Activation of Epidermal Toll-Like Receptor 2 Enhances Tight Junction Function: Implications for Atopic Dermatitis and Skin Barrier Repair

I-Hsin Kuo[1,2], Amanda Carpenter-Mendini[1], Takeshi Yoshida[1], Laura Y. McGirt[3], Andrei I. Ivanov[4,10], Kathleen C. Barnes[5], Richard L. Gallo[6], Andrew W. Borkowski[6], Kenshi Yamasaki[7], Donald Y. Leung[8], Steve N. Georas[9], Anna De Benedetto[1] and Lisa A. Beck[1]

Atopic dermatitis (AD) is characterized by epidermal tight junction (TJ) defects and a propensity for *Staphylococcus aureus* skin infections. *S. aureus* is sensed by many pattern recognition receptors, including Toll-like receptor 2 (TLR2). 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, claudin-1 (CLDN1), claudin-23 (CLDN23), occludin, and Zonulae occludens 1 (ZO-1) in primary human keratinocytes. A TLR2 agonist enhanced skin barrier recovery in human epidermis wounded by tape stripping. $Tlr2^{-/-}$ mice had a delayed and incomplete barrier recovery following tape stripping. AD subjects had reduced epidermal TLR2 expression as compared with nonatopic 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 have a role in their incompetent skin barrier.

Journal of Investigative Dermatology (2013) **133,** 988–998; doi:10.1038/jid.2012.437; published online 6 December 2012

[1]*Department of Dermatology, University of Rochester Medical Center, Rochester, New York, USA;* [2]*Department of Pathology and Laboratory Medicine, University of Rochester Medical Center, Rochester, New York, USA;* [3]*Department of Dermatology, Vanderbilt University Medical Center, Nashville, Tennessee, USA;* [4]*Division of Gastroenterology and Hepatology, Department of Medicine, University of Rochester Medical Center, Rochester, New York, USA;* [5]*Lowe Family Genomics Core, Department of Medicine, Johns Hopkins University School of Medicine, Baltimore, Maryland, USA;* [6]*Division of Dermatology, University of California, San Diego, San Diego, California, USA;* [7]*Department of Dermatology, Graduate School of Medicine, Tohoku University, Sendai, Miyagi, Japan;* [8]*Department of Pediatrics, National Jewish Health, Denver, Colorado, USA and* [9]*Division of Pulmonary and Critical Care Medicine, Department of Medicine, University of Rochester Medical Center, Rochester, New York, USA*

[10]*Current address: Department of Human and Molecular Genetics, Virginia Commonwealth University School of Medicine, Richmond, Virginia, USA.*

Correspondence: Lisa A. Beck, Department of Dermatology, University of Rochester Medical Center, 601 Elmwood Avenue, Box 697, Rochester, New York 14642, USA. E-mail: lisa_beck@urmc.rochester.edu

Abbreviations: AD, atopic dermatitis; CLDN1, claudin-1; CLDN23, claudin-23; LTA, lipoteichoic acid; LPS, lipopolysaccharide; Malp-2, macrophage-activating lipopeptide-2; NOD2, nucleotide-binding oligomerization domain-containing 2; Pam3CSK4, N-palmitoyl-S-[2,3-bis(palmitoyl)-(2RS)-propyl]-(R)cysteinyl-alanyl-glycine; PGN, peptidoglycan; PGLYRP-3, peptidoglycan recognition protein 3; PGLYRP-4, peptidoglycan recognition protein 4; PHK, primary human keratinocyte; SC, stratum corneum; TNF-α, tumor necrosis factor-α; TEER, transepithelial electrical resistance; TEWL, transepidermal water loss; TJ, tight junction; TLR, Toll-like receptor; WT, wild type; ZO-1, Zonulae occludens 1

Received 22 May 2012; revised 7 September 2012; accepted 8 October 2012; published online 6 December 2012

INTRODUCTION

Recent findings have solidified the notion that skin barrier dysfunction has 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; Palmer *et al.*, 2006; Howell *et al.*, 2009), epidermal lipid abnormalities (Murata *et al.*, 1996; Imokawa, 2001; Pilgram *et al.*, 2001), altered protease activity (Cork *et al.*, 2006; Vasilopoulos *et al.*, 2007), more alkaline surface pH (Elias *et al.*, 2008), and 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 that might occur with topical exposure to organic solvents or detergents, scratching, and 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 (Grigoryev *et al.*, 2010; Boguniewicz and Leung, 2011; De Benedetto *et al.*, 2012; Kubo *et al.*, 2012). All 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 2 (TLR2) has received the greatest attention, as it is important for immune responses to a number of microbes, such as *Staphylococcus aureus* and herpes simplex virus (Takeuchi *et al.*, 1999, 2000; Sato *et al.*, 2006; Bochud *et al.*, 2007; Zahringer *et al.*, 2008; De Benedetto *et al.*, 2009), 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 the 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, primary human keratinocytes (PHKs) were stimulated with *S. aureus*–derived peptidoglycan (PGN) and lipoteichoic acid (LTA) (Figure 1a and b). PGN significantly increased the transepithelial electrical resistance (TEER) of PHK monolayers in a dose-dependent manner. In contrast, LTA had no effect on TJ barrier function, despite evidence that it could enhance the

