



Published in final edited form as:

*J Invest Dermatol.* 2013 April ; 133(4): 988–998. doi:10.1038/jid.2012.437.

## Activation of epidermal toll-like receptor 2 enhances tight junction function – Implications for atopic dermatitis and skin barrier repair

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### Abstract

Atopic dermatitis (AD) is characterized by epidermal tight junction (TJ) defects and a propensity for *Staphylococcus aureus* (*S. aureus*) skin infections. *S. aureus* is sensed by many pattern recognition receptors including toll-like receptor (TLR) 2. We hypothesized that an effective innate immune response will include skin barrier repair and that this response is impaired in AD subjects. *S. aureus*-derived peptidoglycan (PGN) and synthetic TLR2 agonists enhanced TJ barrier and increased expression of TJ proteins, CLDN1, CLDN23, occludin and ZO-1 in primary human keratinocytes. A TLR2 agonist enhanced skin barrier recovery in human epidermis wounded by

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### CONFLICT OF INTEREST

The authors state no conflict of Interest.

tape-stripping. *Tlr2*<sup>-/-</sup> mice had a delayed and incomplete barrier recovery following tape-stripping. AD subjects had reduced epidermal TLR2 expression as compared to nonatopic (NA) subjects, which inversely correlated ( $r=0.654$ ,  $P=0.0004$ ) with transepidermal water loss (TEWL). These observations indicate that TLR2 activation enhances skin barrier in murine and human skin and is an important part of a wound repair response. Reduced epidermal TLR2 expression observed in AD patients may play a role in their incompetent skin barrier.

## INTRODUCTION

Recent findings have solidified the notion that skin barrier dysfunction plays a key role in the initiation of atopic dermatitis (AD). Dysfunction of the skin barrier in AD can occur through both genetic and acquired mechanisms. For example, AD subjects may have a loss-of-function mutation in *filaggrin* (Cork *et al.*, 2006; Howell *et al.*, 2009; Palmer *et al.*, 2006), epidermal lipid abnormalities (Imokawa, 2001; Murata *et al.*, 1996; Pilgram *et al.*, 2001), altered protease activity (Cork *et al.*, 2006; Vasilopoulos *et al.*, 2007), more alkaline surface pH (Elias *et al.*, 2008) as well as a defect in tight junction (TJ) function (De Benedetto *et al.*, 2011), all of which can contribute to decreased skin barrier function.

The human epidermis is a multilayered structure that is made up of four progressively differentiated layers. Transient breaches in skin barrier such as might occur with topical exposure to organic solvents or detergents, scratching, endogenous or exogenous proteases (e.g. from allergens or microbes) can temporally disrupt our skin barrier. In healthy subjects, the barrier repair mechanisms normally prevent or minimize the potential for pathogens or allergens to penetrate the host and evoke an immune response. An inadequate barrier repair response would lead to enhanced and more prolonged allergen sensitization, microbial colonization and/or infection in addition to greater transepidermal water loss (TEWL) that leads to skin xerosis (Boguniewicz and Leung, 2011; De Benedetto *et al.*, 2012; Grigoryev *et al.*, 2010; Kubo *et al.*, 2012). All of these are well-recognized features of AD.

The function of innate receptors, conventionally recognized for their antimicrobial effects has recently been extended to include epithelial barrier regulation (Cario *et al.*, 2004, 2007; Rezaee *et al.*, 2011; Yuki *et al.*, 2011b). Toll-like receptor (TLR) 2 has received the greatest attention as it is important for immune responses to a number of microbes such as *Staphylococcus aureus* (*S. aureus*) and herpes simplex virus (HSV) (Bochud *et al.*, 2007; De Benedetto *et al.*, 2009; Sato *et al.*, 2006; Takeuchi *et al.*, 2000; Takeuchi *et al.*, 1999; Zahringer *et al.*, 2008) that more commonly colonize and/or infect the skin of AD patients (Boguniewicz and Leung, 2010; Ong and Leung, 2010). Several groups have shown that AD patients have reduced expression and/or function of TLR2 on their monocytes and macrophages (Hasannejad *et al.*, 2007; Niebuhr *et al.*, 2009). However, the role of TLR2 in modulating epidermal barrier *in vitro* and *in vivo* remains poorly understood. In this study, we observed a reduction of TLR2 expression in AD skin epithelium. In addition, we observed that TLR2 signaling enhances the TJ integrity in cultured keratinocytes, which is in part mediated by enhanced expression of key TJ proteins. This TLR2 barrier repair effect was also confirmed in the TLR2 deficient mouse and in a human wound model. We conclude that TLR2 is important for the maintenance of TJ integrity in response to barrier

insults, and that this barrier repair mechanism may be defective in AD subjects. The consequence of this defect might explain why these patients are susceptible to microbial colonization and infections of the skin, which are thought to perpetuate their chronic skin inflammation.

## RESULTS

### TLR2 agonists enhance TJ function and increase expression of TJ proteins

Several studies have suggested that the antimicrobial barrier and stratum corneum (SC) permeability barrier are coregulated (Aberg *et al.*, 2008; Ahrens *et al.*, 2011; Borkowski and Gallo, 2011; Grether-Beck *et al.*, 2012). We hypothesized that the crosstalk between innate immunity and barrier integrity may extend to TJs, the other epidermal barrier structure important in regulating paracellular permeability of ions and macromolecules. Specifically, we wondered whether epidermal TJ permeability could be regulated by relevant cutaneous pathogens such as *S. aureus*. To test this hypothesis, PHK were stimulated with *S. aureus*-derived peptidoglycan (PGN) and lipoteichoic acid (LTA) (Figure 1a, b). PGN significantly increased the transepithelial electrical resistance (TEER) of PHK monolayers in a dose-dependent fashion. By contrast LTA had no effect on TJ barrier function despite evidence that it could enhance expression of TLR2, cluster of differentiation 14 (CD14), peptidoglycan recognition protein 3 (PGLYRP-3) and human  $\beta$ -defensin 2 (HBD-2) in human keratinocytes (data not shown). Using synthetic ligands targeting specific TLR2 heterodimers, Pam3CSK4 (TLR1/2 agonist) and Malp-2 (TLR2/6 agonist) we found that both increased keratinocyte TEER in dose-dependent manner (Pam3CSK4:  $P < 0.05$ ; Malp-2:  $P < 0.05$  - Figure 1c, d). In contrast LPS, a TLR4 agonist had no effect on TJ barrier integrity even at high concentrations (Figure 1e), which may reflect the low TLR4 expression on human PHK (data not shown) (Baker *et al.*, 2003; Kollisch *et al.*, 2005; Mempel *et al.*, 2003). Interestingly, polyI:C a synthetic TLR3 agonist appeared to consistently enhance epidermal TJ barrier in a wide range of concentrations (0.01–10  $\mu\text{g/ml}$ ) but with substantial variability among KC preparations (Figure 1f). To examine whether TLR2 agonists affect epithelial permeability for larger molecules, we measured paracellular flux of fluorescein (Figure 1g). Incubation of PHK monolayers with natural (PGN) and synthetic TLR2 agonists (Pam3CSK4 and Malp-2) for 24 h significantly decreased their permeability to fluorescein. Collectively, the TEER and paracellular flux data demonstrate that TLR2 signaling enhances TJ barrier function, which nicely complements its other innate defense actions.

