SUPPLEMENTARY MATERIALS

MATERIALS AND METHODS

Reagents and antibodies. The following TLR ligands were used, peptidoglycan derived from *S. aureus* (PGN), lipoteichoic acid from *S. aureus* (LTA), lipopolysaccharide from E. coli 0111:B4 (LPS) (Sigma-Aldrich, St. Louis, MO), N-palmitoyl-S-[2,3-bis(palmitoyl)-(2RS)-propyl]-(R)cysteinyl-alanyl-glycine (Pam3CSK4), macrophage-activating lipopeptides-2 (Malp-2) (Enzo Life Sciences, Plymouth Meeting, PA), polyinosinic-polycytidylic acid (polyI:C) (Amersham/GE Healthcare, Piscataway, NJ). Antibodies used in this study were as follows: TLR2 (Western blot: 24-9028, eBioscience, San Diego, CA; immunohistochemical staining: ab24192, Abcam, Cambridge, MA; for neutralizing: MAB2616, R&D, Minneapolis, MN), TLR1 (sc-30000, Santa Cruz Biotechnology, Santa Cruz, CA), claudin (CLDN) 23 (ab23355, Abcam), β-actin (sc-47778, Santa Cruz Biotechnology), cytokeratin 10 (CK10; PRB-159P, Emeryville, CA, Covance), desmoglein-1 (DSG-1; ab12077, Abcam), desmoglein-3 (DSG-3; ab14416, Abcam), filaggrin (FLG; ab17808, Abcam), loricrin (LOR; PRB-145P, Covance), pan-cadherin (sc-1499, Santa Cruz Biotechnology) and calpain (sc-271856, Santa Cruz Biotechnology). The following antibodies are purchased from Zymed/Invitrogen (San Francisco, CA): CLDN1 (18-7362), occludin (33-1500), and ZO-1 (33-9100).

Primary human keratinocyte culture and stimulation. Primary human keratinocytes (PHK) were isolated from discarded neonatal foreskins, propagated and stimulated as previously described (De Benedetto *et al.*, 2011). The study was approved by the

Research Subject Review Board at the University of Rochester Medical Center. For detecting TJ molecule expression, PHK were cultured in K-SFM (keratinocyte-serum free media, low calcium: 0.09 mM; Invitrogen/Gibco, Carlsbad, CA) with growth supplement (5 ng/mL human recombinant epidermal growth factor and 50 µg/mL bovine pituitary extract) and 1% Pen/Strep, 0.2% Amphotericin B (Invitrogen/Gibco) until confluent, followed by their transfer into DMEM complete media (high calcium: 1.8 mM, with 10% heat-inactivated fetal bovine serum, 1% Pen/Strep and 0.2% Amphotericin B; Invitrogen/Gibco) with or without TLR ligands for 24 or 48 h. mRNA and protein expression from harvested PHK were analyzed by real-time q-PCR and Western blot, respectively.

Real-time quantitative RT-PCR (q-PCR). Total RNA was extracted 4 or 24 h after stimulation of PHK. Complementary DNA (cDNA) was synthesized using the iScript^{TM} cDNA synthesis Kit (Bio-Rad Life Science, Hercules, CA) according to the manufacturer's instructions. Quantitative PCR (q-PCR) was performed using the iQ^{TM} SYBER Green Supermix assay system (Bio-Rad). All PCR amplifications were carried out in triplicate on an iQ5 Multicolor real-time PCR detection system (Bio-Rad). Primers were designed and synthesized by Integrated DNA Technologies (**Table S1**). The normalized Ct value of each sample was calculated using GAPDH as an endogenous control gene. For cultured keratinocyte experiment, relative quantification was performed by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). For the experiment by using patients' samples, absolute quantification was performed by the 2-ΔCt method (Schmittgen and Livak, 2008). RVU (relative value unit)= ($2^{-\Delta Ct}$) x1000.

Preparation of total cell lysate and immunoblotting. Cell lysis and immunoblotting were performed as previously described (De Benedetto *et al.*, 2011).