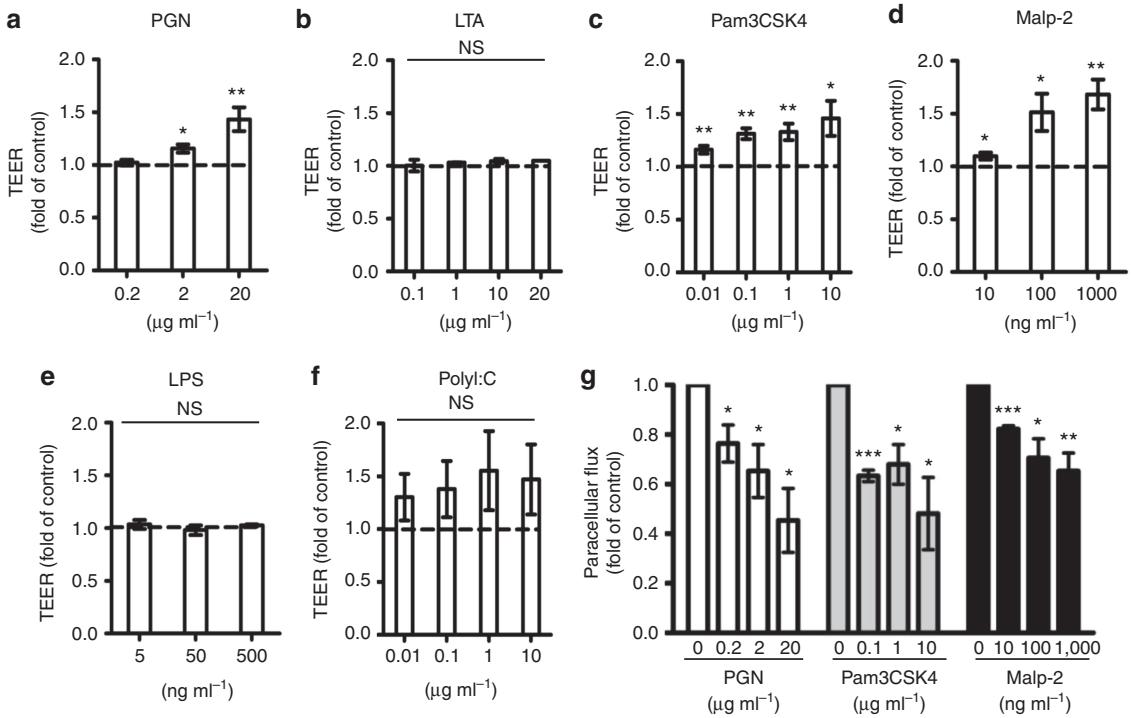

Figure 1. Toll-like receptor 2 (TLR2) agonists enhance tight junction (TJ) function in primary human keratinocytes (PHKs). Transepithelial electrical resistance (TEER) was measured daily until day 8 in PHKs stimulated with (**a**) *Staphylococcus aureus*–derived peptidoglycan (PGN, $n=3$–7), (**b**) *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**) polyinosinic-polycytidylic acid (polyI:C), a TLR3 ligand ($n=3$–9). Malp-2, macrophage-activating lipopeptide-2; Pam3CSK4, N-palmitoyl-S-[2,3-bis(palmitoyl)-(2RS)-propyl]-(R)cysteinyl-alanyl-glycine. Data are presented as the mean ± SEM by analyzing the area under the TEER curve normalized to the mean values for the control group (media alone). (**g**) PHKs grown on Transwell inserts were stimulated with the indicated concentrations of *S. aureus*–derived PGN, Pam3CSK4, and Malp-2 for 48 hours and paracellular flux measured and compared with 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.

expression of TLR2, cluster of differentiation 14, peptidoglycan recognition protein 3 (PGLYRP-3), and human β-defensin 2 in human keratinocytes (data not shown). Using synthetic ligands targeting specific TLR2 heterodimers, Pam3CSK4 (N-palmitoyl-S-[2,3-bis(palmitoyl)-(2RS)-propyl]-(R)cysteinyl-alanyl-glycine; a TLR1/2 agonist) and Malp-2 (macrophage-activating lipopeptide-2; a TLR2/6 agonist), we found that both increased keratinocyte TEER in a dose-dependent manner (Pam3CSK4: $P<0.05$; Malp-2: $P<0.05$; Figure 1c and d). In contrast, lipopolysaccharide (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 PHKs (data not shown) (Baker *et al.*, 2003; Mempel *et al.*, 2003; Kollisch *et al.*, 2005). Interestingly, polyinosinic-polycytidylic acid, a synthetic TLR3 agonist, appeared to consistently enhance epidermal TJ barrier in a wide range of concentrations (0.01–10 μg ml^{-1}) but with substantial variability among PHK 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 hours 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 whether barrier-enhancing effects of TLR2 agonists are associated with altered expression of TJ proteins, we quantified the expression of key transmembrane and cytosolic TJ components in cultured keratinocytes (Lucke *et al.*, 1999; Amagai *et al.*, 2000; Furuse *et al.*, 2001, 2002; Baltes *et al.*, 2004; Kretz *et al.*, 2004; Ma *et al.*, 2004; Tunggal *et al.*, 2005; Djalilian *et al.*, 2006; Gareus *et al.*, 2007; Ohnemus *et al.*, 2008; Yuki *et al.*, 2011a). We found that mRNA expression of claudin-1 (CLDN1), claudin-23 (CLDN23), occludin, and Zonulae occludens 1 (ZO-1) was significantly ($P<0.01$) induced after 24 hours of stimulation of confluent PHKs with PGN, whereas the expression of adherens junction, gap junction, and desmosomal proteins was not significantly affected (Supplementary Figure S1 online). We confirmed these quantitative PCR findings at the protein level by western blot analysis (Figure 2a). TLR2 synthetic agonists (Pam3CSK4 and Malp-2) also enhanced the protein expression of CLDN1, occludin, ZO-1, and CLDN23. It is noteworthy that 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 Supplementary Figure S2 online.

The 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 (Utech *et al.*, 2005; Marchiando *et al.*, 2010). We found that CLDN1, occludin, and CLDN23 accumulated exclusively in the membrane fraction and their level at the membrane was

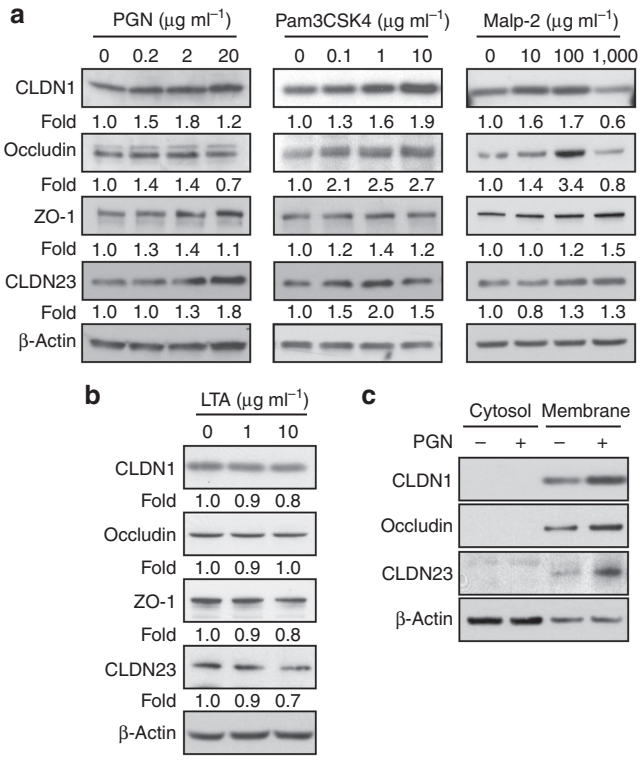

Figure 2. Toll-like receptor 2 (TLR2) agonists induce tight junction (TJ) protein expression and subcellular localization. Primary human keratinocytes (PHKs) were treated with (**a**) *Staphylococcus aureus*–derived peptidoglycan (PGN; 0.2, 2, and 20 μg ml^{-1}), Pam3CSK4 (0.1, 1, and 10 μg ml^{-1}), Malp-2 (10, 100, and 1,000 ng ml^{-1}), and (**b**) lipoteichoic acid (LTA; 1 and 10 μg ml^{-1}) for 48 hours, and claudin-1 (CLDN1), occludin, Zonulae occludens 1 (ZO-1), and claudin-23 (CLDN23) protein levels were detected from whole-cell lysates by western blot analysis. Malp-2, macrophage-activating lipopeptide-2; Pam3CSK4, N-palmitoyl-S-[2,3-bis(palmitoyl)-(2RS)-propyl]-(R)cysteinyl-alanyl-glycine. Quantitative protein expression was determined by densitometry of bands after normalization to the housekeeping protein (β-actin). (**c**) Membrane and cytosolic protein lysates were made and TJ proteins were detected by western blot analysis after treating PHKs with PGN (20 μg ml^{-1}) for 48 hours. Representative blot of $n=3$ experiments.

enhanced by PGN stimulation (Figure 2c). The specificity of our cellular fractionation was confirmed using appropriate markers (Supplementary Figure S3 online).

