



Effect of topical application of lipopolysaccharide on contact hypersensitivity

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ABSTRACT

Lipopolysaccharide (LPS) is the principal component of the outer membrane of gram-negative bacteria. The prior oral administration of LPS attenuates inflammatory responses, such as intestinal injury and atopic dermatitis, in mouse models; however, the underlying mechanism remains unclear. Here, we examined the effect of topical LPS application on allergic contact dermatitis and its mechanism of action using a murine contact hypersensitivity (CHS) model. Prolonged LPS application to the skin significantly suppressed 2,4-dinitrofluorobenzene (DNFB)-induced CHS. LPS application to the skin also reduced the phagocytosis of fluorescein isothiocyanate (FITC)-dextran by Langerhans and dendritic cells. Cutaneous cell migration into the skin-draining lymph nodes (LNs) induced by FITC painting was reduced by LPS application. During the CHS response, DNFB application induced T-cell proliferation and inflammatory cytokine production in skin-draining LNs, whereas prolonged LPS application inhibited DNFB-induced T-cell growth and interferon gamma production, indicating suppression of DNFB-induced sensitization. These results suggest that prolonged LPS application suppressed DNFB-induced sensitization and subsequently CHS response. Our findings imply that topical application of LPS may prevent allergic dermatitis such as CHS.

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1. Introduction

Lipopolysaccharide (LPS) is found in most gram-negative bacteria and induces innate responses by binding to toll-like receptor 4 (TLR4) [1,2]. LPS has long been recognized as a toxic constituent of bacterial endotoxins and has been used to promote inflammatory responses in *in vitro* and *in vivo* studies, such as LPS-induced pneumonia and gut injury in experimental animal models [3,4]. In contrast, epidemiological studies have suggested that environmental exposure to endotoxins, including LPS, during childhood reduces the risk of allergic sensitization, asthma, and pollinosis [5]. Previous studies have shown that the prior oral administration of LPS prevents dextran sulfate sodium-induced gut injury and high-fat diet-induced or diabetes-associated cognitive dysfunction [6,7]. They also suggested that the mechanism by which LPS is effective

for cognitive function is that LPS-trained microglia acquire a molecular expression related to inflammatory regulation, phagocytosis, neuroprotection, anti-apoptosis, and antioxidation [7]. In addition, the oral administration of LPS attenuated symptoms of atopic dermatitis (AD) in a mite extraction-induced AD mouse model [8]. Topical application of LPS-containing cream to patients with mild atopic dermatitis alleviated the symptoms [9]. However, the underlying mechanism by which LPS application affects AD remains unclear.

Allergic contact dermatitis, one of the most common skin diseases, is a delayed-type hypersensitivity response that comprises sensitization and elicitation phases [10]. The contact hypersensitivity (CHS) model in mice is a well-accepted model of allergic contact dermatitis induced by hapten application, including DNFB, and produces a sensitization and an elicitation phase [11,12]. During the sensitization phase induced by hapten application, haptenated proteins are recognized by skin dendritic cells (DCs) or Langerhans cells (LCs) that migrate to skin-draining lymph nodes (LNs), where they present the antigens to naïve T cells followed by T-cell priming.

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Abbreviations

LPS	lipopolysaccharide
CHS	contact hypersensitivity
DNFB	2,4-dinitrofluorobenzene
DNBS	2,4-dinitrobenzenesulfonic acid dehydrate
DC	dendritic cell
LC	Langerhans cell
LN	lymph node
MFI	mean fluorescence intensity
TLR4	Toll-like receptor 4
FITC	fluorescein isothiocyanate
FACS	fluorescence-activated cell sorter
IFN- γ	interferon gamma
AD	atopic dermatitis
PCNA	proliferation-associated nuclear antigen

During the elicitation phase, re-exposure to the same hapten recruits antigen-specific T cells to the allergen-induced sites, causing T-cell-mediated tissue damages such as skin edema [10,11]. Previous studies using the CHS murine model have expanded our understanding of the mechanism of allergic reactions occurring in the skin, especially the specific roles of various immune cells [10,11,13]. In the epidermis, there are some subtypes of immune cells, including Langerhans cells, $\gamma\delta$ T cells, and tissue-resident memory CD8⁺ T cells [14]. Among these immune cells, epidermal Langerhans cells have been thought to play an important role in detecting and transporting antigens to LNs and presenting them to other immune cells such as naïve T cells to initiate an immune response [15,16].

