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# Nucleotide-binding oligomerization domain protein-1 is expressed and involved in the inflammatory response in human sebocytes

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ABSTRACT

Sebocytes express Toll-like receptors (TLRs) and nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), which participate in the innate immune response of the skin. Although the roles of TLRs and NLR family pyrin domain-containing 3 (NLRP3) in inflammatory responses in sebocytes have been reported, the expression and functions of other NLR members, such as NOD protein-1 and -2 (NOD1 and NOD2, respectively), remain unclear. In this study, we showed that, in sebocytes, the expression of NOD1 is higher than that of NOD2, and that NOD1 is involved in inflammatory responses, such as the secretion of proinflammatory cytokines. A NOD1 agonist, L-alanyl-γ-D-glutamyl-meso-diaminopimelic acid (Tri-DAP) induced the expression and secretion of interleukin-8 (IL-8) and activated the nuclear factor-kappa B and mitogen-activated protein kinase signaling pathways. On the other hand, a NOD2 agonist, muramyl dipeptide, did not. Either inhibition with a NOD1 inhibitor, ML130, or knockdown of NOD1 expression abolished Tri-DAP-induced inflammatory responses, suggesting that NOD1 is involved in the immunogenic signaling system of sebocytes. Furthermore, Tri-DAP and an agonist of TLR2 or TLR4 additively increased IL-8 expression compared with each agonist alone. Our results reveal the role of NOD1 in the inflammatory responses of sebocytes and may provide a novel therapeutic target for sebaceous gland inflammatory diseases, such as acne vulgaris.

## 1. Introduction

Innate immunity is the first defense system against pathogenic infections. When pathogens invade the body, pattern recognition receptors (PRRs) sense and respond to microorganism-specific conserved structures called pathogen-associated molecular patterns (PAMPs) [1]. PRRs are roughly classified into five families, among which are the Toll-like receptors (TLRs) and the nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs). PRRs can be either transmembrane receptors or cytosolic receptors [2]. TLRs belong to the former receptor group and recognize specific ligands in the extracellular space and within the endosomes. For example, TLR2 and TLR4 are activated by bacterial cell wall components, such as the lipoteichoic acid (LTA) of Gram-positive bacteria and lipopolysaccharide (LPS) of Gram-negative bacteria, respectively. On the other hand, NLRs belong to the latter receptor group, and NOD protein-1 and -2 (NOD1 and NOD2, respectively) sense the bacterial peptidoglycan motif intracellularly. Ligand binding to these receptors activates various signaling pathways, including nuclear factor-kappa B (NF-κB) and mitogen-activated protein kinase (MAPK), leading to the production of inflammatory cytokines and chemokines [3]. It is known that TLRs and NLRs interact with each other and affect their immune signal activities. Co-stimulation with TLR and NOD1/2 agonists amplifies NF-κB activity and causes a synergistic inflammatory response in several immune cells [4–6]. In contrast, continued NOD2 activation reduces TLR2-or TLR4-mediated tumor necrosis factor-α (TNFα) production in human monocyte-derived macrophages [7].

The expression and functions of PRRs in the skin have been well characterized in immune cells, such as mast cells and neutrophils, and keratinocytes [8]. While sebocytes reside in sebaceous glands attached to hair follicles and physiologically secrete sebum and hormones, the presence of TLR2 and TLR4 in sebocytes indicates that sebocytes are involved in innate immunity [9,10]. In addition, it has been reported that sebocytes contribute to the inflammatory response, together with keratinocytes, against *Cutibacterium acnes*, a major member of the normal skin microbiota [11]. Compared to TLRs, studies on NLRs in sebocytes are still relatively scarce. Although several recent reports have shown that the NLR family pyrin domain containing 3 (NLRP3) is expressed in sebocytes and functions as an innate immune sensor [12], the expression of NOD1 and NOD2 in sebocytes has not yet been investigated. The present study demonstrates for the first time that

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**Abbreviations**

| | |
|---|---|
| *C. acnes* | *Cutibacterium acnes* |
| DEGs | Differentially Expressed Genes |
| ELISA | Enzyme-linked immunosorbent assay |
| ERK | Extracellular signal-regulated kinase |
| FBS | Fetal bovine serum |
| fc | fold change |
| HRP | Horseradish peroxidase |
| IL-8 | Interleukin-8 |
| JNK | c-Jun-N-terminal kinase |
| KEGG | Kyoto Encyclopedia of Genes and Genomes |
| LPS | Lipopolysaccharide |
| LTA | Lipoteichoic acid |
| MAPK | Mitogen-activated protein kinase |
| MDP | Muramyl dipeptide |
| mRNA | Messenger RNA |
| NF-κB | Nuclear factor-kappa B |
| NLR | NOD-like receptor |
| NLRP3 | NLR family pyrin domain-containing 3 |
| NOD | Nucleotide-binding oligomerization domain |
| NOD1 | NOD protein-1 |
| NOD2 | NOD protein-2 |
| NT | Non-targeting |
| PAMPs | Pathogen-associated molecular patterns |
| PRRs | Pattern recognition receptors |
| qPCR | Quantitative polymerase chain reaction |
| RPMI | Roswell Park Memorial Institute |
| RT-PCR | Reverse transcription PCR |
| *S. epidermidis* | *Staphylococcus epidermidis* |
| siRNA | Small interfering RNA |
| TBS-T | Tris-buffered saline containing 0.1% Tween-20 |
| TLR | Toll-like receptor |
| TNFα | Tumor necrosis factor-α |
| Tri-DAP | L-Alanyl-γ-D-glutamyl-meso-diaminopimelic acid |

human sebocytes express higher levels of NOD1 than that of NOD2 and that a NOD1 agonist induces inflammatory responses, such as proinflammatory cytokine expression and secretion, together with activation of NF-κB and MAPK signaling. In addition, the co-activation of NOD1 and TLRs induces a more profound increase in the interleukin-8 (IL-8) production of sebocytes.

## 2. Materials and methods

### 2.1. Materials

L-alanyl-γ-D-glutamyl-meso-diaminopimelic acid (Tri-DAP) and muramyl dipeptide (MDP) were purchased from InvivoGen (San Diego, CA, USA). Lipoteichoic acid (LTA), lipopolysaccharide (LPS), and ML130 were purchased from Sigma-Aldrich (St. Louis, MO, USA). Anti-phospho-NF-κB p65 (p-p65) (#3033, 1:5000), anti-NF-κB p65 (p65) (#8242, 1:5000), anti-phospho-p38 (p-p38) (#9211, 1:5000), anti-p38 (#9212, 1:5000), anti-phospho-c-Jun N-terminal kinase (p-JNK) (#4668, 1:1000), anti-JNK (#9258, 1:1000), anti-phospho-extracellular signal-regulated kinase1/2 (p-ERK) (#4370, 1:5000), anti-ERK (#9102, 1:5000), and horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (#7074) antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). Anti-beta actin antibody (#ab8226, 1:5000) was purchased from Abcam (Cambridge, MA, USA). HRP-conjugated anti-mouse IgG antibody (NA931) was purchased from GE Healthcare Biosciences (Piscataway, NJ, USA). All the other reagents used were of the highest commercially available grade.