Blocking TLR2 prevents PGN-mediated effects on TJ

S. aureus–derived PGN can be sensed by several innate receptors including TLR2, NOD2 (nucleotide-binding oligomerization domain-containing 2), 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 conducted 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). In addition, 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 and Supplementary Figure S2b online) levels.

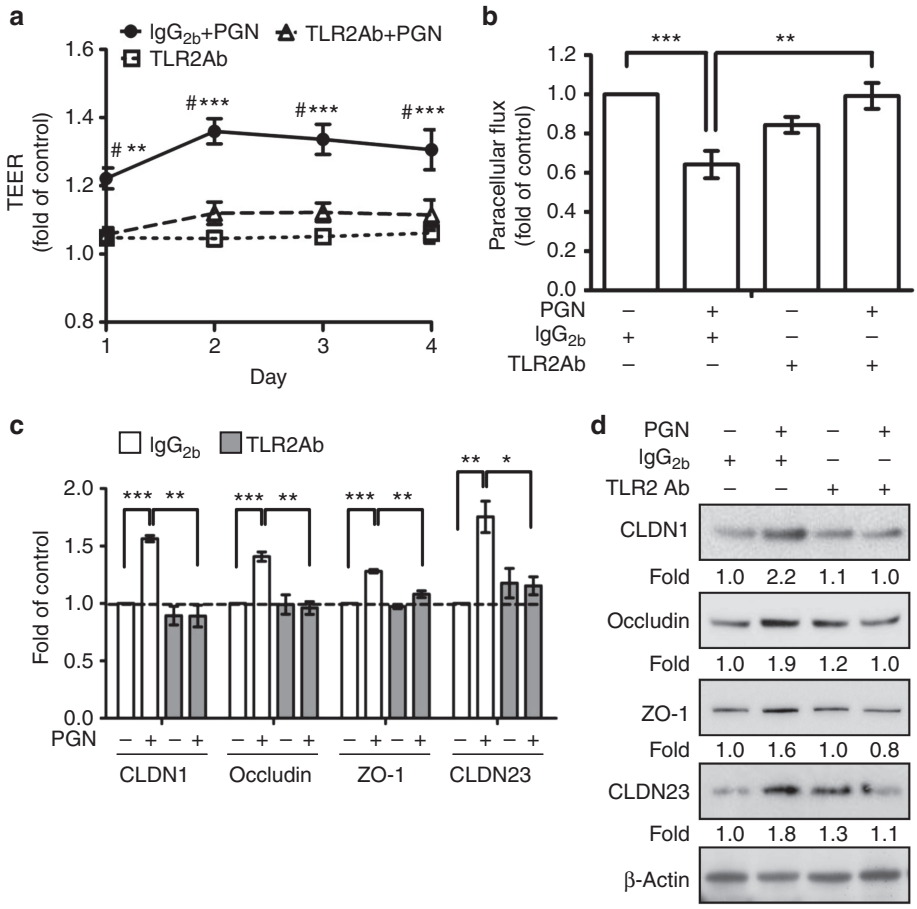

Figure 3. Toll-like receptor 2 (TLR2)-neutralizing antibody inhibited *Staphylococcus aureus*–derived peptidoglycan (PGN) effect on tight junction (TJ) protein function and expression. (**a**) Transepithelial electrical resistance (TEER; $n=5$) and (**b**) paracellular flux ($n=5$) measurement at 48 hours (day 2) in primary human keratinocytes (PHKs) treated with PGN (20 μg ml^{-1}) and/or TLR2-neutralizing antibody (10 μg ml^{-1}). Data are normalized to the control group (IgG_{2b} treatment). (**c**) TJ mRNA ($n=3$) and (**d**) protein expression (representative blot of $n=3$ experiments) was observed in response to treatment with PGN (20 μg ml^{-1}, 24 or 48 hours, respectively) with or without TLR2-neutralizing antibody (5 μg ml^{-1}). The expression was quantified by densitometry of bands and normalization to the housekeeping protein (β-actin). CLDN1, claudin-1; CLDN23, claudin-23; ZO-1, Zonulae occludens 1. *$P<0.05$; **$P<0.01$; ***$P<0.001$; #compared IgG_{2b} + PGN with TLR2Ab + PGN.

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 (Taljebini *et al.*, 1996; Koschwanez and Broadbent, 2011), 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 Materials and 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 an ~50% decrease in the paracellular flux ($P=0.007$; Figure 4c) at 24 hours after wounding.

TLR2 knockout (*Tlr2*$^{-/-}$) 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 wild type (WT) and *Tlr2*$^{-/-}$ mice, and monitored barrier recovery by measuring TEWL. In WT mice, the skin barrier fully recovered 24 hours after tape stripping, whereas *Tlr2*$^{-/-}$ mice had a substantially slower recovery rate and did not reach baseline values even 24 hours after wounding (Figure 4d). At 2 hours after skin barrier disruption, the mRNA expression of *cldn1* and *zo-1* were slightly but significantly increased in WT but not in *Tlr2*$^{-/-}$ mice, whereas *cldn2*, a pore-forming TJ protein, was increased in *Tlr2*$^{-/-}$ mice (Supplementary Figure S4 online). 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 hours after wounding in the WT mice ($P=0.029$). This effect was not observed in wounded