Extraction of membrane and cytosolic proteins. PHK were grown in 6-well plates until confluent, then switched to DMEM complete media with or without *S. aureus*-derived PGN (20 µg/mL) for 48 h. Proteins from different subcellular compartments were isolated using ProteoExtract® Subcellular Proteome Extraction Kit (EMD Biosciences, La Jolla, CA) according to the manufacturer's instructions. Obtained samples were subjected to SDS gel electrophoresis on 4-12% NuPage Bis-Tris gels (Invitrogen) with an equal amount of total protein (10 µg) per lane.

Inactivation of TLR2. PHK were grown in K-SFM until confluent, and switched to DMEM complete media. Mouse monoclonal anti-human TLR2 antibody (5 µg/mL) and mouse IgG_{2B} (R&D) were added 1 h before cells were treated with 20 µg/mL *S. aureus*-derived PGN. mRNA and protein expression were analyzed by q-PCR or Western blot 24 or 48 h later, respectively. TEER in the presence of 10 µg/mL of TLR2 neutralizing antibody was measured every 24 h until 96 h. Paracellular flux of fluorescein was measured at 48 h after treatment.

Study participants. Nonatopic healthy (NA) subjects and patients with AD were enrolled as previously described (De Benedetto *et al.*, 2011). The diagnosis of AD was made using the US consensus conference criteria (Eichenfield, 2004). All subjects

either underwent an epidermal procurement procedure (see below) or a 4-mm punch biopsy of their nonsun-exposed forearm and a TEWL measurement at that same site prior to tissue procurement. These studies were approved by the Research Subject Review Board at the University of Rochester Medical Center. All clinical investigations were conducted according to the Declaration of Helsinki Principles. All subjects provided written informed consent.

Human epidermal procurement and RNA isolation. The blister roof, which consists of full thickness epidermis, was obtained as noted previously (De Benedetto *et al.*, 2011). All epidermal samples were homogenized in Trizol Reagent (Sigma-Aldrich). Total RNA was extracted by RNeasy RNA isolation kits (Qiagen, Valencia, CA).

Immunohistochemical staining. Five µm sections from formalin-fixed, skin biopsy specimens were deparaffinized and rehydrated. Slides were incubated in citrate buffer (invitrogen), pH 6.0, at 95°C for 15 min, and incubated in blocking solution (Dako dual endogenous enzyme block; Dako inc., Carpinteria, CA) for 1 h, followed by overnight incubation at 4°C with TLR2 antibody (1:600) diluted in blocking solution. Samples were incubated with the secondary antibody (biotinylated goat anti-rabbit; Vector Laboratories Inc., Burlingame, CA) diluted 1:100 in PBS for 1 h at room temperature (RT), then slides were covered with alkaline phosphate standard ABC mixture (Vector) for 30 min at RT, and incubated with permanent red (Dako) for 15 min at RT. Slides were then dehydrated and mounted in mounting medium (Richard-Allan Scientific, Thermo Scientific**)**. Fluorescent images were obtained as previously described (De

Benedetto *et al.*, 2011). TLR2 fluorescence staining intensity was quantified only from epidermis by ImageJ version 1.440 (NIH, Bethesda, MD) to exclude the non-epidermal staining signal observed in the dermis.

Keratinocyte propagation from AD subjects. 4-6 blister roofs were collected by the NP-2 negative pressure vacuum apparatus (Electronic Diversities, Finksburg, Maryland, USA) as noted previously.(De Benedetto *et al.*, 2011) The entire epithelium was removed with sterile scissors, washed in Hank's Balanced Salt Solution (Invitrogen/Gibco, Carlsbad, CA), and incubated with 1x Trypsin-EDTA (Invitrogen/Gibco) for 30 minutes at 37 ^{o}C, followed by vigorous pipetting to disperse the keratinocytes. The stratum corneum debris was removed with a 100 μm Falcon strainer (Becton-Dickinson, Franklin Lakes, NJ). Cells were then grown at 37 ^{o}C in Keratinocyte serum-free medium (K-SFM; Invitrogen/Gibco) with added 1% pen/strep and 0.2% Amphotericin B (Invitrogen/Gibco) and were passaged only once. Keratinocytes were stimulated with PGN from *S. aureus* (20 μg/mL; Sigma-Aldrich) for two hours prior to RNA isolation.