### 2.2. Cell cultures

Three lots of human sebocytes (KAC, Kyoto, Japan) were used in this study: Lot.1 (phototype, IV; age, 42; sex, female; localization, abdomen); Lot.2 (phototype, Caucasian; age, 50; sex, female; localization, face); and Lot.3 (phototype, Caucasian; age, 57; sex, female; localization, face). The sebocytes were maintained in Dulbecco's modified Eagle medium/F-12 (Thermo Fisher Scientific, Watham, MA, USA) supplemented with GlutaMAX™ (2 mM, Thermo Fisher Scientific), 2-(4-(2-Hydroxyethyl)-1-piperazinyl)ethanesulfonic acid (10 mM), human epidermal growth factor (50 ng/mL), and 10% heat-inactivated fetal bovine serum (FBS). THP-1 cells (JCRB, Tokyo, Japan) were cultured in Roswell Park Memorial Institute (RPMI) Medium 1640 (Thermo Fisher Scientific) supplemented with 10% heat-inactivated FBS. Both cell lines were grown in a humidified incubator at 37 °C in an atmosphere containing 5% $CO_2$. The medium was changed every alternate day, and the cells were passaged when they reached 80–90% confluency. The medium was changed daily once the cells reached 50% confluency. The cells were not used beyond six passages. Sebocytes were seeded at a density of $1.0 \times 10^5$ cells/well in a 6-well plate or $5.0 \times 10^4$ cells/well in a 12-well plate. Lot. 1 sebocytes were used for all experiments, except for generating the data in Fig. 1, for which all three lots were used. The cellular quality of sebocytes was confirmed by examining their abilities to produce lipids and the steroid hormone, testosterone (Supplementary Fig. 1).

### 2.3. Reverse transcription-polymerase chain reaction (RT-PCR) and quantitative PCR (qPCR)

Total RNA was isolated from cultured cells using an RNeasy Mini Kit (QIAGEN, Valencia, CA, USA), according to the manufacturer's instructions, and the RNA concentrations were measured using an Ultrospec 4300 Pro UV/Visible spectrophotometer (Biochrom, Cambridge, UK). The RNA was stored in ribonuclease-free water at -80 °C until reverse transcription. First-strand cDNA was synthesized with 0.3–1 μg of total RNA using a PrimeScript™ II 1st strand cDNA Synthesis Kit (Takara Bio, Shiga, Japan), according to the manufacturer's instructions. For RT-PCR gene expression studies, cDNA was mixed with KOD Plus (Toyobo, Osaka, Japan) DNA polymerase and gene-specific primer. The primers used were as follows: human *NOD1*, forward 5'-CCACTTCACAGCTGGAGACA-3', and reverse 5'-TGAGTGGAAGCAGCATTTTG-3' for a 209-bp fragment; human *NOD2*, forward 5'-CTCCATGGCTAAGCTCCTTG-3' and reverse 5'-CACACTGCCAATGTTGTTCC-3' for a 244-bp fragment; and human *GAPDH*, forward 5'-GAGTCAACGGATTTGGTCGT-3' and reverse 5'-TTGATTTTGGAGGGATCTCG-3' for a 238-bp fragment. The PCR conditions on a GeneAmp® PCR System 9700 (Applied Biosystems, Foster City, CA, USA) were 30 cycles at 98 °C for 10 s, 60 °C for 5 s and 72 °C for 15 s. PCR products were separated on a 2% agarose gel. For absolute quantification of *NOD1*, *NOD2*, and *NLRP3* by qPCR, the standard curve method was applied. The standard curves of *NOD1*, *NOD2*, *NLRP3*, and *18S* were designed based on known quantities of synthetic DNAs containing the specific sequences of each TaqMan® assay location indicated by Applied Biosystems. For qPCR, target gene messenger RNA (mRNA) expression levels were measured using an Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) with the following TaqMan® Gene Expression Assays: *NOD1* (assay ID Hs01036720_m1), *NOD2* (assay ID Hs01550753_m1), *NLRP3* (assay ID Hs00918082_m1), *CXCL8* (assay ID Hs00174103_m1), and *18S* (assay ID Hs99999901_s1). All reactions were performed in triplicate. *18S* was

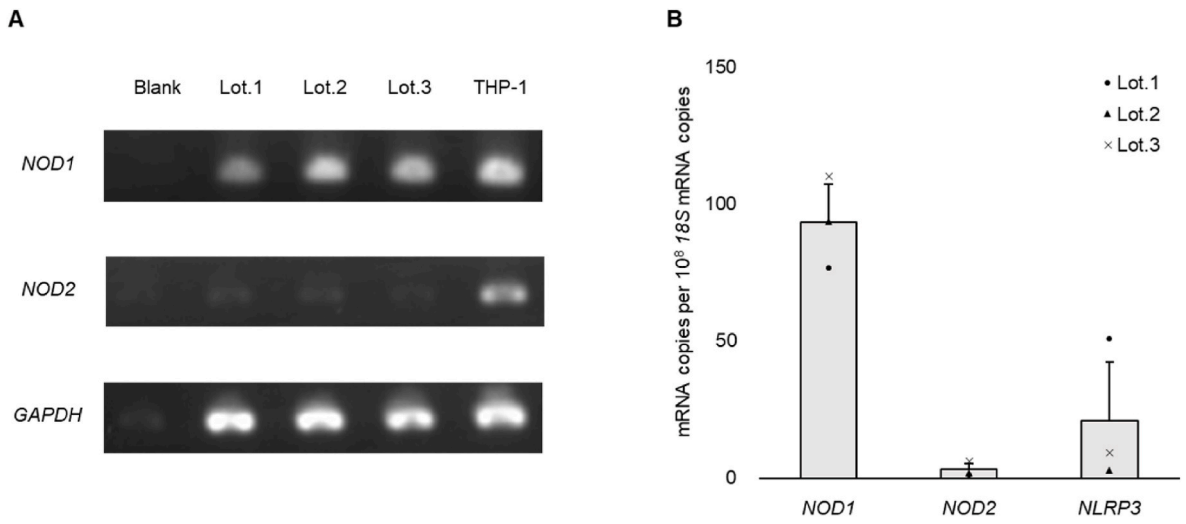



**Fig. 1.** The gene expression levels of nucleotide-binding oligomerization domain (NOD) protein-1 and -2 (*NOD1* and *NOD2*, respectively) in human sebocytes. (A) The gene expression levels of *NOD1*, *NOD2*, and *GAPDH* in three different lots of sebocytes and THP-1 cells were investigated by reverse transcription polymerase chain reaction (RT-PCR). "Blank" refers to a non-template control. (B) The absolute quantification analyses of *NOD1*, *NOD2*, and NOD-like receptor family pyrin domain-containing 3 (*NLRP3*) mRNA expression in sebocytes were performed by quantitative (q)PCR. The mRNA copy number of *NOD1*, *NOD2*, *NLRP3*, and *18S* determined from a standard curve of serial dilution standards is shown in the graph as target mRNA copies per $10^8$ *18S* mRNA copies. Data are presented as mean ± standard deviation of three lots.

used as the housekeeping gene to normalize the amount of RNA. Relative mRNA amounts were calculated with reference to the untreated control group or non-targeting (NT) small interfering (si) RNA-treated group using the comparative $C_T$ method. It was confirmed that no solvent used for reagent preparation affected *CXCL8* mRNA expression at the concentration used in this study by qPCR analysis (Supplementary Fig. 2).

