nature neuroscience

Toll-like receptor 7 mediates pruritus

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Toll-like receptors are typically expressed in immune cells to regulate innate immunity. We found that functional Toll-like receptor 7 (TLR7) was expressed in C-fiber primary sensory neurons and was important for inducing itch (pruritus), but was not necessary for eliciting mechanical, thermal, inflammatory and neuropathic pain in mice. Our results indicate that TLR7 mediates itching and is a potential therapeutic target for anti-itch treatment in skin disease conditions.

Toll-like receptors (TLRs) are important for triggering innate immune responses to pathogen-associated molecular patterns in mammals. With the exception of TLR3, TLRs engage downstream signaling cascades via MyD88 to produce cytokines and chemokines and fight against pathogenic infection[1]. The mammalian TLR family consists of at least 13 members (TLR1–13). TLR7 recognizes single-stranded RNAs from RNA viruses[1]. As innate immunity has been strongly implicated in abnormal pain hypersensitivity[2], we first examined whether thermal and mechanical pain sensitivity or pathological pain are altered in *Tlr7* knockout (*Tlr7*$^{-/-}$) mice.

Compared with wild-type mice, *Tlr7*$^{-/-}$ mice exhibited normal thermal pain sensitivity, as determined by Hargreave's test and the tail-flick test (**Supplementary Fig. 1a**,**b** and **Supplementary Methods**), and normal mechanical pain sensitivity, as assessed by graded von Frey filaments and the Randall-Selitto test (**Supplementary Fig. 1c**,**d**). Acute inflammatory pain elicited by intraplantar injection of capsaicin, mustard oil (**Supplementary Fig. 1e**,**f**) or formalin in both the first and second phases (**Fig. 1a**), as well as carrageenan-induced persistent inflammatory pain (**Fig. 1b**) and spinal nerve ligation-induced neuropathic pain (**Supplementary Fig. 1g**), was unaltered in *Tlr7*$^{-/-}$ mice. Consistently, *Tlr7*$^{-/-}$ mice did not show any developmental defects in the dorsal root ganglia (DRG) and spinal cord, and the expression of neurochemical markers such as transient receptor potential subtype V1 (TRPV1), CGRP and IB4 was normal (data not shown).

Recent studies have shown that distinct molecular mechanisms underlie pain and itching[3–5]. We asked whether TLR7 is involved in itch sensation. We counted the number of scratches (bouts) by a hind-paw of mouse following intradermal injection of pruritogenic agents in the nape of the neck. Notably, scratches induced by histamine-dependent pruritogens, such as histamine, HTMT (histamine H1 receptor agonist) and compound 48/80, which is known to release histamine from mast cells, were comparable between *Tlr7*$^{-/-}$ and wild-type mice (**Fig. 1c** and **Supplementary Fig. 2a–c**). Notably, *Tlr7*$^{-/-}$ mice showed a marked reduction in scratching behaviors in response to nonhistaminergic pruritogens, including chloroquine, an antimalaria drug[5], and SLIGRL-NH2, an agonist of protease-activated receptor 2 (**Fig. 1c** and **Supplementary Fig. 2d**,**e**). Chloroquine-induced scratching in both sexes was reduced in *Tlr7*$^{-/-}$ mice (**Supplementary Fig. 3a**). Notably, chloroquine induced a bell-shaped dose response curve, but scratching elicited by the highest dose (600 μg) was TLR7 independent (**Supplementary Fig. 3b**). Furthermore, scratches induced by serotonin and endothelin-1 were also impaired in *Tlr7*$^{-/-}$ mice (**Fig. 1c** and **Supplementary Fig. 2f**,**g**).