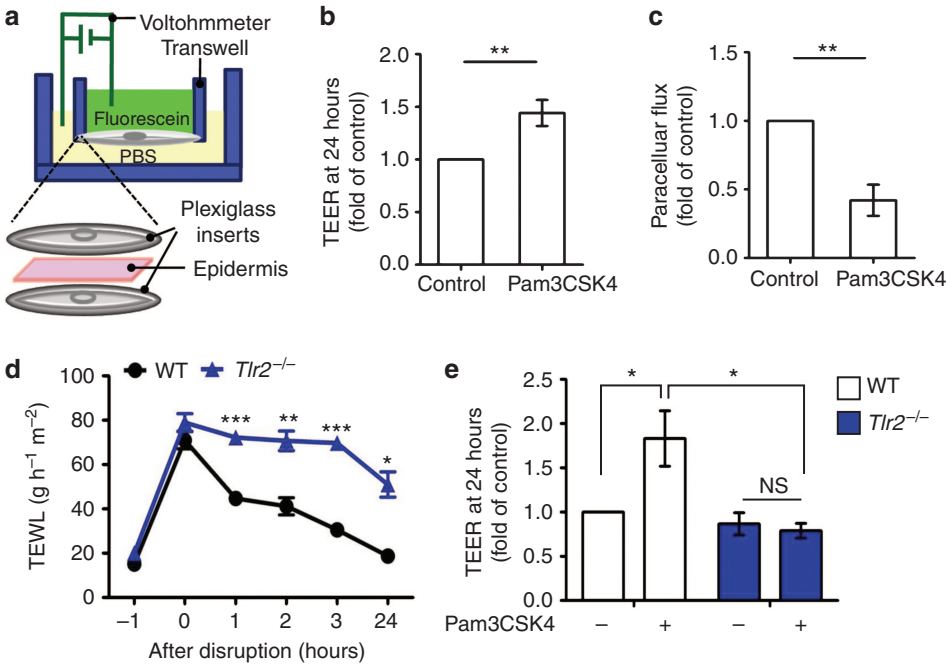

Figure 4. Toll-like receptor 2 (TLR2) agonist enhances skin barrier repair. (**a**) Schema of modified Snapwell system used to measure transepithelial electrical resistance (TEER) and paracellular flux in full-thickness human or mouse epidermis. PBS, phosphate-buffered saline. Epidermal sheets from normal subjects were placed in modified Snapwell following tape stripping and treated with Pam3CSK4 (10 μg ml^{-1}) for 24 hours. (**b**) TEER ($n=7$ per group) and (**c**) paracellular flux ($n=3$ per group) were measured at 24 hours. Pam3CSK4, N-palmitoyl-S-[2,3-bis(palmitoyl)-(2RS)-propyl]-(R)cysteinyl-alanyl-glycine. (**d**) Transepidermal water loss (TEWL) recovery curve after tape stripping mouse skin (wild type (WT), $n=4$; *Tlr2*$^{-/-}$, $n=4$; time −1 hour = baseline TEWL before tape stripping). (**e**) TLR2 agonist effect on TEER restoration at 24 hours measured by modified Snapwell after murine barrier disruption by tape stripping (WT, $n=5$; *Tlr2*$^{-/-}$, $n=5$). TEER absolute value at 24 hours in control group is 227 ± 27 ohms × cm^2 (human epidermis) and 241 ± 44 ohms × cm^2 (WT mice epidermis). NS, not significant. *$P<0.05$; **$P<0.01$; ***$P<0.001$.

epidermis from *Tlr2*$^{-/-}$ 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 TLR2

Keratinocytes respond to *S. aureus* using multiple innate receptors that reside on the cell membrane (TLRs 1, 2, and 6), are intracellular (NOD2), or are secreted (PGLYRP-3 and PGLYRP-4). We hypothesized that the expression of one or more of these key receptors may be reduced in AD, which could explain altered barrier repair responses and the susceptibility of AD subjects to *S. aureus* colonization. To test this hypothesis, epidermal samples were taken from non-sun-exposed volar forearms to control for anatomical differences and photo-induced changes from well-characterized subjects with AD and nonatopic controls. We initially quantified the mRNA expression for different innate receptors in epidermal samples using quantitative PCR. 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 (nonlesional and lesional; median intensity: 76 and 52, $P=0.01$, $P=0.0005$, respectively) as compared with nonatopic samples (median intensity: 96; Figure 6b). Interestingly, PHKs isolated from AD epidermis have a reduced inflammatory response (tumor necrosis factor-α (TNF-α), IL-6, and human β-defensin 2; $P\leq 0.032$) to PGN (Supplementary Figure S7 online).

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 ($r=-0.654$, $P=0.0004$). We also observed that mRNA expression for both TLR1 and TLR2 correlated inversely with TEWL (Supplementary Figure S5 online). 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. TJs are very dynamic structures, loosening and tightening in response to endogenous signals that originate from epithelial and subepithelial compartments, as well as exogenous agents

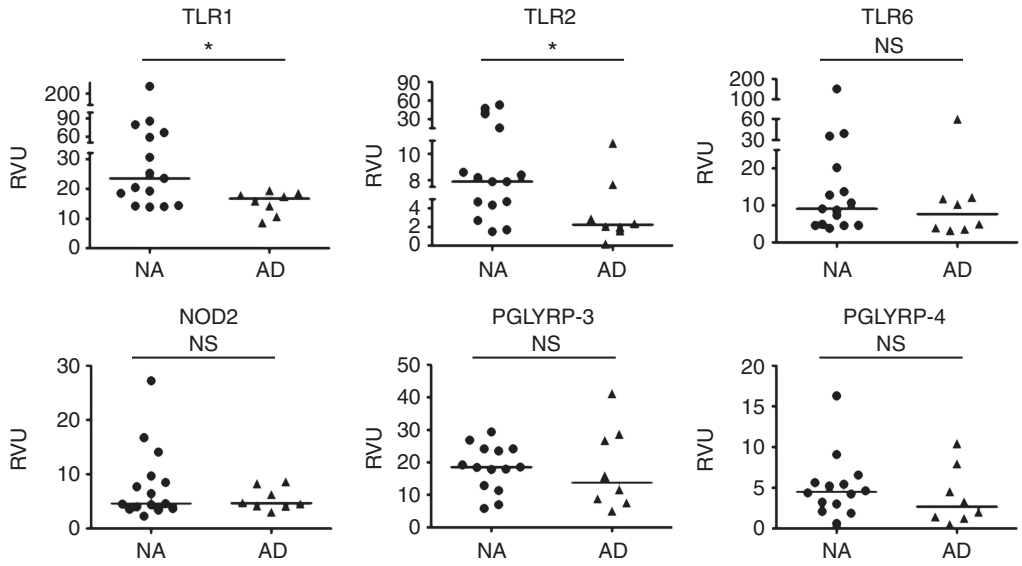

Figure 5. Reduced mRNA expression of Toll-like receptors 1 and 2 (TLR1 and TLR2) in epidermal sheets from atopic dermatitis (AD) subjects. Nonlesional AD epidermis ($n=8$) was compared with epidermis from nonatopic healthy control (NA; $n=14$–15) obtained from the same anatomical location. The relative mRNA expression level was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Mann–Whitney *t*-test). NOD2, nucleotide-binding oligomerization domain-containing 2; PGLYRP-3, peptidoglycan recognition protein 3; PGLYRP-4, peptidoglycan recognition protein 4; RVU, relative value unit. *$P<0.05$; NS, not significant.

