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TLR3 IN CHRONIC HUMAN ITCH: A KERATINOCYTE-ASSOCIATED MECHANISM OF PERIPHERAL ITCH SENSITIZATION

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Keywords: pruritus, toll-like receptor, endothelin, nerve, keratinocyte

Abbreviations: AD, atopic dermatitis; β NGF, beta nerve growth factor; BNP, B-type natriuretic peptide; DAMPs, danger associated molecular patterns; DRG, dorsal root ganglia; ET, endothelin; HC, healthy control; IL, interleukin; NHEK,

normal human epidermal keratinocytes; PAR2, proteinase-activated receptor 2; PN, prurigo nodularis; Poly-(I:C), Polyinosinic:polycytidylic acid; PSO, psoriasis; TLR, Toll like receptor; TNF α tumor necrosis factor α ; TSLP, thymic stromal lymphopoetin;

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Letter

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; Talwalkar et al. 2003; Steinhoff et al. 2018). 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, renal or hepatic disorders (Matterne et al. 2011; Talwalkar et al. 2003). 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 (DRG) 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).

TLR3 plays an important role in epidermal barrier repair and wound healing (Borkowski et al. 2015; Lin et al. 2012). Both processes have pathophysiological relevance in the context of skin injured by scratching. When injured, keratinocytes release danger associated molecular patterns (DAMPs), including self-RNA thereby activating TLR3 in keratinocytes resulting in the production of many pro-inflammatory cytokines and chemokines (Bernard 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 compared to healthy controls. Interestingly barrier disruption by tape stripping downregulated TLR3 mRNA in PSO and AD as compared to normal skin (de Koning et al. 2011), which seems to be at odds with the upregulation of TLR3 expression found in the abovementioned functional studies. A more recent study also found that the mRNA expression of TLR3 is lower in lesional AD and PSO skin compared to non-lesional skin, however, interestingly they show that expression is increased in chronically scratched lesions when compared to 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 DAMPs 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 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).

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 1), 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-scratched) skin obtained from patients with PN, AD and PSO as well as healthy controls (Figure 1 b, d, f and a, respectively). Immunostaining for TLR3 was concentrated in the epidermis of all patients. Quantification of immunofluorescence (Figure 1 c, e, g) showed that staining was significantly increased in the lesional (scratched) skin when compared to healthy controls (HC), as well as compared to nonlesional skin in the case of PN. Notably, TLR3 staining was also increased in perilesional samples, when compared to HC in PN and PSO.

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 (TSLP) at 24 hours of poly-(I:C) stimulation (Figure 1 a, 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 2). In contrast, we did not detect any human beta nerve growth factor (β NGF) mRNA in our samples.

To further explore the production of pruritogens by keratinocytes following activation of TLR3, we examined the release of ET-1, interleukin (IL)-6

and tumor necrosis factor α (TNF α) from normal human epidermal keratinocytes (NHEKs), two known pruritogens that activate high-affinity receptors on sensory nerves and on cells of the innate immune system (Grothe et al. 2000). We found that release of both pruritogens and TNF α was significantly increased (Figure 1 c, d, Supplementary Figure 3 b), while the production of β NGF was not affected (Supplementary Figure 4). Importantly the effect of poly-(I:C) on ET-1 and TNF α production was also blocked by the TLR 3 antagonist CU CPT 4a (Supplementary Figure 3).

In order to compare the secretion of the pruritogens ET-1 and IL-6 from NHEKs treated by poly-(I:C) with other known pruritogens (histaminergic and non-histaminergic), cells were treated with histamine and a proteinase activated receptor 2 (PAR2) agonist (2-FLY) at concentrations of 100 μ M and 10 μ M, respectively. PAR2 activation resulted in similar effects on the release of pruritogens from NHEKs as TLR3 activation, albeit TLR3 activation was the most effective (Supplementary Figure 5).

