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# Combination treatment with lipoteichoic acids isolated from *Lactobacillus plantarum* and *Staphylococcus aureus* alleviates atopic dermatitis via upregulation of CD55 and CD59

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ABSTRACT

The innate immune complement system helps clear invading pathogens by forming membrane attack complexes (MACs) on their surface. Abnormal activation of the complement system may aggravate atopic dermatitis (AD) symptoms in AD patients. Here, we investigated the anti-AD effects of LTAs isolated from *Lactobacillus plantarum* (pLTA) and *Staphylococcus aureus* (aLTA) by examination of complement regulatory proteins (CRPs). Combination treatment with pLTA and aLTA increased CD55 and CD59 production in HaCaT cells. The regulation of CD55 and CD59 was mediated by p38 mitogen-activated protein kinase (p38) signaling pathways in pLTA- and aLTA-treated cells. Complement-dependent cytotoxicity (CDC) and bactericidal assays revealed that combination treatment with pLTA and aLTA down-regulated the complement system. In experiments using an irritant contact dermatitis (ICD)-induced mouse model, the levels of MAC and C3 convertase (C3C) were lower in serum collected from pLTA- and aLTA-injected mice than in serum from mice who were untreated or received pLTA or aLTA alone. Combination treatment also inhibited IgE and CCL2 levels in ICD mice. On the other hand, IFN-γ level was significantly increased, indicating that combination treatment switches the Th2 response to a Th1 response. Our results suggest that combination treatment with LTAs could be a good therapeutic approach to alleviate AD by reducing formation of MACs and inducing a Th1 response.

## 1. Introduction

The complement system is part of the innate immune system that functions to defend the host against infection by foreign pathogens [1]. The complement pathway is regulated by complement regulatory proteins (CRPs), such as membrane cofactor protein (MCP, CD46), decay accelerating factor (DCF, CD55), and protectin (C59) [2]. CD46 regulates complement activation by functioning as a cofactor for factor-I-mediated cleavage of C3b and C4b [3]. CD55 inhibits C3 and C5 cleavage by accelerating the decay of C3 and C5 convertase [4]. CD59 acts as an inhibitor of membrane attack complex (MAC) formation by preventing C9 polymerization with C5b678 [5]. One study found that complement components, including C3, C4, and C3a, are increased in atopic dermatitis (AD) patients compared to non-atopic controls [6]. Overactivation of the complement system has been shown to cause damage to the dermal-epidermal junction [7], which may lead to aggravation of AD. However, the role of CRP in AD has not been elucidated.

Lipoteichoic acid (LTA) is a major immune stimulator of gram-positive bacteria. In general, LTA is composed of a polyglycerophosphate chain and glycolipids that interact with toll-like receptor 2 (TLR2) or CD36, a TLR-independent signaling receptor, but related immune stimulation activities differ among species [8,9]. LTA isolated from pathogenic bacteria such as *S. aureus* (aLTA, LTA isolated from the *S. aureus* cell wall) increases the production of inflammatory cytokines and induces pulmonary inflammation and circulatory failure [10]. Conversely, LTA isolated from probiotics, such as *Lactobacillus plantarum,* induces moderate inflammation. Interestingly, pLTA (LTA isolated from the *L. plantarum* cell wall) strongly inhibits pathogenic ligand-mediated inflammation [11,12]. In the current study, we investigated the effects of LTAs isolated from probiotics and a pathogen on regulation of the complement system by observing expression levels

*Abbreviations:* LTA, Lipoteichoic acid; pLTA, LTA isolated from *L. plantarum* cell wall; aLTA, LTA isolated from *S. aureus* cell wall; DNCB, 24-Dinitrochlorobenzene; ICD, irritant contact dermatitis; AD, atopic dermatitis; CRP, complement regulatory protein; CDC, complement dependent cytotoxicity

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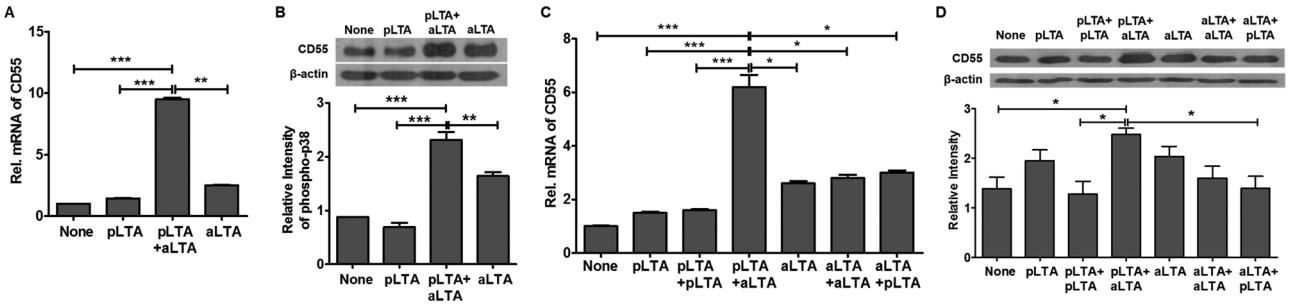


