TLR3 in Chronic Human Itch: A Keratinocyte-Associated Mechanism of Peripheral Itch Sensitization


TO THE EDITOR

The acute itch-scratch reflex, considered a protective and evolutionarily conserved mechanism, can become dysfunctional in the setting of many chronic skin diseases, resulting in chronic itch (Mollanazar et al., 2016; Steinhoff et al., 2018; Talwalkar et al., 2003). The resultant debilitating itch-scratch cycle can occur in inflammatory skin diseases; neurological diseases (Meng et al., 2018; Steinhoff et al., 2012); and systemic conditions such as cancer, diabetes, and renal or hepatic disorders.

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Abbreviations: AD, atopic dermatitis; ET-1, endothelin-1; NHEK, normal human epidermal keratinocyte; PN, prurigo nodularis; poly-Il-1C, polyinosinic-polycytidylic acid; PSO, psoriasis; TLR3, toll-like receptor 3

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Because the exact mechanisms and pathways leading to the chronification of itch still remain unknown, effective treatment still poses many challenges. An interesting candidate in this pathway is toll-like receptor 3 (TLR3). TLR3 was shown to be an important receptor in murine itch signaling and is expressed by sensory nerves and dorsal root ganglia in mice. Indeed, injection of TLR3’s synthetic agonist polyinosinic:polycytidylic acid (poly(I:C)) into the skin of mice results in robust scratching behavior (Liu et al., 2012). In humans, the expression of TLR3 has been investigated at the mRNA level in epidermal sheets from psoriasis (PSO) and atopic dermatitis (AD) lesions (de Koning et al., 2010), where no significant difference was reported between these groups and healthy controls. Interestingly, barrier disruption by tape stripping downregulated TLR3 mRNA in PSO and AD skin compared with normal skin (de Koning et al., 2011), which seems to be at odds with the upregulation of TLR3 expression found in the previously mentioned functional studies. A more recent study also found that the mRNA expression of TLR3 is lower in lesional AD and PSO skin than non-lesional skin; however, interestingly, it shows that expression is increased in chronically scratched lesions compared with healthy skin (Nattkemper et al., 2018).

Because TLR3 plays a role in murine itch and may be activated by skin damage such as scratching, which releases self-RNA, we hypothesize that the TLR3 expressed in lesional skin of chronic pruritic dermatoses may be activated upon keratinocyte injury caused by scratching. Such activation could provide an initiating link between chronic scratching behavior, the innate immune system, and itch. Furthermore, repeated TLR3 activation by danger-associated molecular patterns and the potential release of itch mediators may suggest that the consequences of chronic scratching could by itself potentiate the vicious itch-scratch cycle locally in the epidermis.

Based on this hypothesis, we aimed to investigate the role of TLR3 in human pruritus. First, we determined the expression levels of TLR3 in chronically scratched skin of patients diagnosed with prurigo nodularis (PN), AD, and PSO. Institutional ethical approval was obtained for our experiments, and samples were collected after obtaining written, informed consent. In addition, we evaluated the mRNA expression levels and release of pruritogens from primary normal human epidermal keratinocytes (NHEKs) following stimulation with the TLR3 ligand poly(I:C).

(Matterne et al., 2011; Talwalkar et al., 2003).
We first analyzed TLR3 mRNA expression in the skin of patients with PN, AD, and PSO, and found that there was no significant change in the mRNA expression (Supplementary Figure S1), which coincides with previously reported results (de Koning et al., 2010; Nattkemper et al., 2018). Protein expression patterns were evaluated by immunofluorescence in lesional (scratched) and perilesional (non-lesional) skin obtained from patients with PN, AD, and PSO as well as healthy controls (Figure 1b, d, f, and a, respectively). Immunostaining for TLR3 was concentrated in the epidermis of all patients. Quantification of immunofluorescence (Figure 1c, e, and g) showed that staining was significantly increased in the lesional (scratched) skin compared with healthy controls, as well as non-lesional skin in the case of PN. Notably, TLR3 staining was also increased in perilesional samples in PN and PSO compared with healthy controls.