Table S1. Primers for qPCR.

	Forward	Reverse
CLDN1	5'-TTGACTCCTTGCTGAATCTGAG-3'	5'-TTCTGCACCTCATCGTCTTC-3'
CLDN2	5'-TCTTCCCTGTTCTCCCTGATAG-3'	5'-TCTTGACTTTGGGAGGTTGAC-3'
CLDN23	5'CTTGCCATGCAAACTCTCAA-3'	5'-TTCTCCTCTTGGCTTCTGGA-3'
connexin-26	5'-GTTTAACGCATTGCCCAGTT-3'	5'-GGCCTACAGGGGTTTCAAAT-3'
connexin-43	5'-GGATCGGGTTAAGGGAAAGAG-3'	5'-AGGAGACATAGGCGAGAGG-3'
DSG-1	5'-TCAATCCGAAGGCAGAAACG-3'	5'-TGCGGTATGTAACTTGCTGG-3'
DSG-3	5'-CGAATCTCTGGAGTGGGAATC-3'	5'-AGTCCTTGGGCATTTAGAGC-3'
E-cadherin	5'-CCCAATACATCTCCCTTCACAG-3'	5'-CCACCTCTAAGGCCATCTTTG-3'
GAPDH	5'-GAAGGTGAAGGTCGGAGTC-3'	5'-GAAGATGGTGATGGGATTTC-3'
nectin-1	5'-AGCCATTAAGGAGAAACGA-3'	5'-TTCCCAATTTCTCTGCTCT-3'
NOD2	5'GTCAGCCAGTATGAATGTGAT-3'	5'-TGGCAAGATCAAGCAGCCTT-3'
occludin	5'-GCAAAGTGAATGACAAGCGG-3'	5'-CACAGGCGAAGTTAATGGAAG-3'
PGLYRP-3	5'-TGCACACCCAGGGCTACAA-3'	5'-CGACAGGTGACCCTTCTGGAT-3'
PGLYRP-4	5'-AAGGCCTTCCCACAGATGTCT-3'	5'-GGTGTGACCTCCAGTCCA-3'
TLR1	5'-GTTAGAGGGCTGGCCTGATTC-3'	5'-CACAGCCAACACCAGCATG-3'
TLR2	5'-TGGTAGTTGTGGGTTGAAGC-3'	5'-GACAGAGAAGCCTGATTGGAG-3'
TLR6	5'-TGCAACATAACTCTGCTGATC-3'	5'-GCCCTGCGCCGAGTCT-3'
ZO-1	5'-CGGAAAACATGCTACACAC-3'	5'-CCCATTTACTGGCTGGTAT-3'

