

ORIGINAL ARTICLE

Food Allergy and Gastrointestinal Disease

Toll-like receptor 2 stimulation augments esophageal barrier integrity

Melanie A. Ruffner^{1,2}  | Li Song¹ | Kelly Maurer¹ | Lihua Shi¹ | Margaret C. Carroll¹ | Joshua X. Wang³ | Amanda B. Muir^{2,3}  | Jonathan M. Spergel^{1,2}  | Kathleen E. Sullivan^{1,2} 

¹Division of Allergy and Immunology, Children's Hospital of Philadelphia, Philadelphia, PA, USA

²Department of Pediatrics, Perelman School of Medicine at University of Pennsylvania, Philadelphia, PA, USA

³Division of Gastroenterology, Hepatology and Nutrition, Children's Hospital of Philadelphia, Philadelphia, PA, USA

Correspondence

Melanie A. Ruffner, The Children's Hospital of Philadelphia, Division of Allergy and Immunology, Abramson Research Center, Room 1216, 3515 Civic Center Boulevard, Philadelphia, PA 19104, USA.
Email: ruffnerm@email.chop.edu

Funding information

MAR was funded by NIH T32-HD043021, KL2TR001879, and the ACAAI Young Faculty Award. ABM is funded by NIH K08DK106444. KES is funded by the Wallace Endowed Chair. JMS is funded by Stuart Starr Endowed Chair. JMS and ABM are funded by the Consortium of Eosinophilic Gastrointestinal Disease Researchers (CEGIR U54 AI117804) which is part of the Rare Diseases Clinical Research Network, an initiative of the Office of Rare Diseases Research, NCATS, and is funded through collaboration between NIAID, NIDDK, and NCATS and patient advocacy groups including APFED, CURED, and EFC.

Abstract

Background: Germline-encoded innate immune pattern recognition receptors (PRR) are expressed at epithelial surfaces and modulate epithelial defenses. Evidence suggests that stimulation of the Toll-like receptor (TLR) family of PRR may regulate epithelial barrier integrity by upregulating tight junction (TJ) complex protein expression, but it is not known whether this mechanism is utilized in esophageal epithelial cells. TJ complex proteins maintain intact barrier function and are dysregulated in atopic disorders including eosinophilic esophagitis.

Methods: Pattern recognition receptors expression was assessed in EoE and control primary esophageal epithelial cells, demonstrating robust expression of TLR2 and TLR3. The three-dimensional air-liquid interface culture (ALI) model was used to test whether TLR2 or TLR3 stimulation alters epithelial barrier function using an in vitro model of human epithelium. Transepithelial electrical resistance (TEER) and FITC-Dextran permeability were evaluated to assess membrane permeability. ALI cultures were evaluated by histology, immunohistochemistry, Western blotting, and chromatin immunoprecipitation (ChIP).

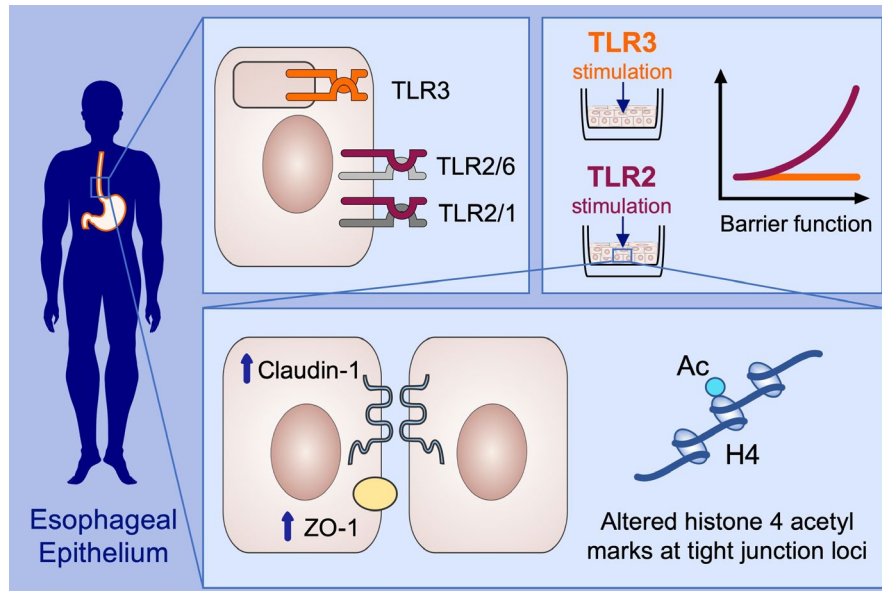
Results: TLR3 stimulation did not change TEER in the ALI model. TLR2 stimulation increased TEER (1.28- to 1.31-fold) and decreased paracellular permeability to FITC-Dextran, and this effect was abolished by treatment with anti-TLR2 blocking antibody. TJ complex proteins claudin-1 and zonula occludens-1 were upregulated following TLR2 stimulation, and ChIP assay demonstrated altered histone 4 acetyl binding at the *TJP1* enhancer and *CLDN1* enhancer and promoter following zymosan treatment, implying the occurrence of durable chromatin changes.

Conclusions: Our findings implicate the TLR2 pathway as a potential regulator of esophageal epithelial barrier function and suggest that downstream chromatin modifications are associated with this effect.

KEYWORDS

eosinophilic esophagitis, epithelium, innate immunity, tight junctions, toll-like receptors

Abbreviations: ALI, air-liquid interface; CAPN14, calpain 14; CLDN1, claudin-1; EPC2-hTERT, primary human esophageal keratinocytes; EPC2 human telomerase immortalized; GST, glutathione-S-transferase; HAT, histone acetyl transferase; HDAC, histone deacetylases; KSFM, keratinocyte serum-free media; PRR, pattern recognition receptor; SNP, single nucleotide polymorphism; TEER, transepithelial electrical resistance; Th2, T-helper type 2; TJ, tight junction; TLR, Toll-like receptor; USA, United States of America; ZO-1, zonula occludens-1.



GRAPHICAL ABSTRACT

Our findings demonstrated robust expression of TLR2 and TLR3 in primary esophageal epithelial cells. We observed increased epithelial barrier function (increased transepithelial electrical resistance, decreased paracellular flux) upon anti-TLR2 stimulation of esophageal epithelium cultured under ALI, while anti-TLR3 treatment had no effect. TLR2 stimulation upregulated expression of tight junction proteins and increased acetylation of histone 4 at the claudin-1 gene enhancer and promoter. Abbreviations: Ac, Acetylation; H4, Histone 4; TEER, Transepithelial electrical resistance; TLR, Toll like receptor; ZO-1, zonula occludens-1.

1 | INTRODUCTION

Eosinophilic esophagitis (EoE) is an allergic disorder of the esophagus. The pathophysiology of EoE is thought to arise from the presence of food allergens in contact with the esophageal mucosa, which causes a T-helper type 2 (Th2) immune response leading to mucosal inflammation and eosinophilia, epithelial dysfunction, and long-term fibrosis.¹ Epithelial barrier dysfunction plays a critical role in this process because it promotes continued exposure to allergens and microbiota in the esophageal lumen, which stimulates epithelial cells and leukocytes to release chemokines and cytokines.^{2,3} Decreased barrier integrity in EoE has been measured by loss of mucosal impedance *in vivo* and transepithelial electrical resistance *in vitro*.⁴⁻⁶

The loss of epithelial barrier integrity in EoE is attributed to the downregulation of important structural proteins in the inflamed mucosa including tight junction (TJ) proteins.^{2,5,7} The tight junction (TJ) complex is a dynamic structure comprised of transmembrane claudins, occludin, and cytosolic proteins (ie, zonula occludens-1, zonula occludens-2, and zonula occludens-3), which connect the TJ to the cytoskeleton.⁸⁻¹⁰ Expression of occludin and claudin-1 is decreased in biopsy tissue of EoE patients both before and after treatment with swallowed corticosteroid.² In addition to tight junctions, multiple structural proteins contribute to the integrity and stratification of the esophageal epithelial barrier. For example, recent descriptions of kindreds with biallelic mutations in *DSG1* and *DSP* implicate desmosomes as a critical structural protein in the esophageal epithelium.^{11,12} Th2 cytokines, including IL-13, have

been implicated as an important signal contributing to the down-regulation of *DSG1*,¹³ but its role in the regulation of other key TJ proteins is less clear.

Innate sensing of microbial products through epithelial pattern recognition receptors (PRR) can regulate epithelial barrier function in human nasal, intestinal, and skin mucosa.¹⁴⁻¹⁹ The mechanisms which have been described vary across epithelial sites, and it can be hypothesized that there may be some degree of tissue specificity in the effector function of different PRR based on their mucosal location. Several studies highlight a role for the PRR family of Toll-like receptors (TLR) as a regulator of TJ integrity in epithelial surfaces. An increasing body of evidence suggests that Toll-like receptor 2 (TLR2) signaling upregulates TJ proteins ZO-1 and claudin-1 in epithelium in the airway, intestine, and skin.¹⁴⁻¹⁹ This mechanism is thought to be one of the many host defenses maintaining balance with commensal bacteria.

In this study, our aim was to determine whether the esophageal epithelium has mechanisms to modulate TJ complex protein expression based on innate immune sensing via TLR. We examined the expression of TLR, observing robust expression of TLR2 and TLR3 in primary isolated epithelial cells. We utilized the three-dimensional, air-liquid interface (ALI) *in vitro* culture model to examine epithelial barrier function following stimulation of TLR2 and TLR3. TLR3 stimulation had little effect on barrier function in the ALI model, whereas TLR2 improved esophageal epithelial barrier function. We hypothesized that TLR2 stimulation was associated with upregulation of TJ proteins, resulting in improved epithelial barrier function.

2 | METHODS

2.1 | Esophageal epithelial cell culture

Immortalized esophageal epithelial cell line (EPC2-hTERT) and isolated primary esophageal epithelial cells were used in this study.²⁰⁻²² Patients were enrolled in the study following informed consent. This study was approved by the Children's Hospital of Philadelphia Institutional Review Board following the US Federal Policy for Protection of Human Subjects.