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 keratinocytes *in vitro*. Furthermore, we also show that ET-1 activates murine DRG cells, which subsequently stimulates release of B-type natriuretic peptide (BNP; Figure 2 e), a known pruritic 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, like in murine itch (Liu et al. 2012). It may also represent an important receptor in human itch associated with innate immune responses and triggers (see Supplementary Discussion; Supplementary Figure 6). Thus, activation of TLR3 in keratinocytes and most likely in infiltrating immune cells of chronic lesions by DAMPs 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 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.

Conflict of Interest:

The authors declare no conflict of interest.

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CRedit STATEMENT:

AGS, IMcD and MS conceptualized the project, AGS, IMcD, ILS, and JM conducted the research presented in the manuscript. AGS, IMcD, and ILS performed formal analysis of the results, EB contributed valuable resources in the form of keratinocytes cultures. The original draft was written by IMcD and AGS. IMcD, ILS, MS and EB contributed significantly to the editing and review of the final version of the manuscript. MS was responsible for funding acquisition.

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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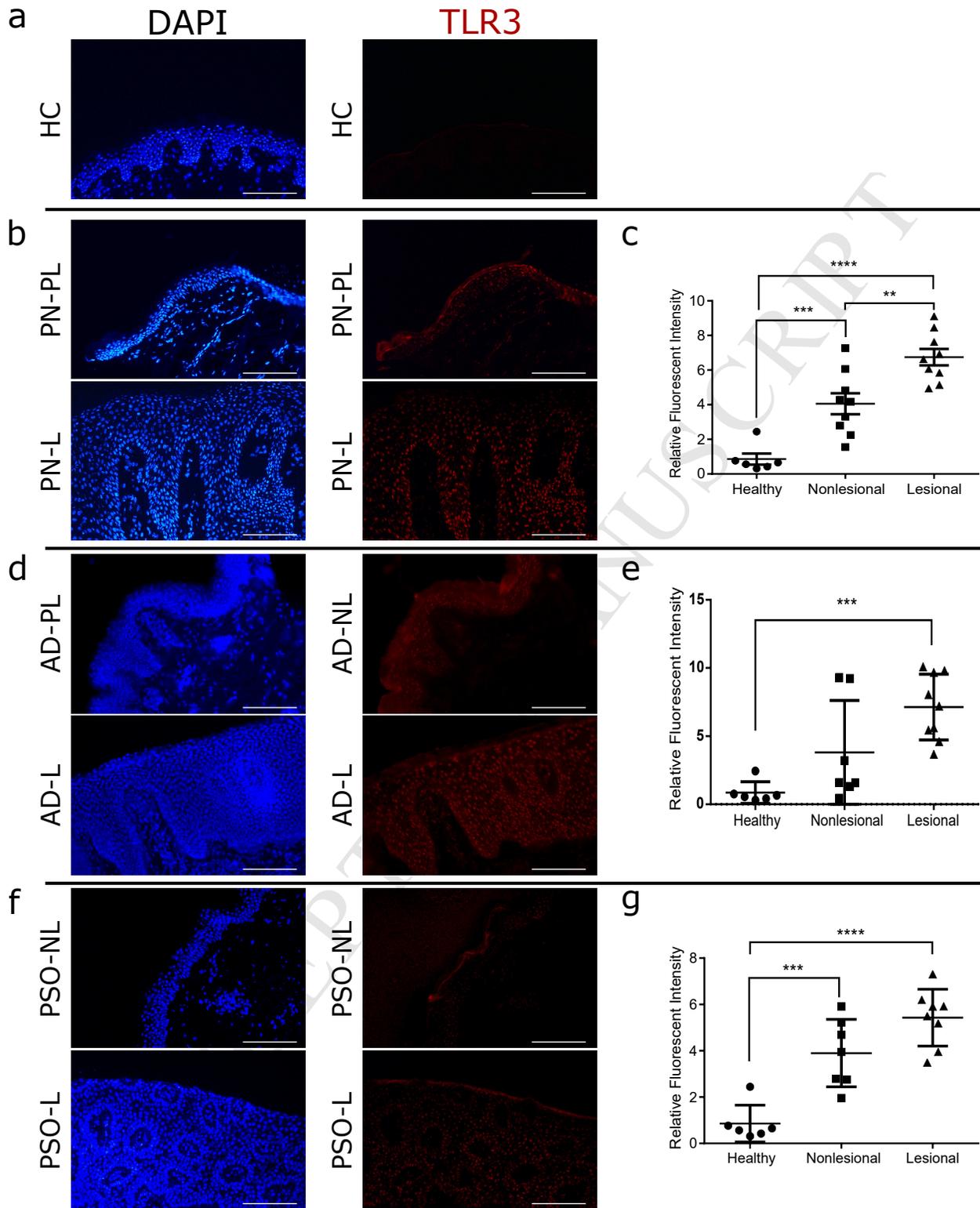
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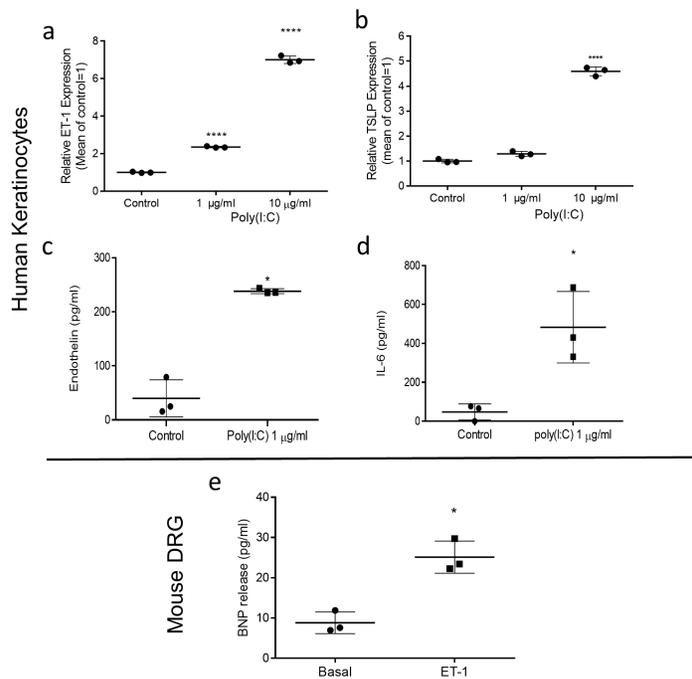
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Figure 1. TLR3 expression is increased in chronically scratched skin lesions of itchy dermatoses. Immunofluorescence detection of TLR3 (red, panels on the right) in skin of (a) healthy controls (HC), perilesional (PL) and lesional (L) skin of patients with (b) prurigo nodularis (PN), (d) atopic dermatitis (AD), (f) psoriasis (PSO). Nuclei were counterstained with DAPI (blue, panels on the left). Relative fluorescence intensity of TLR3 staining was determined with ImageJ software and represented as scatter plots (c, e and g, lines mark mean \pm SD, n= 6-9 independent patient samples per group). Scale bar = 100 μ m. **P<0.01, ***P<0.001 ****P<0.0001 as determined by ANOVA analysis followed by Tukey post-hoc test.