This study aimed to examine the effects of topical application of LPS on allergic contact dermatitis. We determined the effect of LPS on CHS response in a murine CHS model.

2. Materials and methods

2.1. LPS

Purified LPS derived from *Pantoea vagans* (LPSpv) was provided by Macrophi Inc. LPS solution (3 μ g/ml in ethanol) or vehicle (ethanol) was applied to abdomen skin (200 μ l, 4 cm²) or to ear skin (30 μ l), shown in Fig. 1.

2.2. CHS test

C57BL/6 mice were purchased from Japan SLC, Inc. Mice were sensitized with 50 μ l of 0.5% DNFB in acetone/olive oil (4:1) on abdomen skin. On day 6, the ears were challenged by the application of 20 μ l of 0.3% DNFB. Ear swelling was measured with a spring-loaded micrometer (Mitsutoyo) after 24 h after challenge.

All animal experiments were approved by the Institutional Animal Care and Use Committee of Keio University (approval no: 16075).

2.3. Lymphocyte proliferation assay and cytokine production

For DNBS-dependent response, single cell suspensions were prepared from skin draining LNs 5 days post-sensitization with DNFB. The draining LN cells (1×10^6) were cultured with or without DNBS (50 or 100 μ g/ml) for 48 h. The culture supernatants were collected 48 h post-incubation. The amount of IFN- γ was measured by enzyme-linked immunosorbent assay (ELISA; eBiosciences). Cell growth was quantified by CellTiter-Glo Luminescent Cell Viability assay according to manufacturer's instructions (Promega).

2.4. FITC-induced cutaneous cell migration assay

For FITC-induced cutaneous cell migration as described previously [17], mice were painted on the shaved abdomen with 200 μ l of 1% FITC (Dojindo) dissolved in acetone/dibutyl phthalate (1:1, Sigma-Aldrich) mixture. The number of cutaneous cells that migrated into the draining LNs was analyzed by flow cytometry CytoFLEX (Beckman Coulter Life sciences).

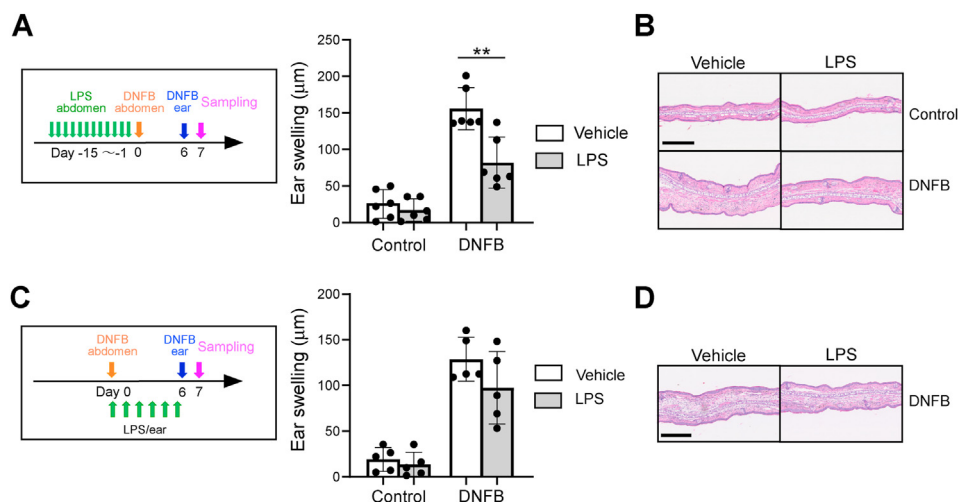


Fig. 1. LPS application suppressed DNFB-induced CHS

A. (left) Schedule of LPS application before sensitization. Either LPS or a vehicle (ethanol) was applied on the mice for 2 weeks (6 times/week), and they were sensitized with DNFB. The ear thickness was measured in micrometers 24 h after the challenge. (right) Ear swelling. **B.** Representative hematoxylin and eosin staining of the ears. **C.** (left) Schedule of LPS application after sensitization. Mice were sensitized with DNFB, and either LPS or the vehicle (ethanol) was applied. (right) Ear swelling. **D.** Representative hematoxylin and eosin staining of the ears. (SE, n = 6, **p < 0.01). Scale bar = 400 μ m.