Primary control patient epithelial lines were derived from esophageal biopsies of pediatric patients (under 18 years of age) during clinically-indicated endoscopic evaluations. Samples were eligible for enrollment in control group if on no treatment at the time of biopsy and found to have normal clinical endoscopy, normal esophageal histology, and no evidence of additional GI pathology. EoE cell lines were derived from pediatric patients with symptoms suggestive of EoE found to have eosinophil count > 15 per high powered field on biopsy.^{23,24} For this study, no patients were on proton-pump inhibitor, swallowed steroid, or dietary therapy at the time of endoscopy. Primary cells were used between passages 2 and 4 for these experiments.

All cells were grown in keratinocyte serum-free media supplemented with bovine pituitary extract and epidermal growth factor (KSFM, Thermo Fischer Scientific). Endotoxin-free reagents and media were used for cell culture and stimulation experiments.

2.2 | Flow cytometry

LSR-Fortessa and FlowJo software (BD Biosciences) were used to examine TLR2 (11G7, Invitrogen mouse anti-human) and Dectin-1 (GE2, Bio-Rad mouse anti-human) expression on EPC2-hTERT cells compared with isotype controls.

2.3 | Air-liquid interface (ALI) culture system

EPC2-hTERT cells were used for ALI experiments.²⁵ Cells were grown submerged in KSFM media on a 0.4- μ m filter (Corning Life Sciences) for three days to reach confluency (protocol schematic, Figure 2A). Cultures were then switched to high-calcium concentration KSFM media (1.8 mmol/L Ca⁺⁺) for 5 days. Media was removed from the upper chamber to promote epithelial differentiation for days 7-10.²⁵

2.4 | TLR stimulation

Toll-like receptor agonists were added to stimulate monolayer cultures or to ALI cultures during days 10-14 of ALI protocol. TLR2 stimulation was assessed using final concentrations of 10 μ g/mL zymosan, 10 μ g/mL peptidoglycan from *Staphylococcus aureus*, and Pam2CSK4 (InvivoGen). To assess response to TLR3 stimulation, Poly(I:C) was annealed per manufacturer's recommendations and used at a final concentration of 10 μ g/mL in ALI cultures from days 10 until 14.

2.5 | TLR2 inhibition

Rat anti-human polyclonal anti-TLR2 neutralizing antibody (InvivoGen) was reconstituted and added to the basolateral chamber media (5 μ g/mL final concentration, InvivoGen) during ALI days 10-14 concurrent with TLR2 stimulants as described above.

2.6 | Transepithelial electrical resistance (TEER)

Air-liquid interface culture resistance was measured with a Millicell ERS-2 Voltohmmeter (Merck Millipore), and the TEER unit area resistance ($\Omega \cdot \text{cm}^2$) was calculated by multiplying the measured sample resistance by the membrane area (0.33 cm^2 for 24-well Millicell inserts). Only epithelial monolayers with TEER > 200 $\Omega \cdot \text{cm}^2$ on day 10 of ALI culture were used for stimulation experiments.

2.7 | Transepithelial Flux Assay

Seventy kDa fluorescein isothiocyanate-dextran (FITC-dextran; 3 mg/mL; Sigma) was added to the upper chamber of day 14 ALI cultures. The initial 3 mg/mL upper solution was serially diluted to generate the assay standard curve. The fluorescein levels in basolateral samples were detected after 4 hours on a Spectramax M5 plate reader (Molecular Devices) and normalized to the mean of the unstimulated control samples.

2.8 | Histology and Immunohistochemistry

Air-liquid interface were harvested on day 14, then fixed in formalin, paraffin-embedded, and serially sectioned. Slides were deparaffinized in xylene and rehydrated thru a series of ethanol washes. Histology slides were stained in hematoxylin, rinsed, and then stained in eosin (Azer Scientific) on a Shandon Gemini automated stainer (Thermo Fisher). Immunohistochemistry slides were incubated with E1 (Leica Biosystems) immunohistochemistry antigen retrieval solution for 20 minutes. Claudin-1 (1:50; LS Bio LS-C415827; 1-hour primary incubation) and ZO-1 (1:50; Sigma HPA001636; 1-hour primary incubation) antibodies were used for staining with Bond Refine polymer staining kit on the Bond-Max automated staining system (Leica Biosystems).

All stained slides were dehydrated in ascending ethanol and xylene washes before coverslipping with cytooseal (Fisher Scientific). Stained slides were digitally scanned at 20x magnification on an Aperio CS-O slide scanner (Leica Biosystems). ALI membrane thickness, number of basolateral nuclei per 100 μ m, percent dilated intracellular space, and percent strong 3,3'-Diaminobenzidine (DAB) staining intensity were calculated using Aperio imaging software algorithms.

2.9 | Western Blot

Day 14 ALI were washed in PBS and lysed in RIPA buffer containing protease cocktail. Bradford assay was used to normalize sample

protein content prior to electrophoresis on a 4%-12% Bis-tris gel, followed by transfer to nitrocellulose membranes. Mouse anti-human β -actin (1:1000, Cell Signaling Technologies 3700S), rabbit anti-human claudin-1 (1:500, LSBio LS-C415827), and rabbit anti-human ZO-1 (1:500, Sigma HPA001636) primary antibodies and secondary HRP-conjugated anti-rabbit and anti-mouse antibodies (1:2000, Cell Signaling Technologies) were used to probe the membrane. Semi-quantitative densitometry analysis was performed in NIH ImageJ software.

2.10 | Quantitative real-time PCR (qRT-PCR)

We harvested RNA using Direct-zol kit with removal of DNA by column (Zymo Research). 0.2 μ g of total RNA was used for reverse-transcription reactions using Advantage RT-for-PCR Kit (TakaRa Bio), and the cDNA was used for qRT-PCR. Taqman gene expression assays (Applied Biosystems, human *TLR1-TLR10*, *CLEC7A*, *CD14*) were normalized to the *GAPDH* internal control signal, and custom SYBR green primer assays (primers, Table S1) were normalized to β -actin control.

2.11 | Chromatin Immunoprecipitation (ChIP)

Five million cells per condition were used to perform ChIP experiments following the protocol from Upstate Biotechnology with some

modifications.²⁶ EPC2-hTERT cells were harvested from monolayer after 30 minutes of stimulation, treated with 1% formaldehyde for 10 minutes at room temperature, sonicated, and immunoprecipitated overnight at 4°C. 5 μ g of anti-H4ac (Histone 4 pan-acetyl, Merck Millipore #06-866), anti-H3K27ac (histone H3 lysine 27 acetyl Abcam ab4729), anti-trimethylated H3 lysine 4 (trimethylated H3 lysine 4, Active Motif 39159), and anti-GST (glutathione-S-transferase, Thermo Fisher 71-7500) antibodies were used for immunoprecipitation. Antibody-bound complexes were collected, eluted, and extracted as previously described,²⁶ then RNase treated, and quantitated before analysis by qRT-PCR (primers, Table S2). The primers for the ChIP assays are listed below. ChIP DNA qRT-PCR results were normalized to *GAPDH* and then to anti-GST signal for each sample.

2.12 | Statistical analysis

Data were collected from a minimum of two independent experiments. Quantitative data are expressed as means $\pm$ standard deviation. Statistical analyses were performed (GraphPad Software) using one-way analysis of variance (ANOVA) to analyze multiple groups followed by Dunnett's post hoc testing to compare samples against control condition. *t* Test was used to compare two means to each other for analysis of ChIP data. *P* values $\leq$ 0.05 were considered significant for all analyses.

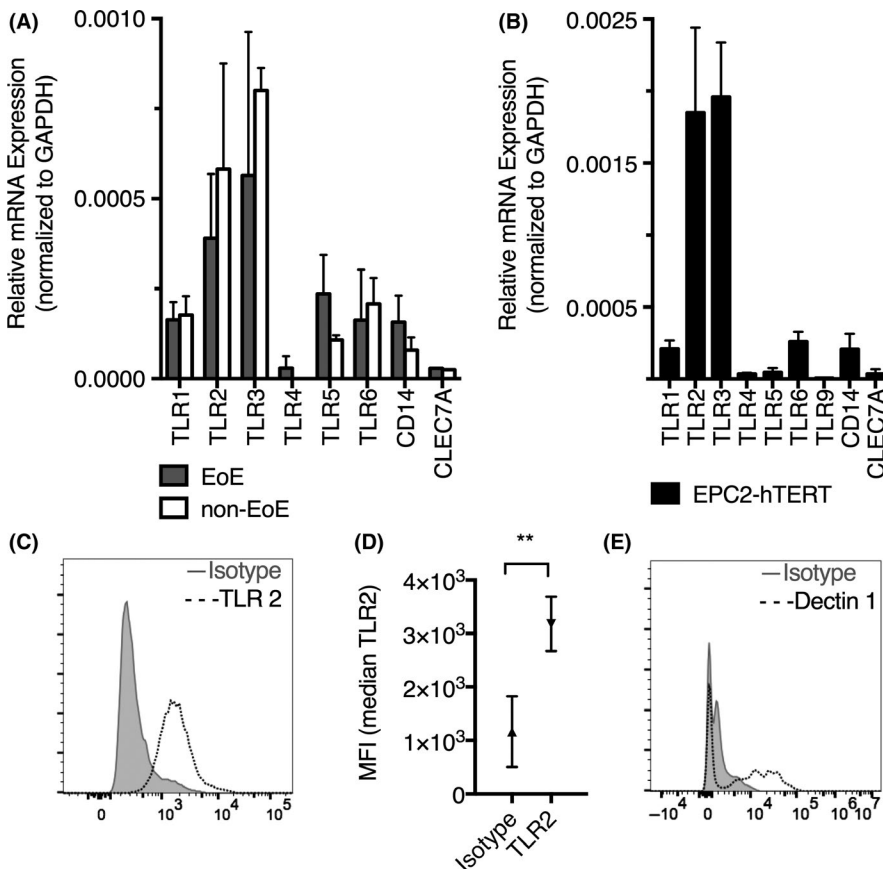


FIGURE 1 Esophageal epithelial cells express high levels of TLR2 and TLR3. TLR expression was determined by qRT-PCR. A, Primary esophageal epithelial cells from EoE patients and controls express *TLR1-TLR6*, *CD14*, and *CLEC7A* (Dectin-1); mean $\pm$ SD, *n* = 3 biologic replicates. *TLR7-TLR10* not shown, resulted as not amplified. B, *TLR1-TLR6*, *TLR9*, *CD14*, and *CLEC7A* (Dectin1) are expressed on immortalized epithelial cell line EPC2-hTERT; mean $\pm$ SD, *n* = 3. *TLR7*, *TLR8*, and *TLR10* not amplified. C, TLR2 surface expression on EPC2-hTERT cells was assessed by flow cytometry, and (D) the median fluorescence intensity is shown (***P* < 0.001 by *t* test, *n* = 3). E, EPC2-hTERT cells evaluated for expression of Dectin-1 by flow cytometry