Figure 2. TLR3 activation results in the production and release of inflammatory and pruritogenic mediators in keratinocytes. Expression of (a) ET-1 and (b) TSLP mRNA in NHEKs treated with the TLR3 agonist poly-(I:C). For both genes the expression of TLR3 is calculated as relative expression to *GAPDH* and normalized to the mRNA level of the vehicle treated control (n=3, mean \pm SD; ****P<0.0001 compared to control). ELISA determination of ET-1 (c) and IL-6 (d) production in supernatant of NHEKs treated with poly-(I:C) (n=3, mean \pm SD; *P<0.01 compared to control). (e) Effect of ET-1 on BNP production of DRG cells n=3; mean \pm SD; *P<0.05, **P<0.01, ***P<0.001 compared to the time matched control as determined by two-tailed unpaired t-test). ET: endothelin, TSLP: thymic stromal lymphopoietin, Poly-(I:C): polyinosinic:polycytidylic acid, NHEK: normal human epidermal keratinocytes, TLR3: toll like receptor 3, GAPDH: glyceraldehyde 3-phosphate dehydrogenase, IL: interleukin, BNP: B-type natriuretic peptide.





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Supplementary 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 earlier (Tjabringa et al. 2008). Cells were maintained at 37 °C, with 5 % CO₂ in a humidified environment with KGM-Gold™ Keratinocyte Growth Medium (Lonza) supplemented with KBM-Gold SingleQuot keratinocyte supplements. Cells were passaged a maximum of 4 times before treatments. Cells were washed with sterile Dulbecco's Phosphate Buffered Saline (DPBS, Thermo Scientific) between medium changes and passaging. Trypsin-EDTA solution (SIGMA) was used to remove adherent cells from the culture surface. Confluence was estimated by visualizing the cells using an inverted microscope (IX2-SLP, OLYMPUS). Cells at 70-80 % confluence were kept in unsupplemented media for 24 hrs before treatment with Polyinosinic-polycytidylic acid (poly(I:C), Invivogen) at concentrations of 1, and 10 µg/mL, 2-Furoyl-LIGRLO-amide (2-FLY, Abcam) at 10 µM and Histamine (Sigma) at 100 µM.

Supernatant and cells were harvested at 4 and 24 hr time points. An extended time line of 48 and 72 hrs was also used in some of the experimental designs.

Dorsal root ganglia isolation and culture:

Dorsal root ganglia (DRG) were isolated from postnatal d5 C57BL/6 mice and dissociated by collagenase I, to investigate BNP release. Neurons were cultured in 24-well plates in the presence of cytosine b-d-arabinofuranoside (Sigma) and nerve growth factor 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 ET-1. BNP release was quantified by using ELISA kit (Sigma).

Enzyme-linked immunosorbent assay (ELISA)

The expression of interleukin 6 (IL-6), Endothelin (ET), human beta Nerve Growth Factor (β -NGF) and B-type natriuretic peptide (BNP) in the supernatant of treated NHEKs and DRGs was assessed according to the manufacturer's instructions (DuoSet ELISA development system, R&D systems for IL-6, ET and β -NGF and Sigma-Aldrich for BNP). Briefly, ELISA plates were coated with the supplied capture antibodies overnight at 4 °C. The following day after washing the plates 4 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 4 times the Streptavidin-HRP solution was added to each well and incubated for 45 minutes at room temperature with gentle shaking. Following 4 more washes the signal was developed using MB One-Step Substrate Reagent which was stopped after 30 minutes using the supplied stop solution and subsequently measured at 450 nm using a SpectraMax plate reader (Molecular Devices). The concentration of the analytes in supernatant was then calculated using a cubic logistic model as recommended by (Herman et al. 2008).

RNA isolation, reverse transcription and qPCR

Total RNA was harvested from NHEKs following treatment using Nucleospin RNA Midi kit (Macherey-Nagel, Nüren, Germany) according to manufacturer's instructions. Following extraction, RNA concentration was quantified by spectrophotometry using a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). Complementary DNA (cDNA) was generated from mRNA transcripts using reverse transcriptase. 1 μ g of total RNA was reverse-transcribed into cDNA using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Real time PCR was performed with predesigned TaqMan Gene Expression Assays (Applied Biosystems) for TLR3 (Hs01551078 m1 TLR3), ET-1 (Hs00174961 m1 EDN1), TSLP (Hs00263639 m1 TSLP) β -NGF (Hs01113193 m1 NGF) and GAPDH (Hs99999905_m1) on a T7900HT Fast Real-Time PCR System (Applied Biosystems). Expression of mRNA was normalized relative to GAPDH as an endogenous

control. The $2^{-\Delta\Delta Ct}$ method was used to calculate relative changes in gene expression, data was 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 was reviewed and subsequently approved by the research and ethics committee of St Vincents University Hospital. Eligible participants were identified from the dermatology department of St. Vincents University Hospital (SVUH). A diagnosis of PN, AD and PSO was made by a consultant dermatologists and study investigator. Healthy volunteers were also recruited to this study as controls. The VAS 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. 4 mm punch biopsies were taken from lesional and perilesional skin.