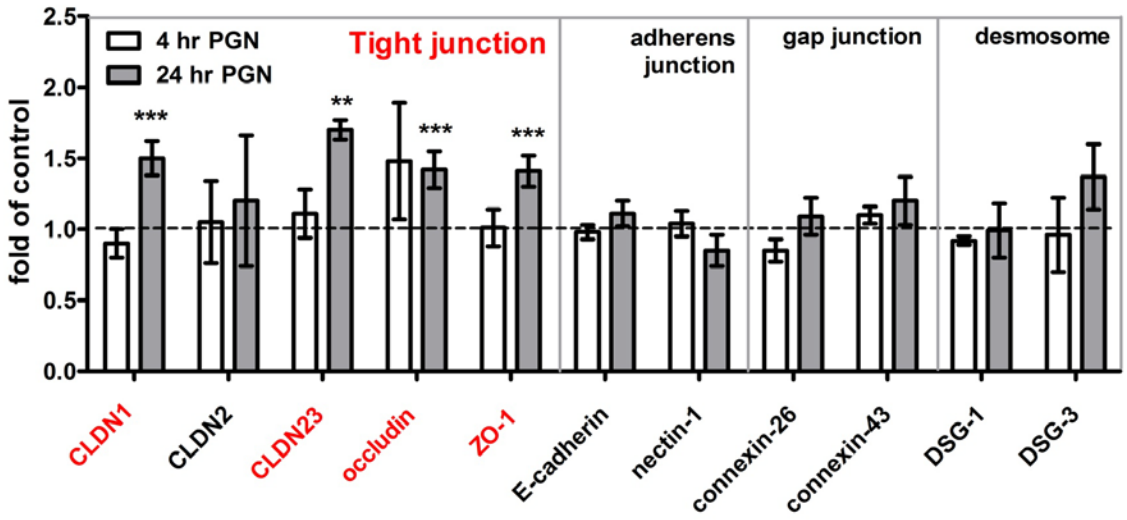

Figure S1. Effect of *S. aureus*-derived PGN on mRNA expression of intercellular junction molecules. Primary human keratinocytes (PHK) were incubated with PGN (20 µg/ml) for 4 and 24 h (n= 4-13) and total mRNA was isolated for qPCR analysis. The mRNA expression of TJ (CLDN1, CLDN2, CLDN4, CLDN23, occludin, and ZO-1), adherens junction (nectin-1 and E-cadherin), gap junction (connexin-26 and connexin-43), and desmosomal (DSG-1 and DSG-3) proteins were quantified and normalized to GAPDH. The dotted line represents expression levels for the control group (media alone) at each time point. $^{**}P$ <0.01; $^{***}P$ <0.001.