To examine if barrier-enhancing effects of TLR2 agonists are associated with altered expression of TJ proteins, we quantified expression of key transmembrane and cytosolic TJ components in cultured keratinocytes (Amagai *et al.*, 2000; Baltes *et al.*, 2004; Djalilian *et al.*, 2006; Furuse *et al.*, 2001; Furuse *et al.*, 2002; Gareus *et al.*, 2007; Kretz *et al.*, 2004; Lucke *et al.*, 1999; Ma *et al.*, 2004; Ohnemus *et al.*, 2008; Tunggal *et al.*, 2005; Yuki *et al.*, 2011a). We found that mRNA expression of CLDN1, CLDN23, occludin and ZO-1 was significantly ( $P < 0.01$ ) induced after 24 h stimulation of confluent PHK with PGN while expression of adherens junction, gap junction, and desmosomal proteins was not significantly affected (Figure S1). We confirmed these qPCR findings at the protein level by

Western blot (Figure 2a). TLR2 synthetic agonists (Pam3CSK4 and Malp-2) also enhance the protein expression of CLDN1, occludin, ZO-1 and CLDN23. Of note, LTA, which had no effect on TJ function, also had no effect on the protein expression of key TJ components (Figure 2b). Quantified western blot results are shown in Figure S2.

Integrity of epithelial barrier depends not only on the total level of TJ proteins but also on their ability to form complexes at the plasma membrane. Therefore, we performed cell fractionation to evaluate the effect of TLR2 signaling on localization of integral membrane TJ proteins (Marchiando *et al.*, 2010; Utech *et al.*, 2005). We found that, CLDN1, occludin and CLDN23 accumulated exclusively in the membrane fraction and their level at the membrane was enhanced by PGN stimulation (Figure 2c). The specificity of our cellular fractionation was confirmed using appropriate markers (Figure S3).

### Blocking TLR2 prevents PGN-mediated effect on TJ

*S. aureus*-derived PGN can be sensed by several innate receptors including TLR2, NOD2 and PGLYRPs. To confirm that the enhanced barrier function and increased expression of TJ protein in PHK monolayers stimulated with PGN was TLR2-dependent, we performed blocking experiments using a specific TLR2-neutralizing antibody. As compared with isotype-matched control, the TLR2-neutralizing antibody completely inhibited the PGN-induced increase in TEER ( $P<0.01$ ) (Figure 3a) and decrease in paracellular flux ( $P<0.01$ ) (Figure 3b). Additionally, blocking TLR2 signaling abrogated the PGN-dependent upregulation of TJ components CLDN1, occludin, ZO-1 and CLDN23 both at the mRNA, (Figure 3c) and protein (Figure 3d, Figure S2b) level.

### A TLR2 agonist enhances human skin barrier repair

A classic hallmark of AD is a chronic itch/scratch cycle that leads to persistent barrier disruption. To mimic this mechanically induced barrier disruption (Koschwanetz and Broadbent) (Taljebini *et al.*, 1996), we performed tape-stripping in human discarded skins. TJ barrier recovery was monitored by measuring TEER and the paracellular fluorescein flux using the modified micro-Snapwell™ system with and without Pam3CSK4 treatment (see Methods and Figure 4a). Interestingly, treatment with the TLR1/2 agonist significantly enhanced TEER recovery (1.4 fold,  $P<0.004$ ) (Figure 4b) and resulted in approximately 50% decrease in the paracellular flux ( $P=0.007$ ) (Figure 4c) at 24 h after wounding.

### TLR2 knockout (*Tlr2*<sup>-/-</sup>) mice have a delayed and incomplete recovery of skin barrier function

To further clarify the importance of TLR2 in epidermal barrier repair *in vivo*, we utilized a tape-stripping wound model in WT and *Tlr2*<sup>-/-</sup> mice, and monitored barrier recovery by measuring transepidermal water loss (TEWL). In WT mice the skin barrier fully recovered 24 h after tape-stripping, whereas *Tlr2*<sup>-/-</sup> mice had a substantially slower recovery rate and did not reach baseline values even 24 h after wounding (Figure 4d). Two hours after skin barrier disruption, mRNA expression of *cldn1* and *zo-1* were slightly but significantly increased in WT but not in *Tlr2*<sup>-/-</sup> mice, while *cldn2*, a pore-forming TJ protein was increased in *Tlr2*<sup>-/-</sup> mice (Figure S4). Because TEWL measurement is an *in vivo* barrier assay that may reflect the integrity of both epidermal barrier structures (SC and TJ) as well

as dermal blood flow, we used our developed micro-Snapwell™ system to clarify whether the changes we observed in TEWL were at least in part reflected in functional changes in TJ *ex vivo*. To determine whether TLR2 agonists affect TJ integrity in this wound model, we measured TEER using the modified micro-Snapwell™ system in murine epidermis with and without Pam3CSK4 treatment. Pam3CSK4 significantly enhanced TEER over media alone 24 h after wounding in the WT mice ( $P=0.029$ ). This effect was not observed in wounded epidermis from *Tlr2*<sup>-/-</sup> mice (Figure 4e). Collectively, these results indicate that TLR2 agonist enhances TJ barrier recovery in human and murine epidermal wound models.

### AD subjects have reduced epidermal expression of TLR1 and 2

Keratinocytes respond to *S. aureus* using multiple innate receptors that reside on the cell membrane (TLR1, 2, 6), are intracellular (NOD2) or are secreted (PGLYRP-3, PGLYRP-4). We hypothesized that expression of one or more of these key receptors may be reduced in AD, which could explain altered barrier repair responses and AD subjects' susceptibility to *S. aureus* colonization. To test this hypothesis, epidermal samples were taken from non-sunexposed, volar forearms to control for anatomical differences and photo-induced changes from well-characterized subjects with AD and nonatopic (NA) controls. We initially quantified mRNA expression for different innate receptors in epidermal samples using qPCR. Both epidermal TLR1, which heterodimerizes with TLR2, and TLR2 mRNA were significantly decreased ( $P=0.03$  and  $0.04$ , respectively) in AD subjects. Expression levels of TLR6, NOD2, PGLYRP-3 and PGLYRP-4 were similar in the two groups (Figure 5). The reduced TLR2 expression was confirmed at the protein level by immunofluorescence staining (Figure 6a). Epidermal TLR2 staining was significantly reduced in all AD samples (non-lesional and lesional; median intensity: 76 and 52,  $P=0.01$ ,  $P=0.0005$ , respectively) as compared to NA (median intensity: 96; Figure 6b). Interestingly, PHKs isolated from AD epidermis have a reduced inflammatory response (TNF- $\alpha$ , IL-6 and HBD-2;  $P=0.032$ ) to PGN (Figure S7).

### TLR2 protein expression inversely correlated with TEWL

To address whether TLR2 epidermal expression might be a determinant of skin barrier function, we evaluated the relationship between TLR2 staining intensity and TEWL, a functional measurement of skin barrier integrity (Figure 6C). Epidermal TLR2 immunoreactivity demonstrated a significant inverse correlation with TEWL ( $r=0.676$ ,  $P=0.0002$ ). We also observed that mRNA expression for both TLR1 and TLR2 correlated inversely with TEWL (Figure S5). In summary, these observations strongly suggest that the decreased expression of key innate receptors TLR1 and TLR2 may adversely affect integrity of the epidermal barrier.