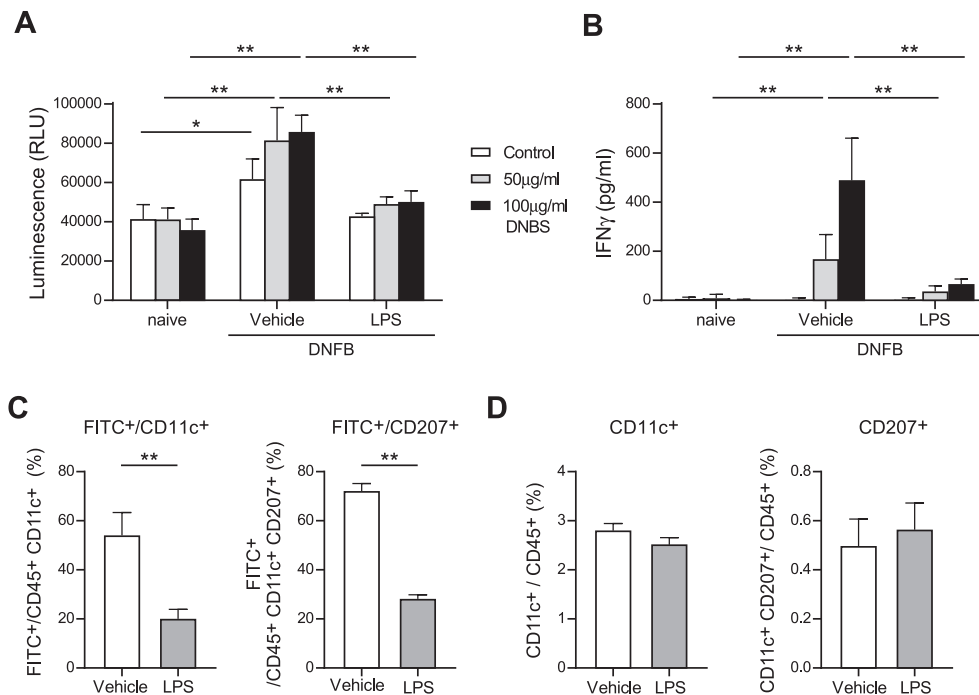


Fig. 2. LPS application suppressed DNFB-induced sensitization during CHS

A. B. Either LPS or the vehicle (ethanol) was applied on the mice for 2 weeks (6 times/week), and they were sensitized with DNFB, corresponding to Fig. 1A and B. Antigen-specific cell proliferation (A) and IFN- γ production (B) were determined in lymphocytes (1×10^5 cells) isolated from the skin-draining LN of mice with or without (naïve mice) DNFB sensitization. (SE, $n = 5$, $*p < 0.05$, $**p < 0.01$). C. D. Either LPS or the vehicle (ethanol) was applied on the mice for 2 weeks (6 times/week) and FITC was applied (10 mg/ml acetone, 200 μ l for 4 cm ^{2}). The draining LNs were collected 24 h later and analyzed by FACS analysis. C. % Ratio of FITC $^+$ cells in CD45 $^+$ CD11c $^+$ (left) or in CD45 $^+$ CD11c $^+$ CD207 $^+$ cells (right). (SE, $n = 5$, $**p < 0.01$) D. % Ratio of CD11c $^+$ to CD45 $^+$ cells (left) and CD11c $^+$ CD207 $^+$ to CD45 $^+$ cells (right).

2.5. Phagocytosis assay

Freshly isolated epidermal cell suspensions were incubated in RPMI-1640 containing 10% FBS for 1 hr and following with FITC-dextran (molecular weight ~10 kDa, 0.5 mg/ml, Sigma-Aldrich) for 45 min (37 $^{\circ}$ C, 5% CO $_2$). The cells were washed four times with cold PBS containing 1% BSA and were stained with monoclonal antibodies against CD45, CD11b, CD11c, and CD207 (eBioscience), and analyzed using the flow cytometry CytoFLEX (Beckman Coulter Life sciences).