### 2.4. RNA sequencing and analysis

Sebocytes were cultured for 4 h in the presence or absence of Tri-DAP at a concentration of 10 μg/mL. Three independent experiments were conducted, each using Lot.1 sebocytes (see section **Cell cultures**). The total RNA of each sample was prepared by the RNA isolation method described above. Each sample was pretreated with RNase-free DNase (QIAGEN) during the RNA isolation process to prevent DNA contamination, according to the manufacturer's instructions.

RNA sequencing was performed by Macrogen Japan (Tokyo, Japan). The TruSeq Stranded mRNA Library Prep Kit (Illumina, San Diego, CA, USA) was used for library preparation. The libraries were sequenced using the NovaSeq6000 (Illumina), producing 40 million reads (100bp paired-end) per sample. DESeq2 was used to analyze Differentially Expressed Genes (DEGs) to compare the expression levels between Tri-DAP-treated and -untreated groups. Data precision and consistency were considered significant when the fold change (fc) and *p*-value were ±2-fold and $<0.05$, respectively. The gene-set enrichment analyses on DEGs were performed using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. The software used in the analyses of DEGs and their gene-set enrichment were follows: FastQC v0.11.7, Trimmomatic v0.38, HISAT2 v2.1.0, Bowtie2 v2.3.4.1, and StringTie v2.1.3b. Sequencing data have been deposited into NCBI's Sequence Read Archive database under BioProject accession no. PRJNA1029725.

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

The concentrations of IL-8 in the supernatants of cultured sebocytes were determined using a Human IL-8 ELISA Kit (Proteintech, Rosemont, IL, USA) according to the manufacturer's instructions. Briefly, the culture supernatant samples were dispensed into each well of an IL-8-specific antibody-coated microplate and incubated for 2 h. The wells were incubated with an IL-8 specific antibody solution for 1 h. After another incubation with HRP-conjugated secondary antibody for 40 min, a 3,3′,5,5′-tetramethylbenzidine solution was added, and the samples incubated at room temperature until adequate color developed. Color development was stopped by adding a stop solution to each well and the absorbance at 450 nm was measured using an Infinite 200 Pro plate reader (Tecan, Männedorf, Switzerland).

### 2.6. Western blotting analysis

Cells were washed twice with phosphate-buffered saline and lysed in lysis buffer (20 mM Tris-HCl [pH 7.5], 150 mM NaCl, 10 mM calcium disodium edetate [pH 8.0], and 1% Triton-X 100) containing protease and phosphatase inhibitor cocktails. The lysates were centrifuged (13,200 rpm for 5 min at 4 °C), and the protein concentration was measured using the BCA protein assay kit (Thermo Fisher Scientific) relative to the albumin standard (Thermo Fisher Scientific). The supernatants were mixed with sample buffer (250 mM Tris-HCl pH 6.8, 20% 2-mercaptoethanol, 8% sodium dodecyl sulfate, 20% sucrose, and 40 μg/μL bromophenol blue). The samples containing 1 μg protein were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis on a 10% gel. Proteins were transferred to polyvinylidene difluoride membranes using a semi-dry blotting apparatus (Bio-Rad Laboratories, Hercules, CA, USA) for 1 h at 100 mV. The membranes were then incubated in a blocking solution (5% non-fat dry milk in Tris-buffered saline containing 0.1% Tween-20 [TBS-T]). After washing thrice with TBS-T, the membranes were incubated with respective primary antibodies overnight at 4 °C. Next day, after washing three times with TBS-T, the membranes were incubated with HRP-conjugated secondary antibodies at room temperature for 2 h. Bands were visualized using ECL Prime Western Blotting Detection Reagent (Cytiva, Tokyo, Japan) or ImmunoStar LD (Wako, Tokyo, Japan) according to the manufacturer's instructions. Band intensity was analyzed using Image J software.

### 2.7. RNA silencing

For siRNA studies, siRNAs targeting *NOD1* were purchased from Horizon Discovery (Cambridge, UK). Non-targeting siRNA (Horizon Discovery) was used as the negative control. Sebocytes were seeded at a density of $5.0 \times 10^4$ cells in six-well plates and transfected with 19.2 nM

of *NOD*1 or scramble siRNA plus Lipofectamine™ 2000 Transfection Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions for mRNA silencing in the cells. The efficiency of siRNA-mediated repression of target mRNA levels was assessed by qPCR.

### 2.8. Statistical analyses

Data are expressed as the mean ± standard deviation from at least three independent experiments. The statistical analyses were performed using the software Statcel3 (OMS, Tokyo, Japan). Data were analyzed using one-way analysis of variance, and differences among means were analyzed using the Tukey–Kramer test. Statistical significance was set at a *p*-value of less than 0.05.

## 3. Results

### 3.1. Human sebocytes express NOD1 more highly than NOD2 in addition to NLRP3

To examine the expression of *NOD1* and *NOD2* in human sebocytes, RT-PCR analyses were performed. The human monocytic cell line THP-1 was used as the positive control for both genes. *NOD1* was clearly