To investigate the role of TLR3 activation in NHEKs, we determined the mRNA expression levels of important known keratinocyte-derived itch mediators (Kido-Nakahara et al., 2014; Wilson et al., 2013) following stimulation with poly-(I:C). We observed a significant and dose-dependent increase in the mRNA expression levels of both endothelin-1 (ET-1) and thymic stromal lymphopoietin at 24 hours of poly-(I:C) stimulation (Figure 1a and b). We also detected that activation of TLR3 resulted in a significant increase in the mRNA levels of TLR3, indicating a positive feedback loop, as reported previously (Borkowski et al., 2015) (Supplementary Figure S2). In contrast, we did not detect any human beta nerve growth factor mRNA in our samples.

To further explore the production of pruritogens by keratinocytes following activation of TLR3, we examined the release of ET-1 and IL-6, two known pruritogens that activate high-affinity receptors on sensory nerves and on cells of the innate immune system (Grothe et al., 2000), and release of tumor necrosis factor-α from NHEKs. We found that release of both pruritogens and tumor necrosis factor-α was significantly increased (Figure 1c and d, Supplementary Figure S3b), whereas the production of beta nerve growth factor was not affected (Supplementary Figure S4). Importantly, the effect of poly-(I:C) on ET-1 and tumor necrosis factor-α production was also blocked by the TLR3 antagonist CU CPT 4a (Supplementary Figure S3).

To compare the secretion of the pruritogens ET-1 and IL-6 from NHEKs treated with poly-(I:C) with other known pruritogens (histaminergic and nonhistaminergic), cells were treated with histamine and a proteinase activated receptor 2 agonist at concentrations of 100 μM and 10 μM, respectively. Activation of proteinase activated receptor 2 resulted in similar effects on the release of pruritogens from NHEKs as TLR3 activation, albeit TLR3 activation was the most effective (Supplementary Figure S5). ET-1 is considered a potent itch mediator in humans, and indeed the expression of ET-1 has been shown to be significantly increased in lesional skin of patients with PN (Kido-Nakahara et al., 2014). Our in vitro and ex vivo results suggest that TLR3 may represent a sentinel receptor for ET-induced chronic itch in humans at a local keratinocyte level. We have demonstrated that its epidermal expression is significantly increased in pruritic dermatoses on the protein level, and that TLR3 activation is efficacious in inducing ET-1 release from healthy controls (Figure 1b, d, f, and a, respectively). Immunostaining for TLR3 was concentrated in the epidermis of all patients. Quantification of immunofluorescence (Figure 1c, e, and g) showed that staining was significantly increased in the lesional (scratched) skin compared with healthy controls, as well as non-lesional skin in the case of PN. Notably, TLR3 staining was also increased in perilesional samples in PN and PSO compared with healthy controls.

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keratinocytes in vitro. Furthermore, we also show that ET-1 activates murine dorsal root ganglia cells, which subsequently stimulates release of B-type natriuretic peptide (Figure 2e), a known pruritoid mediator in the central nervous system (Mishra and Hoon, 2013) and skin (Meng et al., 2018). Thus, we propose that TLR3 represents an innate biosensor in humans, similar to murine itch (Liu et al., 2012). It may also represent an important receptor in human itch associated with innate immune responses and triggers (Supplementary Discussion, Supplementary Figure S6).

Thus, activation of TLR3 in keratinocytes, and most likely in infiltrating immune cells of chronic lesions by danger-associated molecular patterns, provides a pathomechanistic platform to explain chronic itch in the context of the vicious itch-scratch cycle localized in the epidermis that has not been reported previously to our knowledge. Understanding how innate immune mechanisms regulate peripheral itch sensitization on the molecular level will lead to new targeted therapies to treat patients with chronic itch and compulsive scratching.