## DISCUSSION

Epidermal TJs form the barrier preventing paracellular movement between different cell layers of the stratum granulosum. TJ are very dynamic structures, loosening and tightening in response to endogenous signals that originate from epithelial and subepithelial compartments, as well as exogenous agents such as bacterial-derived products and proteolytic allergens. TJ are composed of transmembrane proteins such as occludin and the

claudin family members that directly mediate cell-cell adhesion and contribute to the development of the paracellular barrier. These transmembrane constituents are clustered and stabilized by cytosolic plaque proteins, such as ZO-1. Changes in expression of any one of these TJ components can have significant effects on TJ integrity (Niessen, 2007). We have observed reduced expression of CLDN1 and CLDN23 in nonlesional skin from AD subjects where TJ function is significantly impaired (De Benedetto *et al.*, 2011). Silencing of *CLDN1* in human keratinocytes as well as mice genetically deficient in *CLDN1* confirm the importance of this protein for a competent TJ and skin barrier (De Benedetto *et al.*, 2011; Furuse *et al.*, 2002). Results reported herein arose from experiments designed to identify pathways that would enhance TJ function in hopes that these might be used to repair AD barrier defects. We found that TJ function in keratinocytes was enhanced by activation of the *S. aureus*-responsive innate immune receptor, TLR2 (Figure 1). This TLR2 activation was accompanied by increased expression of several TJ components including those that were reduced in AD epidermis (CLDN1 and CLDN23; Figures 2 and 3). Similarly, exfoliative toxin-negative *S. aureus* enhanced expression of TJ molecules (occludin and ZO-1) in human keratinocytes, but had no effect on the adherens junction proteins (Ohnemus *et al.*, 2008), suggesting that TLR2-mediated effects on TJ integrity in the skin are not mediated by changes in the expression of other intercellular junctional proteins. In keeping with these observations, we found that the *Tlr2*<sup>-/-</sup> mouse had a delayed and incomplete repair response to epidermal injury (Figure 4) suggesting that this innate immune receptor mediates an important barrier repair pathway that is likely operative in AD subjects who suffer from a chronic itch-scratch cycle. This observation corroborates a previous finding that dysregulation of the wound repair response in skin might contribute to the severe phenotype in patients with AD (Grigoryev *et al.*, 2010). Furthermore, we hypothesize that this repair pathway may be defective in AD subjects who express less epidermal TLR2 than controls (Figures 5 and 6). Collectively, these data establish a previously under-appreciated role for innate receptors expressed on epidermal cells in skin barrier maintenance and recovery.

Earlier studies demonstrated that *Tlr2*<sup>-/-</sup> mice also develop more severe intestinal disease in a dextran sulfate sodium (DSS)-mediated ulcerative colitis model, which was due to an impaired intestinal TJ barrier repair response (Cario *et al.*, 2007). Importantly *Tlr4*<sup>-/-</sup> mice did not have the same phenotype suggesting that this was not a global TLR effect in intestinal epithelial cells (Cario *et al.*, 2007). In line with this finding, our results indicate that TLR4 agonist, LPS had no effect on TEER in human keratinocytes (Figure 1e), which is concordant with our unpublished data and that of other groups demonstrating little to no TLR4 expression in human keratinocytes (Baker *et al.*, 2003; Kollisch *et al.*, 2005). On the other hand, TLR3 ligands appeared to have an enhancing effect on TJ function but with tremendous variability from donor to donor (Figure 1f). In contrast, we have shown that TLR3 induces TJ disassembly in bronchial epithelium (Rezaee *et al.*, 2011), suggesting that the functions of these receptors may depend more on the epithelial cell phenotype than the receptor itself. Interestingly, *S. aureus*-derived LTA, which is considered activation through TLR2, had no effect on TJ function (Figure 1b and 2b), but did induce inflammatory gene expression in human keratinocytes (Menziez and Kenoyer, 2006; Olaru and Jensen, 2010). This suggests that the TLR2 signaling pathways may be different for inflammatory mediator

release versus TJ barrier repair. It is also unclear whether LTA is a specific ligand for TLR2 (Schroder *et al.*, 2003; Takeuchi *et al.*, 1999). It has been shown that macrophages isolated from *Tlr2*<sup>-/-</sup> mice stimulated with LTA have similar induction of IL-6 and TNF- $\alpha$  as compared to WT mice. Whereas macrophages from *Tlr4*<sup>-/-</sup> mice stimulated with LTA have severely impaired cytokine production (Takeuchi *et al.*, 1999). This suggests that LTA might act through TLR4. In our studies we found that TLR4 had no effect on TJ function (Figure 1e), and collectively this may explain why we observed no effect with LTA stimulation.

To evaluate the biological relevance of TLR2 in skin barrier repair we evaluated *Tlr2*<sup>-/-</sup> mice using an established mechanical epidermal wound model (i.e. repetitive tape-tripping). Tape-stripping liberates several danger associated molecular patterns such as heat shock proteins and hyaluronic acid some of which are recognized as TLR2 ligands (Dickel *et al.*, 2010; Ozinsky *et al.*, 2000; Schwandner *et al.*, 1999; Tsan and Gao, 2004; Yoshimura *et al.*, 1999). Additionally in this model murine commensal bacteria are also activating epidermal TLR2. In this model, we observed that *Tlr2*<sup>-/-</sup> mice had a markedly delayed and incomplete barrier recovery in response to a mechanically induced epidermal wound as measured by TEWL, an *in vivo* measurement that may reflect changes in either or both epidermal barrier structures, SC and/or TJ (Figure 4d). We found that TLR2 agonists help repair epidermal barrier in human and WT mice skin after tape-stripping (Figure 4b, c and e). To determine whether TLR2 agonist are acting on epidermal TJs, we developed a method to study human and murine epidermal TJ function (i.e. TEER and paracellular flux) using the micro-Snapwell™ system. We took advantage of the fact that prolonged epidermal hydration (i.e. 24 hr) results in disruption of intercellular lamellar lipid bilayers, degradation of corneodesmosomes, and formation of amorphous regions within the intercellular lipid (Warner *et al.*, 1999; Warner *et al.*, 2003), which would arguably significantly compromise SC barrier function. This observation combined with the fact that the skin samples had undergone repetitive tape-stripping, lead to our assertion that we are primarily measuring TJ function in this micro-Snapwell™ assay. We cannot entirely rule out the possibility that this significantly disturbed SC may contribute to our readouts but we think that is highly unlikely. In summary, we have found that TLR2 agonists enhance TJ functions in full thickness epidermal samples from both mice and humans that reaffirm our observations made in keratinocyte monolayers. We observed a delay in barrier repair in our *Tlr2*<sup>-/-</sup> murine wound model which we think is explained at least in part to an impairment in TJ recovery.

We speculate that the enhanced expression of TLR2 observed in adjacent epidermal cells following barrier disruption (Jin *et al.*, 2009; Schaubert *et al.*, 2007) serves two purposes. The first is to respond to and contain pathogens that proliferate in wounded skin. The second function is to promote early TJ formation in keratinocytes that form a monolayer over the dermal wound base. The early formation of this TJ would prevent further serum leakage and thereby limit the nutrients and adhesion molecules that would promote microbial overgrowth, and additionally this would also minimize insensible water loss preventing wound desiccation. We have observed that TLR2 is only expressed on keratinocytes in the cellular layers below TJs (unpublished data). Therefore, the development of a competent TJ

would also serve to limit TLR2-mediated inflammation that might be harmful at these later stages of wound repair.