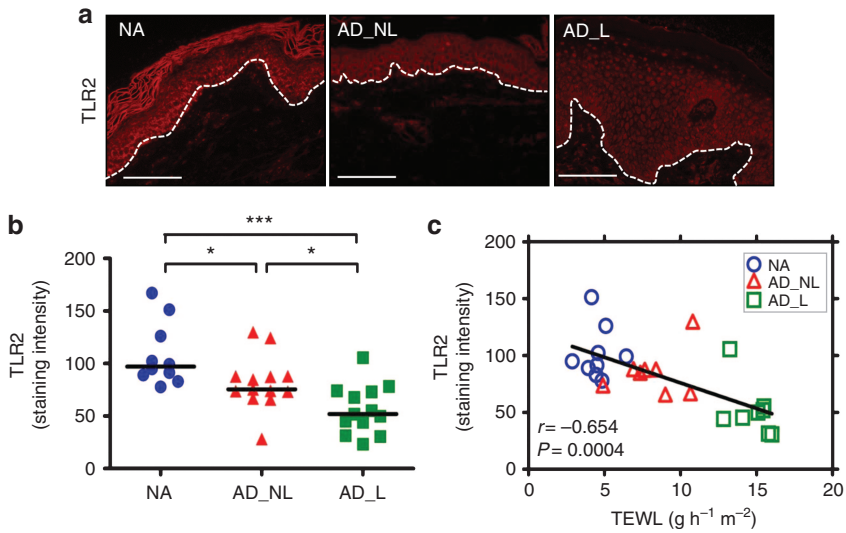

Figure 6. The reduced Toll-like receptor 2 (TLR2) expression observed in atopic dermatitis (AD) epithelium inversely correlates with measures of barrier integrity. (**a**) Representative paraffin-embedded skin biopsy samples from a nonatopic healthy control (NA; $n=10$), nonlesional AD (AD_NL; $n=13$), and lesional AD (AD_L; $n=13$) subjects stained for TLR2 are shown. The white dotted lines denote the epidermal–dermal junction. Bar = 100 μm. (**b**) Epidermal TLR2 staining intensity is shown (Mann–Whitney *t*-test). (**c**) The line represents the linear least square fit for TLR2 epidermal staining intensity versus transepidermal water loss (TEWL; Spearman's nonparametric correlation ($n=25$); $r=-0.654$, $P=0.0004$). *$P<0.05$; ***$P<0.001$.

such as bacterial-derived products and proteolytic allergens. TJs 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 the 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 and in mice genetically deficient in *CLDN1* confirm the importance of this protein for a competent TJ and skin barrier (Furuse *et al.*, 2002; De Benedetto *et al.*, 2011). Results reported herein arose from experiments designed to identify pathways that would

enhance TJ function in the hope 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 the 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 *Tlr2*$^{-/-}$ 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 underappreciated role for innate receptors expressed on epidermal cells in skin barrier maintenance and recovery.

Earlier studies demonstrated that *Tlr2*$^{-/-}$ mice also develop more severe intestinal disease in a dextran sulfate sodium–mediated ulcerative colitis model, which was due to an impaired intestinal TJ barrier repair response (Cario *et al.*, 2007). Importantly, *Tlr4*$^{-/-}$ 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).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 an activation through TLR2, had no effect on TJ function (Figures 1b and 2b), but did induce inflammatory gene expression in human keratinocytes (Menzies 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 (Takeuchi *et al.*, 1999; Schroder *et al.*, 2003). It has been shown that macrophages isolated from *Tlr2*$^{-/-}$ mice stimulated with LTA have similar induction of IL-6 and TNF-α as compared with WT mice, whereas macrophages from *Tlr4*$^{-/-}$ 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*$^{-/-}$ 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 (Schwandner *et al.*, 1999; Yoshimura *et al.*, 1999; Ozinsky *et al.*, 2000; Tsan and Gao, 2004; Dickel *et al.*, 2010). In addition, in this model, murine commensal bacteria may activate epidermal TLR2. In this model, we observed that *Tlr2*$^{-/-}$ 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 agonists 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 hours) results in disruption of intercellular lamellar lipid bilayers, degradation of corneodesmosomes, and formation of amorphous regions within the intercellular lipid (Warner *et al.*, 1999, 2003), which would arguably significantly compromise SC barrier function. This observation, combined with the fact that the skin samples had undergone repetitive tape stripping, led 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 it 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*$^{-/-}$ murine wound model, which 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 (Schauber *et al.*, 2007; Jin *et al.*, 2009) 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 (Yoshida, 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.* (2011b) found that adding TLR2 agonists to highly differentiated adult human keratinocytes enhances TJ barrier function within 3 hours, but no change in TJ protein expression was observed. There are several critical differences in their methodology as compared with ours. They used primary keratinocytes isolated from adult skin samples and differentiated them in high calcium media (1.8 mM) for 4 days before treating with TLR2 agonist, whereas 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 effect when added to highly differentiated keratinocytes (Kuo, unpublished data). This most likely reflects the reduced expression of TLR2 observed in highly differentiated keratinocytes (Supplementary Figure S6 online), and suggests that TLR2 signaling has 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. In addition, 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 $\mu g\,ml^{-1}$) induced TEER, whereas we used lower doses of LPS (5–500 $ng\,ml^{-1}$) and did not observe an effect on TJ function. It is worth noting that high concentrations of LPS (1 $\mu g\,ml^{-1}$) have been shown to induce TLR2 expression and its downstream signaling (NF-κB activation) (Liu *et al.*, 2001). In addition, the concentrations of both LTA and PGN (ug range) as well as of LPS (ng range) we used in our experiments are comparable when they are transposed to bacterial equivalents. Based on this, the doses of LPS used in the study of Yuki *et al.* (2011b) are potentially supraphysiologic. Therefore, it is possible that the TEER effect they observed in LPS may be through TLR2 signaling.