Data availability statement
The data that support the findings of this study are available from the corresponding author upon reasonable request.

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CONFLICT OF INTEREST
The authors state no conflict of interest.

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AUTHOR CONTRIBUTIONS
Conceptualization: AGS, IMcD, MS; Formal Analysis: AGS, IMcD, ILS; Funding Acquisition: MS; Investigation: AGS, IMcD, ILS, JM; Resources: EB; Writing — Original Draft Preparation: IMcD, AGS; Writing — Review and Editing: IMcD, ILS, MS, EB.

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SUPPLEMENTARY MATERIAL
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REFERENCES
SUPPLEMENTARY MATERIALS AND METHODS

Keratinocyte culture
Normal human epidermal keratinocytes (NHEKs) from adult donors were obtained from skin derived from patients undergoing abdominal skin reconstruction. Isolation was performed as described previously (Tjabringa et al., 2008). Cells were maintained at 37 °C, with 5% CO2 in a humidified environment with KBM-Gold Keratinocyte Growth Medium (Lonza Biosciences, Basel, Switzerland) supplemented with KGM-Gold SingleQuot keratinocyte supplements. Cells were passaged a maximum of four times before treatments. Cells were washed with sterile Dulbecco’s Phosphate Buffered Saline (Thermo Fisher Scientific, Waltham, MA) between medium changes and passaging. Trypsin-EDTA solution (Sigma-Aldrich, St. Louis, MO) was used to remove adherent cells from the culture surface. Confluence was estimated by visualizing the cells using an inverted microscope (IX2-SLP, Olympus, Tokyo, Japan). Cells at 70–80% confluence were kept in unsupplemented media for 24 hours before treatment with polyinosinic:polycytidylic acid (Invivogen, San Diego, CA) at concentrations of 1 and 10 μg/ml, 2-Furoyl-LIGRLamide (Abcam, Cambridge, United Kingdom) at 10 μM, and histamine (Sigma-Aldrich) at 100 μM. Supernatant and cells were harvested at 4- and 24-hour time points. An extended time line of 48 and 72 hours was also used in some of the experimental designs.

Dorsal root ganglia isolation and culture
Dorsal root ganglia were isolated from postnatal d5 C57BL/6 mice and dissociated by collagenase I to investigate B-type natriuretic peptide (BNP) release. Neurons were cultured in 24-well plates in the presence of cytosine β-d-arabinofuranoside (Sigma-Aldrich) and nerve growth factor at 100 ng/ml for 7 days in vitro. Basal low-potassium release buffer (22.5 mmol/L HEPES, 135 mmol/L NaCl, 3.5 mmol/L KCl, 1 mmol/L MgCl2, 2.5 mmol/L CaCl2, 3.3 mmol/L glucose, and 0.1 % BSA, pH 7.4) was added into each well, followed by a 30-minute incubation at 37 °C. Cells were then stimulated for 30 minutes by 1 μmol/L endothelin-1 (ET-1). BNP release was quantified by using an ELISA kit (Sigma-Aldrich).

ELISA
The expression of IL-6, ET, human beta nerve growth factor (βNGF), and BNP in the supernatant of treated NHEKs and dorsal root ganglia was assessed according to the manufacturer’s instructions (DuoSet ELISA development system, IL-6, ET, and βNGF: R&D Systems, Minneapolis, MN; BNP: Sigma-Aldrich). Briefly, ELISA plates were coated with the supplied capture antibodies overnight at 4 °C. The following day, after washing the plates four times with the supplied wash solution, buffer standards and samples were added to the appropriate wells and incubated at room temperature for 2.5 hours. After washing the plates four times, the Streptavidin-HRP solution was added to each well and incubated for 45 minutes at room temperature with gentle shaking. Following four more washes, the signal was developed using TMB One-Step Substrate Reagent (RayBiotech, Peachtree Corners, GA), which was stopped after 30 minutes using the supplied stop solution and subsequently measured at 450 nm using a SpectraMax plate reader (Molecular Devices, San Jose, CA). The concentration of the analytes in the supernatant was then calculated using a cubic logistic model as recommended by Herman et al. (2008).