Recently, Yuki et al. found that adding TLR2 agonists to highly differentiated adult human keratinocytes enhances TJ barrier function within 3 h but no change in TJ protein expression was observed (Yuki *et al.*, 2011b)). There are several critical differences in their methodology as compared to ours. They used primary keratinocytes isolated from adult skin samples and differentiated them in high calcium media (1.8mM) for 4 days before treating with TLR2 agonist, while in our study we stimulated our neonatal primary keratinocytes with TLR2 ligands at the point the cells were placed in high calcium media. Interestingly, we found that TLR2 agonists had little effects when they were added to highly differentiated keratinocytes (unpublished data). This most likely reflects the reduced expression of TLR2 observed in highly differentiated keratinocytes (Figure S6) and suggests that TLR2 signaling plays a major role not in a steady-state maintenance of epidermal barrier integrity but rather in epidermal repair. These methodological differences may explain why they did not observe any changes in TJ protein expression. Additionally, we utilized both TEER measurement and a paracellular flux assay, which is more sensitive to assess changes in TJ integrity. Moreover, we evaluated this finding in full thickness human epidermis and murine skins both of which were wounded by tape-stripping and observed this same TLR2-mediated effect. Tape-stripping is a model for the chronic itch-scratch cycle commonly observed in subjects with AD and therefore our findings provide more direct evidence that this is a clinically relevant effect. The other discrepancy between our findings is that they observed that LPS treatment (10  $\mu\text{g}/\text{mL}$ ) induced TEER, whereas we used lower doses of LPS (5–500  $\text{ng}/\text{mL}$ ) and did not observe an effect on TJ function. It is worth noting that high concentration of LPS (1 $\mu\text{g}/\text{mL}$ ) have been shown to induce TLR2 expression and its' downstream signaling (NF- $\kappa\text{B}$  activation) (Liu *et al.*, 2001). Additionally, the concentrations we employed in our experiments of both LTA and PGN ( $\mu\text{g}$  range) as well as of LPS ( $\text{ng}$  range) are comparable when they are transposed to bacterial equivalents. Based on this, the doses of LPS used in Yuki et al., paper are potentially super-physiologic. Therefore it is possible that the TEER effect they observed in LPS may be through TLR2 signaling.

Lastly, keratinocytes from AD subjects have reduced mRNA expression of TNF- $\alpha$  and IL-6 upon PGN stimulation (Figure S7). Interestingly, it has been shown that *Il-6*<sup>-/-</sup> mice (Wang *et al.*, 2004) and *Tnfr1*<sup>-/-</sup> mice (Jensen *et al.*, 1999) have delayed TEWL recovery after tape-stripping, suggesting that IL-6 and TNF- $\alpha$  might be critical downstream mediators of TLR2 mediated skin barrier repair. Studies are ongoing to understand the mechanism by which TLR2 improves TJ barrier function. It is possible that the reduced response to PGN observed in AD keratinocytes is due in part to the reduced TLR2 expression observed in AD epidermis (Figure 5, 6a and 6b). This finding is also consistent with the observation that TLR2 expression is decreased in circulating monocytes from AD subjects (Antiga *et al.*, 2011; Niebuhr *et al.*, 2009), suggesting that AD subjects may have a genetic or epigenetic defect that affects expression and/or function of TLR2 on multiple cell types (Antiga *et al.*, 2011; Hasannejad *et al.*, 2007; Mrabet-Dahbi *et al.*, 2008; Niebuhr *et al.*, 2009). Indeed, we examined 11 haplotype-tagging single nucleotide polymorphisms in the *TLR2* gene, and found none of these were significantly associated, after correction for multiple comparisons,



with AD in two independent groups of patients with AD (Caucasians: n=258; African Americans: n=176) and healthy control subjects (n=156; n= 176, respectively) (unpublished data). This suggests that the observed changes in TLR2 expression in AD subjects likely develop as the consequence of local inflammatory mediators or on an epigenetic basis. Recent reports indicate that several microRNAs (miRNA) could inhibit TLR2 protein translation and thereby decrease its expression and likely also its function (Benakanakere *et al.*, 2009; Jurkin *et al.*, 2010; Liu *et al.*, 2009; Nahid *et al.*, 2011). Studies are ongoing to evaluate this important hypothesis.

In conclusion, keratinocytes provide the first line of defense against microbes and dynamically respond to tissue injury. This involves the production of a tightly orchestrated inflammatory response aimed at enhancing epidermal barrier recovery and limiting microbial overgrowth. Our data demonstrate that the innate immune function of the keratinocytes also includes the preservation of TJ integrity. Therefore, in healthy subjects TLR2 will have a reparative effect on disrupted TJ, and we hypothesize that this barrier protective response is diminished in AD subjects who express less TLR2. Defective TLR2 responses would also enable pathogenic bacteria like *S. aureus* to persist on the skin surface and perpetuate inflammation. Strategies that boost TLR2 expression or function may hold promise in restoring epidermal integrity in AD.

## MATERIALS AND METHODS

### Transepithelial Electric Resistance (TEER)

Primary human keratinocytes (PHK) were isolated from discarded neonatal foreskins (also see Supplementary materials). PHK were plated in K-SFM in 24-well Costar® Transwell inserts (polyester membranes, 0.4- $\mu$ m pore size; Corning Life Sciences, Corning, NY). After cells were confluent, media were switched to DMEM media allowing PHK differentiation and TJ formation. At the same time, TLR ligands were placed in upper wells for 8 days. Culture media was changed every other day. TEER was measured as previously described (De Benedetto *et al.*, 2011). TEER absolute values at day 1 in control groups range from  $87\pm 15$  to  $305\pm 120$  ohms  $\times$  cm<sup>2</sup>. The study was approved by the Research Subject Review Board at the University of Rochester Medical Center and was conducted according to Declaration of Helsinki Principles.

### Paracellular flux assay

PHK were seeded in Transwell inserts and treated as described above. After 48 h, 0.02% fluorescein sodium (Sigma-Aldrich) in PBS was added to the upper well, while PBS alone was added to the lower well. Samples were collected from the lower well after 30 mins. The amount of fluorescein sodium (fluorescein) that diffused from across the filter was measured with the iQ5 Multicolor real-time PCR detection system (Bio-Rad). Paracellular flux was presented as follows: Paracellular flux (fold of control)= fluorescein intensity of treatment groups/ fluorescein intensity of control group.

### Barrier disruption of murine skin

Age-matched (12–16 weeks old), female Tlr2<sup>-/-</sup> mice (C57BL6 background; kindly provided by Dr. Jian-Dong Li) (Shuto *et al.*, 2002) and WT mice (C57BL6; purchased from National Cancer Institute) were anesthetized with ketamine/xylazine, back hair was shaved and depilatory applied for 3 min (Veet, Reckitt Benckiser Inc; NJ) to completely remove all hair. To disrupt the skin barrier, Cellotape (Nichiban, Tokyo, Japan) was pressed firmly against the skin, removed, and repeated with a new piece of tape each time until TEWL reached the same value ( $\sim 75 \pm 10$  g/h/m<sup>2</sup>) on both mice, and then barrier recovery was monitored by TEWL measurement (Tewameter 300; Courage-Khazaka Electronics, Koln, Germany) at 0, 1, 2, 3 and 24 h. Animal experiments were approved by the University Committee on Animal Resources at the University of Rochester Medical Center.

### Micro-Snapwell™ barrier function assay on *ex vivo* mouse and human skin

The Snapwell™ system was used to measure TEER from human and murine epidermis with and without exposure to the TLR2 agonist, Pam3CSK4. The protocol used was a modification of one previously described (El Asmar *et al.*, 2002). In brief, skin from the back was harvested from mice immediately following tape-stripping (time 0 h), and rinsed in PBS. For human skins, full thickness epidermis was obtained from discarded skin samples after fifteen tape-strippings, using a Weck blade (Goulian Skin Graft Knife Set, George Tiemann & Co. Hauppauge, NY) and then rinsed in PBS. Skin samples were mounted on filter supports (Whatman Nuclepore Track-Etch Membrane; GE Healthcare Biosciences, Pittsburgh, PA) with the epidermal side oriented upward, and sandwiched between two sterile Plexiglas discs (Rohm & Haas Co., Philadelphia, PA) with an opening of 3 mm and placed in modified Snapwell™ chambers (Corning, Corning, NY, USA). Samples were submerged in DMEM complete media and kept at 37°C, 5% CO<sub>2</sub> for 30 min. Pam3CSK4 (10 µg/mL) or media alone were then added to both sides of the transwell, and TEER was measured at 0 and 24 h by using Endohm with a planar electrode (Endohm-Snap; World Precision Instruments, Sarasota, FL, USA). TEER (ohms × cm<sup>2</sup>) = (measured value) × (transwell area; 0.07 cm<sup>2</sup>). The percentage change in TEER between time 0 (100 percent) and time 24 h was expressed as follows:  $(TEER_{24h} / TEER_{0h}) \times 100$ . Data were normalized to control group, and presented as fold of control. Paracellular flux of fluorescein in human epidermis was measured at 24 h as described above. Using human discarded skins was approved by the Research Subject Review Board at the University of Rochester Medical Center. This study was conducted according to Declaration of Helsinki Principles.