3 | RESULTS

3.1 | TLR2 and TLR3 are expressed in esophageal epithelial cells

We examined gene expression of TLRs 1-10, CD14, and Dectin-1 (CLEC7A) using qRT-PCR in primary esophageal epithelial cells (Figure 1A) and in the EPC2-hTERT esophageal epithelial cell line (Figure 1B). Both primary esophageal epithelial cells and the EPC2-hTERT cell line express *TLR1* through *TLR6*, with minimal expression of *TLR7* through *TLR10*, *CD14*, and *CLEC7A*. As in prior studies, we observe robust mRNA expression of TLR2 and TLR3.²⁷⁻²⁹ We did not observe significant differences in PRR expression between cell lines from EoE patients and controls (Figure 1A). TLR2 forms heterodimers with TLR6 and TLR1, both of which were also transcribed in the esophageal cells (Figure 1A & B). TLR2 protein was detected on the epithelial cell surface using flow cytometry (Figure 1C & D). We assessed levels of Dectin-1 expression on the epithelial surface (Figure 1E). Dectin-1 has been shown to have functional overlap with TLR2 in macrophages, resulting in enhanced TLR2 signaling.³⁰⁻³³ We observe low levels of Dectin-1 surface expression on esophageal epithelial cells (Figure 1E), confirming that esophageal epithelial cells express multiple families of PRR which may be capable of signaling crosstalk.

3.2 | TLR2 stimulation improved epithelial barrier function in air-liquid interface culture

We next set out to test the effect of TLR2 and TLR3 stimulation on esophageal epithelial cell barrier function. These were chosen for further investigation due to their robust expression in esophageal epithelium. We used the three-dimensional ALI model and added TLR ligands to the basolateral chamber media on days 10-14 of culture (Figure 2A, schematic).

TLR3 on esophageal epithelial cells has been shown to respond to synthetic ligands and necrotic cell debris, resulting in NF- κ B activation, and IL-8 and thymic stromal lymphopoietin secretion.^{27,34,35} We stimulated ALI with the TLR3 ligand Poly(I:C); however, we did not observe any change in the epithelial barrier function (Figure S1). Following treatment with Poly(I:C), transepithelial electrical resistance (TEER) and FITC-Dextran permeability were unchanged compared with control untreated ALI cultures.

In contrast, stimulation of ALI cultures with TLR2 agonists resulted in a significant increase in epithelial barrier function measured by both TEER (Figure 2B) and FITC-Dextran flux assays (Figure 2C). Several TLR2 ligands were utilized in order to determine whether this effect varied by targeting the TLR2/6 or TLR2/1 complex. However, increased TEER and decreased FITC-Dextran permeability were seen using irrespective of whether the ligand targeted the TLR2/1 heterodimer or TLR2/6 heterodimer. TLR2 forms a heterodimer with TLR 1 to recognize triacylated lipopeptides, and heterodimerizes with TLR6 to recognize zymosan and other diacylated lipopeptides. The average TEER was increased by 26.8% in zymosan-treated ALI, 32.2% in peptidoglycan-treated ALI, and 40.3% over control in

Pam2CSK4-treated ALI. The FITC-Dextran assay (Figure 2C) confirms these findings with TLR2 agonist-treated samples allowing less flux of FITC-Dextran through the ALI membrane (Figure 2C). These results are consistent with improved barrier function in the TLR2 agonist-treated samples.

3.3 | Improved barrier function is TLR2-specific

The full complement of PRR in esophageal epithelium has not been defined, and their functions are incompletely understood. For example, although zymosan is recognized by the TLR 2/6 heterodimer, it can also be recognized by Dectin-1³¹⁻³³ and potentially by epithelial-specific, innate receptors such as Ephrin A2.³⁶ Our data demonstrate that EPC2-hTERT cells do express low levels of Dectin-1 (Figure 1E); therefore, we next set out to determine whether the observed improvement in epithelial barrier function was TLR2-specific. To do so, we utilized PAb-hTLR2, which is a polyclonal rat anti-human blocking antibody specific for TLR2.^{37,38} Co-incubation of ALI with PAb-hTLR2 during addition of TLR2 agonists into the culture media abrogated improvement in epithelial TEER (Figure 2E). This demonstrates a specific requirement for TLR2 to achieve improved esophageal epithelial barrier function in vitro.

3.4 | TLR2 stimulation alters epithelial membrane architecture

We next performed histology to examine epithelial architectural changes associated with zymosan and peptidoglycan treatment in ALI culture (Figure 3A). We observe the expected stratification of the epithelial membrane in unstimulated samples, with thinning of the epithelial membrane following TLR2 agonist treatment (Figure 3B, $24.8 \pm 5.6 \mu\text{m}$ untreated vs $17.6 \pm 6.5 \mu\text{m}$ zymosan-treated, $P < 0.001$, and $18.7 \pm 3.9 \mu\text{m}$ peptidoglycan-treated $P < 0.05$). The density of basolateral nuclei was decreased in TLR2 agonist-treated cultures (Figure 3C). Dilated intracellular spaces are characteristic findings of epithelial barrier dysfunction in EoE. However, there was no difference in the amount of dilated intracellular space between treated and untreated ALI cultures (Figure 3D). Dilated intracellular spaces have been shown to correlate with poor barrier function.³⁹ Therefore, this corresponds with data obtained in our barrier function assays as we observed improved barrier function with TLR2 stimulation.^{40,41}

3.5 | TLR2 stimulation upregulated TJ complex gene and protein expression

Tight junction complex proteins are known to be a primary mediator of epithelial barrier integrity.⁴² Stimulation of TLR2 in intestinal and airway epithelium increases expression of TJ complex proteins^{15,17,19}; however, this association has not previously been examined in the esophageal epithelium. In light of our data demonstrating improved ALI barrier function following TLR2 stimulation, we hypothesized that TLR2 stimulation may upregulate TJ complex protein expression in ALI cultures. EPC2-hTERT cells in monolayer