2.6. Cell lines

The murine epidermal keratinocytes PAM212 were grown in RPMI-1640 medium containing 10% FBS and penicillin-streptomycin (50 u/ml, and 50 μ g/ml).

XS52 and NS47 cells were provided by Dr. Takashima and Dr. Kojima. XS52 is the Langerhans cell-like line established from epidermis of a newborn BALB/c mouse [18]. XS52 cells were incubated in RPMI-1640 medium supplemented with the culture supernatant (10% v/v) of the NS47 fibroblastic stromal cell line as described previously [19]. Both cells were stimulated with LPS for 24 h (0.1, 1 or 10 μ g/ml).

2.7. Flow cytometry analysis

The dorsal halves of the mouse ears were digested with Liberase TL (0.3 mg/ml, Roche) dissolved in Hanks' Balanced Salt Solution containing 10% FBS and 0.05% DNase I (Sigma-Aldrich, St Louis, Mo) for 1 h at 37 $^{\circ}$ C. LN was minced to collect single cells. Single cell suspensions from skin and LNs were stained with monoclonal antibodies against CD45, CD11b, CD11c, F4/80, CD207, MHCII, CD80

and CD86 (eBioscience). The samples were analyzed using the flow cytometry CytoFLEX (Beckman Coulter Life sciences).

2.8. RNA extraction and real-time quantitative RT-PCR

Total RNA was extracted using TRIZOL (Invitrogen Life Technologies). The cDNA was reverse transcribed from total RNA using the Prime Script RT reagent kit (Takara Bio). Quantitative RT-PCR was performed using SYBR Green I (Takara Bio) and the StepOne Plus real-time PCR apparatus (Thermo Fisher Scientific).

2.9. Histochemistry

Paraffin-embedded sections were stained with hematoxylin and eosin (Wako chemical).

2.10. Statistical analysis

Statistical analysis was performed using the two-tailed Student's t-test, one way or two-way ANOVA by GraphPad Prism8.

3. Results

3.1. LPS application suppressed DNFB-induced CHS

To determine the effect of LPS application on cutaneous immune reactions, we utilized a well-established hapten-induced CHS murine model [11,20,21]. Either LPS or a vehicle was applied on abdominal skin for 2 weeks, and the mice were sensitized by applying DNFB to evaluate the effect of LPS on the sensitization phase (Fig. 1A, left). No signs of toxicity or inflammation were observed in mice and their skin treated with LPS. Ear swelling in