### Statistical Analysis

Data are expressed as mean ± standard error (SEM) and are representative of at least three separate experiments. One-way or two-way analysis of variance (ANOVA) was used to determine significance unless otherwise stated. Mann-Whitney t-test was used to compare gene expression and immunofluorescence intensity between AD and NA skin. Spearman nonparametric correlation was used to evaluate the correlation between TLR2 staining intensity and TEWL. The statistical analysis was performed using GraphPad Prism 4 (GraphPad Software, Inc, San Diego, CA).  $P < 0.05$  was considered significant.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## ACKNOWLEDGMENTS

This work was funded by Atopic Dermatitis Research Network (contract HHSN272201000020C and HHSN272201000017C) (L.A.B., R.L.G., D.Y.L. and K.C. DHHS/PHS/NIH 5 T32 AR007472-21 (A.D.B.), National Eczema Association research grant (A.D.B. and L.A.B.), Dermatology Foundation research grant (A.D.B.), National Institute of Diabetes Digestive and Kidney Diseases grants DK083968 and DK084953 A.I.I.) and NIH R01 HL071933 (S.N.G) and NIEHS P30 ES01247 (S.N.G). Authors thankful to the Department of Pathology at the University of Rochester Medical Center for providing discarded skin samples for the study. We also thank Dr. Jian-Dong Li, PhD, Dr. Jae Hyang Lim, PhD at Georgia State University (Atlanta, GA), and Dr. Xiangbin Xu, PhD at the University of Rochester Medical Center (Rochester, NY) provided the *Tlr2*<sup>-/-</sup> mice.

## Abbreviations

<b>AD</b>	Atopic Dermatitis
<b>CLDN1</b>	Claudin-1
<b>CLDN23</b>	Claudin-23
<b>LTA</b>	Lipoteichoic acid
<b>LPS</b>	Lipopolysaccharide
<b>Malp-2</b>	Macrophage-activating lipopeptide-2
<b>NA</b>	Nonatopic
<b>NOD2</b>	Nucleotide-binding oligomerization domain containing 2
<b>Pam3CSK4</b>	N-palmitoyl-S-[2,3-bis(palmitoyl)-(2RS)-propyl]-(R)cysteinyl-alanyl-glycine
<b>PGN</b>	Peptidoglycan
<b>PGLYRP-3</b>	Peptidoglycan recognition protein 3
<b>PGLYRP-4</b>	Peptidoglycan recognition protein 4
<b>PolyI:C</b>	Polyinosinic-polycytidylic acid
<b>PHK</b>	Primary human keratinocytes
<b>qPCR</b>	Quantitative PCR
<b>TJ</b>	Tight junction
<b>TEER</b>	Transepithelial electrical resistance
<b>TEWL</b>	Transepidermal water loss
<b>TLR</b>	Toll-like receptor
<b>ZO-1</b>	Zonulae occludens 1

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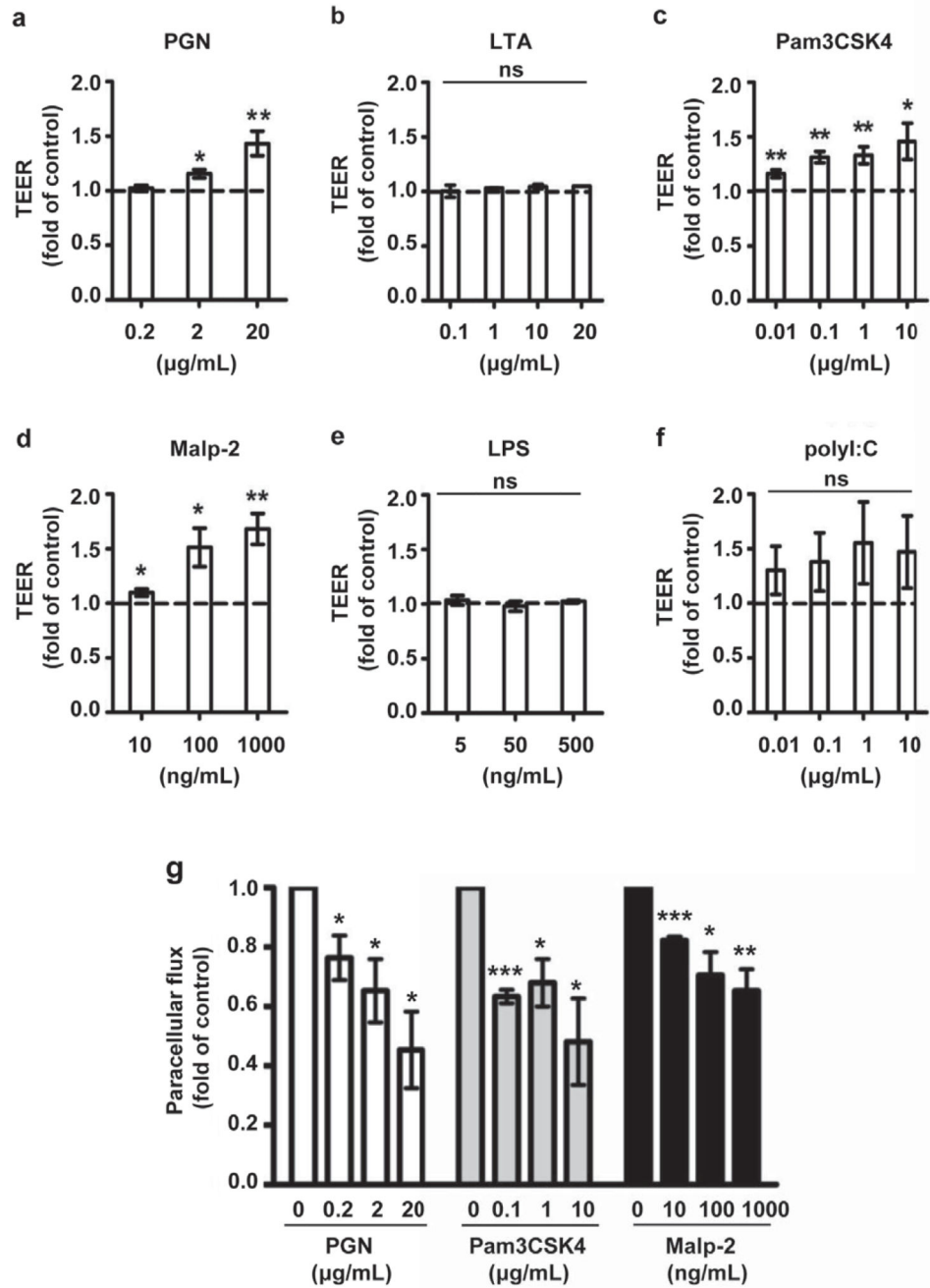
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### Figure 1. TLR2 agonists enhance TJ function in PHK