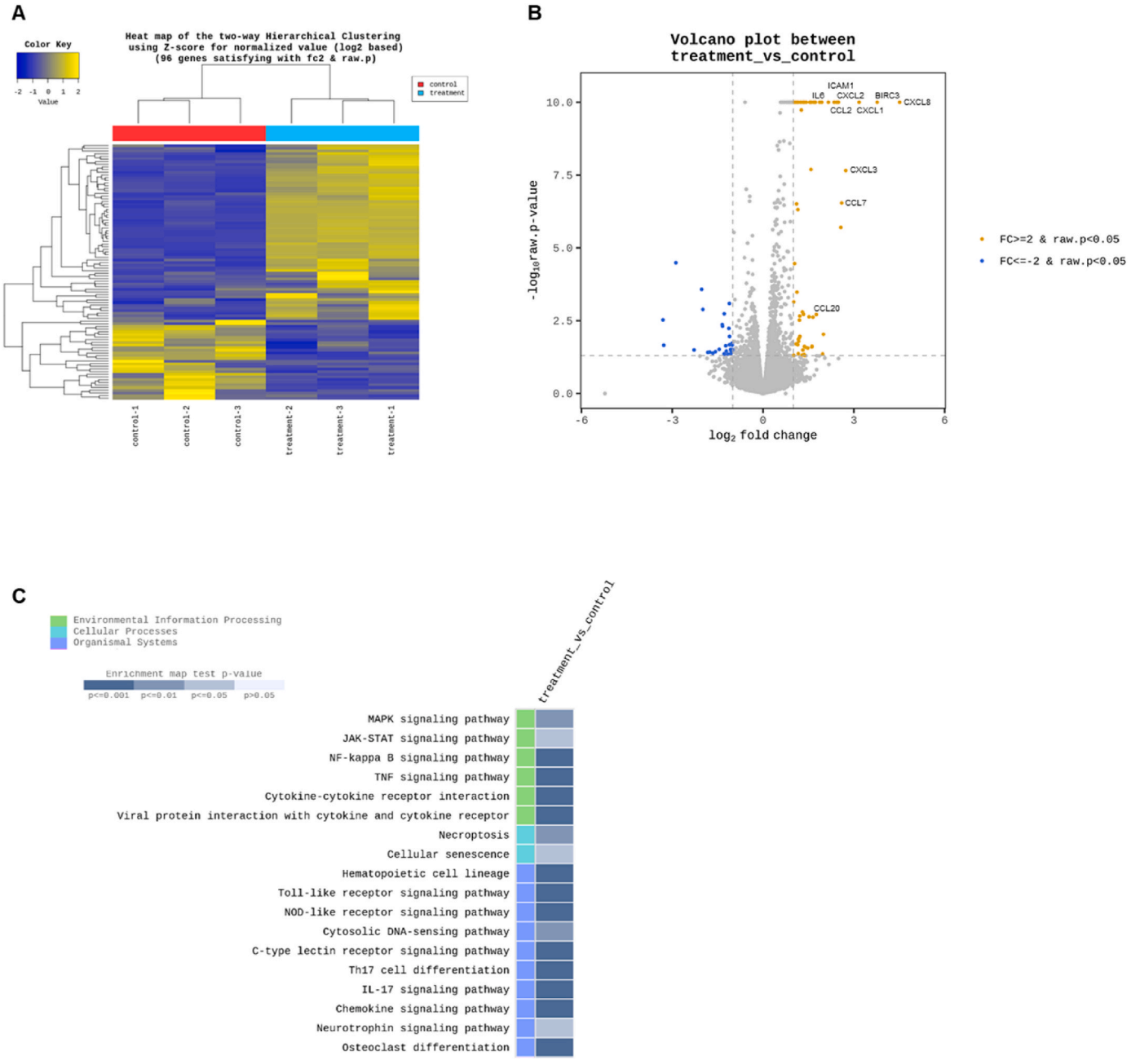



**Fig. 2.** Graphical depiction of Differentially Expressed Genes (DEGs) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses. (A) Hierarchical clustering analysis of untreated- (represented as control) and L-alanyl-γ-D-glutamyl-meso-diaminopimelic acid (Tri-DAP)-treated (represented as treatment) sebocytes based on the DEG list. Gene expression level, satisfying |fold change (fc)| ≥ 2 and raw *p*-value <0.05 conditions, is indicated by the color scales. (B) Volcano plotting of significantly altered genes in the treatment vs. control comparison. Log2 fc and *p*-value obtained from the comparison between the two groups is plotted. (X-axis: log2 fc, Y-axis: −log10 *p*-value). The yellow and blue plots represent genes with significantly upregulated or downregulated expression in the treatment vs. control comparison. (C) KEGG pathway enrichment analysis of DEGs. KEGG enrichment map score (*p*-value) is indicated by the color scale. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

detected in all three sebocyte lots, whereas *NOD2* was barely detectable (Fig. 1A). Furthermore, the expression levels of *NOD1* and *NOD2* were compared using qPCR for absolute quantification. As shown in Fig. 1B, *NOD1* expression was higher than that of *NOD2*. In addition, sebocytes were found to constitutively express *NOD1* at a level equal to or higher than that of the other NLR member, *NLRP3*, which is reported to be primarily expressed in human sebocytes.

### 3.2. Whole transcriptome sequencing reveals that NOD1 activation induces innate immune responses in human sebocytes

NOD1 is an important receptor with roles in the innate immune system including sensing and responding to the bacterial peptidoglycan motif, such as Tri-DAP. To investigate the immune-related functions of NOD1 in human sebocytes, we analyzed the changes in intracellular genes during NOD1 activation by Tri-DAP via an RNA sequencing experiment. The analysis of DEGs between untreated and treated sebocytes with Tri-DAP (control and treatment groups, respectively) showed differential expression of 66 upregulated and 30 downregulated mRNAs,