**Fig. 1.** Combination treatment with pLTA and aLTA increased CD55 expression. (A and B) HaCaT cells were pretreated with pLTA for 18 h followed by treatment with aLTA for 6 h or 24 h. CD55 mRNA (A) and protein levels (B, upper panel) were measured by real-time PCR and Western blot, respectively. Densitometry analysis for CD55 (B, lower panel). (C and D) HaCaT cells were treated with a combination of pLTA and aLTA (pretreating with one and then followed by treatment with the other). The levels of CD55 mRNA (C) and CD55 protein (D, upper panel) were examined. Densitometry analysis for CD55 (D, lower panel). CD55 mRNA level was examined by qRT-PCR. Levels of mRNA were normalized to GAPDH. Data are displayed as the mean ± SD of three independent experiments. Statistical analysis was conducted with one-way ANOVA and Tukey's statistical tests. $*p < 0.05$; $**p < 0.01$; $***p < 0.001$.

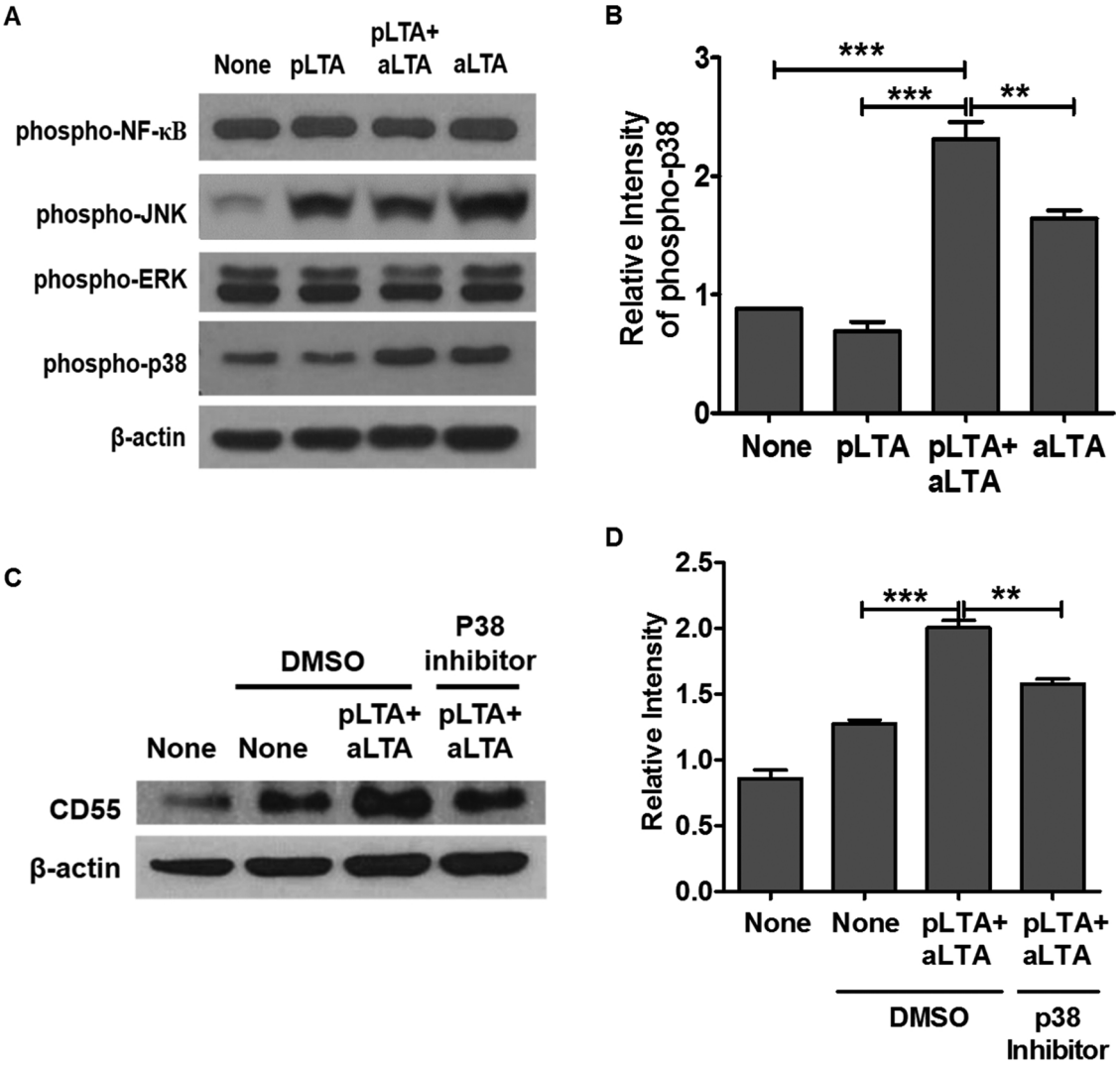


**Fig. 2.** CD55 induction was mediated by p38 phosphorylation. (A) The protein levels of p-NF-κB, p-JNK, p-ERK, and p-p38 were determined by Western blot. (B) Densitometry analysis for phosphor-p38. (C) 10 μM p38 MAPK inhibitor (SB203580) was used to pretreat cells for 30 min before treatment with pLTA followed by aLTA. (D) Densitometry analysis for CD55. β-actin was used as an internal control. Data are displayed as the mean ± SD of three independent experiments. Statistical analysis was conducted with one-way ANOVA and Tukey's statistical tests. $**p < 0.01$; $***p < 0.001$.