Finally, keratinocytes from AD subjects have reduced mRNA expression of TNF-α and IL-6 upon PGN stimulation (Supplementary Figure S7 online). Interestingly, it has been shown that *Il-6*$^{-/-}$ mice (Wang *et al.*, 2004) and *Tnfr1*$^{-/-}$ mice (Jensen *et al.*, 1999) have delayed TEWL recovery after tape stripping, suggesting that IL-6 and TNF-α 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 partly because of the reduced TLR2 expression observed in AD epidermis (Figures 5 and 6a, b). This finding is also consistent with the observation that TLR2 expression is decreased in circulating monocytes from AD subjects (Niebuhr *et al.*, 2009; Antiga *et al.*, 2011), suggesting that AD subjects may have a genetic or epigenetic defect that affects expression and/or function of TLR2 on multiple cell types (Hasannejad *et al.*, 2007; Mrabet-Dahbi *et al.*, 2008; Niebuhr *et al.*, 2009; Antiga *et al.*, 2011). Indeed, we examined 11 haplotype-tagging single-nucleotide polymorphisms in the *TLR2* gene, and found no association with AD in two independent groups of patients with AD (Caucasians: $n=258$; African Americans: $n=176$) and healthy control subjects ($n=156$; $n=152$, respectively; Barnes, 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 could inhibit TLR2 protein translation and thereby decrease its expression and likely also its function (Benakanakere *et al.*, 2009; Liu *et al.*, 2009; Jurkin *et al.*, 2010; 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, such as *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

PHKs were isolated from discarded neonatal foreskins (also see Supplementary Materials online). PHKs were plated in K-SFM in 24-well Costar Transwell inserts (polyester membranes, 0.4-μm 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 ranged from 87 ± 15 to 305 ± 120 ohms $\times$ cm^2. The study was approved by the Research Subject Review Board at the University of Rochester Medical Center and was conducted according to the Declaration of Helsinki Principles.

Paracellular flux assay

PHKs were seeded in Transwell inserts and treated as described above. After 48 hours, 0.02% fluorescein sodium (Sigma-Aldrich, St Louis, MO) in phosphate-buffered saline was added to the upper well, whereas phosphate-buffered saline alone was added to the lower well. Samples were collected from the lower well after 30 minutes. 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, Hercules, CA). 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 $Tlr2^{-/-}$ mice (C57BL6 background; kindly provided by Dr Jian-Dong Li) (Shuto *et al.*, 2002) and WT mice (C57BL6; purchased from the National Cancer Institute, Bethesda, MD) were anesthetized with ketamine/xylazine, back hair was shaved, and depilatory applied for 3 minutes (Veet; Reckitt Benckiser, Wayne, 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 ($\sim 75 \pm 10\,g\,h^{-1}\,m^{-2}$) 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 hours. 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 hours), and rinsed in phosphate-buffered saline. For human skin, full-thickness epidermis was obtained from discarded skin samples after 15 tape strippings, using a Weck blade (Goulian Skin Graft Knife Set; George Tiemann, Hauppauge, NY) and then rinsed in phosphate-buffered saline. 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, Philadelphia, PA), with an opening of 3 mm and placed in modified Snapwell chambers (Corning, Corning, NY). Samples were submerged in DMEM complete media and kept at 37 °C, 5% CO_2 for 30 minutes. Pam3CSK4 ($10\,\mu g\,ml^{-1}$) or media alone were then added to both sides of the transwell, and TEER was measured at 0 and 24 hours by using Endohm with a planar electrode (Endohm-Snap; World Precision Instruments, Sarasota, FL). TEER ($ohms \times cm^2$) = (measured value) × (transwell area; 0.07 cm^2). The percentage change in TEER between time 0 (100%) and time 24 hours was expressed as follows: $(TEER_{24\,hours}/TEER_{0\,hours}) \times 100$. Data were normalized to control group, and presented as fold of control. Paracellular flux of fluorescein in human epidermis was measured at 24 hours 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 the Declaration of Helsinki Principles.

Statistical analysis

Data are expressed as mean ± SEM and are representative of at least three separate experiments. One-way or two-way analysis of variance was used to determine significance unless otherwise stated. Mann–Whitney *t*-test was used to compare gene expression and immunofluorescence intensity between AD and nonatopic skin. Spearman's nonparametric correlation was used to evaluate the correlation between TLR2 staining intensity and TEWL. The statistical analysis was performed using GraphPad Prism 5 (GraphPad Software, San Diego, CA). $P < 0.05$ was considered significant.

CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

This work was funded by the Atopic Dermatitis Research Network (contract HHSN272201000020C and HHSN272201000017C; to LAB, RLG, DYL, and KCB), DHHS/PHS/NIH 5 T32 AR007472-21 (to ADB), National Eczema Association research grant (to ADB and LAB), Dermatology Foundation research grant (to ADB), National Institute of Diabetes and Digestive and Kidney Diseases grants DK083968 and DK084953 (to AII) and NIH R01 HL071933 (to SNG), and NIEHS P30 ES01247 (to SNG). We thank 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 and Dr Jae Hyang Lim at the Georgia State University (Atlanta, GA) and Dr Xiangbin Xu at the University of Rochester Medical Center (Rochester, NY) for providing the *Tlr2*$^{-/-}$ mice.

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

REFERENCES

Aberg KM, Man MQ, Gallo RL *et al.* (2008) Co-regulation and interdependence of the mammalian epidermal permeability and antimicrobial barriers. *J Invest Dermatol* 128:917–25

Ahrens K, Schunck M, Podda GF *et al.* (2011) Mechanical and metabolic injury to the skin barrier leads to increased expression of murine beta-defensin-1, -3, and -14. *J Invest Dermatol* 131:443–52

Amagai M, Matsuyoshi N, Wang ZH *et al.* (2000) Toxin in bullous impetigo and staphylococcal scalded-skin syndrome targets desmoglein 1. *Nat Med* 6:1275–7

Antiga E, Volpi W, Torchia D *et al.* (2011) Effects of tacrolimus ointment on Toll-like receptors in atopic dermatitis. *Clin Exp Dermatol* 36:235–41

Baker BS, Ovigne JM, Powles AV *et al.* (2003) Normal keratinocytes express Toll-like receptors (TLRs) 1, 2 and 5: modulation of TLR expression in chronic plaque psoriasis. *Br J Dermatol* 148:670–9

Baltes S, Nau H, Lampen A (2004) All-trans retinoic acid enhances differentiation and influences permeability of intestinal Caco-2 cells under serum-free conditions. *Dev Growth Differ* 46:503–14

Benakanakere MR, Li Q, Eskan MA *et al.* (2009) Modulation of TLR2 protein expression by miR-105 in human oral keratinocytes. *J Biol Chem* 284:23107–15

Bochud PY, Magaret AS, Koelle DM *et al.* (2007) Polymorphisms in TLR2 are associated with increased viral shedding and lesional rate in patients with genital herpes simplex virus Type 2 infection. *J Infect Dis* 196:505–9

Boguniewicz M, Leung DY (2010) Recent insights into atopic dermatitis and implications for management of infectious complications. *J Allergy Clin Immunol* 125:4–13

Boguniewicz M, Leung DY (2011) Atopic dermatitis: a disease of altered skin barrier and immune dysregulation. *Immunol Rev* 242:233–46

Borkowski AW, Gallo RL (2011) The coordinated response of the physical and antimicrobial peptide barriers of the skin. *J Invest Dermatol* 131:285–7