RNA isolation, reverse transcription, and quantitative reverse transcriptase PCR
Total RNA was harvested from NHEKs following treatment using a Nucleospin RNA Midi kit (Macherey-Nagel, Düren, Germany) according to the manufacturer’s instructions. Following extraction, RNA concentration was quantified by spectrophotometry using a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific). cDNA was generated from mRNA transcripts using reverse transcriptase. Using a high capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA), 1 μg of total RNA was reverse-transcribed into cDNA. Real-time PCR was performed with predesigned TaqMan Gene Expression Assays (Applied Biosystems) for TLR3 (Hs01551078 m1 TLR3), ET-1 (Hs00174961 m1 EDN1), thymic stromal lymphopoietin (TSLP; Hs00263639 m1 TSLP), βNGF (Hs01113193 m1 NGF), and glyceraldehyde-3-phosphate dehydrogenase (Hs99999905_m1) on a T9000HT Fast Real-Time PCR System (Applied Biosystems). Expression of mRNA was normalized relative to glyceraldehyde-3-phosphate dehydrogenase as an endogenous control. The 2-DΔCt method was used to calculate relative changes in gene expression, and data were expressed as fold difference relative to control samples.

Patients
Before initiating the study, the study protocol, the formal ethics application form, patient information leaflet, and consent form were reviewed and subsequently approved by the research and ethics committee of St. Vincent’s University Hospital. Eligible participants were identified from the dermatology department of St. Vincent’s University Hospital. A diagnosis of prurigo nodularis (PN), atopic dermatitis (AD), or psoriasis (PSO) was made by a consultant dermatologist and study investigator. Healthy volunteers were also recruited to this study as controls. The visual analogue scale for itch was used to measure pruritus intensity in patients. Skin biopsies were obtained from lesional and perilesional skin of patients following written informed consent. Lesional skin was identified by the investigator as representative of the underlying inflammatory skin disease with evidence of excoriations (acute and chronic). Perilesional skin was defined as uninvolved skin 10 cm away from the lesional area. Punch biopsies 4 mm in diameter were taken from lesional and perilesional skin.

Immunofluorescence
Snap-frozen skin biopsies were placed in Tissue-Tek O.C.T. (Sakura Finetek, Torrance, CA) before sectioning. Cryosectioning was performed using the CRYOSTAR NX70 (Thermo Fisher Scientific). Tissue sections of 4 μm were placed onto Superfrost Plus slides (Thermo Fisher Scientific), and fixed with 4% paraformaldehyde. Sections were blocked with donkey serum (MiliporeSigma, Burlington, MA), after which a monoclonal antibody against...
TLR3 (NBP2-24875, Novus Biologicals, Centennial, CO) was used as the primary antibody diluted 1:50 in DCS antibody diluent (DCS Innovative Diagnostik-systeme, Hamburg, Germany). Staining was visualized with Alexa Fluor 568 conjugated secondary antibody (donkey anti-Mouse IgG (H+L), Thermo Fisher Scientific) diluted to a concentration of 1:500 in DCS antibody diluent. For nuclear counter staining, DAPI Fluorescent Dye (Thermo Fisher Scientific) was used, after which sections were covered with Fluoromount Aqueous Mounting Medium (Sigma-Aldrich). Sections were imaged on a LEICA DFC7000T inverted microscope (Leica Microsystems, Wetzlar, Germany). Image J software (National Institutes of Health, Bethesda, MD) was used to quantify immunofluorescence.

Statistics
Data were statistically analyzed using GraphPad Prism 5.0 using two-tailed unpaired t-test or one-way analysis of variance with Tukey or Dunnett multiple comparisons tests, where applicable. Significant differences were considered when \( P \leq 0.05 \).