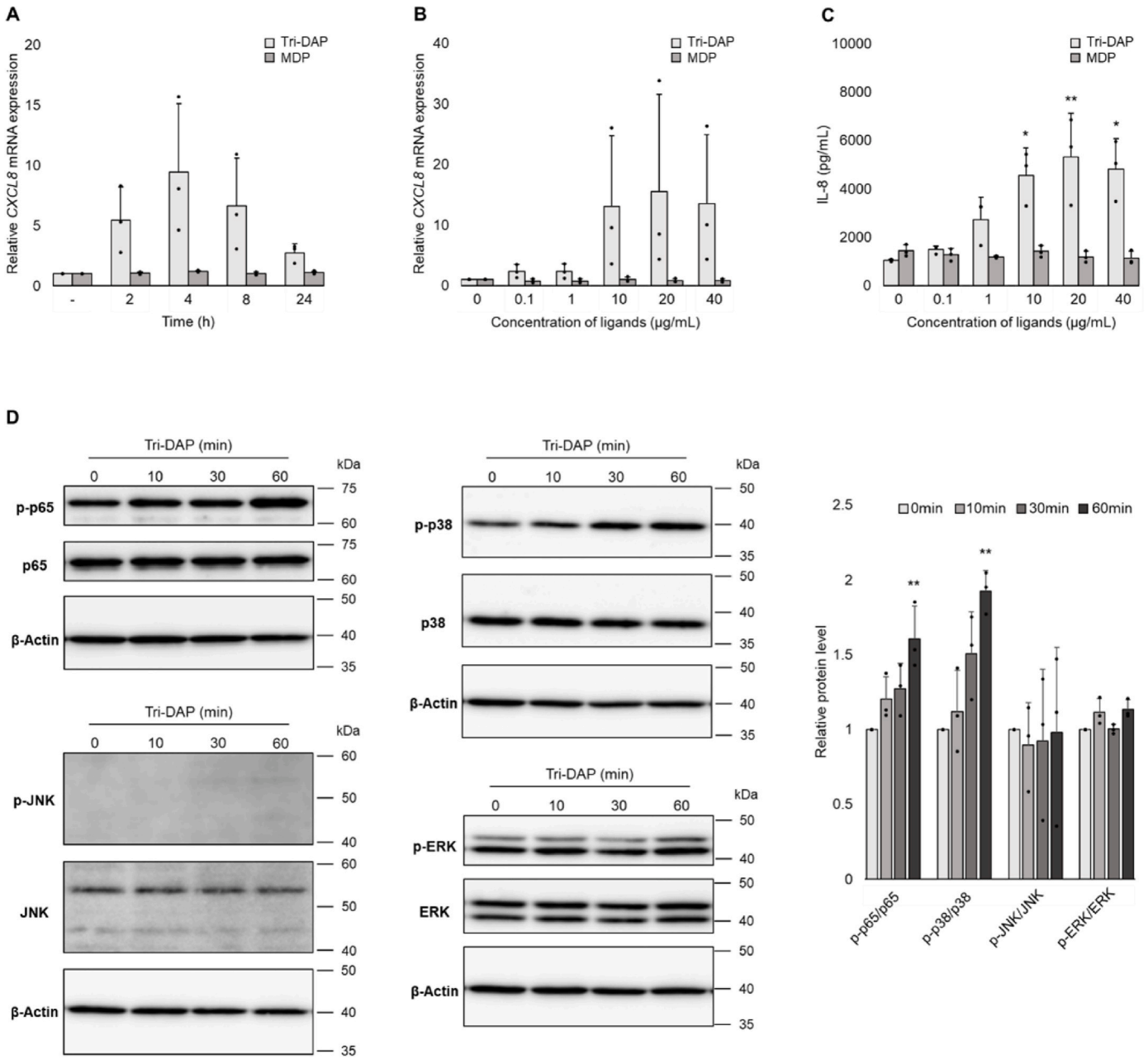



**Fig. 3.** The inflammatory responses in sebocytes induced by L-alanyl-γ-D-glutamyl-meso-diaminopimelic acid (Tri-DAP) or muramyl dipeptide (MDP) stimulation. (A, B) Sebocytes were stimulated with Tri-DAP or MDP at 10 μg/mL concentration for the indicated hours or at the indicated concentrations for 4 h. The expression levels of *CXCL8* were examined using qPCR. Data are presented as mean ± standard deviation (SD) of three independent experiments from one lot. (C) Sebocytes were stimulated with the indicated doses of Tri-DAP or MDP for 24 h. Interleukin-8 (IL-8) concentration in the cell media was measured by ELISA. Data are presented as mean ± SD of three independent experiments from one lot. (D) Sebocytes were stimulated with 10 μg/mL Tri-DAP for the indicated minutes. The phosphorylation of p65, p38, c-Jun N-terminal kinase (JNK), and extracellular signal-regulated kinase (ERK) proteins was detected by western blotting. β-Actin was used as a loading control. Data are presented as mean ± SD of three independent experiments from one lot. *$p<0.05$; **$p<0.01$.

which satisfied $|fc| \geq 2$ and raw $p$-value $<0.05$ conditions (Fig. 2A and B and Supplementary Table 1). Tri-DAP treatment induced increased expression of several immune-related genes, including inflammatory cytokines and chemokines, such as *CXCL8* and *CCL7*. Furthermore, as shown in Fig. 2C, the signaling pathway enrichment of the DEGs was examined by KEGG enrichment analysis, which revealed that Tri-DAP treatment significantly changed inflammation-related signaling pathways such as MAPK, NF-κB, and TLR signaling pathways. These results suggest that NOD1 activation causes immune responses in human sebocytes.

### 3.3. A ligand for NOD1 induces the expression of proinflammatory cytokine IL-8 and activates the NF-κB and MAPK signaling pathways

Next, we evaluated inflammatory reactions in response to NOD1 or NOD2 stimuli in sebocytes. NOD1 and NOD2 recognize the peptidoglycan motifs Tri-DAP and MDP, respectively. After stimulation of sebocytes with Tri-DAP or MDP for 2–24 h, mRNA levels of the proinflammatory cytokine IL-8 were examined. Tri-DAP treatment increased the gene expression of *CXCL8* (the gene that encodes IL-8) with a peak at 4 h, whereas stimulation with MDP did not show any observable expression of *CXCL8* up to 24 h (Fig. 3A). Dose-dependent experiments also demonstrated that Tri-DAP induced *CXCL8* mRNA expression and significantly increased IL-8 secretion at concentrations of ≥10 μg/mL (Fig. 3B and C). In contrast, MDP had no effect on either mRNA expression or protein release of IL-8 at any concentration. Furthermore, 0.1–40 μg/mL Tri-DAP did not affect cell viability, proliferation, and sebaceous lipogenesis (Supplementary Figs. 3A–C). These data suggest that NOD1 contributes more decisively to the inflammatory system in sebocytes than does NOD2. NOD1 stimulation is known to activate the NF-κB and MAPK signaling pathways to provoke immune responses in various cell types [13]. To investigate whether NOD1 stimulation induces the activation of NF-κB and MAPK in sebocytes as well, we determined the phosphorylation of the NF-κB subunit p65 and MAPKs (p38, JNK, and ERK). Western blotting analyses demonstrated that Tri-DAP significantly induced the phosphorylation of p65 and p38 within 60 min (Fig. 3D). However no significant change in phosphorylation of JNK and ERK was observed by Tri-DAP treatment. These results suggest that the detection of bacterial peptidoglycan motifs by NOD1 induces IL-8 expression by activating NF-κB and MAPK signaling in sebocytes.

### 3.4. Pharmacological inhibition or knockdown of NOD1 suppresses Tri-DAP-induced inflammatory events

To clarify whether NOD1 mediates the inflammatory responses induced by Tri-DAP in sebocytes, we examined the effects of the NOD1 inhibitor ML130 on IL-8 expression and NF-κB/MAPK signaling in Tri-DAP-stimulated cells. ML130 pretreatment for 30 min significantly suppressed Tri-DAP-induced increase in *CXCL8* mRNA expression and IL-8 protein secretion (Fig. 4A and B). ML130 also inhibited Tri-DAP-induced p65 and p38 phosphorylation (Fig. 4C). To address this issue further, we transfected sebocytes with NT siRNA or siRNA targeting NOD1. NOD1 siRNA treatment decreased *NOD1* mRNA and protein expression levels by approximately 50% and 30%, respectively (Supplementary Figs. 4A and B) and significantly abolished the increase in IL-8 mRNA expression and protein release induced by Tri-DAP (Fig. 4D and E). Furthermore, there was no significant difference in cell viability between NT or NOD1 siRNA treated cells (Supplementary Fig. 5). These results indicate that inhibition of NOD1, either pharmacologically by ML130 or by NOD1-targeting siRNA, suppresses the Tri-DAP-induced inflammatory responses, suggesting that NOD1 is involved in the immunogenic inflammatory signaling system in human sebocytes.