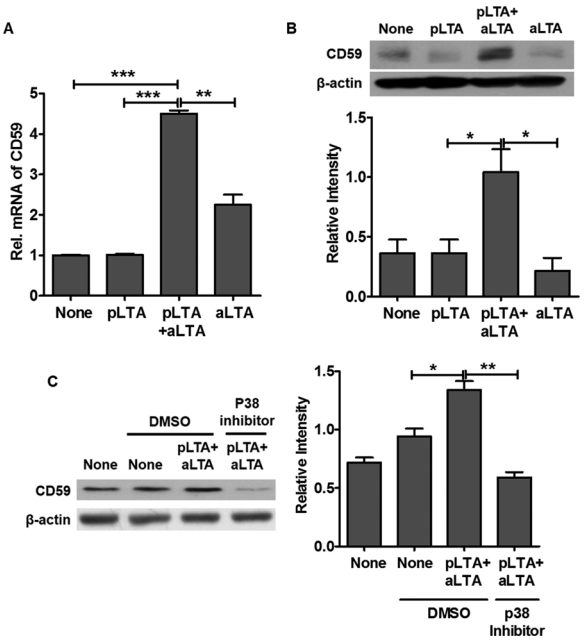


**Fig. 3.** Combination treatment with pLTA and aLTA increased CD55 expression via p38 activation. CD59 mRNA (A) and protein (B) levels were determined by real-time PCR and Western blot, respectively. Densitometry analysis for CD55 (B, lower panel). Involvement of p38 in CD59 expression was examined with 10 μM p38 inhibitor by Western blot (C, left panel). Densitometry analysis for CD59 (C, right panel). mRNA level was normalized to GAPDH, and β-actin was used as an internal control. Data are displayed as the mean ± SD of three independent experiments. Statistical analysis was conducted with one-way ANOVA and Tukey's statistical tests. $*p < 0.05$; $**p < 0.01$; $***p < 0.001$.

of CD55 and CD59. We also examined the alleviating effects of LTAs using an ICD mouse model.

## 2. Materials and methods

### 2.1. LTA preparation

LTAs were isolated from *L. plantarum* K8 (KCTC 10887BP; pLTA) and *S. aureus* (ATCC 25923; aLTA) as previously described [11]. Protein and endotoxin contamination were examined using silver staining and an endotoxin assay kit (GenScript, NJ, USA), respectively.

| | aLTA | pLTA |
|---|---|---|
| Silver staining | No bands detected | No bands detected |
| Endotoxin (EU/ml) | 0.039 | 0.0257 |

### 2.2. Quantitative PCR

After stimulation of HaCaT cells with pLTA and/or aLTA, total RNA was extracted using RNA-Bee reagent (AMS Biotechnology, MA, USA), and cDNA was synthesized using the iScript cDNA Synthesis kit (Bio-Rad, CA, USA) according to the manufacturer's instructions. To quantify messenger RNA expression, real-time PCR was performed with the CFX Connect™ Real-Time PCR Detection system (Bio-Rad). PCR products were detected with SYBR® Premix Ex Taq™ II (TaKaRa, Otsu, Japan). The following sequences for the forward and reverse primer pairs were used: 5′-CAGCACCACCACAAATTGAC-3′ and 5′-CTGAACT GTTGGTGGGACCT-3′ for CD55, 5′−CCGCTTGAGGGAAAATGAG-3′ and 5′-CAGAAATGGAGTCACCAGCA-3′ for CD59, and 5′-AAGGTCGG AGTCAACGGATT-3′ and 5′-GCAGTGAGGGTCTCTCTCCT-3′ for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). mRNA was normalized to GAPDH.

### 2.3. Enzyme-linked immunosorbent assay (ELISA)

Blood collected from mice was used to examine secreted IFN-γ and CCL2 levels. Sandwich ELISA was performed with capturing antibodies including anti-mouse IFN-γ (#37801) and anti-mouse CCL2 (Gln24-Arg96) antibodies and biotinylated anti-mouse IFN-γ and biotinylated CCL2 polyclonal antibodies (R & D Systems, MN, USA). The quantities of MAC and C3 convertase (C3C) were measured with mouse serum collected from LTA-injected mice. MAC and C3C kits were purchased from MyBioSource (CA, USA). The assay was performed according to the manufacturer's instructions. The absorbance was measured at 450 nm using an ELISA reader. The concentrations of MAC and C3C were calculated using the standards obtained from the kit. For detection of soluble CD55 from mouse serum, rabbit anti-CD55 (Santa Cruz, CA, USA) was coated onto an ELISA plate (Corning Costar flat-bottom high-binding EIA/RIA 3690 plate) in PBS (pH 7.4; 0.4 μg/well) at 37 °C for 2 h. The plate was blocked with 2% BSA in PBS. Mouse serum was added in triplicate to experimental or control wells. After incubation at 4 °C overnight, wells were washed, and bound CD55 was detected by serial addition of a biotin-labeled secondary antibody, avidin-horseradish peroxidase (HRP) conjugate (Pierce Immunopure streptavidin-HRP conjugate), and peroxidase substrate (Pierce Chemicals, IL). The absorbance at 450 nm was read and presented after subtraction of reagent-control values reacting against BSA-coated negative controls.