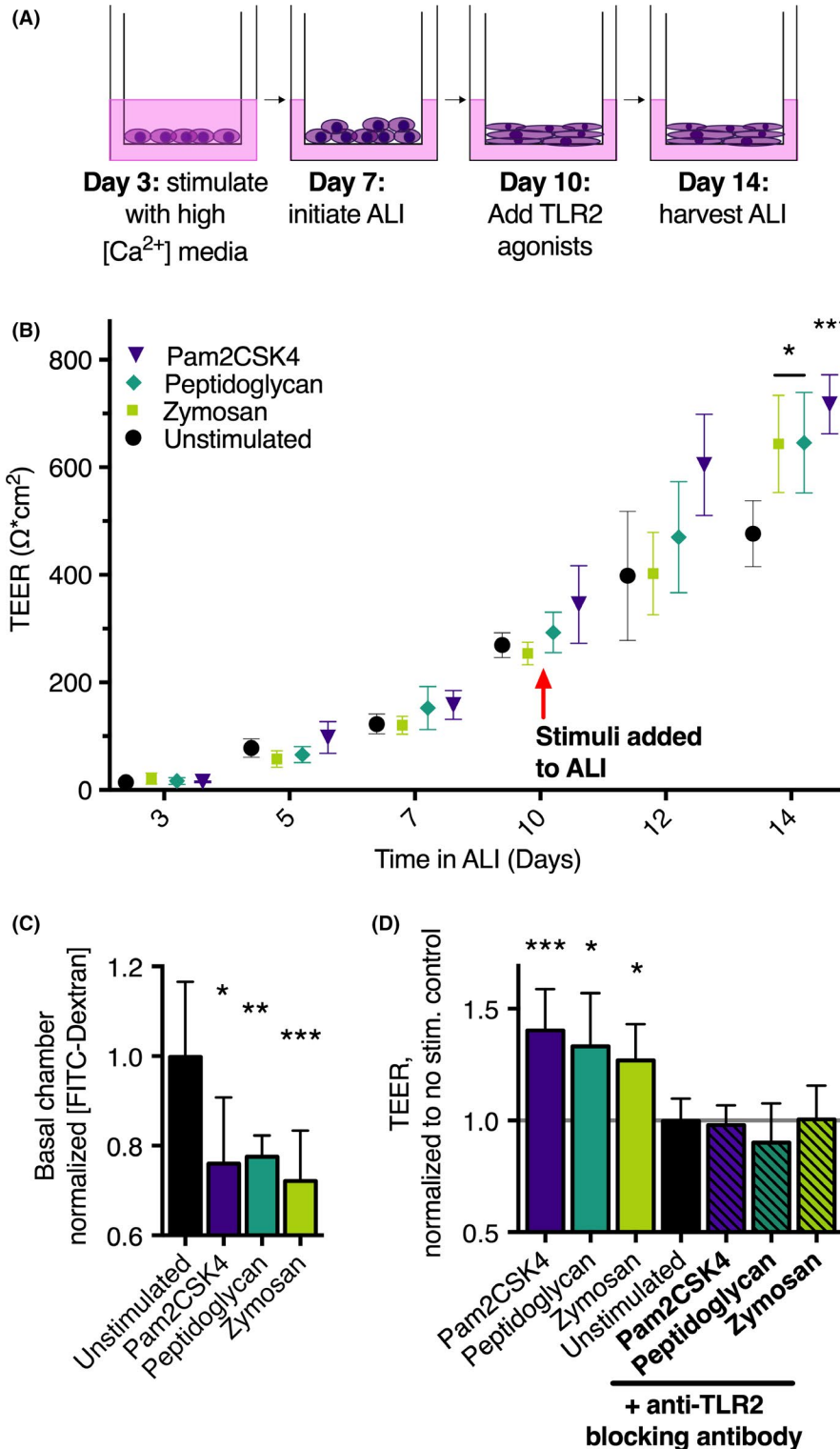


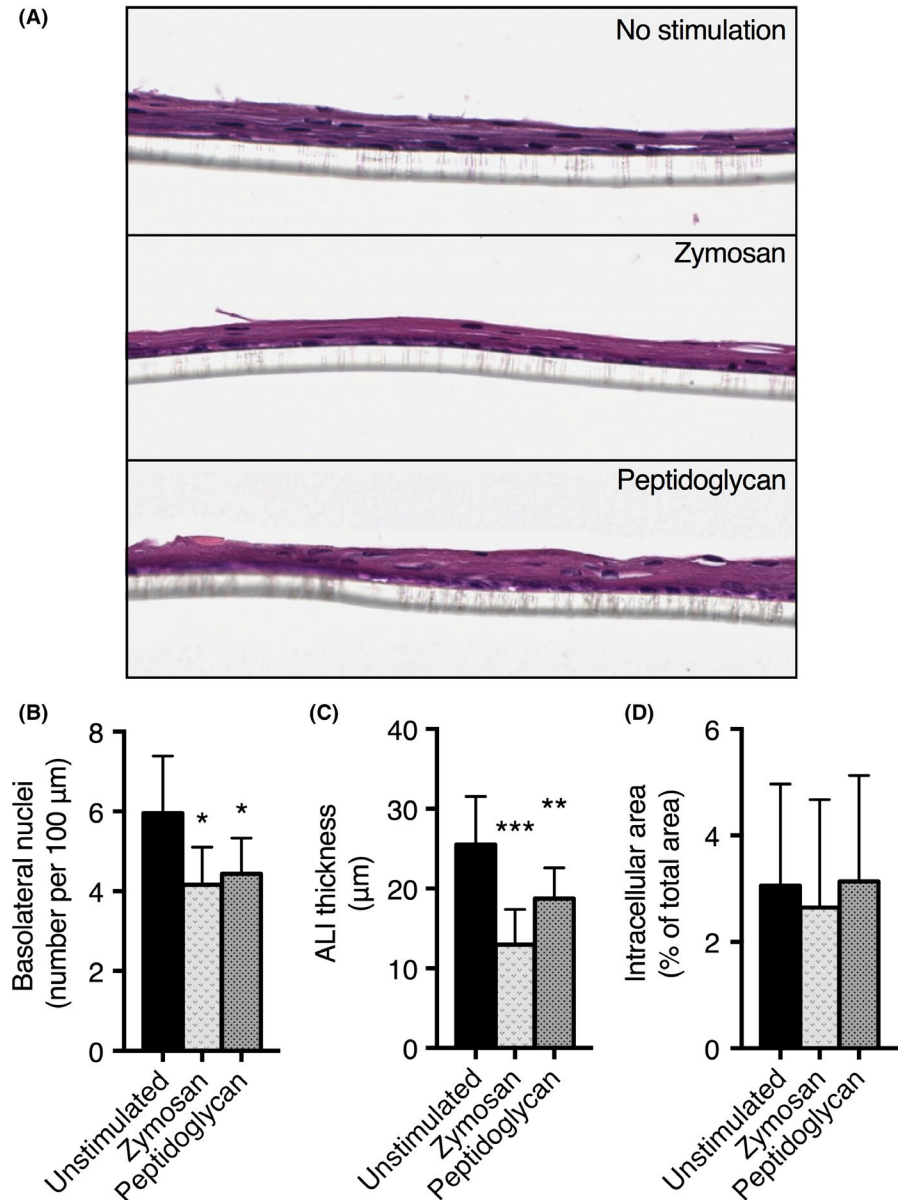
FIGURE 2 Toll-like receptor 2 (TLR2) stimulation of EPC2-hTERT cells in air-liquid interface (ALI) culture results in improved barrier function. A, Schematic of ALI epithelial culture model. Culture days 1-7 allow for proliferation and initial differentiation, and days 7-10 permit terminal differentiation. Stimuli applied in the basolateral media chamber during days 10-14 allow assessment of effects on epithelial barrier function. B, ALI cultures were stimulated with TLR2 agonists zymosan (10 $\mu g/mL$), peptidoglycan (10 $\mu g/mL$), or Pam2CSK4 (10 ng/mL) from days 10-14 in ALI culture. Transepithelial electrical resistance (TEER, $\Omega \cdot cm^2$) was measured by ohmmeter and calculated by multiplying by the area of the ALI membrane. TLR2 agonist-treated cultures demonstrate significantly increased day 14 TEER compared with unstimulated ALI cultures. $n = 10$ wells per group, representative of three experiments. C, On day 14, translocation of 70 kDa FITC-Dextran across ALI membranes into the basolateral culture chamber was decreased in the zymosan- and peptidoglycan-treated groups ($n = 6$ wells per group, mean $\pm$ SD), representative of two experiments. D, Addition of pAB-hTLR2 and anti-TLR2 blocking antibody to ALI cultures stimulated with 10 $\mu g/mL$ of zymosan block zymosan-induced TEER increase. $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$ using ANOVA followed by Dunnett's test compared with unstimulated control throughout

culture were stimulated with zymosan or peptidoglycan, and qRT-PCR was used to assess expression of TJ complex genes *CLDN1* and *TJP1* (claudin-1 and zonula occludens-1 ZO-1, Figure 4). *TJP1* was significantly upregulated over 2-fold following 6 hours of TLR2 stimulation with zymosan and peptidoglycan. mRNA levels of *CLDN1* rose to 2-fold over control unstimulated samples by 30 minutes of stimulation with zymosan and after 6 hours of stimulation with peptidoglycan. We did not see significant

upregulation desmoglein 1 mRNA following TLR2 stimulation with either zymosan or peptidoglycan (Figure S2), or following stimulation of TLR3 (Figure S1C).

To test the effect of TLR2 stimulation on TJ complex protein expression in esophageal epithelial cells, we examined claudin-1 and ZO-1 protein expression by immunohistochemistry (IHC) and Western blotting (Figure 5). ALI cultures were harvested on day 14 for IHC (Figure 5A), demonstrating an increased staining intensity

FIGURE 3 Zymosan stimulation of esophageal ALI alters epithelial morphology. EPC2-hTERT cells grown in ALI were harvested on day 14 of culture, fixed, paraffinized, and stained with hematoxylin and eosin. A, Images were captured at 20x. We quantified (B) ALI thickness, (C) basolateral nuclei (expressed per every 100 μm of basolateral membrane adjacent to the transwell membrane), and (D) the percent of total area dilated intracellular space quantified using Aperio software imaging algorithms ($n = 5$ slides per condition; mean $\pm$ SD, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, ANOVA, post hoc Dunnett's test) [Color figure can be viewed at wileyonlinelibrary.com]



for claudin-1 and ZO-1 in TLR2 agonist samples. 3,3'-diaminobenzidine staining intensity was quantified in Aperio imaging software to confirm upregulation of ZO-1 and claudin-1 in TLR2 agonist-treated samples (Figure 5B,C). We additionally observed increased expression of claudin-1 and ZO-1 protein from TLR2-stimulated ALI using Western blot (Figure 5D). Semi-quantitative analysis of Western blot band intensity in ImageJ software (Figure 5E) shows a mean 2.3- to 2.4-fold increase in claudin-1 and mean 2.9- to 4.2-fold increase in ZO-1 expression with stimulation. From these studies, we conclude that TLR2 stimulation is associated with increased expression of TJ proteins in the ALI model of esophageal epithelium.

3.6 | Histone modifications in zymosan-treated epithelial cells

Modification to the chromatin regulatory landscape alters gene expression in response to changes in the cellular environment. We

hypothesized TLR2 stimulation may induce regulatory histone modifications at tight junction complex gene loci.⁴³⁻⁴⁵ We performed chromatin immunoprecipitation (ChIP) assays of EPC2-hTERT cells following stimulation with zymosan (Figure 6) to assess for changes in the chromatin environment at the promoter and enhancer of *CLDN1* and *TJP1*. Acetylation of histone H4 was significantly increased at the *CLDN1* enhancer and promoter and decreased in the *TJP1* promoter of zymosan-treated samples. This suggests changes in acetylation of histone H4 occur subsequent to TLR2 stimulation with zymosan and may be a novel mechanism for regulation of esophageal tight junction proteins.

4 | DISCUSSION

The pathophysiology of eosinophilic esophagitis (EoE) is not solely defined by eosinophil-predominant inflammation, but additionally

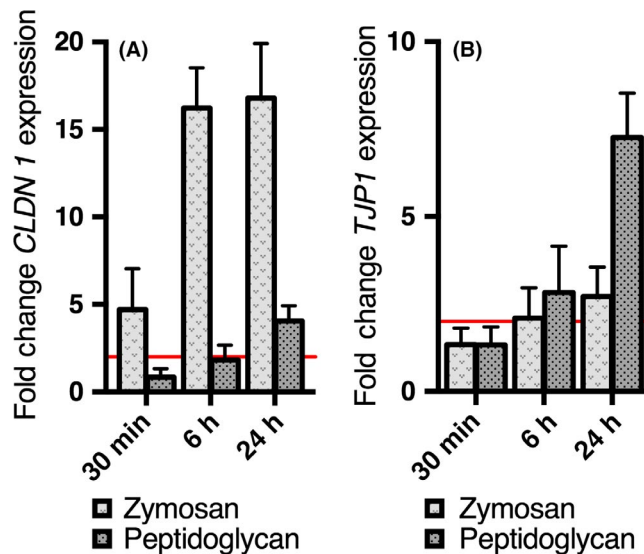


FIGURE 4 Zymosan treatment of EPC2-hTERT cells upregulates expression of cell adhesion molecules. EPC2-hTERT cells stimulated with zymosan or peptidoglycan (10 µg/mL) in culture result in greater than 2-fold upregulation of (A) *CLDN1* and (B) *TJP1* by 6 h of stimulation. mRNA signal was normalized to β -actin and expressed as fold change compared with unstimulated control cells ($n = 4$; mean $\pm$ SD)

by abnormal epithelial barrier morphology.³⁹ Increased esophageal epithelial permeability is a major aspect of the pathophysiology of EoE, which perpetuates disease by allowing allergens and microbes to penetrate the mucosal barrier.^{4,6} Little is known about innate esophageal epithelial responses to stimuli. In this study, we explore the role of TLR stimulation on epithelial barrier function utilizing the air-liquid interface model.