Ethical conduct of the study
The study was carried out in accordance with the study protocol and with adherence to good clinical practice guidelines as described in the Declaration of Helsinki, concerning medical research in humans (1964), including all amendments up to and including the 2008 revision; the Good Clinical Practice commission directive 2005/28/EC; and in adherence with national laws.

SUPPLEMENTARY DISCUSSION
In this work, we show that TLR3 may act as a central player in the itch-scratch cycle in several distinct pruritic dermatoses, namely, AD, PN, and PSO (Figure 1). We have also shown that TLR3 activation leads to the release of ET-1 from in vitro NHEK cultures (Figure 1c), with concomitant release of IL-6 and tumor necrosis factor-\( \alpha \) (Figure 1d, Supplementary Figure S3). This dose-dependent increase in the production of IL-6 from NHEKs is consistent with previous studies (Miller, 2008; Rana et al., 2015). Interestingly, Nordlind et al. (1996) previously demonstrated IL-6 to be increased in the lesional skin of patients with PN. It may be that TLR3 is a significant contributor to this finding (Nordlind et al., 1996). Notably, we were unable to demonstrate an increase in BNGF production in keratinocytes (either with ELISA or quantitative reverse transcriptase PCR). This is a significant negative result in the context of itch. BNGF was previously shown to be increased in the pruritic, dry skin of mice, and significantly, the increased expression was absent in TLR3\(-/-\) mice (Liu et al., 2012). This observation has led some commentators to surmise that TLR3 may play a role in the production of BNGF in skin in humans (Taves and Ji, 2015). We suggest, given our findings, that this might not be the case, or that there are other factors that contribute to BNGF production. It is also interesting to note that in PN, there is a significant decrease in epidermal sensory C fibers in the nodules in addition to the perilesional skin of patients (Schuhknecht et al., 2011). In addition, Cameron et al. previously showed that activation of TLR3 by polynosinic:polycytidylic acid or by mRNA rapidly causes growth cone collapse and irreversibly inhibits neurite extension independent of NF-\( \alpha \)B (Cameron et al., 2007). Combined, these data suggest that TLR3 activation may in fact have a negative impact on nerve growth and, significantly, may explain the finding observed in patients with PN.

Although the production of BNGF was absent, TSLP transcription was significantly increased upon TLR3 activation. TSLP is a chemokine that is important in initiating dendritic cell–mediated T helper type 2 immune responses seen in AD, for example. It results in the production of many important itch cytokines such as IL-4 and IL-13 (Zhu et al., 2011). Wilson et al. (2013) has also shown TSLP to be an important itch mediator in mice, capable of activating TRPA1 and stimulating nonhistaminergic itch (Wilson et al., 2013a).

Of the other investigated cytokines, IL-6 is a proinflammatory cytokine that has been linked to psoriasis for 25 years (Blauvelt, 2017). IL-6 also has been shown to be increased in the serum of patients with PN (Konda et al., 2015) and has been linked to the development of AD. In addition, IL-6 production is increased in T cells from patients with AD (Haragozolou et al., 2013; Toshitani et al., 1993). TSLP has been shown to be expressed in PSO lesions (Volpe et al., 2014) and is implicated as a trigger factor for AD (Indra, 2013) and pruritus (Wilson et al., 2013b). ET-1 has been shown to be increased in AD and PN (Aktar et al., 2015; Kido-Nakahara et al., 2014) and also has been implicated in PSO (Simeone et al., 2004). There are admittedly fewer results with BNP; however, as a neurotransmitter in the central nervous system, this is not surprising. Even so, there are intriguing data because an increase of the N-terminal pro-form of the peptide (Pietrzak et al., 2013) was shown to be increased in the serum of patients with PSO, as well as from our own workgroup, where we showed that BNP is implicated in IL-31–induced AD (Meng et al., 2018).