### 2.4. Western blot analysis

Protein samples were prepared with 2X reducing sample buffer (Laemmli buffer) after cell stimulation. The denatured proteins were separated by 10%–12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in Tris/glycine buffer (25 mM Tris, 250 mM glycine, 0.1% SDS) and transferred onto a nitrocellulose membrane (40 V, 4 °C, overnight). The membrane was incubated with TBS-T buffer (20 mM Tris−HCl, 150 mM NaCl, 0.05% Tween-20, 5% skim milk) for 1 h at room temperature (RT) and washed three times with TBS-T. Primary antibodies diluted in TBS-T buffer were added, and membranes were incubated for 2 h at RT or overnight at 4 °C. After washing with TBS-T buffer, membranes were incubated with TBS-T containing HRP-conjugated secondary antibodies for 1 h at RT. After three washes, membranes were treated with enhanced chemiluminescence (ECL) reagents (GE Healthcare, Buckinghamshire, UK) and exposed to x-ray film. Anti-phospho-p38, anti-phospho-JNK, anti-phospho-ERK, anti-phospho-IkBα, anti-phospho-p65, anti-phospho-c-Jun (Cell Signaling Technology, MA, USA), anti-CD55, anti-CD59, and anti-β-actin (Santa Cruz Biotechnology, TX, USA) antibodies were used in this study.

### 2.5. Mice

BALB/c mice (6 weeks old) were purchased from Central Lab Animal Inc. (Seoul, Korea). They were kept in individual cages at 24 ± 2 °C and 50 ± 10% moisture conditions and fed nutritionally balanced rodent food (Central Lab Animal Inc.) and sterilized water. The mice were cared for and treated in accordance with the guidelines of the Animal Ethics Committee of Kyung Hee University (KHU14-021). To develop an atopy-like mouse model, the shaved back skin on each mouse was exposed to 200 μl immune-disturbing material containing acetone: olive oil (3:1) and 2.5% 2, 4-dinitrochloro benzene (DNCB; Sigma). After 3 days of exposure, mice were treated with 150 μl 1.0% DNCB at 3-day intervals. After 12 days of treatment with DNCB, generation of irritant contact dermatitis (ICD) was confirmed by the naked eye, and mice were randomly allocated to five groups ($n = 4$/group): normal control, ICD control, ICD-pLTA + aLTA treatment, ICD-pLTA treatment, and ICD-aLTA treatment. Mice from each experimental