Immunofluorescence

Snap frozen skin biopsies were placed in O.C.T. (O.C.T.TM, Tissue-Tek) prior to sectioning. Cryo sectioning was performed using the CRYOSTAR NX70 (Thermo Fisher Scientific). Tissue sections of 4 μ m were placed onto Superfrost[®] Plus slides (Thermo Fisher Scientific), and fixed with 4% paraformaldehyde (PFA). Sections were blocked with donkey-serum (Merck/Millipore), after which a monoclonal antibody against TLR3 (NBP2-24875, Novus Biologicals) was used as the primary antibody diluted 1:50 in DCS antibody diluent (DCS Innovative Diagnostik-systeme). 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. Image J software (NIH, Bethesda, MA) was used to quantify immunofluorescence.

Statistics

Data was statistically analyzed using graph pad prism 5.0 using two-tailed unpaired t-test or one way ANOVA with Tukey's or Dunnetts multiple comparisons tests, where applicable. Significant differences were considered when the p value was: ≤ 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 to and including the 2008 revision, the GCP commission directive 2005/28/EC and in adherence with National laws.

Supplementary Figure Legends:

Supplementary Figure 1. **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 \pm SD).

Supplementary Figure 2: **TLR3 activation leads to increased TLR3 expression:** Expression of TLR3 mRNA in NHEKs treated with the TLR3 agonist poly(I:C) for 24 hrs. The gene expression is calculated as relative expression to *GAPDH* and normalized to the mRNA level of the vehicle treated control (n=3, mean \pm SEM; **P<0.01, ***P<0.001 compared to control as determined by two-tailed unpaired t-test; representative result of three independent donors).

Supplementary Figure 3. **Poly (I:C)-induced endothelin and TNF α 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 μ g/ml, CU CPT 4a 30 μ M. **(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, which effect is abrogated by the coapplication of the antagonist. **(b)** represents the concentration of TNF α 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, which effect is abrogated by the coapplication of the antagonist. *P<0.05, **P<0.01, ****P<0.0001. Statistical analysis using ANOVA with Tukey post hoc multiple comparisons test. All the Data are mean \pm SD.

Supplementary Figure 4: **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 hrs (n=3, mean \pm SD; * representative result of three independent donors). Statistical analysis using ANOVA with Tukey post hoc multiple comparison test.

Supplementary Figure 5. **Poly (I:C) results in greater endothelin and IL-6 secretion from NHEKs compared to 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 μ g/ml, histamine 100 μ M and 2-FLY at 10 μ M. **(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 endothelin when compared to 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 to histamine and 2-FLY. **P<0.01, ****P<0.0001. Statistical analysis using ANOVA with Tukey post hoc multiple comparisons test. All the Data are mean \pm SD

Supplementary Figure 6: 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 neighbouring 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 neighbouring 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.

Discussion

In our current 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 1 c), with concomitant release of IL-6 and TNF α (Figure 1 d, Supplementary Figure 3). 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 previously demonstrated IL-6 to be increased in the lesional skin of patients with prurigo nodularis. 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 β NGF production in keratinocytes (either with ELISA or qPCR). This is a significant negative result in the context of itch. β NGF 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 lead some commentators to surmise that TLR3 may play a role in the production β NGF 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 β NGF 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 poly(I:C) or by mRNA rapidly causes growth cone collapse and irreversibly inhibits neurite extension independent of nuclear factor κ B (Cameron et al. 2007). Combined, this data suggests that TLR3 activation may in fact have a negative impact on nerve growth and significantly may explain the finding observed in patients with PN.