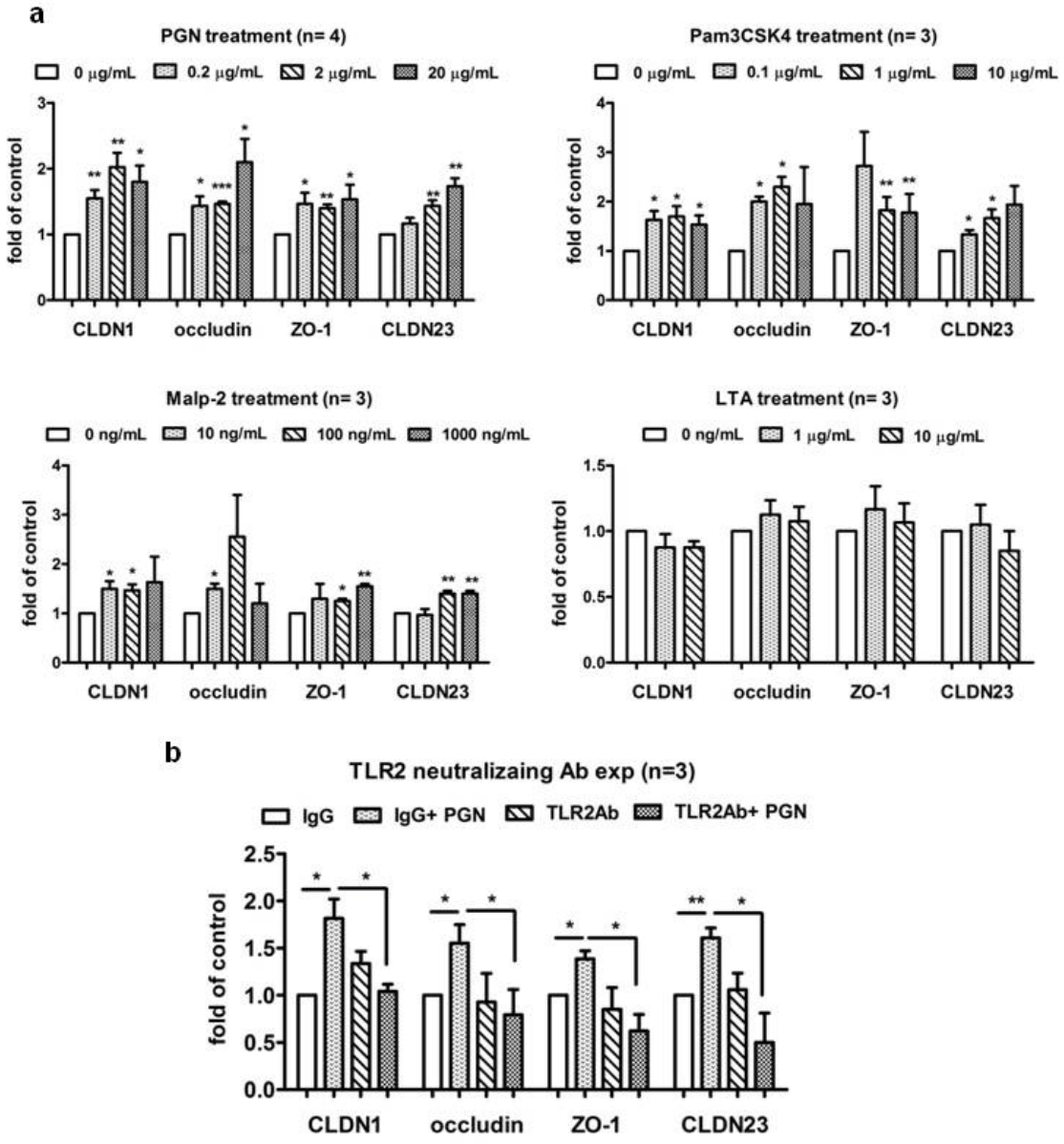

Figure S2. Densitometric quantification of the immunoblots in Figure 2a, b and 3d.

Protein levels in **(a)** Figure 2a, b and **(b)** Figure 3d were quantified by densitometry and normalized to media treatment alone or IgG treatment group. **P* <0.05; ***P* <0.01; ****P* < .001

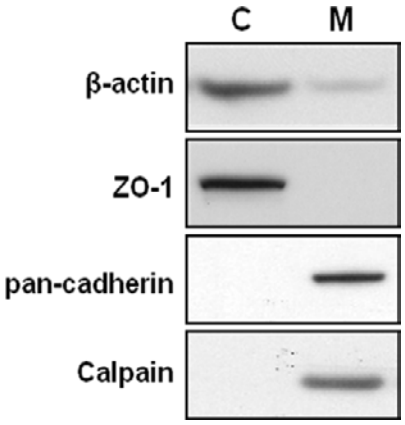

Figure S3. The specificity of cellular fractionation by using ProteoExtract® Subcellular Proteome Extraction Kit. β-actin and ZO-1 are detected in cytosolic fraction (C). Pan-cadherin and activated form of Calpain are detected in membrane fraction (M) of PHK.