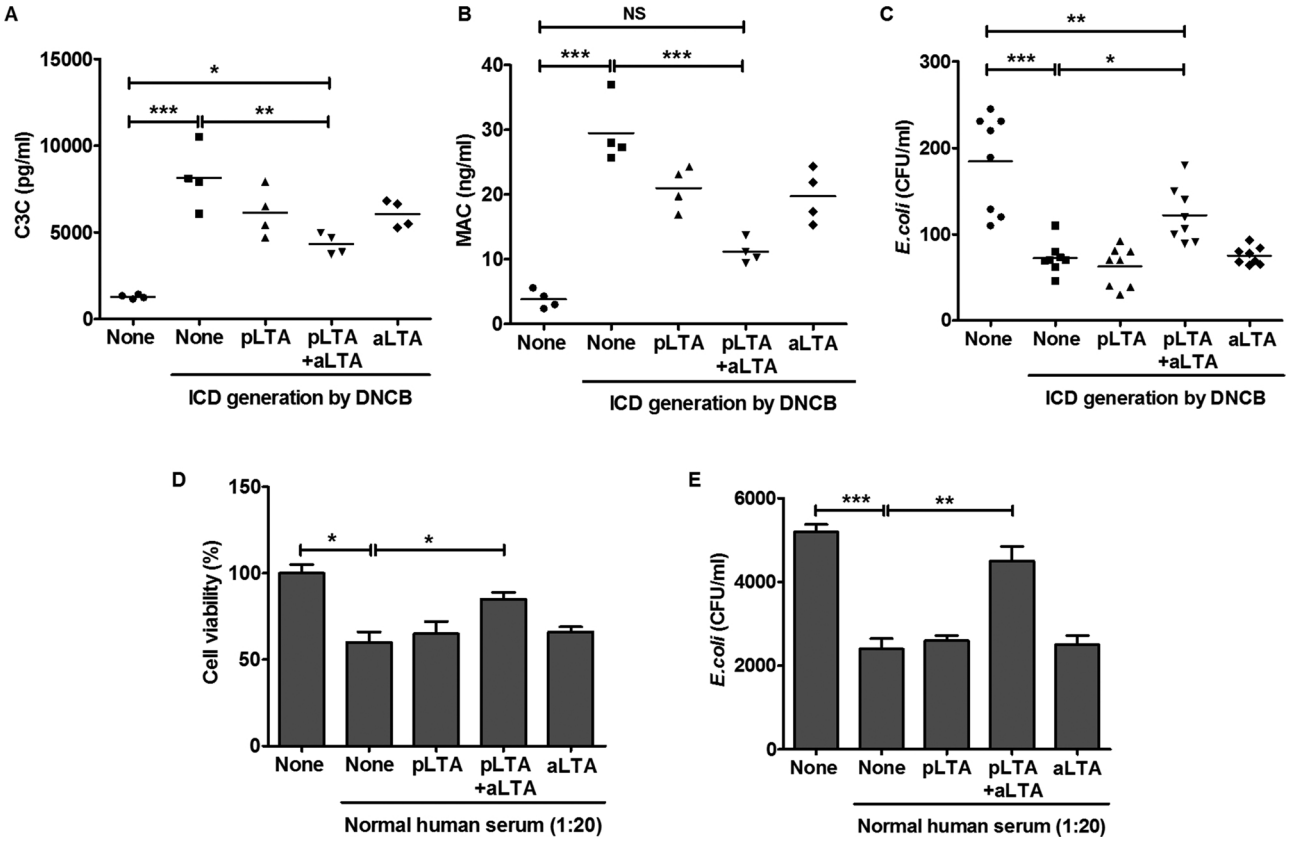


**Fig. 4.** Combination treatment with pLTA and aLTA inhibited complement activity. (A to C) DNCB-induced ICD mice were intraperitoneally injected with pLTA at 5 mg/kg for 24 h followed by treatment with either pLTA or aLTA. After 24 h, blood from the mice was collected, and the serum was separated. The quantities of C3C (A) and MAC (B) were measured by ELISA assay. The concentrations of MAC and C3C were calculated using the standard sample from commercial ELISA kits. (C) Bactericidal assays were performed to examine the ability of sera from LTA-injected ICD mice to kill bacteria. P values were determined by one-way ANOVA and Tukey's statistical tests, and values are expressed as mean ± SD of 4 animals for each group. (D and E) HaCaT cells were treated with pLTA and aLTA. Human serum was diluted 1:20 with serum-free medium, and the cells were incubated for 6 h. Cell viability was analyzed by Calcein-AM assay, and results are shown as the percentage of surviving cells compared with untreated cells (D). Bactericidal assays were performed with NHS (1:20) and HaCaT cell culture supernatants after stimulation with LTAs (E). Values are expressed as mean ± SD of three or more independent experiments. Student's *t*-test was used for comparison between treated groups. $*p < 0.05$; $**p < 0.01$; $***p < 0.001$.

group were injected with 50 mg/kg pLTA and/or aLTA at 3-day intervals. Both control and experimental mice received 0.2% DNCB to prevent natural cure until sacrifice. Blood from the mice was collected, and then the serum was separated and used to examine the quantities of MAC and C3C through the bacterial killing assay.

### 2.6. Clinical skin score

Mice in each experimental group were photographed using a digital camera to analyze AD symptoms and the clinical appearance of the skin. AD symptoms were evaluated by scoring scaling and dryness, hemorrhage and excoriation, and edema and redness. The sum of the individual symptom scores was calculated (0 = normal, 1 = mild, 2 = moderate, 3 = severe). The total score for each animal ranged from 0 to 9 points.

### 2.7. Complement-dependent cytotoxicity (CDC) assay

HaCaT cells were seeded in 96-well plates with DMEM supplemented with 10% FBS and P/S. The cells were pretreated with LTAs and washed with DPBS. Normal human serum (NHS) was diluted 1:20 with DMEM without supplement, and the cells were incubated with NHS for 6 h. The cells were washed with DPBS, and the viability of the HaCaT cells was measured with the Calcein AM cell viability assay system (EMD Millipore, #206700, MA, USA). Briefly, 2 μM Calcein AM (final concentration) was added to each well and incubated for 15 min at 37 °C under CO2. Fluorescence was examined at 490 nm excitation and 520 nm emission wavelengths. NHS was isolated from blood supplied by the Blood Center of the Korean Red Cross.

### 2.8. Bactericidal assay

*E. coli* was cultured overnight in LB broth, and the bacteria were washed and diluted with PBS. *E. coli* at $1 \times 10^4$ cells was cultured with mouse serum (1:20) or NHS (1:20) at 37℃ for 60 min. Incubated bacteria were washed with DPBS and spread on LB plates. After overnight culture, CFUs were calculated. The mouse serum was obtained from mice injected with LTAs.

### 2.9. Data analysis

All experiments were repeated at least three times. The data shown are representative results of the mean ± SD of triplicate experiments. Statistical analyses were conducted with an unpaired two-tailed *t*-test, then one-way ANOVA, followed by Tukey's honestly significant difference (HSD) post hoc test or with two-way ANOVA. Prism 5 software was used for the analysis (Graphpad Software Inc., Version 5.01, CA, USA). $p < 0.05$ was considered significant.