### 3.5. Co-stimulation of NOD1 and TLR2/TLR4 agonists induces higher IL-8 expression than either of the agonists alone

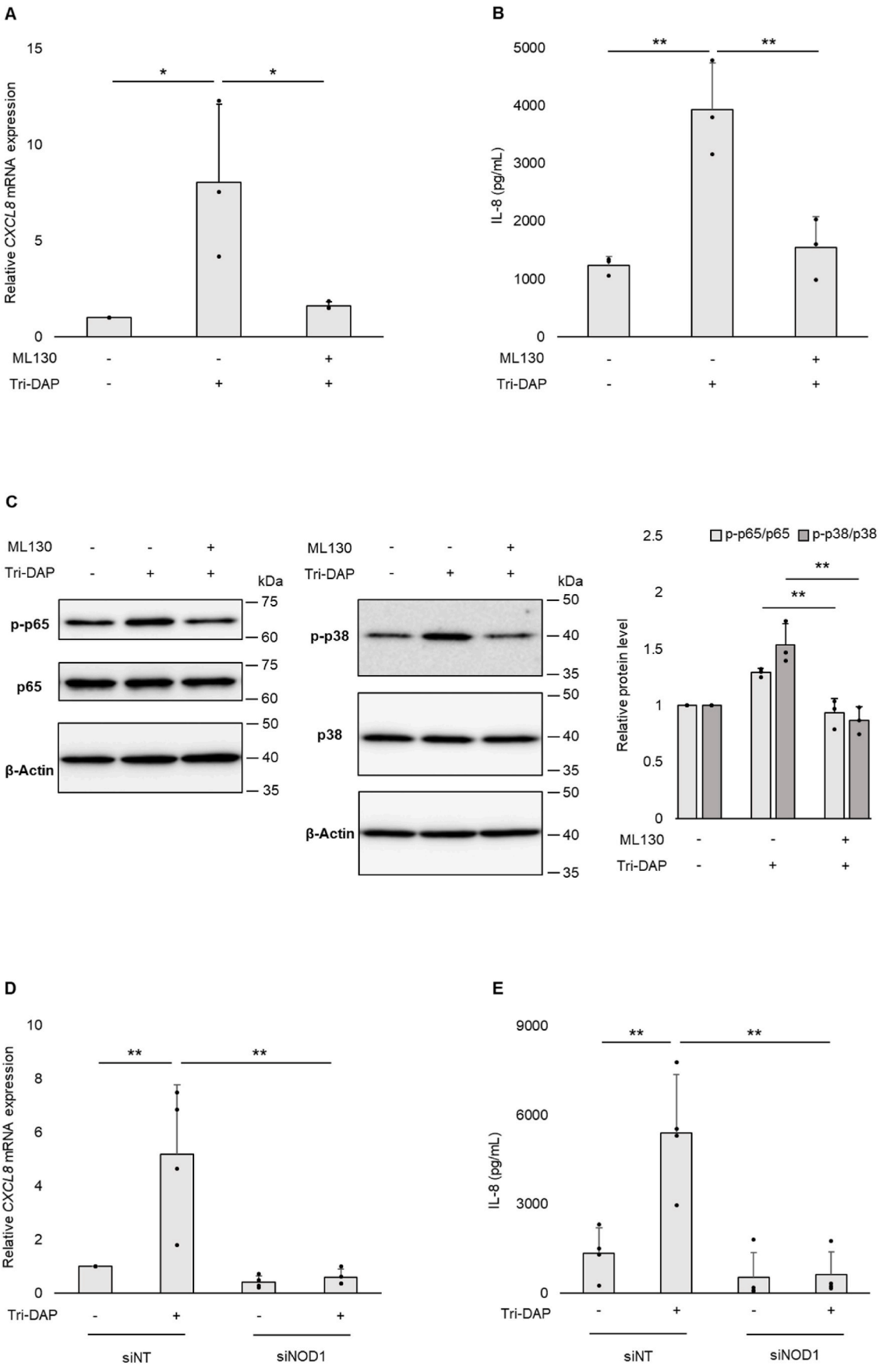
To evaluate the crosstalk between NOD1 and TLR2 signaling for proinflammatory cytokine production in sebocytes, cells were stimulated with Tri-DAP, LTA (a TLR2 agonist), or a combination of both agents. The combination of Tri-DAP and LTA stimulation increased IL-8 mRNA expression and significantly increased protein secretion compared with either stimulus alone (Fig. 5A and B). Furthermore, we also examined the crosstalk between NOD1 and TLR4 signaling. Co-stimulation with Tri-DAP and LPS (a TLR4 agonist) augmented IL-8 expression and release compared to either stimulus alone (Fig. 5C and D). These results show that the co-activation of NOD1 and TLRs coordinately increased proinflammatory cytokine IL-8 production in sebocytes.

## 4. Discussion

It has been widely reported that sebocytes contribute to innate immunity in the skin as one of the immunocompetent cells. Although the role of TLRs in inflammation of sebaceous glands has been well documented, data regarding NLRs in sebocytes are still missing. In recent years, several reports have shown that NLRP3 may also act as one of the main PRRs in sebocytes, together with TLRs; therefore, the expression and function of NLRs in sebocytes has received increased attention. To the best of our knowledge, this is the first evidence that human sebocytes express *NOD1*. We showed that a NOD1 agonist, Tri-DAP, activated the NF-κB and MAPK signaling pathways and increased proinflammatory cytokine IL-8 expression (Fig. 6). In contrast, *NOD2* expression was also detected but was much weaker than that of *NOD1* and *NLRP3*. In addition, the NOD2 agonist MDP failed to induce IL-8 secretion. Taken together, these results suggest that NOD1 plays an important role in the detection of microbial components in the sebocytes.

To understand in more detail the coordinated enhancement of the inflammatory response by NOD1 and TLRs shown in Fig. 5, we focused on the map frames of the NF-κB signaling pathway that showed significant change in the KEGG-enriched pathway analysis. NF-κB is activated via two parallel pathways, canonical and non-canonical. Although NOD1 is known to activate the canonical NF-κB pathway [27], as shown in Fig. 3D, The KEGG pathway map frame of the NF-κB signaling pathway revealed that the stimulation of NOD1 induces *p100* and *RelB* gene expression classified as non-canonical pathways of NF-κB (Supplementary Fig. 6A). Therefore, NOD1 activation in human sebocytes might activate both the canonical and non-canonical pathways of NF-κB. TLRs also activate the NF-κB canonical pathway, as NOD1 [28]. Regarding the TLR signaling pathway map frame, the NOD1 activation in human sebocytes only slightly changed the expression of many TLR downstream genes ($|fc| \geq 1$) and did not activate the NF-κB canonical pathway (Supplementary Fig. 6B). These results suggested that the co-activation of NOD1 and TLR2/TLR4 additively increased NF-κB transcription activity by activating both NF-κB canonical and non-canonical pathways, resulting in coordinately increased proinflammatory cytokine production shown in Fig. 5. In addition, it has been reported that TLR2 or TLR4 stimulation in immortalized human SZ95 sebocytes induces an increase in *CXCL8* expression [9], supporting the possibility that co-stimulation of NOD1 and TLR2/TLR4 resulted in an additive increase in IL-8 production as shown in Fig. 5.