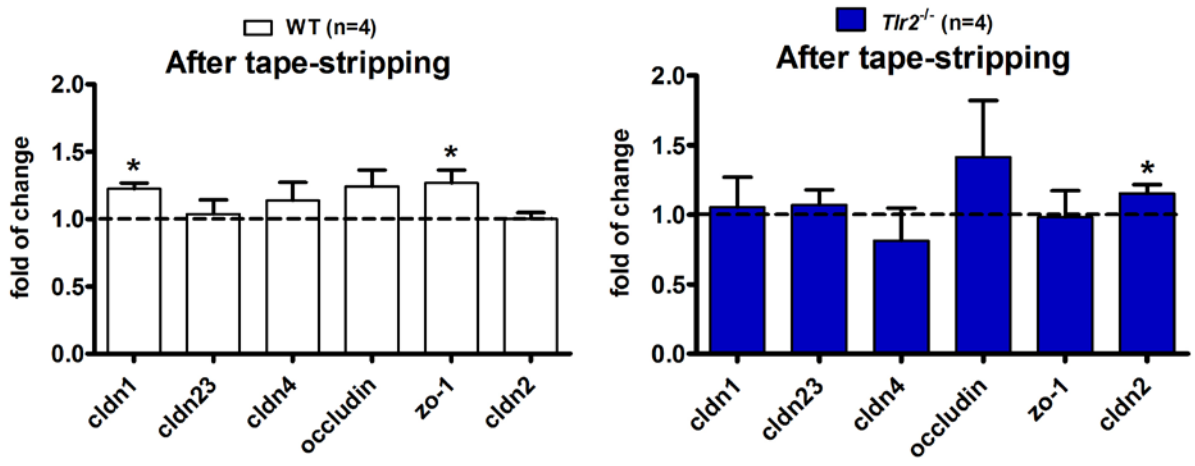

Figure S4. mRNA expression of *cldn1*, *zo-1* in WT mice and *cldn2 in Tlr2*$^{-/-}$ mice were significant changed after tape-stripping. Mouse skins were isolated immediately at 2 h time point after tape-stripping. mRNA expression of TJ proteins were measured and normalized to non-tape stripping groups. **P* <0.05; ns: not significant.

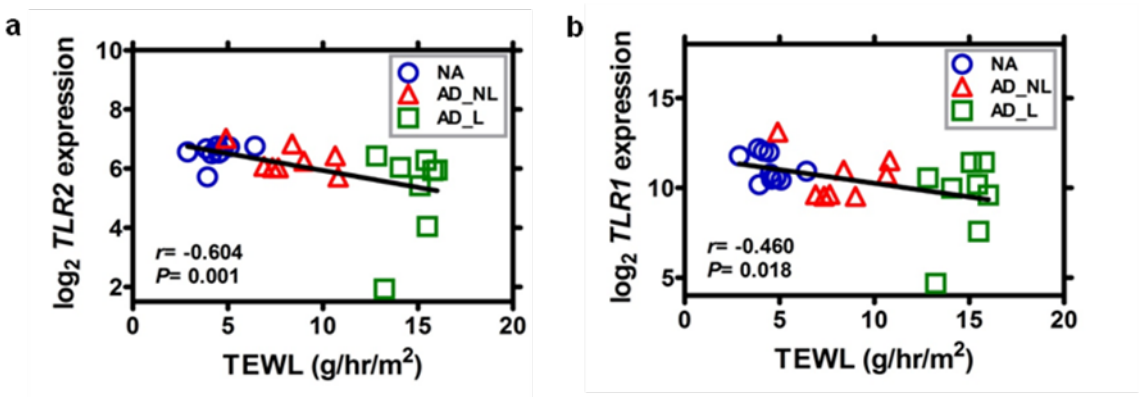

Figure S5. Epidermal expression of TLR2 and TLR1 inversely correlates with TEWL in human subjects. **(a)** The *line* represents the linear least square fit for $\log_2$ *TLR2* expression level versus TEWL (Spearman nonparametric correlation, [n = 26]: $r = -0.604$, $P = 0.001$). **(b)** The plot of $\log_2$ *TLR1* expression level versus TEWL ([n = 26]; $r = -0.460$, $P = 0.018$). The disease phenotypes (nonatopic control subjects [NA]; non-lesional AD [AD_NL]; lesional AD [AD_L]) are denoted by unique symbols.