TLR7 was originally found to recognize imidazoquinoline derivatives, such as imiquimod and resiquimod (R848), and guanine analogs, such as loxoribine, all of which have anti-viral and anti-tumor properties[1,6,7]. Intradermal injection of imiquimod, R848 and loxoribine induced inverted U–shaped dose response curves of scratching in mice (**Fig. 2a** and **Supplementary Fig. 4a**,**b**). As expected, scratches induced by imiquimod, R848 and loxoribine were reduced in *Tlr7*$^{-/-}$ mice (**Fig. 2b** and **Supplementary Figs. 2h–j** and **4c**,**d**).

To further investigate the involvement of TLR7 in itching and pain, we used a recently developed 'cheek model' of itch[8]. The TLR7 ligands imiquimod, R848 and loxoribine all elicited itch-like scratching, but not pain-indicative wiping behavior (**Supplementary Fig. 5**), suggesting that TLR7 mediates itching rather than pain.

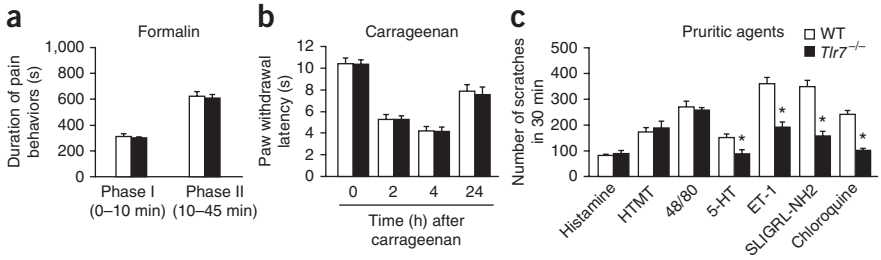

Figure 1 Intact pain, but impaired itch, in *Tlr7*$^{-/-}$ mice. (**a**,**b**) Acute and persistent inflammatory pain induced by intraplantar formalin (5%, $n = 5$) and carrageenan (1%, $n = 5$) ($P > 0.05$ compared with wild-type control). (**c**) Total number of scratches in 30 min following intradermal injection of 50 μl of pruritic agents, including histamine (500 μg), HTMT (100 μg), compound 48/80 (48/80, 100 μg), serotonin (5-HT, 20 μg), endothelin-1 (ET-1, 25 ng), SLIGRL-NH2 (PAR2 agonist, 100 μg) and chloroquine (200 μg) in *Tlr7*$^{-/-}$ and wild-type (WT) mice (*$P < 0.05$, versus wild-type control, $n = 5$–8 mice). All data are means ± s.e.m.

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Received 30 August; accepted 4 October; published online 31 October 2010; doi:10.1038/nn.2683

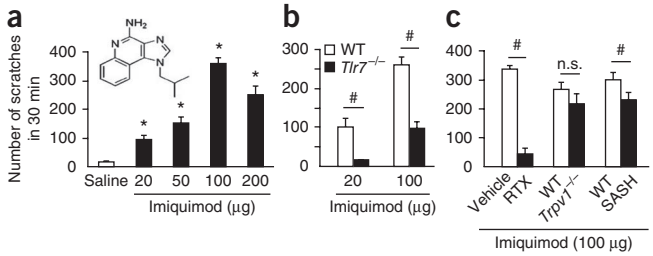

Figure 2 Scratches induced by imiquimod. (**a**) Dose-dependent scratches after intradermal imiquimod treatment (n = 5–8). Inset, structure of imiquimod. (**b**) Imiquimod-induced scratches in wild-type and *Tlr7*$^{-/-}$ mice (n = 5). (**c**) Imiquimod-induced scratches after RTX and vehicle treatment in *Trpv1*$^{-/-}$ mice and wild-type control mice, as well as in mast cell–deficient SASH mice. $^{*}P < 0.05$ versus saline; $^{\#}P < 0.05$; n.s., not significant (n = 5). All data are means ± s.e.m.