Our data demonstrate that esophageal epithelial cells express multiple pattern recognition receptors, including TLR1-TLR6, Dectin-1, and CD14. Our findings demonstrate robust expression of TLR2 and TLR3 in primary epithelium isolated from patients with and without EoE. This is similar to prior findings in primary esophageal epithelial cells from pediatric patients.²⁷ We do not observe gene expression differences in the PRRs on primary epithelial cells from EoE and control patients. In contrast, Arias et al have recently published gene expression data from whole biopsy tissue of adults with EoE demonstrating significant upregulation of TLR1, TLR2, TLR4, and TLR9 when compared to control patient biopsy tissue.⁴⁶ The overexpression of these TLR improved with six-food elimination diet.⁴⁶ Our current study used isolated cells between passages 2 and 4, which may have allowed for some normalization of expression between the EoE and control cell lines in vitro. To our knowledge, there are no published data comparing expression of PRR in esophageal biopsy tissue and freshly isolated esophageal epithelial cells to determine the gene expression signal from inflammatory cells present in the biopsy transcriptome. Data from Rochman et al examining the overlap of the EoE biopsy transcriptome and esophageal-specific proteome demonstrate a functional enrichment of IL-1 cytokine pathways in EoE, further supporting a role for epithelial innate signaling in EoE.⁴⁷

In the ALI model, we have tested whether stimulation of TLR2 and TLR3 can improve barrier function. Our data show that recognition of lipoprotein agonists by TLR2 is a mechanism that increases epithelial barrier integrity (Figure 2), with a modest increase in ALI TEER following stimulation with TLR2 ligands zymosan, peptidoglycan, and Pam2CSK4. It is not known what magnitude of in vitro barrier effect corresponds to a meaningful in vivo effect in this system. In contrast, stimulation of TLR3 with Poly(I:C) resulted in no change in barrier function compared with unstimulated control ALI cultures (Figure S1). TLR3 stimulation of esophageal epithelial cells has previously been shown to activate NF- κ B, resulting in secretion of IL-8 and TSLP.^{34,35} Therefore, although stimulation of the TLR3 pathway does not affect barrier function in this model, this does not exclude the possibility that the TLR3 pathway engages additional epithelial innate effector mechanisms which were not examined.

The esophagus is not a sterile environment, and changes in microbiome composition have been reported in patients with reflux and Barrett's esophagus as well as EoE patients with active inflammation.⁴⁸⁻⁵⁰ Further, tissue damage from inflammation or reflux causes release of endogenous damage-associated molecular patterns from necrotic cells which can also interact with esophageal PRR.^{27,34} Our findings describe one potential mechanism for esophageal barrier modulation in response to these changes. Our data show that TLR2 stimulation increases expression of TJ-associated proteins claudin-1 and ZO-1 (Figures 4 and 5). TJ complex molecules claudin-1 and ZO-1 are upregulated within 24 hours following TLR2 stimulation (Figure 4). Our results are consistent with findings from other epithelial types, including intestinal, airway, and skin keratinocytes, where TLR2 agonism has been associated with increased expression of TJ complex molecules claudin-1 and ZO-1.^{14-17,19,51}

Extrapolating upon these findings, we hypothesized that signaling thru innate immune receptors may play a critical role in epithelial cell fate. We find that stimulation of TLR2 with zymosan results in significant enrichment of H4ac at both the *CLDN1* promoter and enhancer (Figure 6). H4ac is an activation mark with a widespread distribution at regulatory regions. TLR2-induced chromatin remodeling could be secondary to an overall shift in the balance of histone acetyltransferases and deacetylase enzymes or more likely due to targeting of chromatin modification complexes to the regulatory regions.⁵² Novel therapies with histone deacetylase inhibitors could be investigated to target this pathway within the mucosal for disorders like EoE.

In this study, we focused on the response of claudin-1 and ZO-1 because of their critical role in tight junction complex formation.^{42,53} A number of epithelial proteins including desmosomes, serine peptidase inhibitors, filaggrin, and calpains are involved in maintenance of esophageal epithelial barrier integrity.^{13,25,54,55} We did not see alterations in the expression of desmoglein 1; however, further work will be required to determine the full scope of epithelial change in response to TLR2 stimulation. We hypothesize that TLR2 stimulation induces additional alterations in esophageal

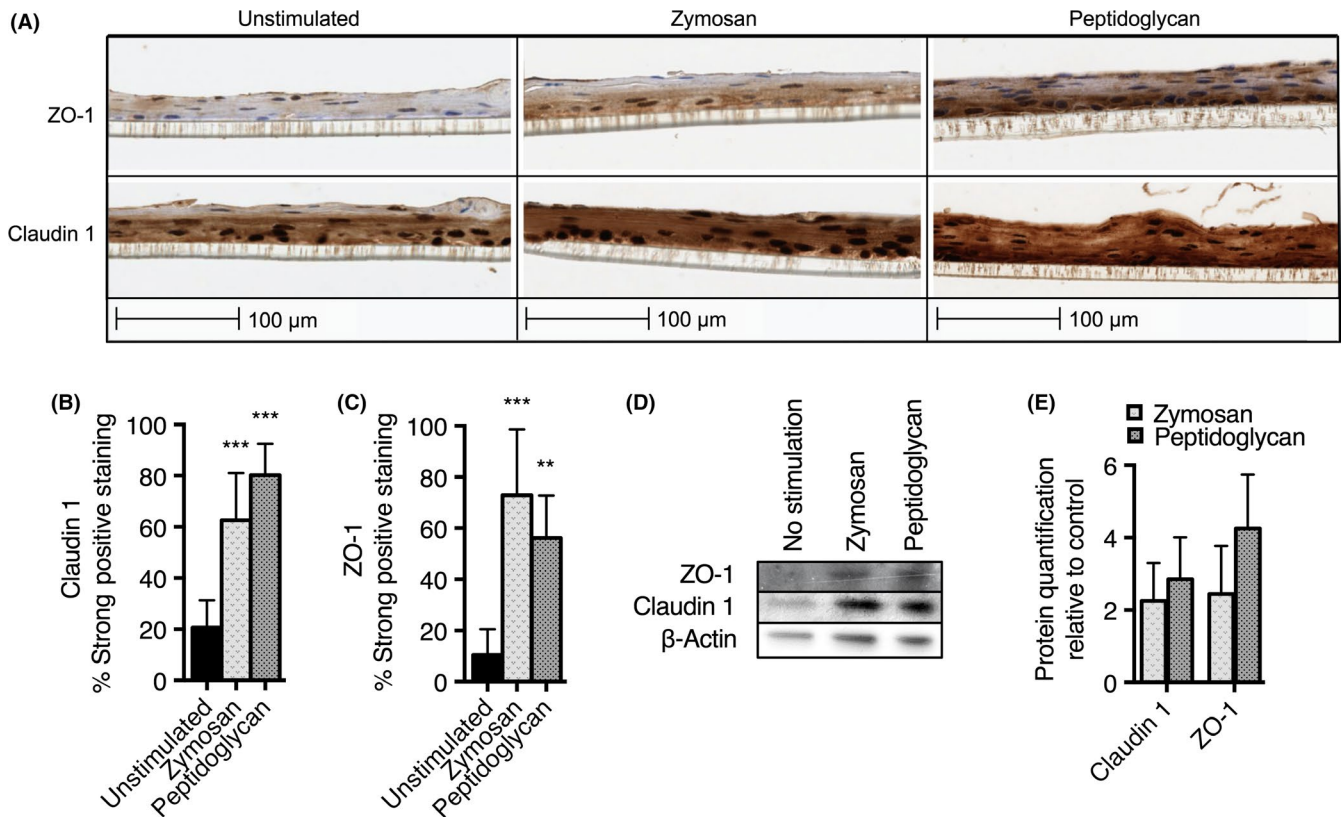


FIGURE 5 Air-liquid interface culture (ALI) cultures treated with zymosan upregulate epithelial cell-cell adhesion molecules. A, EPC2-hTERT ALI culture was stimulated with zymosan and peptidoglycan and then stained for immunohistochemistry with anti-claudin-1 and anti-zonula occludens-1 (ZO-1). Claudin-1 is diffusely upregulated while the pattern of ZO-1 staining is more dense in the basal layer of ALI culture. Using DAB staining quantification algorithm in Aperio, we find that (B) claudin-1 and (C) ZO-1 staining are significantly upregulated (mean $\pm$ SD ** P < 0.01 or *** P < 0.001 ANOVA, post hoc Dunnett's test compared with unstimulated control). D, Western blotting of day 14 ALI cultures confirms upregulation of claudin-1 and ZO-1, with (E) densitometry normalization to beta-actin (NIH ImageJ) demonstrating 2- to 4-fold upregulation of protein following TLR2 stimulation. Results shown are representative of three experiments