While the production of β NGF was absent, TSLP transcription was significantly increased upon TLR3 activation. TSLP is a chemokine that is important in initiating dendritic cell-mediated Th2 immune responses seen in AD for example. It results in the production of many important itch cytokines such as IL-4 and 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 non-histaminergic 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 also been linked to the development of AD. In addition, IL-6 production is increased in T cells from AD patients (Gharagozlou 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). Endothelin-1 has been shown to be increased in AD and PN (Aktar et al. 2015; Kido-Nakahara et al. 2014), and has also been implicated in PSO (Simeone et al. 2004). There are admittedly fewer results with B-type natriuretic peptide; however, as a neurotransmitter in the central nervous system, this is not surprising. Even so, there is intriguing data since an increase of the N-terminal pro-form of the peptide (Pietrzak et al. 2015) 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 psoriasis 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 TNF α secretion (Supplementary Figure 4), as described previously (Bernard et al. 2012). While TNF α is typically linked to pain and not itch, its production by keratinocytes can contribute to the inflammatory milieu of pruritic skin lesions.

While 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 non-lesional 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 DAMPs released by injured cells including keratinocytes activate it 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 (Danial et al. 2015). Chronic venous ulceration is also associated with significant itch, which can impact upon healing and add to patient morbidity (Paul et al. 2011).

We should also 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. While the positive feedback loop outlined in Supplementary Figure 6 is alluring in its simplicity, we should take into account the high degree of crosstalk 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.