Perhaps our most important finding, however, is the release of ET-1 from NHEKs, which conceivably occurs in all three dermatoses where TLR3 levels are elevated. Indeed, in PSO, where itch is thought to occur in 80% of patients and can cause significant additional morbidity (Reich et al., 2010; Taves and Ji, 2015), it is also established that ET-1 is increased in the lesional skin of these patients and that it correlates with disease severity. Our data, showing increased TLR3 expression in the lesional skin of patients with PSO, may provide a pathomechanistic explanation for the increase in ET-1 and, importantly, the symptom of itch in these patients.

We have also shown that TLR3 activation also results in tumor necrosis factor-\( \alpha \) secretion (Supplementary Figure S4), as described previously (Bernard et al., 2012). Although tumor necrosis factor-\( \alpha \) is typically linked to pain and not itch, its production by keratinocytes can contribute to the inflammatory milieu of pruritic skin lesions.

Although it may be possible that the increase in TLR3 expression is not linked specifically to scratching, but to an overall decrease in the barrier function, our finding that TLR3 expression is not increased in AD nonlesional skin seems to argue against this. The barrier defect alone is present...
in non-lesional AD skin, and if this would be enough to cause a rise in TLR3 expression, this would be reflected in our results.

Therefore, it appears that, in addition to murine itch, TLR3 also has a role to play in human itch. The fact that danger-associated molecular patterns released by injured cells, including keratinocytes, activate TLR3 provides a pathomechanistic platform to explain itch in the context of other pruritic dermatoses. For example, healing wounds in patients with epidermolysis bullosa were noted to be the itchiest of skin lesions.

Itch in this setting has very significant implications for patients, leading to further significant injury and complications from scratching (Daniel et al., 2015). Chronic venous ulceration is also associated with significant itch, which can impact healing and add to patient morbidity (Paul et al., 2011).

We also should not neglect the fact that TLR3 is present at very high levels in many immune cells, both those that reside in the skin at rest and those that are part of the infiltrate of chronic skin lesions. Although the positive feedback loop outlined in Supplementary Figure S6 is alluring in its simplicity, we should take into account the high degree of cross-talk possible between keratinocytes and immune cells in developing the final phenotype of any given pruritic lesion.

Targeting TLR3 in these and other pruritic diseases may indeed provide a new avenue for future itch therapy.

**SUPPLEMENTARY REFERENCES**


Supplementary Figure S1. mRNA expression level of TLR3 shows no significant difference between lesional and non-lesional skin of patients with PN. The gene expression is calculated as relative expression to ACTB (n = 5, mean ± SD). PN, prurigo nodularis; SD, standard deviation; TLR3, toll-like receptor 3.

Supplementary Figure S2. TLR3 activation leads to increased TLR3 expression. Expression of TLR3 mRNA in NHEKs treated with the TLR3 agonist poly-(I:C) for 24 hours. The gene expression is calculated as relative expression to GAPDH and normalized to the mRNA level of the vehicle-treated control (n = 3, mean ± SEM; **P < 0.01, ***P < 0.001 compared with control as determined by two-tailed unpaired t-test; representative result of three independent donors). GAPDH, glyceraldehyde-3-phosphate dehydrogenase; NHEK, normal human epidermal keratinocyte; poly-(I:C), polyinosinic:polycytidylic acid; SEM, standard error of the mean; TLR3, toll-like receptor 3.
Supplementary Figure S3. Poly-(I:C)–induced endothelin and TNFα secretion is blocked by the TLR3 antagonist CU CPT 4a. Analysis of supernatant from primary NHEKs treated in vitro with poly-(I:C) and CU CPT 4a at 24 hours using ELISA. Cells were treated with poly-(I:C) 1 μg/ml and CU CPT 4a 30 μM. (a) represents the concentration of ET-1 secretion from NHEKs treated with the agonist or the combination of both agonist and antagonist and shows a significant increase in poly-(I:C) treated cells, the effect of which is abrogated by the coapplication of the antagonist. (b) represents the concentration of TNFα secretion from NHEKs treated with the agonist or the combination of both agonist and antagonist and shows a significant increase in poly-(I:C) treated cells, the effect of which is abrogated by the coapplication of the antagonist. *P < 0.05, **P < 0.01, ****P < 0.0001. Statistical analysis was performed using ANOVA with Tukey post hoc multiple comparisons test. All the data are mean ± SD. ANOVA, analysis of variance; CU CPT 4a, ; ET-1, endothelin-1; NHEK, normal human epidermal keratinocyte; poly-(I:C), polyinosinic:polycytidylic acid; SD, standard deviation; TLR3, toll-like receptor 3; TNFα, tumor necrosis factor-α.