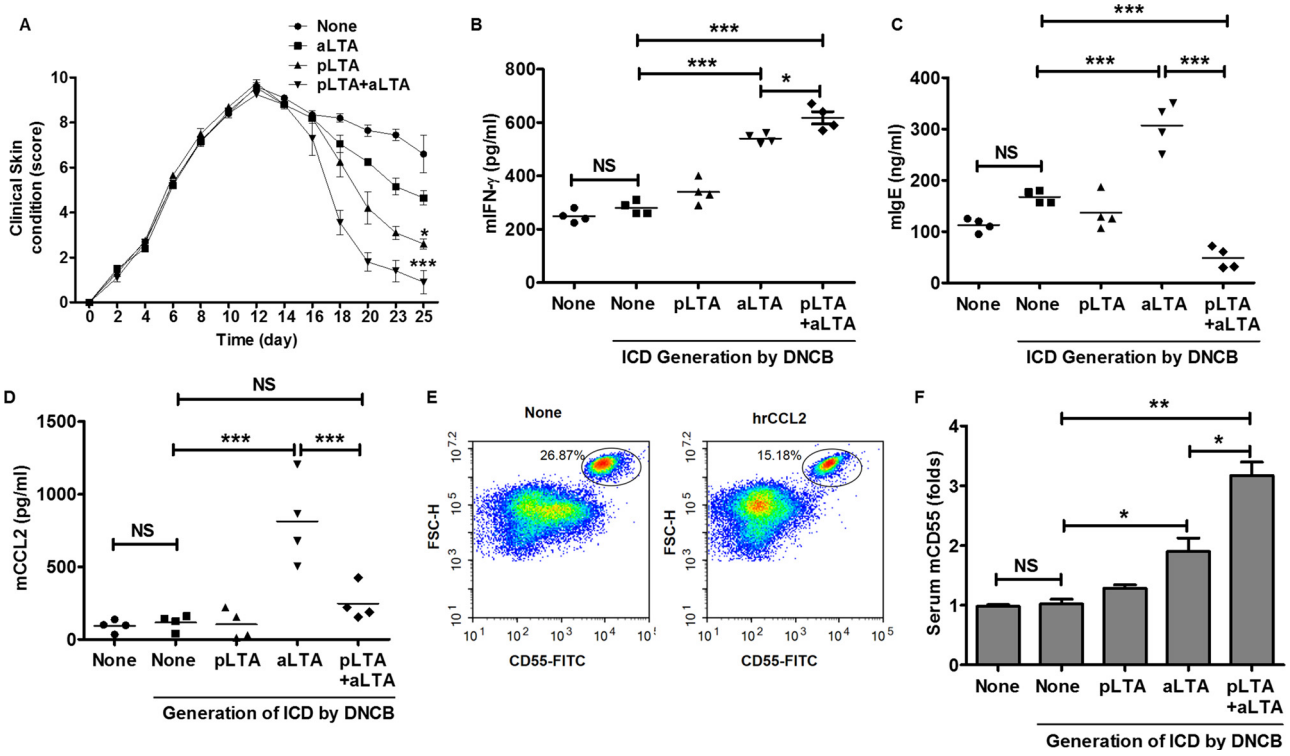


**Fig. 5.** pLTA pretreatment followed by aLTA treatment alleviated ICD symptoms. Atopy-like mice generated by DNCB were pretreated with 50 mg/kg pLTA by intraperitoneal injection. After 24 h, 50 mg/kg aLTA was administered for co-treatment. Control mice were injected with 50 mg/kg pLTA or aLTA alone. (A) The graph shows the clinical condition of skin lesions in ICD mice intraperitoneally injected with LTAs. Serum IFN-γ (B) and IgE (C), CCL2 (D), and secreted CD55 (F) were examined by ELISA with blood collected from ICD mice intraperitoneally injected with LTAs. (E) CD55 cell surface expression was examined by FACS analysis using anti-CD55-FITC in HaCaT cells stimulated with rhCCL2. P values were determined by one-way ANOVA and Tukey's statistical tests, and values are expressed as mean ± SD of 4 animals for each group. $*p < 0.05$; $**p < 0.01$; $***p < 0.001$.

## 3. Results

### 3.1. pLTA and aLTA combination treatment increases CD55 and CD59 expression

Previous studies have shown that pretreatment with pLTA induces tolerance effects against LPS- or aLTA-mediated production of inflammatory cytokines [11,12]. In this study, we pretreated cells with pLTA and then treated them with aLTA to determine if CRPs are regulated by both LTAs. CD46 was not changed by LTA combination treatment (data not shown). However, CD55 mRNA level was significantly increased by pretreatment with pLTA followed by treatment with aLTA in HaCaT cells (Fig. 1A). The protein level of CD55 was also increased by combination treatment compared to others (Fig. 1B, upper panel). The densitometry analysis also indicated that combination treatment significantly increased CD55 protein level (Fig. 1B, lower panel). Fig. 1C and D show that the increased levels of CD55 mRNA and protein were not due to the amount of LTA used to treat the cells. Combination treatment with pLTA/pLTA, aLTA/aLTA, or aLTA/pLTA did not increase the level of CD55; only pretreatment with pLTA followed by treatment with aLTA markedly increased CD55 mRNA (Fig. 1C) and protein (Fig. 1D upper panel) expression levels. Similar results from the densitometry analysis of CD55 protein are shown in Fig. 1D, lower panel.