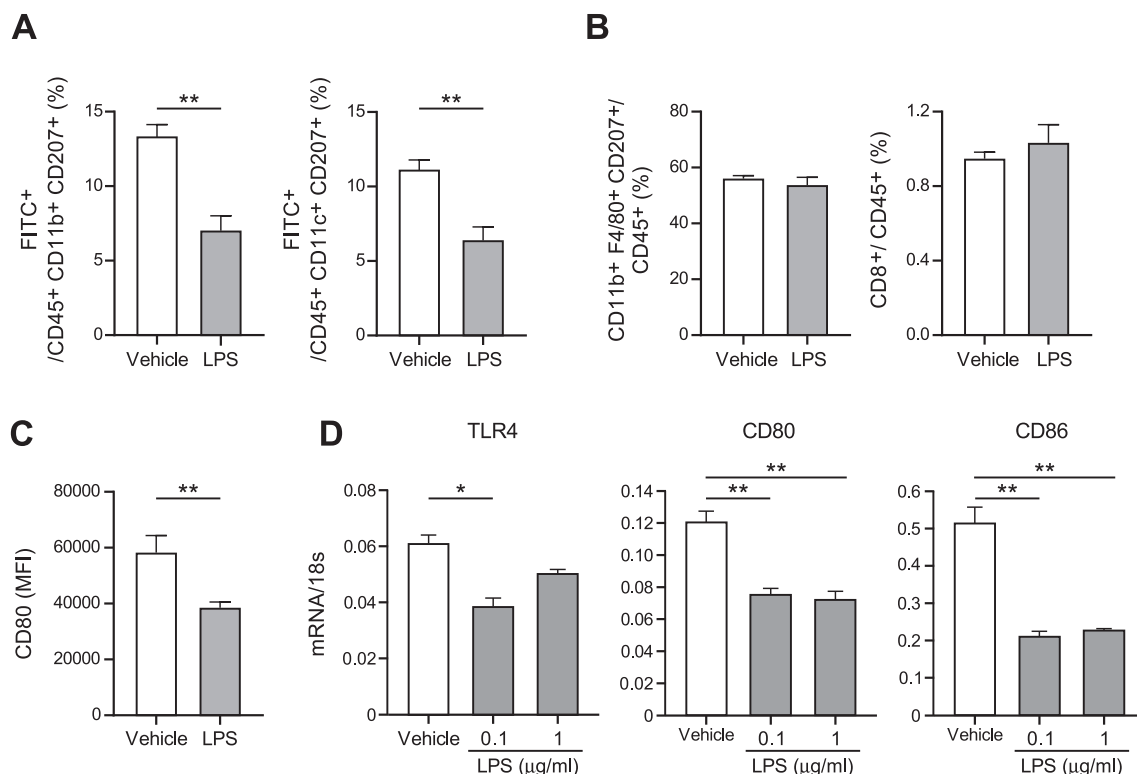


Fig. 3. LPS decreased phagocytosis and maturation markers of LCs

A. Phagocytosis assay. LPS was applied on the ear skin for 2 weeks. Skin cell suspension was incubated with FITC-Dextran (10 kDa, 0.5 mg/ml) for 45 min. Cells were analyzed using FACS analysis. % of FITC⁺ cells in CD45⁺ CD11b⁺ CD207⁺ cells (left) and CD45⁺ CD11c⁺ CD207⁺ cells (right). **B.** Single cell suspension of epidermis was analyzed using FACS analysis. % of CD11b⁺ F4/80⁺ CD207⁺ cells (left) and CD8⁺ cells (right) among CD45⁺ cells. (SE, n = 5). **C.** MFI of CD80 in CD45⁺ CD11b⁺ CD207⁺ cells using FACS analysis (mean ± SE, n = 5, **p < 0.01). **D.** XS52 cells were incubated with LPS (0.1 or 1 µg/ml) for 24 h. mRNA expression of indicated genes was quantified by real-time PCR and showed the expression level as ratio to 18s ribosome (mean ± SE, n = 5, *p < 0.05, **p < 0.01).

response to the DNFB challenge, evidence of CHS, was significantly decreased in LPS-treated mice compared to vehicle-treated mice (Fig. 1A, right). Histological examination confirmed that edema and infiltration of lymphocytes into the dermis were attenuated by LPS application (Fig. 1B). Next, LPS was applied on the ear skin for 6 days after DNFB treatment of the abdomen to examine the effect of LPS on the elicitation phase (Fig. 1C, left). In this setting, LPS application did not affect ear swelling as a CHS response (Fig. 1C, 1D). These results indicated that the application of LPS before sensitization with DNFB could suppress CHS response, suggesting that LPS specifically affected the sensitization phase during CHS response.

3.2. LPS application suppressed DNFB-induced sensitization during CHS

To examine the effect of LPS on sensitization during CHS response, antigen-specific cell proliferation and IFN-γ production were examined. LPS was applied for 2 weeks before DNFB treatment, as shown in Fig. 1A. Lymphocytes from the skin-draining LN of mice with DNFB or without DNFB (naïve) were isolated 5 days after DNFB sensitization and incubated with DNBS, a water-soluble compound with the same antigenicity as DNFB. Cell proliferation (Fig. 2A) and IFN-γ production (Fig. 2B) in response to DNBS stimulation were remarkably higher in the lymphocytes from DNFB-treated mice than in those from naïve mice. We observed a significant repression of antigen-induced cell proliferation and IFN-γ production in LPS-treated mice compared to vehicle-treated mice, indicating that T-cell activation during the sensitization phase is suppressed by LPS application (Fig. 2A and B).