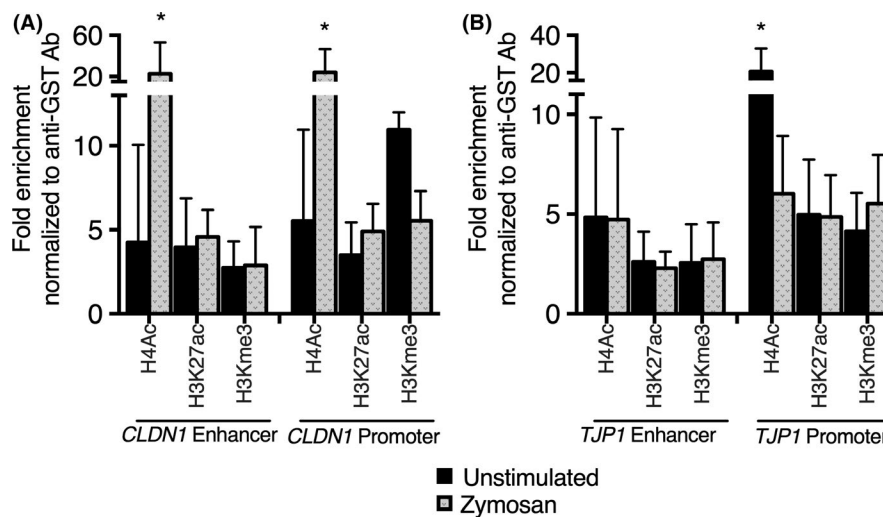


FIGURE 6 EPC2-hTERT cells stimulated with zymosan alter chromatin at *CLDN1* and *TJP1* promoters and enhancers. Following a 30-min incubation with zymosan, chromatin immunoprecipitation assays were performed using antibodies specific to H4ac, H3K27ac, and H3Kme3. PCR was performed to amplify immunoprecipitated DNA, and results were normalized to *GAPDH* and displayed as fold enrichment over anti-glutathione-S-transferase antibody. A, We observe significant enrichment of H4ac at *CLDN1* enhancer and promoter locus following zymosan treatment, whereas (B) there is decreased H4ac signal following zymosan treatment at the *TJP1* enhancer (n = 4, mean $\pm$ SD * P < 0.05, t test)

epithelial cell gene expression that were not fully captured in our experiments, and future experiments characterizing these additional effects will clarify the full impact of TLR2 stimulation on the esophageal mucosa.

A TLR3 single nucleotide polymorphism (SNP) was recently linked to increase risk of developing EoE in a Spanish cohort.⁵⁶ TLR2 SNPs have not been associated directly with EoE, but have been associated with closely linked atopic diseases. Specifically, there are TLR2 SNPs which predispose to the development of asthma in Puerto Rican, Norwegian, Danish, and Caucasian African populations⁵⁷⁻⁶³ and development of severe atopic dermatitis in Italian and German populations.^{64,65} Interestingly, several of the SNPs decrease TLR2 signaling activity.⁵⁷ Further studies elucidating how PRRs such as TLRs contribute to host barrier responses may help clarify mechanisms of atopic disease including EoE.

In conclusion, we have shown that esophageal epithelial cells express TLR1-TLR6, TLR9, Dectin-1, and CD14, with membrane expression of TLR2. Stimulation of epithelial TLR2 with lipoproteins Pam2CSK4, zymosan, and peptidoglycan upregulate claudin-1 and ZO-1, resulting in enhanced esophageal epithelial barrier function in the ALI model. This effect is TLR2-specific and abrogated by TLR2 blocking antibody. Our data highlight a potential homeostatic role of esophageal epithelial TLR2 activity in upregulating tight junction complex protein expression. This has implications for esophageal mucosal responses to infection, colonizing microbiota, and endogenous DAMPs released in the context of reflux and inflammation. TLR2 agonism appears to convey a protective effect on the esophageal mucosal barrier, and further investigation is warranted to determine whether this response is intact during mucosal inflammatory diseases such as EoE.

ACKNOWLEDGMENTS

EPC2-hTERT cells were the kind gift of Dr Hiro Nakagawa. We wish to thank Drs. De'broski Herbert, Rob Rubenstein, and Yann Bikard for advice regarding the ALI model and to acknowledge support from the Children's Hospital of Philadelphia Center for Epithelial Excellence. We are grateful to Swathi Raman and the Children's Hospital of Philadelphia Flow Cytometry and Pathology Core facilities for technical assistance.

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

AUTHOR CONTRIBUTIONS

MAR conceived of the presented idea. MAR, ABM, JMS, and KES, developed idea, reviewed, and interpreted data. ABM performed endoscopies and isolated epithelial cells. MAR, LS, LHS, MCC, KM, and JXW performed experiments, analyzed, and data. MAR wrote article with review and comments from all other authors.

ORCID

Melanie A. Ruffner  <https://orcid.org/0000-0002-2943-9944>

Amanda B. Muir  <https://orcid.org/0000-0003-2616-7063>

Jonathan M. Spergel  <https://orcid.org/0000-0002-4658-5353>

Kathleen E. Sullivan  <https://orcid.org/0000-0003-4018-1646>

REFERENCES

1. Rochman M, Azouz NP, Rothenberg ME. Epithelial origin of eosinophilic esophagitis. *J Allergy Clin Immunol*. 2018;142:10-23.
2. Simon D, Page B, Vogel M, et al. Evidence of an abnormal epithelial barrier in active, untreated and corticosteroid-treated eosinophilic esophagitis. *Allergy*. 2018;73:239-247.
3. Simon D, Straumann A, Dahinden C, Simon HU. Frequent sensitization to *Candida albicans* and profilins in adult eosinophilic esophagitis. *Allergy*. 2013;68:945-948.
4. Warners MJ, van Rhijn BD, Verheij J, Smout A, Bredenoord AJ. Disease activity in eosinophilic esophagitis is associated with impaired esophageal barrier integrity. *Am J Physiol Liver Physiol*. 2017;313:G230-G238.
5. Warners MJ, Vlieg-Boerstra BJ, Verheij J, et al. Esophageal and small intestinal mucosal integrity in eosinophilic esophagitis and response to an elemental diet. *Am J Gastroenterol*. 2017;112:1061-1071.
6. Katzka DA, Ravi K, Geno DM, et al. Endoscopic mucosal impedance measurements correlate with eosinophilia and dilation of intercellular spaces in patients with eosinophilic esophagitis. *Clin Gastroenterol Hepatol*. 2015;13:1242-1248.
7. Katzka DA, Tadi R, Smyrk TC, et al. Effects of topical steroids on tight junction proteins and spongiosis in esophageal epithelia of patients with eosinophilic esophagitis. *Clin Gastroenterol Hepatol*. 2014;12:1824-1829.
8. Zeisel MB, Dhawan P, Baumert TF. Tight junction proteins in gastrointestinal and liver disease. *Gut*. 2019;68(3):547-561.
9. Zihni C, Mills C, Matter K, Balda MS. Tight junctions: from simple barriers to multifunctional molecular gates. *Nat Rev Mol Cell Biol*. 2016;17:564-580.
10. Farquhar MG, Palade GE. Junctional complexes in various epithelia. *J Cell Biol*. 1963;17:375-412.
11. Samuelov L, Sarig O, Harmon RM, et al. Desmoglein 1 deficiency results in severe dermatitis, multiple allergies and metabolic wasting. *Nat Genet*. 2013;45:1244-1248.
12. McAleer MA, Pohler E, Smith F, et al. Severe dermatitis, multiple allergies, and metabolic wasting syndrome caused by a novel mutation in the N-terminal plak domain of desmoplakin. *J Allergy Clin Immunol*. 2015;136:1268-1276.
13. Sherrill JD, Kc K, Wu D, et al. Desmoglein-1 regulates esophageal epithelial barrier function and immune responses in eosinophilic esophagitis. *Mucosal Immunol*. 2014;7:718-729.
14. Kuo I-H, Carpenter-Mendini A, Yoshida T, et al. Activation of epidermal toll-like receptor 2 enhances tight junction function: implications for atopic dermatitis and skin barrier repair. *J Invest Dermatol*. 2013;133:988-998.
15. Cario E, Gerken G, Podolsky DK. Toll-like receptor 2 controls mucosal inflammation by regulating epithelial barrier function. *Gastroenterology*. 2007;132:1359-1374.
16. Tunis MC, Marshall JS. Toll-like receptor 2 as a regulator of oral tolerance in the gastrointestinal tract. *Mediators Inflamm*. 2014;2014:606383.
17. Cario E, Gerken G, Podolsky DK. Toll-like receptor 2 enhances ZO-1-associated intestinal epithelial barrier integrity via protein kinase C. *Gastroenterology*. 2004;127:224-238.