Cario E, Gerken G, Podolsky DK (2004) Toll-like receptor 2 enhances ZO-1-associated intestinal epithelial barrier integrity via protein kinase C. *Gastroenterology* 127:224–38

Cario E, Gerken G, Podolsky DK (2007) Toll-like receptor 2 controls mucosal inflammation by regulating epithelial barrier function. *Gastroenterology* 132:1359–74

Cork MJ, Robinson DA, Vasilopoulos Y *et al.* (2006) New perspectives on epidermal barrier dysfunction in atopic dermatitis: gene-environment interactions. *J Allergy Clin Immunol* 118:3–21

De Benedetto A, Agnihothri R, McGirt LY *et al.* (2009) Atopic dermatitis: a disease caused by innate immune defects? *J Invest Dermatol* 129:14–30

De Benedetto A, Kubo A, Beck LA (2012) Skin barrier disruption: a requirement for allergen sensitization? *J Invest Dermatol* 132:949–63

De Benedetto A, Rafaels NM, McGirt LY *et al.* (2011) Tight junction defects in patients with atopic dermatitis. *J Allergy Clin Immunol* 127:773–86.e1-7

Dickel H, Gambichler T, Kamphowe J *et al.* (2010) Standardized tape stripping prior to patch testing induces upregulation of Hsp90, Hsp70, IL-33, TNF-alpha and IL-8/CXCL8 mRNA: new insights into the involvement of 'alarmins'. *Contact Dermatitis* 63:215–22

Djalilian AR, McGaughey D, Patel S *et al.* (2006) Connexin 26 regulates epidermal barrier and wound remodeling and promotes psoriasiform response. *J Clin Invest* 116:1243–53

El Asmar R, Panigrahi P, Bamford P *et al.* (2002) Host-dependent zonulin secretion causes the impairment of the small intestine barrier function after bacterial exposure. *Gastroenterology* 123:1607–15

Elias PM, Hatano Y, Williams ML (2008) Basis for the barrier abnormality in atopic dermatitis: outside-inside-outside pathogenic mechanisms. *J Allergy Clin Immunol* 121:1337–43

Furuse M, Furuse K, Sasaki H *et al.* (2001) Conversion of zonulae occludentes from tight to leaky strand type by introducing claudin-2 into Madin-Darby canine kidney I cells. *J Cell Biol* 153:263–72

Furuse M, Hata M, Furuse K *et al.* (2002) Claudin-based tight junctions are crucial for the mammalian epidermal barrier: a lesson from claudin-1-deficient mice. *J Cell Biol* 156:1099–111

Gareus R, Huth M, Breiden B *et al.* (2007) Normal epidermal differentiation but impaired skin-barrier formation upon keratinocyte-restricted IKK1 ablation. *Nat Cell Biol* 9:461–9

Grether-Beck S, Felsner I, Brenden H *et al.* (2012) Urea uptake enhances barrier function and antimicrobial defense in humans by regulating epidermal gene expression. *J Invest Dermatol* 132:1561–72

Grigoryev DN, Howell MD, Watkins TN *et al.* (2010) Vaccinia virus-specific molecular signature in atopic dermatitis skin. *J Allergy Clin Immunol* 125:153–9.e28

Hasannejad H, Takahashi R, Kimishima M *et al.* (2007) Selective impairment of Toll-like receptor 2-mediated proinflammatory cytokine production by monocytes from patients with atopic dermatitis. *J Allergy Clin Immunol* 120:69–75

Howell MD, Kim BE, Gao P *et al.* (2009) Cytokine modulation of atopic dermatitis filaggrin skin expression. *J Allergy Clin Immunol* 124:R7–12

Imokawa G (2001) Lipid abnormalities in atopic dermatitis. *J Am Acad Dermatol* 45:S29–32

Jensen JM, Schutze S, Forl M *et al.* (1999) Roles for tumor necrosis factor receptor p55 and sphingomyelinase in repairing the cutaneous permeability barrier. *J Clin Invest* 104:1761–70

Jin H, Kumar L, Mathias C *et al.* (2009) Toll-like receptor 2 is important for the T(H)1 response to cutaneous sensitization. *J Allergy Clin Immunol* 123:875–82 e1

Jurkin J, Schichl YM, Koeffel R *et al.* (2010) miR-146a is differentially expressed by myeloid dendritic cell subsets and desensitizes cells to TLR2-dependent activation. *J Immunol* 184:4955–65

Kollisch G, Kalali BN, Voelcker V *et al.* (2005) Various members of the Toll-like receptor family contribute to the innate immune response of human epidermal keratinocytes. *Immunology* 114:531–41

Koschwanez HE, Broadbent E (2011) The use of wound healing assessment methods in psychological studies: a review and recommendations. *Br J Health Psychol* 16:1–32

Kretz M, Maass K, Willecke K (2004) Expression and function of connexins in the epidermis, analyzed with transgenic mouse mutants. *Eur J Cell Biol* 83:647–54

Kubo A, Nagao K, Amagai M (2012) Epidermal barrier dysfunction and cutaneous sensitization in atopic diseases. *J Clin Invest* 122:440–7

Liu G, Friggeri A, Yang Y *et al.* (2009) miR-147, a microRNA that is induced upon Toll-like receptor stimulation, regulates murine macrophage inflammatory responses. *Proc Natl Acad Sci USA* 106:15819–24

Liu Y, Wang Y, Yamakuchi M *et al.* (2001) Upregulation of toll-like receptor 2 gene expression in macrophage response to peptidoglycan and high concentration of lipopolysaccharide is involved in NF-kappa b activation. *Infect Immun* 69:2788–96

Lucke T, Choudhry R, Thom R *et al.* (1999) Upregulation of connexin 26 is a feature of keratinocyte differentiation in hyperproliferative epidermis, vaginal epithelium, and buccal epithelium. *J Invest Dermatol* 112:354–61

Ma TY, Iwamoto GK, Hoa NT *et al.* (2004) TNF-alpha-induced increase in intestinal epithelial tight junction permeability requires NF-kappa B activation. *Am J Physiol Gastrointest Liver Physiol* 286:G367–76

Marchiando AM, Shen L, Graham WV *et al.* (2010) Caveolin-1-dependent occludin endocytosis is required for TNF-induced tight junction regulation in vivo. *J Cell Biol* 189:111–26

Mempel M, Voelcker V, Kollisch G *et al.* (2003) Toll-like receptor expression in human keratinocytes: nuclear factor kappaB controlled gene activation by Staphylococcus aureus is toll-like receptor 2 but not toll-like receptor 4 or platelet activating factor receptor dependent. *J Invest Dermatol* 121: 1389–96

Menzies BE, Kenoyer A (2006) Signal transduction and nuclear responses in Staphylococcus aureus-induced expression of human beta-defensin 3 in skin keratinocytes. *Infect Immun* 74:6847–54