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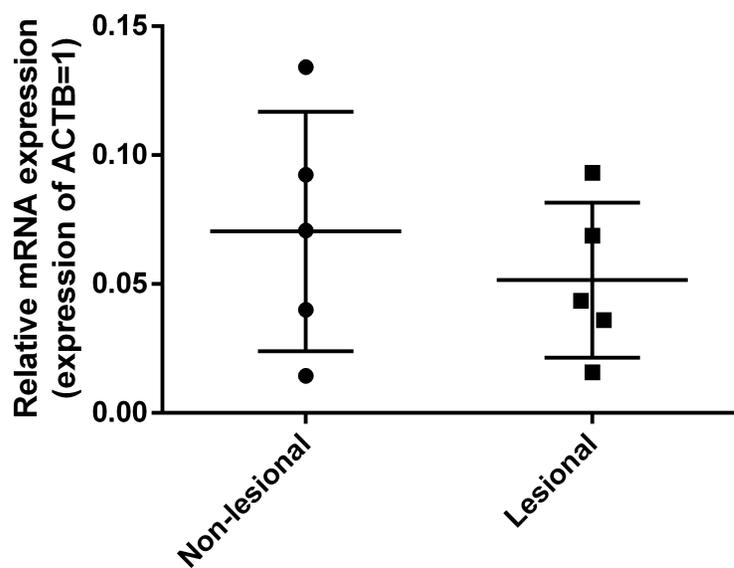
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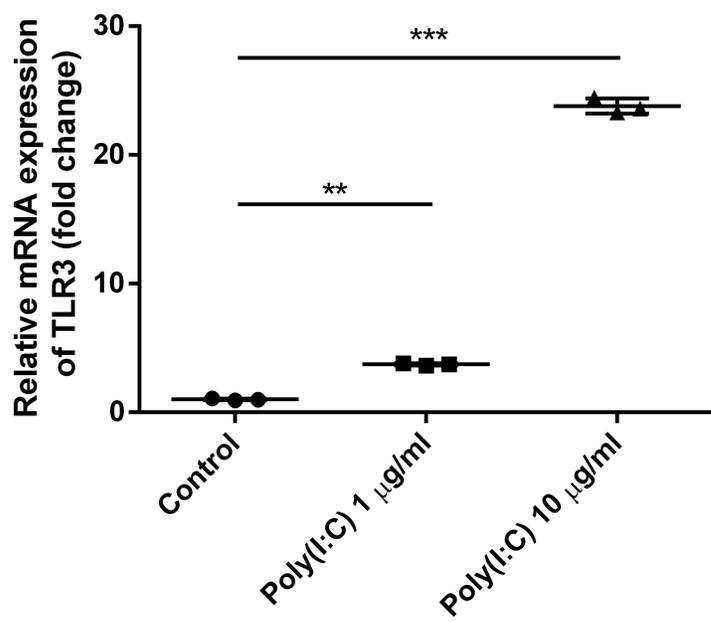
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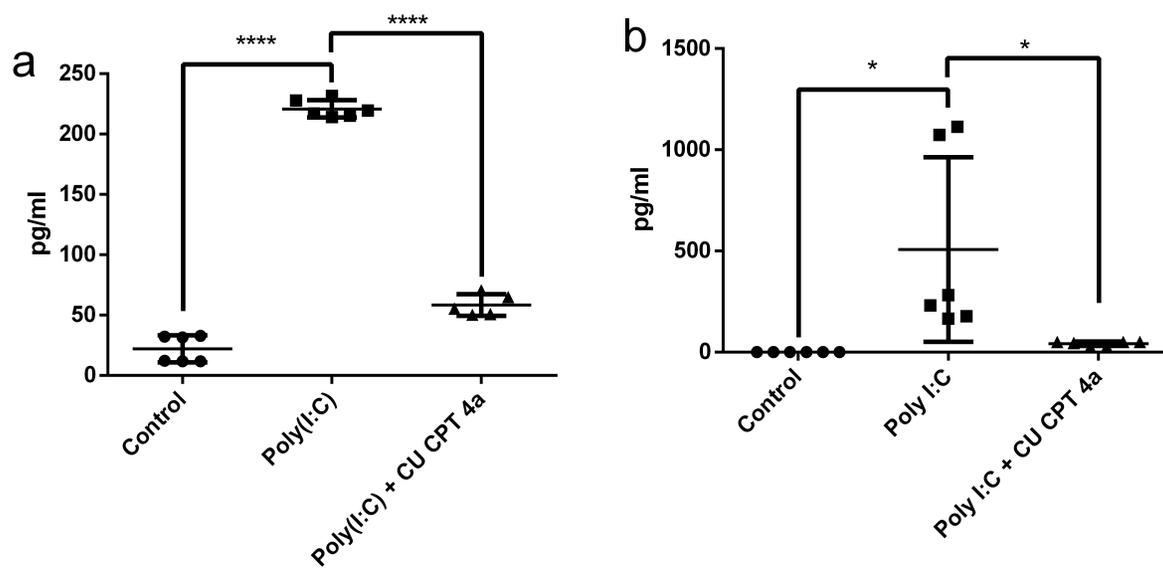
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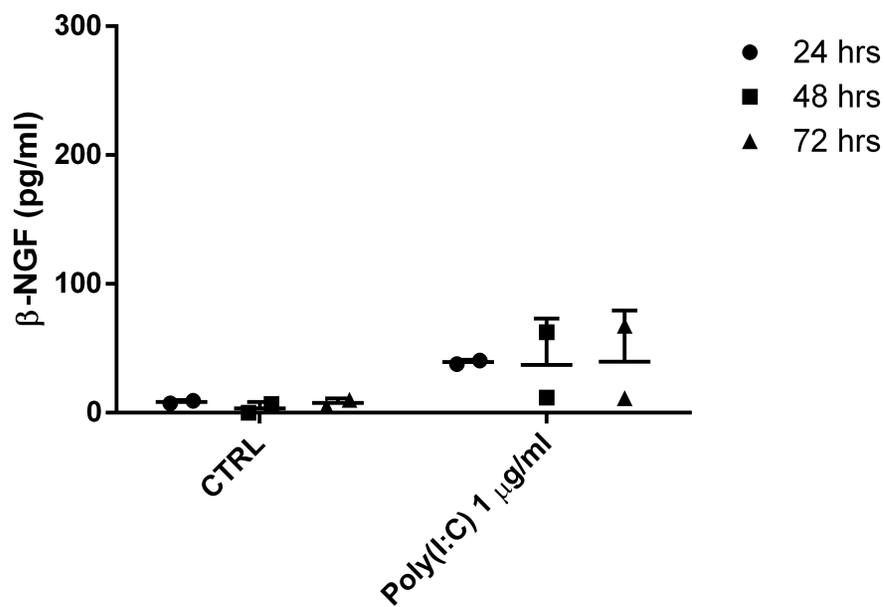
Supplementary Figure 1.