Gram-negative bacteria are generally known to be highly pathogenic, but inflammatory diseases caused by them in the sebaceous glands have rarely been investigated. Though TLR4 is the main component for dealing with Gram-negative bacteria, NOD1 may also have substantial role in the sebocytes. TLRs and NLRs are known to interact with each other [14]. Indeed, Fig. 5 shows that simultaneous stimulation of TLR4 and NOD1 induces a stronger inflammatory response than stimulation of either of them alone in sebocytes. Although the mechanism of NOD–TLR interactions remains unclear, several




*(caption on next page)*

**Fig. 4.** The effects of nucleotide-binding oligomerization domain protein-1 (NOD1) inhibition on the inflammatory responses in sebocytes. (A–C) Sebocytes were stimulated with 10 μg/mL L-alanyl-γ-D-glutamyl-meso-diaminopimelic acid (Tri-DAP), in the absence or presence of 5 μM ML130. (A) The expression levels of *CXCL8* were examined at 4 h stimulation. Data are presented as mean ± standard deviation (SD) of three independent experiments from one lot. (B) Interleukin-8 (IL-8) concentration in the cell media was detected at 24 h stimulation. Data are presented as mean ± SD of three independent experiments from one lot. (C) The phosphorylation of p38 and p65 proteins was detected at 1 h stimulation. β-Actin was used as a loading control. Data are presented as mean ± SD of three independent experiments from one lot. (D, E) After transfection with the indicated 19.2 nM siRNA plus Lipofectamine™ 2000 Transfection Reagent for 48 h, sebocytes were exposed to the culture medium for 24 h and stimulated with 10 μg/mL Tri-DAP. *CXCL8* mRNA levels after 4 h and IL-8 protein levels in the cell media after 24 h were quantified. Data are presented as mean ± SD of four independent experiments from one lot. *$p<0.05$; **$p<0.01$.

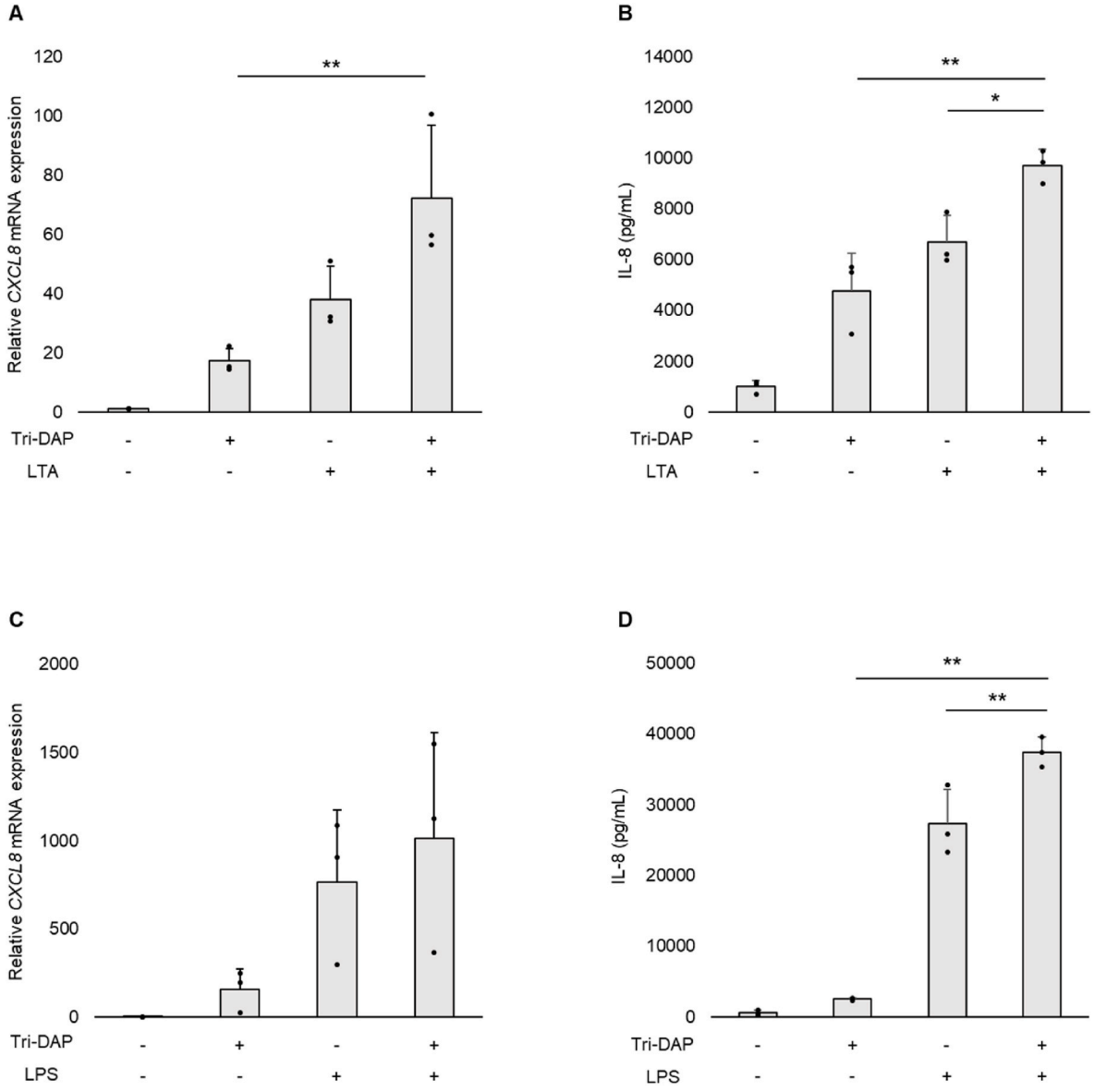



**Fig. 5.** The expression of proinflammatory cytokine induced by nucleotide-binding oligomerization domain protein-1 (NOD1) and/or Toll-like receptor (TLR) activation. Sebocytes stimulated with 10 μg/mL L-alanyl-γ-D-glutamyl-meso-diaminopimelic acid (Tri-DAP) and/or 10 μg/mL lipoteichoic acid (LTA) or 100 ng/mL lipopolysaccharide (LPS). (A, C) *CXCL8* mRNA levels after 4 h. Data are presented as mean ± standard deviation (SD) of three independent experiments from one lot. (B, D) Interleukin-8 (IL-8) protein levels after 24 h. Data are presented as mean ± SD of three independent experiments from one lot. *$p<0.05$; **$p<0.01$.

mechanisms have been proposed. For example, NOD and TLR signaling-related proteins favor inflammation together, and TLRs support the post-transcriptional translation of inflammatory cytokine genes derived from NOD signaling [14]. Therefore, to protect against Gram-negative bacteria in sebaceous glands, it may be necessary to consider not only TLR4 but also NOD1. Simultaneous inhibition of the TLR4 and NOD1 receptor, or their signaling pathways may impede excessive inflammation in sebaceous glands more efficiently than inhibition of either receptor alone.