Mrabet-Dahbi S, Dalpke AH, Niebuhr M *et al.* (2008) The Toll-like receptor 2 R753Q mutation modifies cytokine production and Toll-like receptor expression in atopic dermatitis. *J Allergy Clin Immunol* 121:1013–9

Murata Y, Ogata J, Higaki Y *et al.* (1996) Abnormal expression of sphingomyelin acylase in atopic dermatitis: an etiologic factor for ceramide deficiency? *J Invest Dermatol* 106:1242–9

Nahid MA, Satoh M, Chan EK (2011) Mechanistic role of microRNA-146a in endotoxin-induced differential cross-regulation of TLR signaling. *J Immunol* 186:1723–34

Niebuhr M, Lutat C, Sigel S *et al.* (2009) Impaired TLR-2 expression and TLR-2-mediated cytokine secretion in macrophages from patients with atopic dermatitis. *Allergy* 64:1580–7

Niessen CM (2007) Tight junctions/adherens junctions: basic structure and function. *J Invest Dermatol* 127:2525–32

Ohnemus U, Kohrmeyer K, Houdek P *et al.* (2008) Regulation of epidermal tight-junctions (TJ) during infection with exfoliative toxin-negative Staphylococcus strains. *J Invest Dermatol* 128:906–16

Olaru F, Jensen LE (2010) Staphylococcus aureus stimulates neutrophil targeting chemokine expression in keratinocytes through an autocrine IL-1alpha signaling loop. *J Invest Dermatol* 130:1866–76

Ong PY, Leung DY (2010) The infectious aspects of atopic dermatitis. *Immunol Allergy Clin North Am* 30:309–21

Ozinsky A, Underhill DM, Fontenot JD *et al.* (2000) The repertoire for pattern recognition of pathogens by the innate immune system is defined by cooperation between toll-like receptors. *Proc Natl Acad Sci USA* 97:13766–71

Palmer CN, Irvine AD, Terron-Kwiatkowski A *et al.* (2006) Common loss-of-function variants of the epidermal barrier protein filaggrin are a major predisposing factor for atopic dermatitis. *Nat Genet* 38:441–6

Pilgram GS, Vissers DC, van der Meulen H *et al.* (2001) Aberrant lipid organization in stratum corneum of patients with atopic dermatitis and lamellar ichthyosis. *J Invest Dermatol* 117:710–7

Rezaee F, Meednu N, Emo JA *et al.* (2011) Polyinosinic:polycytidylic acid induces protein kinase D-dependent disassembly of apical junctions and barrier dysfunction in airway epithelial cells. *J Allergy Clin Immunol* 128:1216–24 e11

Sato A, Linehan MM, Iwasaki A (2006) Dual recognition of herpes simplex viruses by TLR2 and TLR9 in dendritic cells. *Proc Natl Acad Sci USA* 103:17343–8

Schauber J, Dorschner RA, Coda AB *et al.* (2007) Injury enhances TLR2 function and antimicrobial peptide expression through a vitamin D-dependent mechanism. *J Clin Invest* 117:803–11

Schroder NW, Morath S, Alexander C *et al.* (2003) Lipoteichoic acid (LTA) of Streptococcus pneumoniae and Staphylococcus aureus activates immune cells via Toll-like receptor (TLR)-2, lipopolysaccharide-binding protein (LBP), and CD14, whereas TLR-4 and MD-2 are not involved. *J Biol Chem* 278:15587–94

Schwandner R, Dziarski R, Wesche H *et al.* (1999) Peptidoglycan- and lipoteichoic acid-induced cell activation is mediated by toll-like receptor 2. *J Biol Chem* 274:17406–9

Shuto T, Imasato A, Jono H *et al.* (2002) Glucocorticoids synergistically enhance nontypeable Haemophilus influenzae-induced Toll-like receptor 2 expression via a negative cross-talk with p38 MAP kinase. *J Biol Chem* 277:17263–70

Takeuchi O, Hoshino K, Akira S (2000) Cutting edge: TLR2-deficient and MyD88-deficient mice are highly susceptible to Staphylococcus aureus infection. *J Immunol* 165:5392–6

Takeuchi O, Hoshino K, Kawai T *et al.* (1999) Differential roles of TLR2 and TLR4 in recognition of gram-negative and gram-positive bacterial cell wall components. *Immunity* 11:443–51

Taljebini M, Warren R, Mao-Oiang M *et al.* (1996) Cutaneous permeability barrier repair following various types of insults: kinetics and effects of occlusion. *Skin Pharmacol* 9:111–9

Tsan MF, Gao B (2004) Endogenous ligands of Toll-like receptors. *J Leukoc Biol* 76:514–9

Tunggal JA, Helfrich I, Schmitz A *et al.* (2005) E-cadherin is essential for in vivo epidermal barrier function by regulating tight junctions. *EMBO J* 24:1146–56

Utech M, Ivanov AI, Samarin SN *et al.* (2005) Mechanism of IFN-gamma-induced endocytosis of tight junction proteins: myosin II-dependent vacuolarization of the apical plasma membrane. *Mol Biol Cell* 16:5040–52

Vasilopoulos Y, Cork MJ, Teare D *et al.* (2007) A nonsynonymous substitution of cystatin A, a cysteine protease inhibitor of house dust mite protease, leads to decreased mRNA stability and shows a significant association with atopic dermatitis. *Allergy* 62:514–9

Wang XP, Schunck M, Kallen KJ *et al.* (2004) The interleukin-6 cytokine system regulates epidermal permeability barrier homeostasis. *J Invest Dermatol* 123:124–31

Warner RR, Boissy YL, Lilly NA *et al.* (1999) Water disrupts stratum corneum lipid lamellae: damage is similar to surfactants. *J Invest Dermatol* 113:960–6

Warner RR, Stone KJ, Boissy YL (2003) Hydration disrupts human stratum corneum ultrastructure. *J Invest Dermatol* 120:275–84

Yoshimura A, Lien E, Ingalls RR *et al.* (1999) Cutting edge: recognition of Gram-positive bacterial cell wall components by the innate immune system occurs via Toll-like receptor 2. *J Immunol* 163:1–5

Yuki T, Hachiya A, Kusaka A *et al.* (2011a) Characterization of tight junctions and their disruption by UVB in human epidermis and cultured keratinocytes. *J Invest Dermatol* 131:744–52

Yuki T, Yoshida H, Akazawa Y *et al.* (2011b) Activation of TLR2 enhances tight junction barrier in epidermal keratinocytes. *J Immunol* 187:3230–7

Zahringer U, Lindner B, Inamura S *et al.* (2008) TLR2 - promiscuous or specific? A critical re-evaluation of a receptor expressing apparent broad specificity. *Immunobiology* 213:205–24