An essential step in the sensitization phase for CHS is the migration of hapten-bearing cutaneous cells into the skin-draining LNs [11,13]. Currently, Langerhans cells and four types of DCs are identified in the skin, of which some types of cells can migrate through the lymphatic ducts into the skin-draining LNs in response to stimulation [22]. To assess the effect of LPS application on cutaneous cell migration into skin-draining LNs, we used the FITC-induced cutaneous cell migration assay model [17]. Mice were painted with FITC on the abdominal skin, and the FITC-capturing cells were detected in the skin-draining LNs after by fluorescence activated cell sorting (FACS) analysis. We found that in LPS-treated mice, the number of FITC-positive cells gated on the CD11c⁺ or CD11c⁺ CD207⁺ DCs that migrated from the skin to the draining LNs after FITC application significantly decreased (Fig. 2C). However, the total number of CD11c⁺ or CD11c⁺ CD207⁺ DCs draining into the LNs was unaffected by LPS application (Fig. 2D). These results suggested that LPS application impaired the migration of antigen-capturing cells, mainly DCs, into the skin-draining LNs, and subsequently impaired T-cell activation in the skin-draining LNs.

3.3. LPS decreased phagocytosis and maturation of LCs

Next, we determined the effect of LPS on LCs and DCs. First, we examined whether LPS application affected phagocytosis in DCs and LCs since *in vivo* migration of antigen-capturing cells had decreased by LPS application (shown in Fig. 2C). We utilized FITC-labeled dextran (10 kDa), a commonly used model substance for pinocytosis and phagocytosis [23]. The ear skin was collected after