TEER was measured daily until day 8 in PHK stimulated with (a) *S. aureus*- derived peptidoglycan (PGN, n= 3–7), (b) *S. aureus*-derived lipoteichoic acid (LTA, n= 3), (c) Pam3CSK4, a TLR1/2 ligand (n= 3–6), (d) Malp-2, a TLR2/6 ligand (n= 5), (e) Lipopolysaccharide (LPS), a TLR4 ligand (n=3) and (f) poly I:C, a TLR3 ligand (n= 3–9). Data are presented as the mean  $\pm$  SEM by analyzing the area under the TEER curve normalized to the mean values for the control group (media alone). (g) PHK grown on Transwell™ inserts were stimulated with the indicated concentrations of *S. aureus*-derived

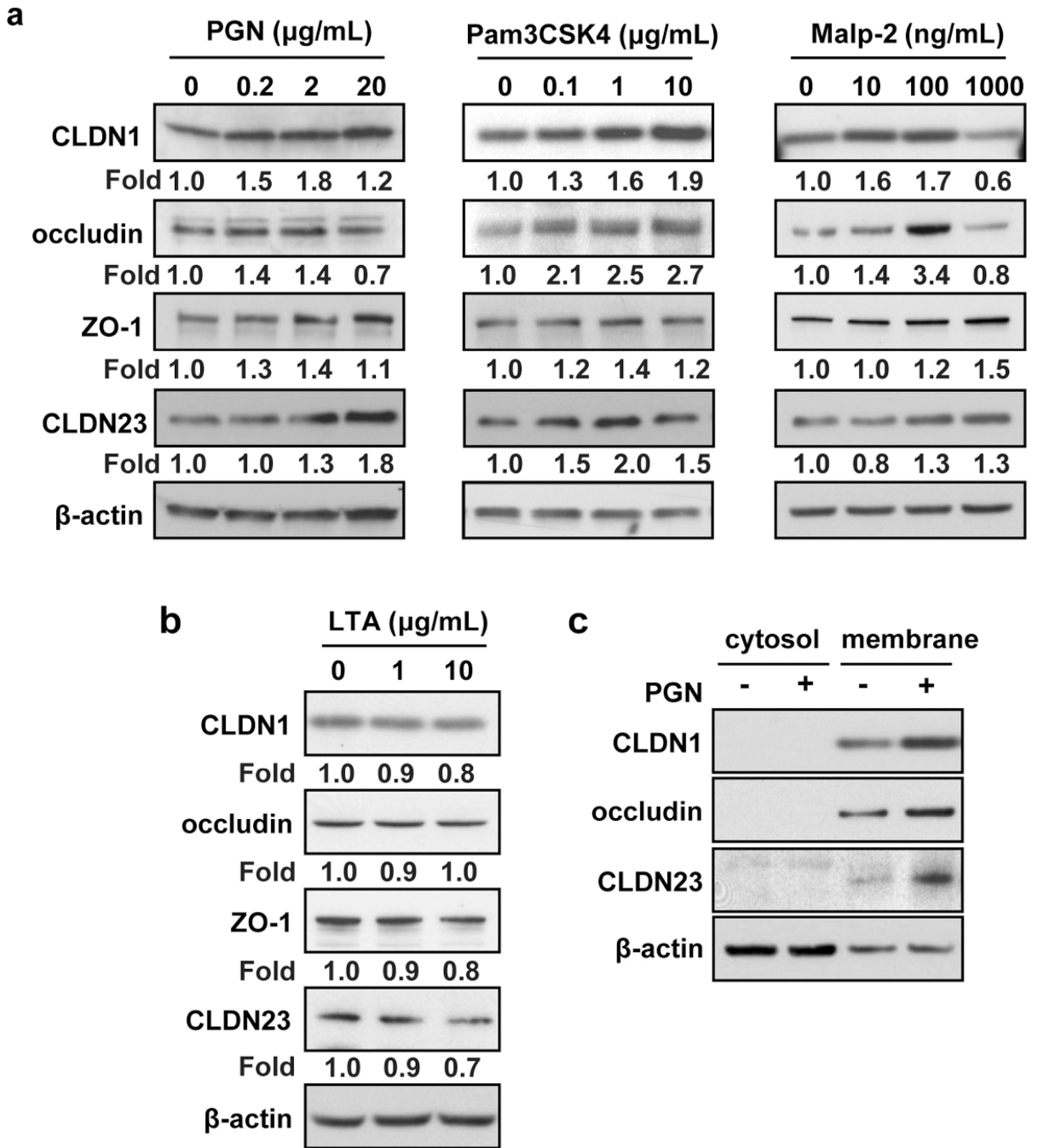
PGN, Pam3CSK4 and Malp-2 for 48 h and paracellular permeability measured and compared to media alone. Data are expressed as a fold of media alone group. n=3; \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < .001$ ; ns: not significant.

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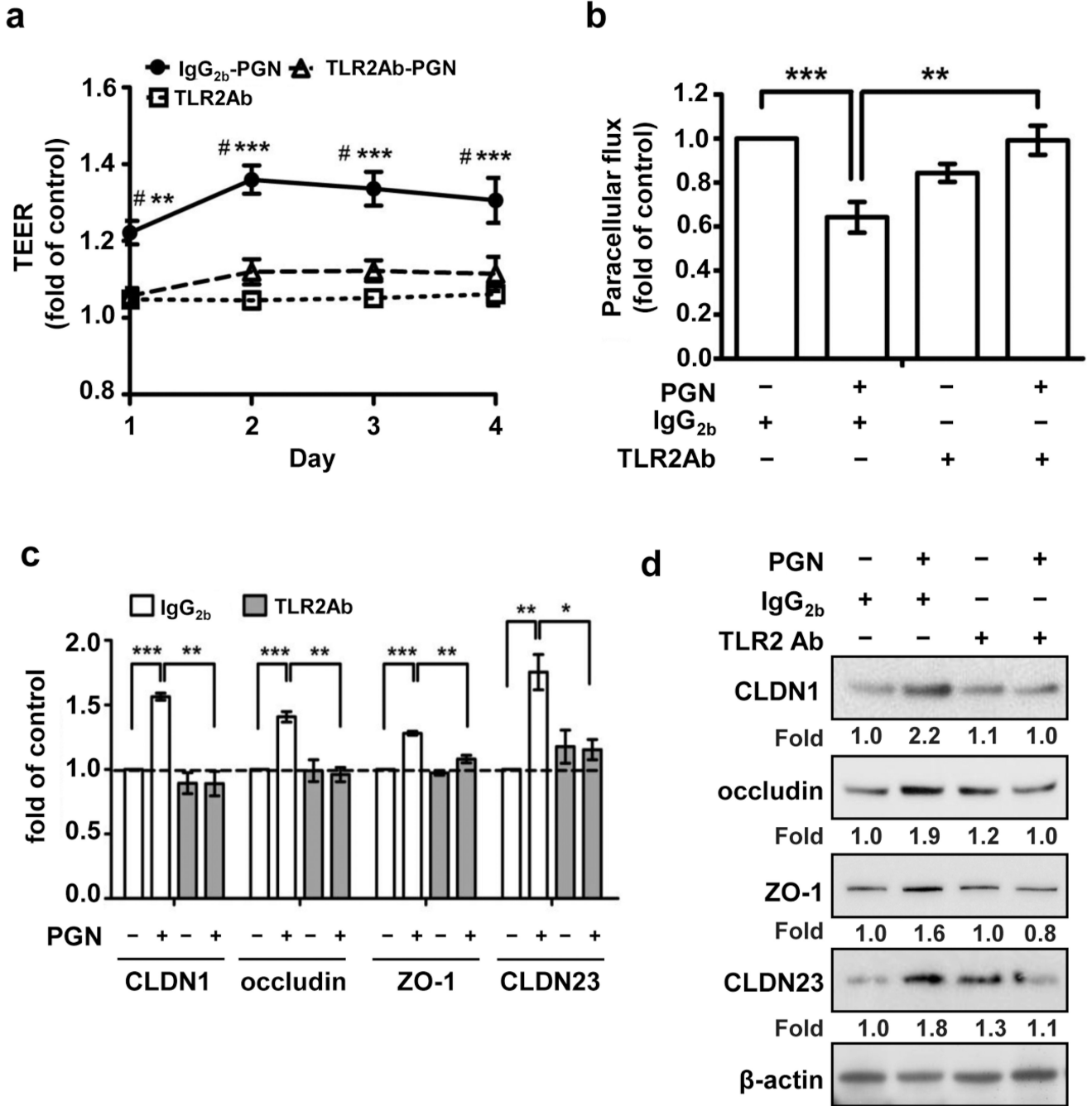
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**Figure 2. TLR2 agonists induce TJ protein expression and subcellular localization**  
 PHK were treated with (a) *S. aureus*-derived PGN (0.2, 2, 20  $\mu\text{g/ml}$ ), Pam3CSK4 (0.1, 1, 10  $\mu\text{g/ml}$ ), Malp-2 (10, 100, 1000 ng/mL) and (b) LTA (1, 10  $\mu\text{g/ml}$ ) for 48 h and CLDN1, occludin, ZO-1 and CLDN23 protein levels were detected from whole cell lysates by Western blot. Quantitative protein expression was determined by densitometry of bands after normalization to the housekeeping protein ( $\beta$ -actin). (c) Membrane and cytosolic protein lysates were made and TJ proteins were detected by Western blot after treating PHK with PGN (20  $\mu\text{g/ml}$ ) for 48 h. Representative blot of n=3 experiments.