LTA is recognized by TLR2 [13] and activates NF-κB and MAPK, including p38 mitogen-activated protein kinases (p38), c-Jun N-terminal kinases (JNK), and extracellular signal-regulated kinases (ERK) [14]. Thus, we examined whether LTA combination treatment would alter the signaling variation of NF-κB and MAPK. We found that phosphorylation of p38 was increased by LTA combination treatment (Fig. 2A), similar to the CD55 results. The densitometry analysis of phosphor-p38 showed that combination treatment with pLTA and aLTA activated the p38 pathway (Fig. 2B). In contrast, phosphorylation of NF-κB p65 and JNK decreased. Next, to determine if p38 signaling is associated with expression of CD55, cells were blocked using a p38 MAPK inhibitor (SB203580). When the p38 pathway was blocked, the level of CD55 was inhibited in cells treated with pLTA and aLTA (Fig. 2C). The relative intensity of CD55 protein is displayed in Fig. 2D. These results suggest that expression of CD55 is mediated by the p38 signaling pathway and not by other MAPK signals.

The expression pattern of CD59, an inhibitor of MAC formation, was also examined. As shown in Fig. 3A, combination treatment with pLTA and aLTA significantly increased CD59 mRNA level. The protein level of CD59 was also increased by combination treatment (Fig. 3B, upper panel). The densitometry analysis is shown in Fig. 3B, lower panel. Interestingly, similar to CD55 expression, CD59 expression was also regulated by the p38 pathway in HaCaT cells treated with the pLTA and aLTA combination. A representative WB result (Fig. 3C, left panel) and densitometry analysis (Fig. 3C, right panel) are shown. Because CD59 directly inhibits MAC activity, we assume that increased CD59 is associated with alleviation of AD.

### 3.2. Combination treatment with pLTA and aLTA inhibits complement activity

To identify the effect of LTAs *in vivo*, irritant contact dermatitis (ICD) mice generated using DNCB were pretreated with pLTA 24 h before aLTA treatment. After 24 h of aLTA treatment, blood was collected, and serum was separated. The quantities of C3C and MAC in the sera were measured by ELISA assay. C3C and MAC levels in the group treated with both pLTA and aLTA showed the greatest decline among the groups (Fig. 4A and B). Given our previous results, the decrease in

C3C and MAC levels based on pretreatment with pLTA followed by aLTA treatment could be explained by upregulation of CD55.

Next, to confirm complement system activity, we performed bactericidal assays using mouse serum from ICD mice injected with LTAs. Serum from mice injected with both pLTA and aLTA had the weakest ability to kill bacteria, as predicted (Fig. 4C). Although combination treatment with pLTA and aLTA decreased complement activity, serum from that group still showed more activity than serum from untreated normal mice, indicating that complement activity was not completely inhibited but was still high enough to affect infected bacteria in pLTA- and aLTA-treated mice. Inhibition of complement activity was shown in the CDC assay using normal human sera (NHS) and HaCaT cells. As shown in Fig. 4D, about 60% HaCaT cell death was seen in NHS-treated cells, while the viability was increased by 85% in HaCaT cells treated with pLTA and aLTA followed by NHS treatment. Bactericidal activity of NHS was also decreased by pLTA and aLTA combination treatment (Fig. 4E). These results suggest that the combination treatment with pLTA and aLTA can inhibit the excessive complement activation.

### 3.3. Combination treatment of pLTA and aLTA alleviated irritant contact dermatitis (ICD)

Clinical assessment of ICD was performed by observing skin conditions of dryness, hemorrhage, excoriation, edema, and redness. Clinical skin score of the pLTA- and aLTA-treated group was significantly decreased at day 12, when mice were intraperitoneally injected with LTAs at 3-day intervals until day 27 (Fig. 5A). On the last day of experiments, blood was collected from all mice, and cytokine expression levels were examined. IFN-γ was significantly higher in the ICD mice treated with aLTA only and in those with pLTA and aLTA treatment (Fig. 5B), while IgE level was increased by aLTA only but was significantly decreased by the combination of pLTA and aLTA (Fig. 5C), indicating that combination treatment with both pLTA and aLTA switched the Th2 response to a Th1 response. Serum CCL2 level was also high in aLTA only-treated ICD mice but was decreased by combination treatment with pLTA and aLTA (Fig. 5D). Unlike IgE level, serum CCL2 was not altered by pLTA and aLTA combination treatment as compared to ICD mouse only (None). It might be because CCL2 expression was not affected by DNCB. CCL2 is known to stimulate IL-4 production and is involved in Th2 polarization [15]. Recombinant human CCL2 (rhCCL2) reduced CD55 cell surface expression by 43.5% in HaCaT cells, indicating that the complement system is activated by CCL2 (Fig. 5E). Thus, our data suggest that aLTA induces CCL2 production, which aggravates AD symptoms by polarizing the Th2 response as well as activating the complement system. On the other hand, serum CD55 level was increased in ICD mice treated with aLTA only and the aLTA and pLTA combination, but combination treatment induced higher levels than aLTA alone (Fig. 5F). The results suggest that CCL2 and IgE levels increased by aLTA injection are associated with AD generation. In addition, we assumed that increased CD55 following combination treatment with pLTA and aLTA also alleviated ICD.