C fibers express TRPV1 and are indispensable for induction of itching by various pruritogens[9]. Pretreatment with resiniferatoxin (RTX), an ultrapotent TRPV1 agonist, resulted in a loss of heat sensitivity (**Supplementary Fig. 6a**) and almost abolished imiquimod-induced scratches (**Fig. 2c**). Despite a marked reduction of histamine-induced scratching in *Trpv1*$^{-/-}$ mice[10], imiquimod-induced scratching remained unaltered (**Fig. 2c**). Thus, TRPV1-expressing C fibers, but not TRPV1 *per se*, are required for imiquimod-elicited itching. We also tested itch responses in mast cell–deficient SASH mice[5] and found a moderate reduction (28%) in imiquimod-induced scratching (**Fig. 2c**).

It is virtually unknown whether primary sensory neurons express functional TLR7. Immunohistochemistry revealed that TLR7 was mainly expressed in small-size DRG neurons (**Fig. 3a** and **Supplementary Fig. 7a**). TLR7 was highly colocalized with TRPV1 and gastrin-releasing peptide (GRP), a neuropeptide that is known to elicit itch via GRP receptor expressed by spinal cord superficial dorsal horn neurons[3] (**Supplementary Fig. 7b**,**c**). TLR7 staining was absent in DRG sections of *Tlr7*$^{-/-}$ mice, confirming the specificity of the TLR7 antibody (**Supplementary Fig. 7d**). *In situ* hybridization also revealed that *Tlr7* mRNA was expressed in DRG neurons (**Supplementary Fig. 7e**). Single-cell real-time (RT)-PCR analysis, conducted selectively in small DRG neurons, indicated that TLR7-positive population also expressed GRP and TRPV1 (**Fig. 3b** and **Supplementary Fig. 8a**). Notably, the G protein–coupled receptor MrgprA3, which is known to mediate chloroquine-induced itch[5], was colocalized with TLR7 (**Fig. 3c**). In contrast, TLR8, the family member that is phylogenetically most similar to TLR7, was not expressed in adult mouse DRG (**Fig. 3c** and **Supplementary Fig. 8a**–**e**).

Patch-clamp recording showed that imiquimod (20–500 μM) induced dose-dependent inward currents in capsaicin-responsive DRG neurons of wild-type, but not *Tlr7*$^{-/-}$, mice (**Fig. 3d**,**e**). Imiquimod also induced action potentials in DRG neurons of wild-type, but not *Tlr7*$^{-/-}$, mice (**Fig. 3f**). Thus, imiquimod was able to directly excite DRG neurons in a TLR7-dependent manner. R848, a dual ligand for TLR7 and TLR8, was also able to induce TLR7-dependent inward currents (**Supplementary Fig. 9**).

Immunohistochemistry revealed TLR7 expression in nerve branches in the dermis and nerve terminals in the epidermis of skin tissues (**Supplementary Fig. 10a**–**c**). RTX treatment ablated TLR7-positive fibers in the skin (**Supplementary Fig. 6b**,**c**). We also found TLR7 in spinal cord axonal terminals (**Supplementary Fig. 10d**). Thus, it is likely that TLR7 is transported from DRG cell bodies to skin nerve terminals to mediate pruritus.

In summary, we found that TLR7 mediates itch sensation, whereas all of the previously identified itch receptors are G protein-coupled receptors[3,5,11]. We found functional TLR7 receptors in small DRG neurons that coexpress TRPV1, GRP and MrgprA3. In particular, TLR7 is important for pruritus elicited primarily by nonhistaminergic pruritogens, although we do not exclude a partial role of TLR7 in histamine-dependent itch. Because TLR7 activation by imiquimod and R848 not only induced marked scratching, but also generated inward currents and actions potentials in DRG neurons, TLR7 ligands likely elicit itch via a direct action on sensory neurons. However, we should not rule out a role for non-neuronal cells in the skin, such as keratinocytes and mast cells, in TLR7-mediated pruritus. Notably, topical application of imiquimod, a clinically used antiviral and anti-tumor drug, frequently elicits pruritus in human[12] and also induces psoriasis-like skin inflammation in mice[13], indicating that TLR7's role in pruritus is conserved across different species. Chronic itch is frequently associated with skin diseases and is often