**Figure 3. TLR2 neutralizing antibody inhibited *S. aureus*-derived PGN effect on TJ protein function and expression**

TEER (n=5) (a) and paracellular permeability (n=5) at 48 h (day 2) (b) measurement in PHK treated with PGN (20 µg/ml) and/or TLR2-neutralizing antibody (10 µg/ml). Data are normalized to the control group (IgG<sub>2b</sub> treatment). (c) TJ mRNA (n= 3) and (d) protein expression (representative blot of n=3 experiments) observed in response to treatment with PGN (20 µg/ml, 24 or 48 h, respectively) with or without TLR2-neutralizing antibody (5 µg/ml). Expression was quantified by densitometry of bands and normalization to the

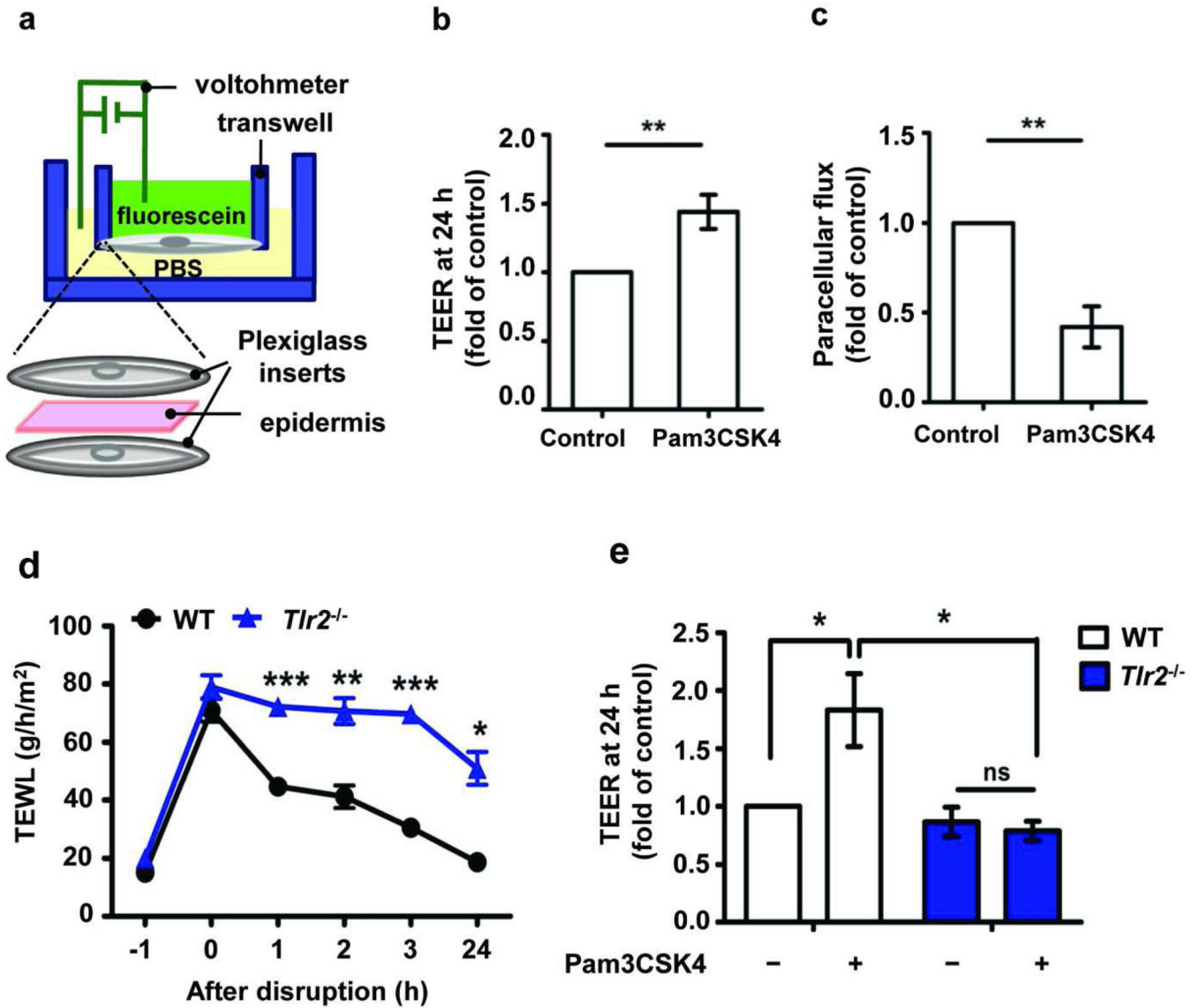
housekeeping protein ( $\beta$ -actin). \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ ; #: compared IgG<sub>2b</sub>+PGN vs TLR2Ab+PGN.