## 4. Discussion

AD is the most common allergic skin disease, but its pathogenesis is complex and not fully understood. Researchers have shown that the complex immune reaction with a Th2 response and IgE production affects AD, but Th22, Th17, and Th1 activation also occurs in AD [16]. Th2 lymphocytes secrete IL-4, IL-5, and IL-13 into the skin, while Th1 lymphocytes produce mainly IFN-γ, TNF-α, IL-8, and IL-12, which activate the complement system and cause skin damage as AD lesions [17,18]. It is also reported that the serum levels of C3, C4, and C1-inh are higher in AD patients compared to healthy non-atopic controls [19]. In addition, expression of the anaphylatoxin C5a receptor increases in AD mice, and treatment with a C5aR antagonist decreases IL-4 and IFN-γ levels in skin tissue, as well as the levels of IL-4, IFN-γ, histamine, and IgE in serum, indicating that a C5aR antagonist can inhibit AD [20]. In this study, we established the following mechanism: (i) LTA combination treatment induced CD55 and CD59 production through the activation of p38 pathways; (ii) Increased CD55 expression inhibited the formation of C3C, which resulted in reduction of MAC formation; (iii) Induction of CD59 directly inhibited MAC activity.

Previous studies have shown that pLTA has beneficial effects such as anti-aging, immune response regulation, and skin whitening. On the other hand, aLTA induces severe inflammation [8,21]. The anti-inflammatory effects of pLTA were mediated by tolerance in immune cells. For example, pLTA inhibits the production of pro-inflammatory cytokines induced by LPS or aLTA [11,12]. Unlike the tolerance effects of pLTA on LPS- or aLTA-mediated cytokine production, production of CD55 and CD59 significantly increased after pLTA treatment followed by aLTA. Signaling molecules, including NF-κB and MAPKs, were also down-regulated by pLTA and aLTA combination treatment in THP-1 cells. On the other hand, in HaCaT cells, combination treatment with pLTA and aLTA increased the phosphorylation of p38, which regulated CD55 and CD59 production. In THP-1 cells, aLTA increased p38 phosphorylation [12], while it did not affect p38 phosphorylation in HaCaT cells [22], suggesting that immune cells and epithelial cells respond differently to LTAs.

The synergistic effects of pLTA and aLTA combination treatment were uniquely induced by pretreatment with pLTA followed by aLTA treatment. Other combinations, including aLTA pretreatment followed by pLTA treatment, pLTA pretreatment followed by pLTA retreatment, and aLTA pretreatment followed by aLTA retreatment, did not affect the synergistic production of CD55 (Fig. 1D). Fig. 1D indicates that the synergy of CD55 production was not due to the dosage of LTAs used for treatment. Since pLTA itself has low immune activation properties [11], the combination of pLTA with pLTA may have no ability to induce activation of the p38 pathway. In addition, given that 100 μg/ml aLTA does not affect p38 phosphorylation in HaCaT cells [22], it makes sense that the aLTA plus aLTA combination might not affect the p38 pathway.

Unlike CD55 and CD59, the complement component C3 is inhibited by pretreatment with pLTA followed by TNF-α treatment, and the downregulation is associated with dephosphorylation of NF-kB p65 and p38 [23]. Although we do not fully understand how combination treatment with pLTA and aLTA activates p38, it may be caused by cross tolerance or synergy between signaling pathways. Sato and colleagues have reported that co-stimulation of mouse peritoneal macrophages with MALP-2 and LPS results in synergistic production of TNF-a, suggesting synergy between TLR2- and TLR4-mediated signaling pathways [24]. In the current study, however, both LTAs use the same TLR2 signaling pathway, suggesting that cross-tolerance or synergy occurred in a single signaling pathway. TLR2-associated receptors or adaptor proteins might be involved in the activation of p38. Ligand-specific recognition and signaling through TLR2 occurs via heterodimerization with TLR1 or TLR6. LTA is recognized by TLR2/TLR6, and accessory molecules, including CD14, CD36, and mannose binding lectin (MLB), are required [25]. It is possible that the pLTA-mediated TLR2 receptor complex is different than the aLTA-mediated complex, since the two LTAs have different LTA structures. In the previous studies, we have reported that LTAs have different glycolipid structure, which contributes to individual differences in MAPK signaling [9,26]. The TLR2 adaptor protein MyD88 interacts with members of the IL-1R-associated kinase (IRAK) family, which dissociate from MyD88 and interact with tumor necrosis factor (TNF) receptor–associated factor 6 (TRAF6) [27]. Among IRAK members, IRAK1 and IRAK2 respond differentially to the two LTAs. Unfortunately, we have little knowledge about possible synergy between pLTA and aLTA, and more studies are needed to reveal differences in signaling initiated by pLTA-TLR2 and aLTA-TLR2 interactions.

## 5. Conclusion

We showed that aLTA can aggravate AD, while pLTA can reduce both the aLTA-mediated excessive inflammatory response and complement activity. Our findings suggest that a combination treatment using pLTA and aLTA shows promise as a therapeutic treatment for AD.

## Authors' contributions

H designed the study and wrote the manuscript. Y, YD, and MS contributed to data collection. DK performed the statistical analysis and interpretation of the results. All authors read and approved the final manuscript.

## Declaration of Competing Interest

We have no conflicts of interest to declare.

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