18. Chun J, Prince A. TLR2-induced calpain cleavage of epithelial junctional proteins facilitates leukocyte transmigration. *Cell Host Microbe*. 2009;5:47-58.
19. Ragupathy S, Esmaeili F, Paschoud S, Sublet E, Citi S, Borchard G. Toll-like receptor 2 regulates the barrier function of human bronchial epithelial monolayers through atypical protein kinase C zeta, and an increase in expression of claudin-1. *Tissue Barriers*. 2014;2:e29166.
20. Takaoka M, Harada H, Deramandt TB, et al. Ha-RasG12V induces senescence in primary and immortalized human esophageal keratinocytes with p53 dysfunction. *Oncogene*. 2004;23:6760-6768.
21. Harada H, Nakagawa H, Oyama K, et al. Telomerase induces immortalization of human esophageal keratinocytes without p16INK4a inactivation. *Mol Cancer Res*. 2003;1:729-738.
22. Muir AB, Lim DM, Benitez AJ, et al. Esophageal epithelial and mesenchymal cross-talk leads to features of epithelial to mesenchymal transition in vitro. *Exp Cell Res*. 2013;319:850-859.
23. Lucendo AJ, Molina-Infante J, Arias Á, et al. Guidelines on eosinophilic esophagitis: evidence-based statements and recommendations for diagnosis and management in children and adults. *United European Gastroenterol J*. 2017;5:335-358.
24. Dellon ES, Liacouras CA, Molina-Infante J et al. Updated international consensus diagnostic criteria for eosinophilic esophagitis: proceedings of the AGREE conference. *Gastroenterology*. 2018; 155(4):1022-1033.
25. Davis BP, Stucke EM, Khorki ME, et al. Eosinophilic esophagitis-linked calpain 14 is an IL-13-induced protease that mediates esophageal epithelial barrier impairment. *JCI Insight*. 2016; 1(4):e86355.
26. Maurer K, Ramen S, Shi L, Song L, Sullivan KE. Rapid induction of expression by LPS is accompanied by favorable chromatin and rapid binding of c-Jun. *Mol Immunol*. 2018;95:99-106.
27. Lim DM, Narasimhan S, Michaylira CZ, Wang M-L. TLR3-mediated NF- κ B signaling in human esophageal epithelial cells. *Am J Physiol Gastrointest Liver Physiol*. 2009;297:G1172-G1180.
28. Verbeek RE, Siersema PD, Vleggaar FP, et al. Toll-like receptor 2 signalling and the lysosomal machinery in Barrett's esophagus. *J Gastrointest Liver Dis*. 2016;25:273-282.
29. Huhta H, Helminen O, Lehenkari PP, Saarnio J, Karttunen TJ, Kauppila JH. Toll-like receptors 1, 2, 4 and 6 in esophageal epithelium, Barrett's esophagus, dysplasia and adenocarcinoma. *Oncotarget*. 2016;7:23658-23667.
30. Zhao G-Q, Qiu X-Y, Lin J, et al. Co-regulation of Dectin-1 and TLR2 in inflammatory response of human corneal epithelial cells induced by *Aspergillus fumigatus*. *Int J Ophthalmol*. 2016;9:185-190.
31. Yadav M, Schorey JS. The β -glucan receptor dectin-1 functions together with TLR2 to mediate macrophage activation by mycobacteria. *Blood*. 2006;108:3168-3175.
32. Shin DM, Yang CS, Yuk JM, et al. Mycobacterium abscessus activates the macrophage innate immune response via a physical and functional interaction between TLR2 and dectin-1. *Cell Microbiol*. 2008;10:1608-1621.
33. Ferwerda G, Meyer-Wentrup F, Kullberg BJ, Netea MG, Adema GJ. Dectin-1 synergizes with TLR2 and TLR4 for cytokine production in human primary monocytes and macrophages. *Cell Microbiol*. 2008;10:2058-2066.
34. Lim DM, Wang M-L. Toll-like receptor 3 signaling enables human esophageal epithelial cells to sense endogenous danger signals released by necrotic cells. *Am J Physiol Gastrointest Liver Physiol*. 2011;301:G91-G99.
35. Chandramouleeswaran PM, Shen D, Lee AJ, et al. Preferential secretion of thymic stromal lymphopoietin (TSLP) by terminally differentiated esophageal epithelial cells: relevance to eosinophilic esophagitis (EoE). *PLoS One*. 2016;11:e0150968.
36. Swidergall M, Solis NV, Lionakis MS, Filler SG. EphA2 is an epithelial cell pattern recognition receptor for fungal β -glucans. *Nat Microbiol*. 2018;3:53-61.
37. Brandt KJ, Fickentscher C, Boehlen F, Kruihof E, De Moerloose P. NF- κ B is activated from endosomal compartments in antiphospholipid antibodies-treated human monocytes. *J Thromb Haemost*. 2014;12:779-791.
38. Vogt LM, Meyer D, Pullens G, et al. Toll-like receptor 2 activation by 2-% α 1-fructans protects barrier function of T84 human intestinal epithelial cells in a chain length-dependent manner. *J Nutr*. 2014;144:1002-1008.
39. Collins MH, Martin LJ, Alexander ES, et al. Newly developed and validated eosinophilic esophagitis histology scoring system and evidence that it outperforms peak eosinophil count for disease diagnosis and monitoring. *Dis Esophagus*. 2017;30:1-8.
40. Kiran KC, Rothenberg ME, Sherrill JD. In vitro model for studying esophageal epithelial differentiation and allergic inflammatory responses identifies keratin involvement in eosinophilic esophagitis. *PLoS One*. 2015;10:e0127755.
41. Björkman E, Edebo A, Oltean M, Casselbrant A. Esophageal barrier function and tight junction expression in healthy subjects and patients with gastroesophageal reflux disease: functionality of esophageal mucosa exposed to bile salt and trypsin in vitro. *Scand J Gastroenterol*. 2013;48:1118-1126.
42. Furuse M, Hata M, Furuse K, et al. Claudin-based tight junctions are crucial for the mammalian epidermal barrier: a lesson from claudin-1-deficient mice. *J Cell Biol*. 2002;156:1099-1111.
43. Stebe-Frick S, Ostaff MJ, Stange EF, Malek NP, Wehkamp J. Histone deacetylase-mediated regulation of the antimicrobial peptide hBD2 differs in intestinal cell lines and cultured tissue. *Sci Rep*. 2018;8:12886.
44. Neagos J, Standiford TJ, Newstead MW, Zeng X, Huang SK, Ballinger MN. Epigenetic regulation of tolerance to toll-like receptor ligands in alveolar epithelial cells. *Am J Respir Cell Mol Biol*. 2015;53:872-881.
45. Perkins DJ, Patel MC, Blanco J, Vogel SN. Epigenetic mechanisms governing innate inflammatory responses. *J Interferon Cytokine Res*. 2016;36:454-461.
46. Arias Á, Vicario M, Bernardo D, et al. Toll-like receptors-mediated pathways activate inflammatory responses in the esophageal mucosa of adult eosinophilic esophagitis. *Clin Transl Gastroenterol*. 2018;9:147.
47. Rochman M, Travers J, Miracle CE, et al. Profound loss of esophageal tissue differentiation in patients with eosinophilic esophagitis. *J Allergy Clin Immunol*. 2017;140:738-749.
48. May M, Julian Abrams BA. Emerging insights into the esophageal microbiome. *Curr Treat Options Gastroenterol*. 1938;16:72-85.
49. Benitez AJ, Hoffmann C, Muir AB, et al. Inflammation-associated microbiota in pediatric eosinophilic esophagitis. *Microbiome*. 2015;3:23.
50. Harris JK, Fang R, Wagner BD, et al. Esophageal microbiome in eosinophilic esophagitis. *PLoS One*. 2015;10(5):e0128346.
51. Ren C, Zhang Q, De Haan BJ, Zhang H, Faas MM, De Vos P. Identification of TLR2/TLR6 signalling lactic acid bacteria for supporting immune regulation. *Sci Rep*. 2016;6:34561.
52. Allis CD, Jenuwein T. The molecular hallmarks of epigenetic control. *Nat Rev Genet*. 2016;17:487-500.
53. Matter K, Balda MS. Functional analysis of tight junctions. *Methods*. 2003;30:228-234.
54. Wu L, Oshima T, Li M, et al. Filaggrin and tight junction proteins are crucial for IL-13-mediated esophageal barrier dysfunction. *Am J Physiol Gastrointest Liver Physiol*. 2018;315:341-350.
55. Azouz NP, Ynga-Durand MA, Caldwell JM, et al. The antiprotease SPINK7 serves as an inhibitory checkpoint for esophageal epithelial inflammatory responses. *Sci Transl Med*. 2018;10:82-88.
56. Ávila-Castellano R, García-Lozano J-RR, Cimbollek S, et al. Genetic variations in the TLR3 locus are associated with eosinophilic esophagitis. *United European Gastroenterol J*. 2018;6:349-357.

57. Kormann M, Ferstl R, Depner M, et al. Rare TLR2 mutations reduce TLR2 receptor function and can increase atopy risk. *Allergy*. 2009;64:636-642.
58. Smit L, Bongers S, Ruven H, et al. Atopy and new-onset asthma in young Danish farmers and CD14, TLR2, and TLR4 genetic polymorphisms: a nested case-control study. *Clin Exp Allergy*. 2007;37:1602-1608.
59. Kormann M, Depner M, Hartl D, et al. Toll-like receptor heterodimer variants protect from childhood asthma. *J Allergy Clin Immunol*. 2008;122:86-92.
60. Potaczek DP, Nastalek M, Okumura K, Wojas-Pelc A, Undas A, Nishiyama C. An association of TLR2-16934A>T polymorphism and severity/phenotype of atopic dermatitis. *J Eur Acad Dermatol Venereol*. 2011;25:715-721.
61. Lau M, Dharmage SC, Burgess JA, et al. The interaction between farming/rural environment and TLR2, TLR4, TLR6 and CD14 genetic polymorphisms in relation to early- and late-onset asthma. *Sci Rep*. 2017;7:43681.
62. Eder W, Klimecki W, Yu L, et al. Toll-like receptor 2 as a major gene for asthma in children of European farmers. *J Allergy Clin Immunol*. 2004;113:482-488.
63. Tizaoui K, Kaabachi W, Hamzaoui K, Hamzaoui A. Association of single nucleotide polymorphisms in toll-like receptor genes with asthma risk: a systematic review and meta-analysis. *Allergy Asthma Immunol Res*. 2014;7:130-140.
64. Salpietro C, Rigoli L, Miraglia Del Giudice M et al. TLR2 and TLR4 gene polymorphisms and atopic dermatitis in Italian children: a multicenter study. *Int J Immunopathol Pharmacol*. 2011;24(4_suppl):33-40.
65. Oh D-YY, Schumann RR, Hamann L, Neumann K, Worm M, Heine G. Association of the toll-like receptor 2 A-16934T promoter polymorphism with severe atopic dermatitis. *Allergy*. 2009;64:1608-1615.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

How to cite this article: Ruffner MA, Song L, Maurer K, et al. Toll-like receptor 2 stimulation augments esophageal barrier integrity. *Allergy*. 2019;74:2449–2460. <https://doi.org/10.1111/all.13968>

