a novel therapy against fibrosis and vasculopathy in SSc, because the drug can reverse the expression of Fli1, which is a key transcription factor controlling fibrosis and vasculopathy as well as immune dysfunction of this disease (5, 54, 55). Side effects include myelosuppression, headache, nausea, vomiting, diarrhea, and edema.

TGF-β and PDGF signaling is also involved in the fibrosis seen in SSc, and the drug may be able to inhibit fibrotic process of SSc by blocking them both. The inhibition of the pathway could decrease the mRNA and protein expression of collagen in both normal and SSc dermal fibroblasts (8, 56). Furthermore, imatinib reduced skin thickening of tight-skin mice, and prevented the differentiation of resting fibroblasts into myofibroblasts (57). Notably, imatinib not only stopped further progression of established dermal fibrosis, but also induced regression of existing fibrosis. (49).

Case reports have indicated the effect of imatinib on skin sclerosis. Several open-label studies also reported significant decrease of skin score (58). However, the effects were not significant in a six-month randomized double-blind control study (400 mg/day of imatinib) of 28 patients (P=0.098) (59), double-blind study (400 mg/day) of 10 SSc patients, and open-label study (200 mg/day) of 30 patients (60, 61).

3. Future treatments

According to ClinicalTrials (https://clinicaltrials.gov), placebo-controlled randomized clinical trials of several drugs other than those described above, including abatacept or ethanol extract physalis angulata linn, targeted skin sclerosis, and have already been completed.

Trial of brentuximab vedotin, riociguat study for dcSSc (RISE-SSc) (62) or pirfenidone is still ongoing.

On the other hand, several drugs have been proven to have therapeutic potential by open-label studies. For example basiliximab, CD25 monoclonal antibody, has improved skin sclerosis (63). Furthermore, belimumab (antibody for B-lymphocyte stimulator), dasatinib, and nilotinib (Abl tyrosine kinase and PDGF receptor inhibitors) (49), antibody against lymphotoxin-α, -β, CD40L, CD27L, FasL, OX40L, or tumor necrosis factor superfamily (TRAIL) (8) are speculated to have anti-fibrotic effects. Guideline of SSc was published by a Japanese group in 2018 (64), and evidence of various treatments has been analyzed and evaluated. Further investigation of the effects of novel drugs will contribute to the treatment of skin sclerosis in SSc.

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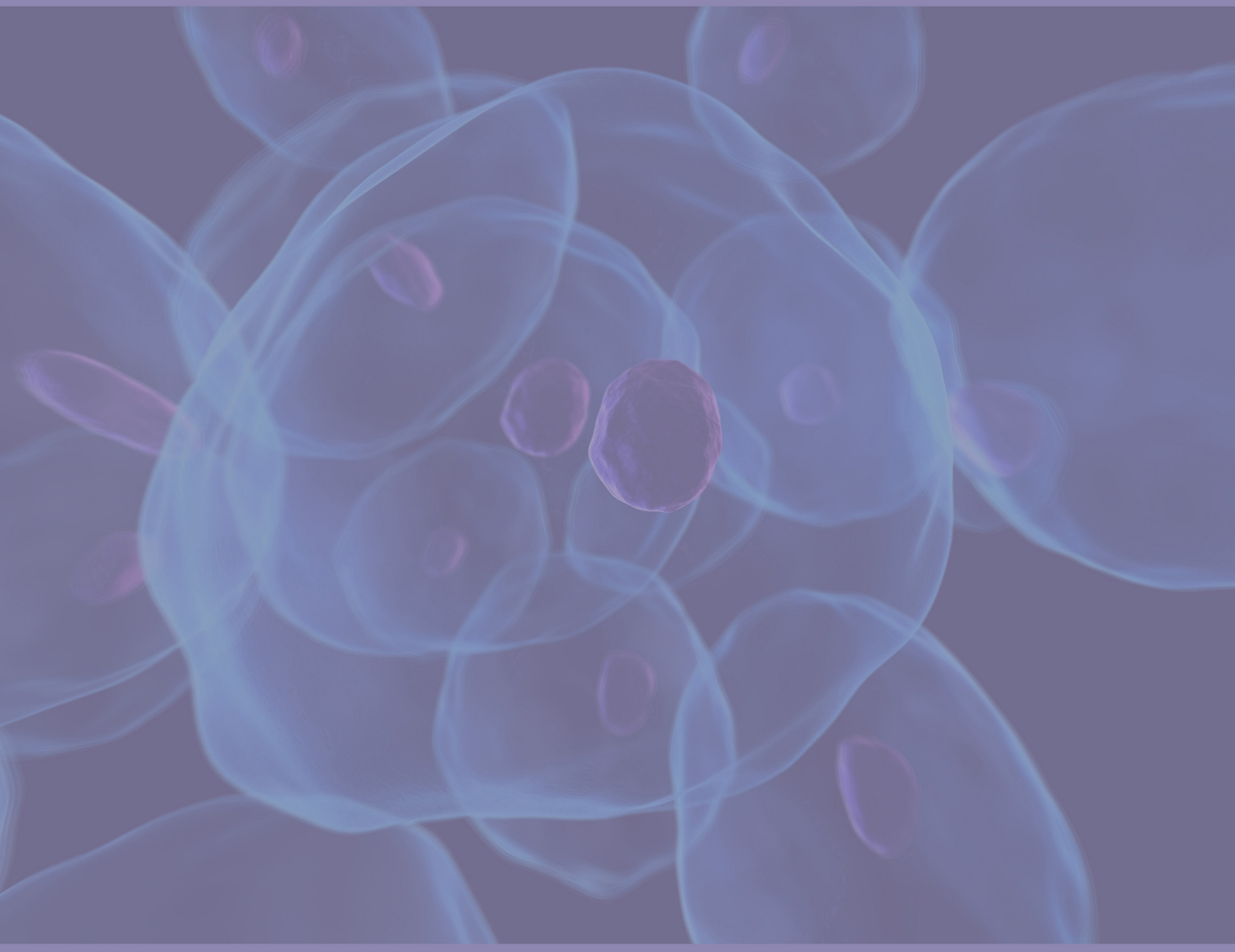

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