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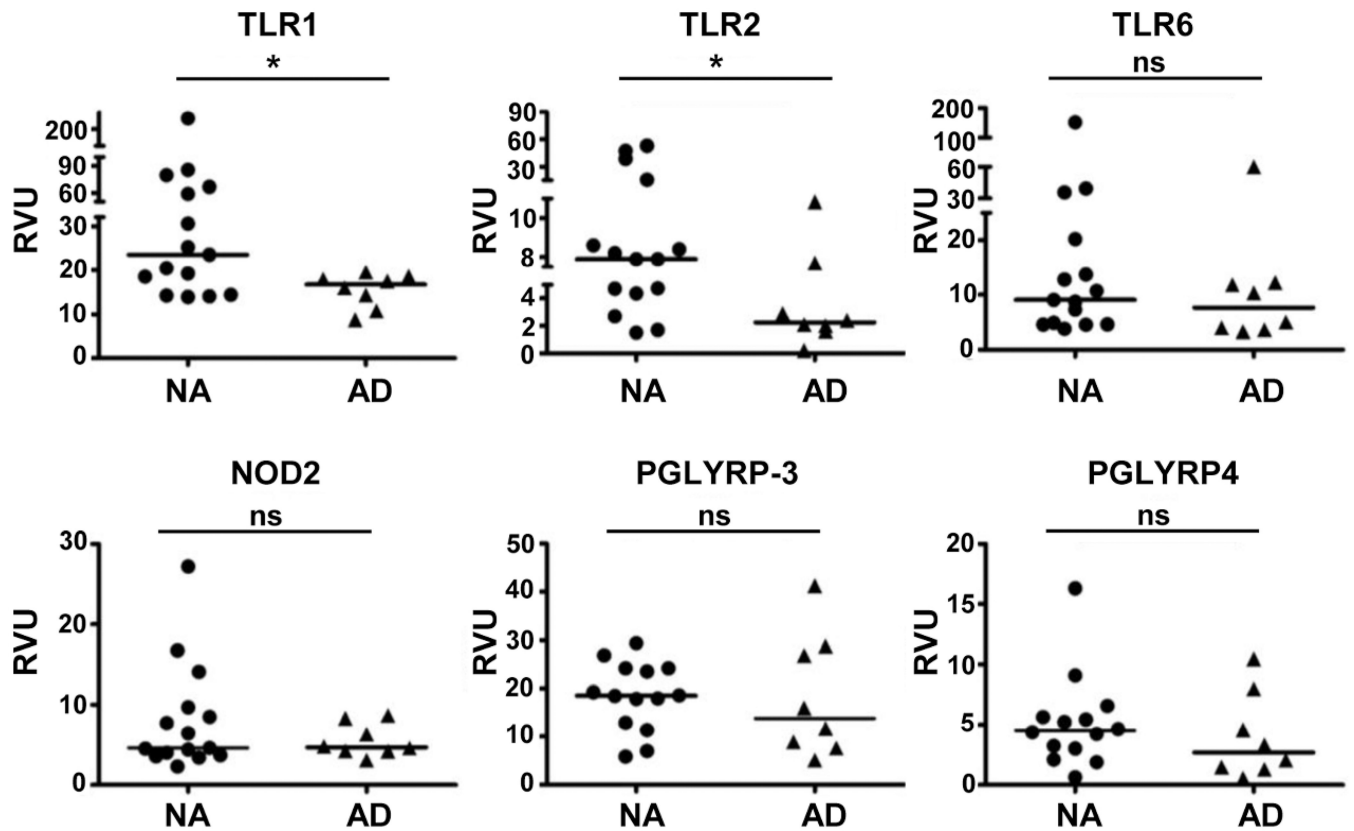
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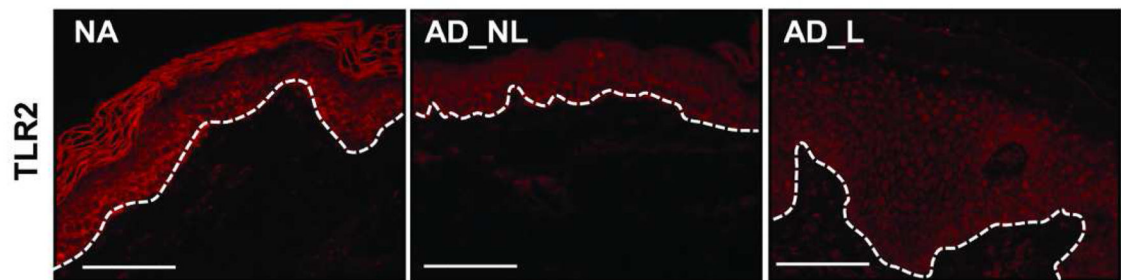
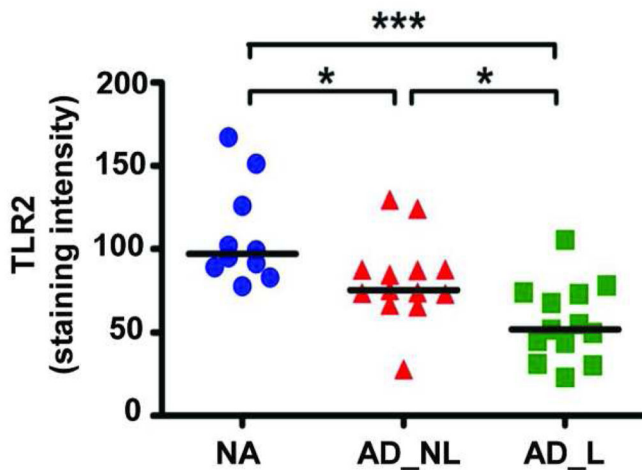
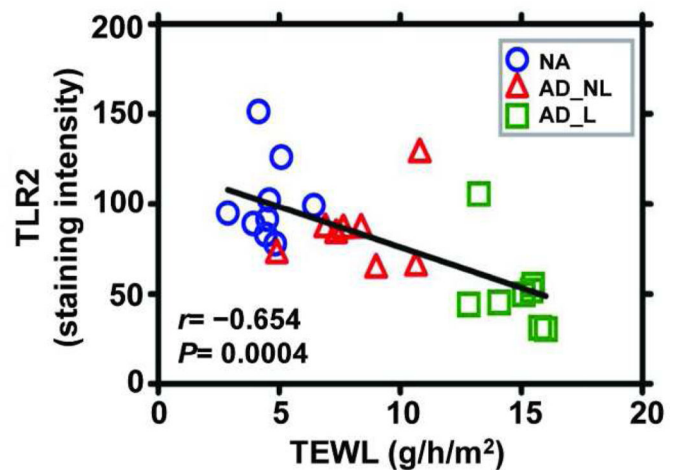


**Figure 4. TLR2 agonist enhances skin barrier repair**

(a) Schema of modified Snapwell™ system used to measure TEER and paracellular flux in full thickness human or mouse epidermis. Epidermal sheets from normal subjects were placed in modified Snapwell following tape-stripping and treated with Pam3CSK4 (10 µg/mL) for 24 h. TEER (n=7/group) (b) and paracellular flux (n=3/group) (c) were measured at 24 h. (d) TEWL recovery curve after tape-stripping mouse skin. (WT, n= 4; *Tlr2*<sup>-/-</sup>, n= 4; Time -1 h= baseline TEWL before tape-stripping). (e) TLR2 agonist effect on TEER restoration at 24 h measured by modified Snapwell™ after murine barrier disruption by tape-stripping (WT, n= 5; *Tlr2*<sup>-/-</sup>, n= 5). TEER absolute value at 24 h in control group is 227±27 ohms × cm<sup>2</sup> (human epidermis) and 241± 44 ohms × cm<sup>2</sup> (WT mice epidermis). ns: not significant; \**P* <0.05; \*\**P* <0.01; \*\*\* *P* <0.001.



**Figure 5. Reduced mRNA expression of TLR1 and 2 in epidermal sheets from AD subjects** Nonlesional AD epidermis (n= 8) was compared with epidermis from nonatopic healthy control (NA; n= 14–15) obtained from the same anatomical location. The relative mRNA expression level was normalized to GAPDH (Mann-Whitney t-test). RVU= relative value unit. \* $P < 0.05$ ; ns: not significant.

**a****b****c**

**Figure 6. The reduced TLR2 expression observed in AD epithelium inversely correlates with measures of barrier integrity**

(a) Representative paraffin-embedded skin biopsy samples from a nonatopic healthy control (NA; n= 10), non-lesional AD (AD\_NL; n= 13), and lesional AD (AD\_L; n= 13) subjects stained for TLR2 are shown. The white dotted lines denote the epidermal-dermal junction. Bar= 100  $\mu$ m. Epidermal TLR2 staining intensity is shown in (b) (Mann-Whitney t-test). (c) The line representing the linear least square fit for TLR2 epidermal staining intensity versus TEWL (Spearman nonparametric correlation, [n= 25];  $r= 0.654$ ,  $P= 0.0004$ ).