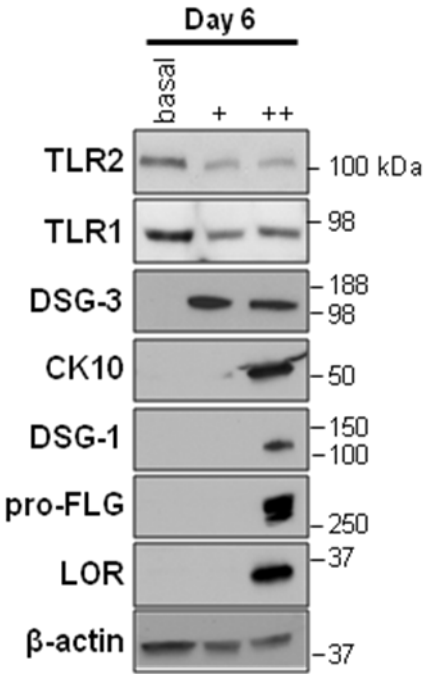

Figure S6. TLR1 and 2 protein expression decreases with human keratinocyte differentiation. PHK were incubated in keratinocyte serum free growth media (**basal**), DMEM with (**+**), or without (**++**) 10% FBS for 6 days. Protein expression of TLR2, TLR1 and several differentiation markers including CK10, DSG-1, DSG-3, LOR and pro-FLG were measured by Western blot. Representative blot of n=2.

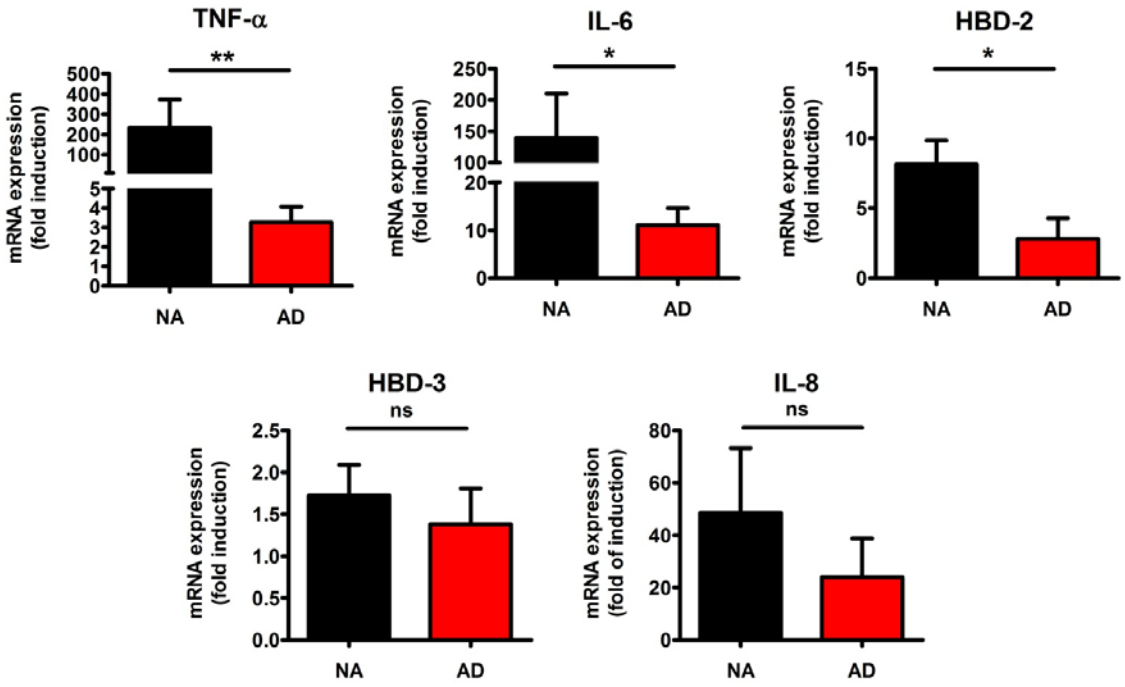

Figure S7. PGN stimulation induces a greater fold-induction of TNF-α, IL-6 and HBD-2 in NA keratinocytes. PGN stimulation (20 μg/mL for 2 h) induced a greater fold-induction of TNF-α, IL-6 and HBD-2 in NA keratinocytes as compared to AD keratinocytes. There was also a trend of increased IL-8 mRNA response in NA compared to AD keratinocytes, but this did not reach statistical significance (AD: n=5; NA: n=5). **P* <0.05, ***P* <0.01.

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