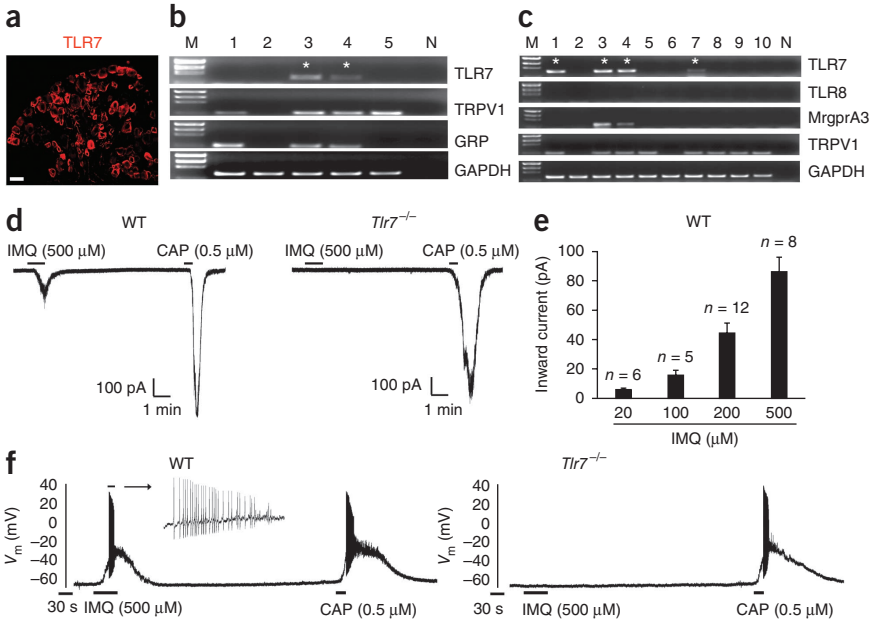

Figure 3 Expression of functional TLR7 in DRG neurons. (**a**) Immunohistochemistry showing TLR7 expression in DRG neurons. Scale bar represents 50 μm. (**b**,**c**) Single-cell RT-PCR showing colocalization of TLR7 with TPRV1, GRP and MrgprA3. M, molecular weights; N, negative controls from pipettes that did not harvest any cell contents, but were submerged in the bath solution. Asterisk indicates TLR7-positive neurons. Full-length gels are shown in **Supplementary Figure 8a**. (**d**) Inward currents evoked by imiquimod (IMQ, 500 μM) and capsaicin (0.5 μM) in small DRG neurons from wild-type mice; 8 of 17 neurons responded to imiquimod. All ten neurons from *Tlr7*$^{-/-}$ mice failed to respond to imiquimod. (**e**) Amplitude of inward currents evoked by imiquimod (20–500 μM). The numbers over the bars indicate the number of responsive neurons. Error bars represent mean ± s.e.m. (**f**) Action potentials evoked by imiquimod (500 μM) and capsaicin (0.5 μM) in small DRG neurons from wild-type mice. Note that imiquimod-induced action potentials were lost in *Tlr7*$^{-/-}$ mice (n = 8 neurons).

resistant to antihistamine treatment[14,15]. Given its involvement in pruritus, targeting TLR7 may be promising for anti-itch treatment of skin disease.

Note: Supplementary information is available on the Nature Neuroscience website.

ACKNOWLEDGMENTS

The work was supported by US National Institutes of Health grants R01-DE17794, R01-NS54362 and R01-NS67686 to R.-R.J.

AUTHOR CONTRIBUTIONS

T.L. conducted behavioral tests for itch and acute pain and participated in experimental design and manuscript preparation. Z.-Z.X. performed immunohistochemistry and behavioral tests of pain. C.-K.P. conducted single-cell PCR and electrophysiology. T.B. performed *in situ* hybridization. R.-R.J. supervised the project, designed experiments and wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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