Supplemental Information

Table S1: Primers used for SYBR qRT-PCR experiments

<i>CLDN1</i>	Forward	5'-TCCCAGAAGGCAGAGAGAA-3'
	Reverse	5'-GGTATGGCAATAGAATCGTTCAAG-3'
<i>TJP1</i>	Forward	5'-GGATGGGTTTCATAGCGTTTCT-3'
	Reverse	5'-AAAGCCAGCGCATTCTCA-3'
<i>DSG1</i>	Forward	5'-AGCCACCATATGGGATCTTTG-3'
	Reverse	5'-TCAGAGCTCGGCAGTAGATAA-3'
<i>ACTB</i>	Forward	5'-AAGGGACTTCCTGTAACAATGCA-3'
	Reverse	5'-CTGGAACGGTGAAGGTGACA-3'

Table S2: Primers and probes for chromatin immunoprecipitation assay

<i>CLDN1</i> promoter	Forward	5'-CGGTTCTGCGTCTCAGTT-3'
	Reverse	5'-CGCTGGAGAAGCTCTGG-3'
	Probe	5'-/56- FAM ATACTAGAA/ZEN/GCTGCGGTTGCTCCC/36-TAMSp/-3'
<i>CLDN1</i> enhancer	Forward	5'-GATCTGGAGAAGTGCCAAGAG-3'
	Reverse	5'-CTGAAGAAGCTAAGACCAGATAGG-3'
	Probe	5'-/56- FAM CCACTTCCA/ZEN/AGTCTGCCACTCACA/36-TAMSp/-3'
<i>TJP1</i> promoter	Forward	5'-AGGAAACAACCTGTCGGGTATC-3'
	Reverse	5'-GTCAGGTATGTAGCAGGTCAATAG-3'
	Probe	5'-/56- FAM CTTTCCAGC/ZEN/CGCACTCATGCATG/36-TAMSp/-3'
<i>TJP1</i> enhancer	Forward	5'-AGTGGCTCATGCCTGTAATC-3'
	Reverse	5'-GTAGAGACAGGGTTTCATCATGT-3'
	Probe	5'-/56- FAM TTTGGGAGG/ZEN/CTGAGGAGGGTAGAT/36-TAMSp/-3'
<i>GAPDH</i>	Forward	5'-CGGTGCGTGCCCAAGTT-3'
	Reverse	5'-CCCTACTTTCTCCCCGCTTT-3'
	Probe	5'-/56-FAM/ACCAGGCGGCTGCGGAAAAAA/36-TAMSp/-3'

Supplemental Figure 1:

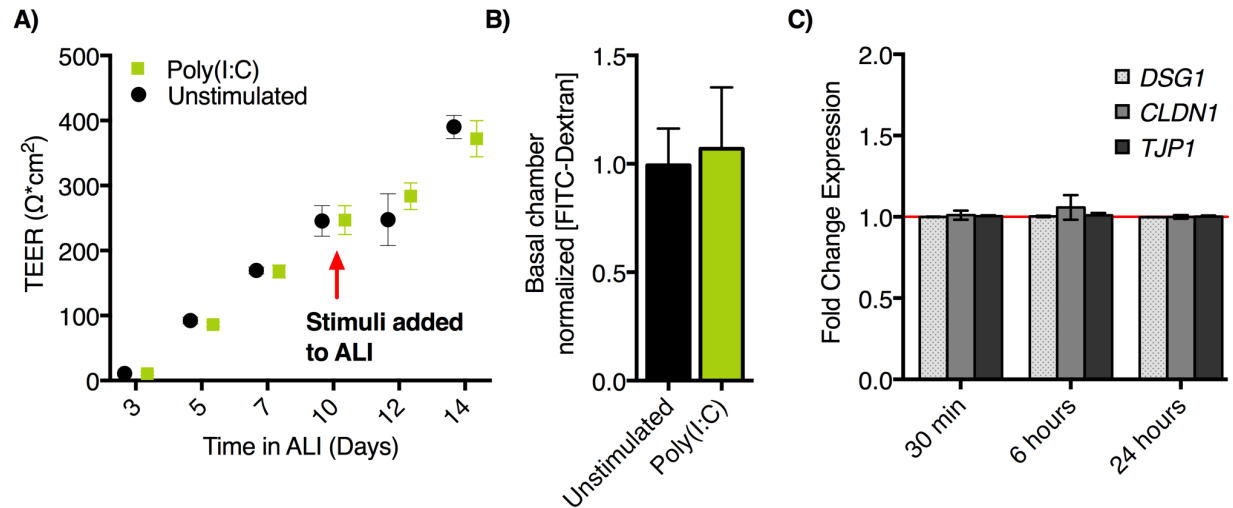


Figure S1: TLR3 stimulation does not alter barrier function of EPC2-hTERT in air-liquid

interface (ALI) model. ALI cultures were stimulated with TLR3 ligand poly(I:C) from days 10-14, with no change in **A)** TEER or **B)** FITC-Dextran permeability observed ($n=7$ wells per group, representative of two experiments). **C)** EPC2-hTERT were stimulated with Poly(I:C) in culture. mRNA signal was normalized to β -actin and expressed as fold change compared to unstimulated control cells and no differences were seen in levels of *CLDN1*, *TJP1* or *DSG1* when compared to unstimulated control cells. ($n=4$; mean $\pm$ SD, red horizontal line at Fold Change =1)

Supplemental Figure 2:

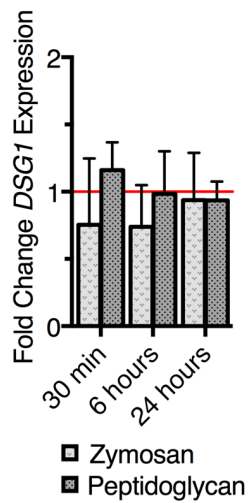


Figure S2: TLR2 stimulation of EPC2-hTERT cells does not alter expression of *DSG1*. EPC2-hTERT cells stimulated with zymosan and peptidoglycan (each at 10 μ g/ml) in culture were analyzed by qRT-PCR. mRNA signal was normalized to β -actin and expressed as fold change compared to unstimulated control cells ($n=4$; mean $\pm$ SD, red horizontal line at Fold Change =1)

Supplemental Figure 3:

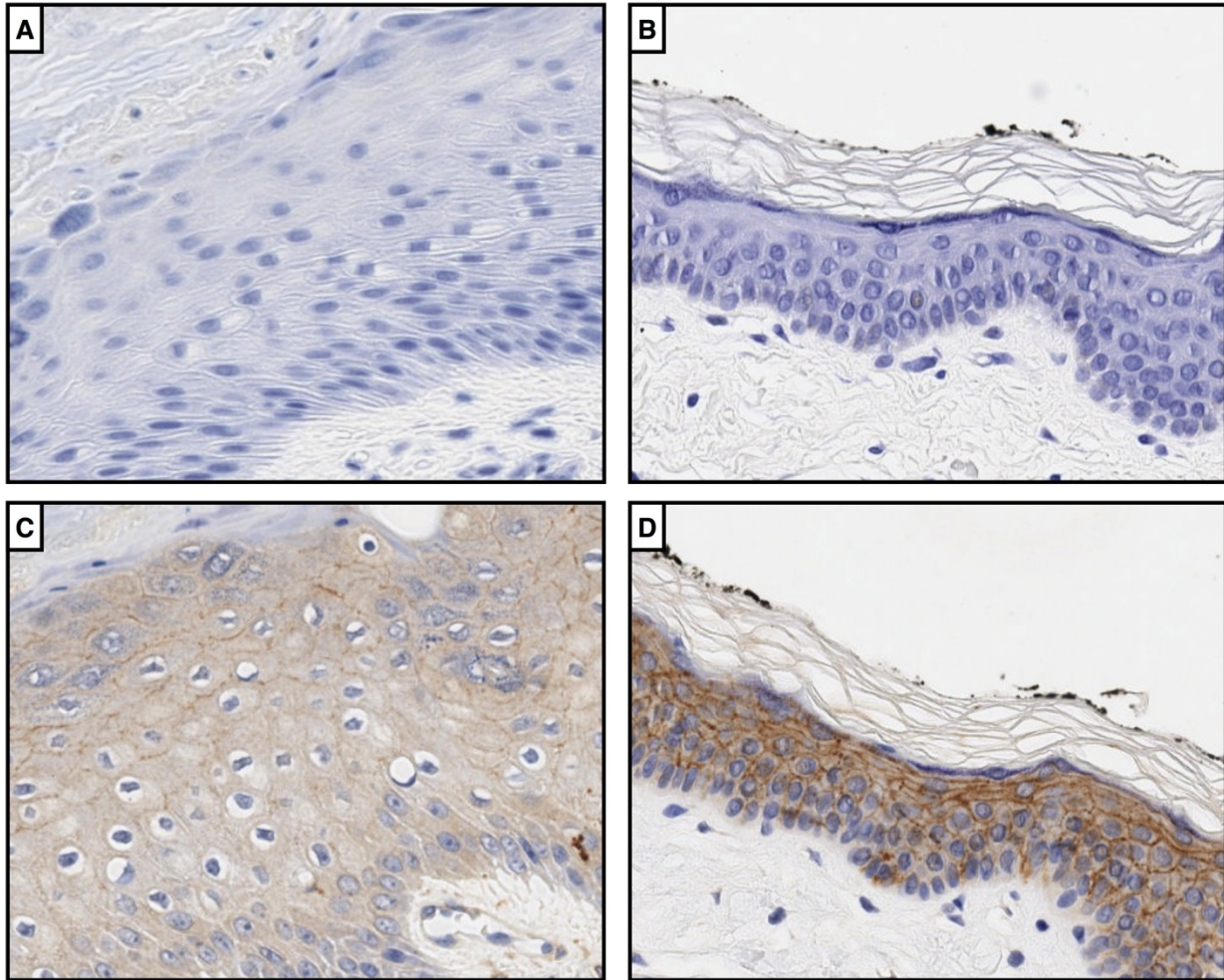


Figure S3: Immunohistochemistry controls. Immunohistochemical staining for the expression of claudin-1 and zonula occludens-1 (ZO-1) was optimized using sections of human skin as negative and positive controls. **A)** no primary ZO-1 control; **B)** no primary claudin-1 control; **C)** 1:50 anti-ZO-1 primary antibody **D)** 1:50 anti-claudin-1 primary antibody.