In recent years, several studies have reported that activation of NOD1 affects not only the inflammatory response but also various cellular events. In adipocytes, which have properties similar to sebocytes, NOD1 activation inhibits the insulin signaling pathway and cell differentiation [15–17]. Insulin-like growth factor 1 increases sebum production in sebocytes [18]. As sebocytes differentiate, lipid droplets accumulate inside the cells and are released as sebum in the form of a

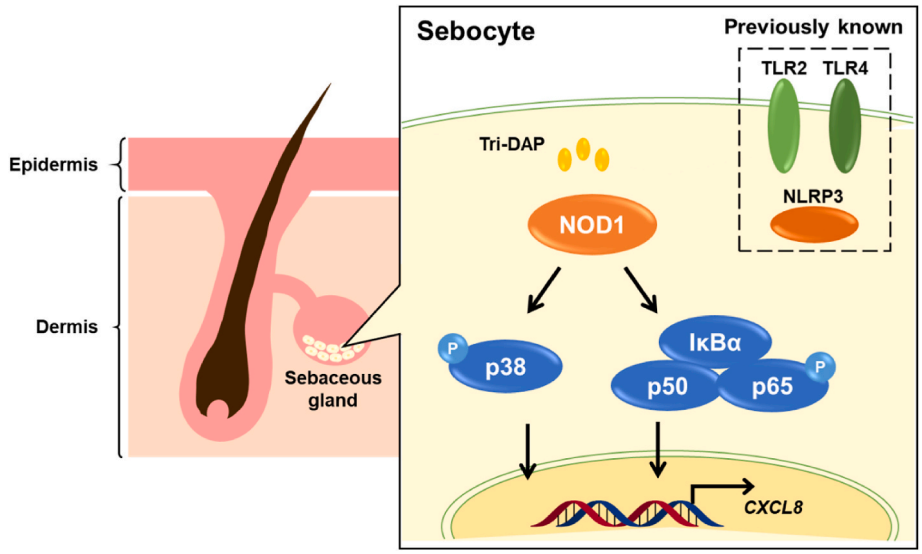



**Fig. 6.** Schematic illustration of previously known pattern recognition receptor expression and nucleotide-binding oligomerization domain protein-1 (NOD1)-mediated inflammatory signaling by L-alanyl-γ-D-glutamyl-meso-diaminopimelic acid (Tri-DAP) in human sebocyte. The expression of toll-like receptor (TLR) 2, TLR4, and NOD-like receptor (NLR) family pyrin domain-containing 3 (NLRP3) have been previously reported. Tri-DAP activation of NOD1 induces *CXCL8* mRNA expression by phosphorylating p38, one of the mitogen-activated protein kinases, and p65, which forms a complex with the inhibitor of nuclear factor-kappa B (IκB) and p50.

holocrine secretion. Excessive secretion of sebum due to hormonal imbalance or abnormal differentiation causes several skin diseases. For example, overproduced sebum and hyperkeratosis in the infundibulum contribute to the formation of non-inflammatory comedones. Subsequently, *C. acnes* proliferates on sebum, which triggers comedonal acne vulgaris [19]. One of the etiological factors of seborrheic dermatitis is *Malassezia*, a fungal genus, thought to thrive on skin as sebum increases [20]. Therefore, if NOD1 activation suppresses insulin signaling and cell differentiation in sebocytes, regulation of sebum production via NOD1 might be useful in the prevention of comedone formation and the treatment of seborrheic skin conditions.

Acne vulgaris is the most commonly known inflammatory disease of sebaceous follicles. Conventional therapies for acne vulgaris at the inflammatory stage mainly use antimicrobials and microbicides. However, long-term use of these drugs can result in development of bacterial resistance and change in the composition of the bacteria, resulting in disruption of homeostasis between the normal cutaneous microbiota and the host [21,22]. The cutaneous bacterial flora is mainly composed of Gram-positive bacteria, such as *Staphylococcus epidermidis*, *C. acnes*, and *S. aureus*. *S. epidermidis* particularly contributes to the prevention of skin diseases by accelerating wound healing and promoting the production of antibacterial peptides [23]. Thus, alternative strategies that do not affect the normal skin bacterial flora are required. According to previous reports, at least three PRRs, TLR2, NLRP3, and protease-activated receptor 2, are involved in inflammatory events at the lesion site of acne vulgaris [10,12,24]. Because *C. acnes'* excess growth within the sebaceous gland induces inflammation through PRRs to exacerbate symptoms, the inhibition of PRRs has recently attracted attention as a target for the treatment of acne vulgaris inflammation. However, PRRs have been reported to not only regulate inflammation but also mediate the production of antibacterial peptides by *S. epidermidis* [23]. NOD1 senses Gram-negative and some Gram-positive bacteria, including *C. acnes* [25,26]. Inhibition of NOD1 is expected to suppress some of the *C. acnes*-derived inflammation while maintaining the benefits of many other Gram-positive bacteria. Therefore, NOD1 might be a potential target as an alternative treatment for acne vulgaris.

Further experiments using multiple lots lead to understanding of donor individual difference because most of experimental data in this study were obtained from one type of lot. To further understand the function of NOD1 in human sebocytes, investigation using bacteria such as *C. acnes* is necessary in addition to experiments using model peptides. Additional research is also needed to elucidate the inflammation mechanism by NOD1 activation. For example, examining the expression of other inflammation-associated genes, such as TANK-binding kinase 1 and I kappa B kinase-epsilon would lead to understanding NOD1-mediated interferon-β signaling pathways in human sebocytes.

In conclusion, we have shown that human sebocytes express NOD1 whose stimulation can activate NF-κB and MAPK induced inflammatory signaling pathways. NOD1 has been reported to be involved in various biological events in addition to inflammation. Further research on NOD1 in sebocytes might contribute to the basis for the regulation of sebum production and the treatment of acne vulgaris.

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## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Data availability

Data will be made available on request.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.

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