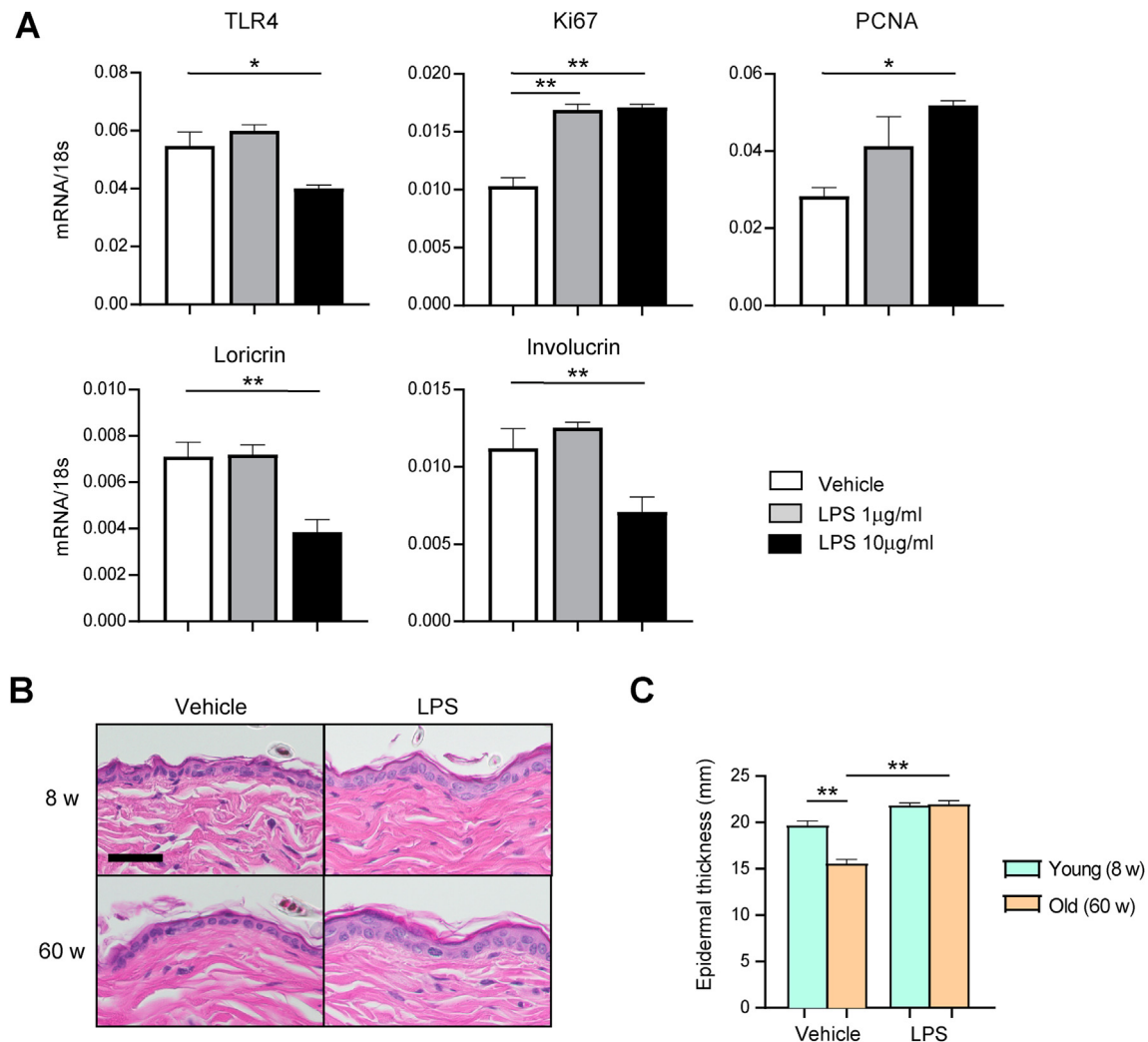


Fig. 4. LPS increased proliferation-associated genes in keratinocytes

A. Mouse keratinocytes PAM212 were incubated with LPS (1 or 10 µg/ml) for 24 h. mRNA expression of indicated genes was quantified by real-time PCR and showed the expression level as ratio to 18s ribosome (mean ± SE, n = 5, *p < 0.05, **p < 0.01). **B.** LPS was applied to the flank skin of young (8 weeks) or old (60 weeks) mice for 10 days. **B.** Representative hematoxylin and eosin staining of the ears. Scale bar = 200 µm. **C.** Thickness of epidermal layer (mean ± SE, n = 5, **p < 0.01).

2 weeks of LPS application. Single cell suspensions from the skin were incubated with FITC-dextran, and the mean fluorescence intensity (MFI) of the ingested FITC gated on CD45⁺ CD11b⁺ CD207⁺ LCs and CD45⁺ CD11c⁺ CD207⁺ DCs was analyzed by FACS analysis [16]. Fig. 3A shows that LPS-treated cutaneous cells had reduced cellular uptake of FITC-dextran, indicating reduced phagocytosis owing to LPS application. We next analyzed the number of LCs and the expression levels of LC activation markers in LPS-treated skin. FACS analysis showed that the number of LCs (CD45⁺ CD11b⁺ F4/80⁺ CD207⁺ cells) and CD8⁺ T cells in the epidermis were unaffected by LPS treatment (Fig. 3B). In contrast, LPS application significantly reduced the MFI of maturation markers CD80 on gated CD45⁺ CD11b⁺ F4/80⁺ CD207⁺ LCs (Fig. 3C), suggesting that LPS application suppresses LC maturation.

To directly examine the effect of LPS on LCs, we used the murine LC line XS52, which was derived from the epidermis of BALB/c mice [18]. XS52 cells were incubated with LPS for 24 h, and the mRNA expression levels were analyzed using quantitative real-time RT-PCR. We confirmed that XS52 expressed TLR4 (Fig. 3D), which is the LPS receptor, although it is still controversial whether TLR4 is expressed on LCs [24–27]. Supplementation of XS52 cells with LPS

reduced CD80 and CD86 expression levels (Fig. 3D). These results suggested the involvement of LPS in the LC maturation.

3.4. LPS increased proliferation-associated genes in keratinocytes

Keratinocytes are the major cells of the epidermis and may be affected by topical application of LPS. The TLR4 expression in keratinocytes remains controversial [25,28–30]. Therefore, we determined the effect of LPS on keratinocytes using the murine epidermal keratinocyte PAM212. PAM212 cells were incubated with LPS for 24 h, and the mRNA expression levels were analyzed using quantitative real-time RT-PCR. First, we confirmed that PAM212 expressed TLR4 (Fig. 4A). Fig. 4A shows that LPS increased the expression levels of Ki-67, a proliferation-associated nuclear antigen, and proliferation-associated nuclear antigen (PCNA). In contrast, LPS reduced the expression levels of loricrin and involucrin, both of which are major precursor proteins to the cornified cell envelope expressed in differentiated keratinocytes [31].