Supplementary Figure S4. TLR3 activation does not result in β-NGF production. ELISA determination of β-NGF production in supernatant of NHEKs treated with poly-(I:C) for 24, 48, and 72 hours (n = 3, mean ± SD; *representative result of three independent donors). Statistical analysis was performed using ANOVA with Tukey post hoc multiple comparisons test. ANOVA, analysis of variance; β-NGF, beta nerve growth factor; NHEK, normal human epidermal keratinocyte; poly-(I:C), polyinosinic:polycytidylic acid; TLR3, toll-like receptor 3.
Supplementary Figure S5. Poly-(I:C) results in greater ET and IL-6 secretion from NHEKs compared with histamine and 2-FLY. Analysis of supernatant from primary NHEKs treated in vitro with poly-(I:C), histamine, and 2-FLY at 24 hours using ELISA. Cells were treated with poly-(I:C) 1 μg/ml, histamine 100 μM, and 2-FLY at 10 μM. (a) represents the concentration of ET-1 secretion from NHEKs treated with the agonists and shows a significant increase in poly-(I:C) and 2-FLY but not histamine-treated cells. Poly-(I:C) resulted in the greatest release of ET when compared with histamine and 2-FLY. (b) represents the concentration of IL-6 secretion from NHEKs treated with the agonists and shows a significant increase in poly-(I:C) and 2-FLY but not histamine-treated cells. Poly-(I:C) resulted in the greatest release of IL-6 when compared with histamine and 2-FLY. **P < 0.01, ****P < 0.0001. Statistical analysis was performed using ANOVA with Tukey post hoc multiple comparisons test. All the data are mean ± SD. 2-FLY, 2-Furoyl-LIGRLO-amide; ANOVA, analysis of variance; ET, endothelin; NHEK, normal human epidermal keratinocyte; poly-(I:C), polyinosinic:polycytidylic acid.

Supplementary Figure S6. Chronic pruritus leads to recurrent scratching with resultant injury and epidermal damage. Damage to keratinocytes results in the release of DAMPs including self-RNA. These DAMPs are capable of activating TLR3 on intact neighboring keratinocytes, which results in the release of pruritic mediators (chiefly ET-1 and TSLP), as well as the induction of TLR3 expression. Keratinocyte-derived mediators then activate their receptors on neighboring nerve endings and infiltrating immune cells such as mast cells and macrophages (TSLPR and ETAR for TSLP and ET-1, respectively), resulting in release of further inflammatory mediators, which is then followed by BNP release from the DRG. BNP has been previously shown to be a pruritic mediator in the CNS (Mishra and Hoon, 2013). These processes may form a molecular basis for the itch-scratch cycle. BNP, B-type natriuretic peptide; CNS, central nervous system; DAMP, danger-associated molecular pattern; DRG, dorsal root ganglia; ET-1, endothelin-1; ETAR, endothelin receptor type A; TLR3, toll-like receptor 3; TSLP, thymic stromal lymphopoietin; TSLPR, thymic stromal lymphopoietin receptor.