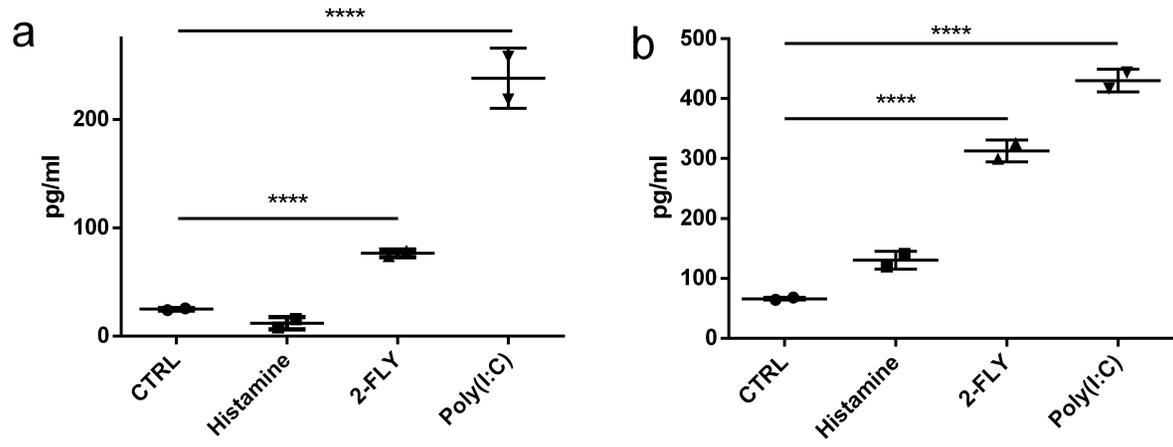
Supplementary Figure 2



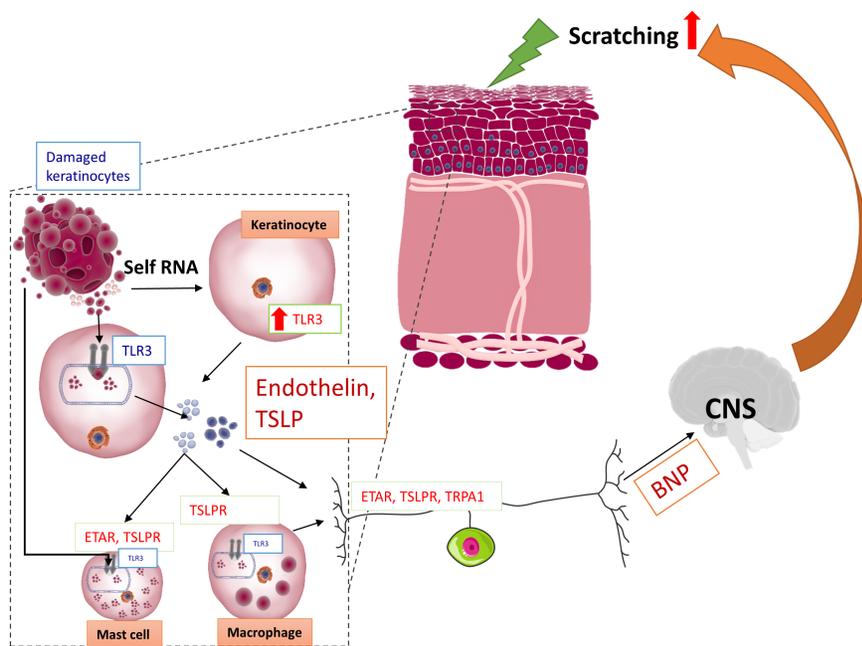
Supplementary Figure 3



Supplementary Figure 4



Supplementary Figure 5



Supplementary Figure 6