Next, we examined whether LPS application affects keratinocyte proliferation in young (8 weeks) and old (60 weeks) mouse skin. In general, the thickness of the epidermis is thinner in aged skin than

in young skin, which is attributed to impaired cell proliferation with aging [32]. We verified that the epidermis was thinner in aged skin than in young skin using hematoxylin and eosin staining (Fig. 4B and C). Quantification of epidermal thickness showed that LPS application restored the epidermal thinning found in older skin (Fig. 4C), while LPS had less effect on the epidermal thickness of young skin. These results suggest the involvement of LPS in keratinocyte proliferation.

4. Discussion

In this study, we found that prolonged topical application of LPS suppressed CHS response in the experimental mouse CHS model. In contrast, short-term application (data not shown) or application after sensitization did not affect CHS (Fig. 1C and D). Together, these results indicate that prolonged application of LPS was effective during the sensitization phase. Moreover, LPS application reduced antigen phagocytosis by LCs and DCs and decreased the number of migrated cells carrying antigens from the skin into the skin-draining LNs, resulting in the suppression of T-cell activation in LNs. Consequently, the swelling of the ear, as a response to CHS, was suppressed by LPS application. Our results imply that prolonged topical application of LPS can be applied for treating inflammatory skin diseases, including CHS and AD. Previous studies have reported that the oral administration of LPS attenuated the symptoms of AD with less inflammation in a murine AD experimental model, but the underlying mechanism of action remained unclear [8]. In patients with mild atopic dermatitis, topical application of an LPS-containing cream alleviated AD symptoms [9]. Further studies on the mechanism by which topical LPS acts on the skin may reveal the optimal amount of LPS to be used in skin diseases, including AD, leading to the development of new therapies.

Previous studies have identified that DCs in the skin can be classified into LCs, which reside in the epidermis, and other types of DCs located in the dermis [14,33]. LCs have long been considered to play an important role in detecting and transporting antigens to LNs and presenting them to other immune cells such as naive T cells to initiate an immune response [11,13,15]. In contrast, a recent study revealed that the newly identified Langerhans-like cells in the dermis migrate to skin-draining LNs, while the LCs do not migrate to the LNs [22]. In this study, we found that after the application of FITC on the abdominal skin, the CD11c⁺ CD207⁺ cells that captured the painted FITC were detected in skin-draining LNs. Our analysis does not allow us to determine whether these FITC-positive cells were derived from the epidermis or dermis, as CD11c⁺ CD207⁺ cells are derived from both the epidermis and dermis [22]. Nevertheless, our results showed that the number of FITC-incorporated cells that migrated into the skin LNs had decreased following LPS application. This result implies that LPS application may reduce the migration of DNFB-incorporated cells into the skin-draining LN during DNFB-induced CHS response, leading to suppressed sensitization. Further studies are needed to identify the cells involved in suppressing CHS response following LPS application and to elucidate the mechanism of LPS effects through these cells.

Here, we showed that the application of LPS to the aged skin restored the epidermal thinning. *In vitro* experiments with cultured keratinocytes, LPS increased the expression levels of cell proliferation markers (at concentrations above 1 µg/ml, shown in Fig. 4A), suggesting the direct effect of LPS on keratinocyte proliferation. On the other hand, LPS affected LC cells at a 10-fold lower concentration than that for keratinocytes (Fig. 3D). These results implicate that applied LPS might have directly affected the keratinocytes or promoted keratinocyte proliferation via LC cells in the skin. Although further studies are necessary to determine the underlying mechanism, our results may lead to the development of an

application of LPS to improve the aged skin.

In summary, our data suggested that a prolonged topical application of LPS suppresses hapten-induced CHS with impaired sensitization. Future studies may provide an optimal treatment strategy for allergic dermatitis using LPS.

Declaration of competing interest

The authors declare no competing interests.

Data availability

No data was used for the research described in the article.

Acknowledgements

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