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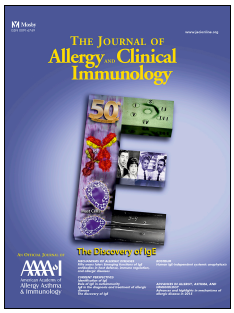

The antimicrobial peptide hBD2 promotes itch through Toll-like receptor 4 signaling in mice

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TITLE PAGE

(1) Title

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The antimicrobial peptide hBD2 promotes itch through Toll-like receptor 4 signaling in mice

Capsule Summary:

The psoriasis biomarker hBD2 produces a robust scratching response in a TLR4-dependent manner in mice. TRPV1 is a downstream mediator of hBD2-induced itch. These findings suggest that hBD2 might act as an endogenous pruritogen in psoriatic itch.

Keywords:

human beta-defensin 2, CCR2, CCR6, TLR4, chronic itch, psoriasis, TRPV1, TRPA1

Abbreviations:

AMPs: antimicrobial peptides

$[Ca^{2+}]_i$: intracellular Ca^{2+}

CCR2: C-C chemokine receptor type 2

CCR6: C-C chemokine receptor type 6

hBD2: human beta-defensin 2

hBD3: human beta-defensin 3

mBD2: murine beta-defensin 2

mBD4: murine beta-defensin 4

NGF: nerve growth factor

TRPA1: transient receptor potential cation channel subfamily A member 1

TRPV1: transient receptor potential cation channel subfamily V member 1

To the Editor:

Chronic skin inflammation is considered the most prominent feature for clinical diagnosis of psoriasis, a long-lasting autoimmune disease characterized by patches of red, itchy and scaly skin. Besides skin inflammation, up to 84% of psoriatic patients also suffer from chronic itch, which significantly impairs quality of life [1]. Although recent exciting studies have identified a positive correlation between the intensity of psoriatic itch and the expression levels of nerve growth factor (NGF), neuropeptides, and many cytokines [2], the molecular and cellular mechanisms underlying psoriatic itch are not fully understood.

In response to Th1 or Th17 cytokines, excessive antimicrobial peptides (AMPs) are locally released by rapidly differentiating psoriatic keratinocytes. Among them, human beta-defensin 2 (hBD2) is increased by nearly 400-fold in patients with severe psoriasis and serves as a biomarker for psoriasis activity [3]. Besides potent antimicrobial activity, hBD2 has diversified roles in regulating adaptive immunity, wound healing, and male fertility [4]. Interestingly, hBD2 also promotes inflammation by recruiting multiple types of immune cells through interacting with both C-C chemokine receptor type 2 (CCR2) and 6 (CCR6) *in vitro* [5,6]. However, the role of hBD2 in itch sensation has not been determined. We therefore investigated whether hBD2 could elicit scratching in *wild-type C57BL/6J* mice. Strikingly, intradermal injections of hBD2 produced a robust scratching response in *wild-type* mice in a dose-dependent manner (Fig 1, *A-B*). In addition, mBD4, the hBD2 ortholog in mouse, and hBD3 could also elicit scratching in mice although the itch intensity varied among different AMPs (see Fig E1 in this article's Online Repository at www.jacionline.org), which provides a proof of concept that both human and mouse AMPs could serve as endogenous pruritogens.

Previous studies have demonstrated that sensory transient receptor potential cation channel subfamily V member 1 (TRPV1) and subfamily A member 1 (TRPA1) channels are selectively expressed by a subpopulation of primary afferent nociceptors and serve as molecular integrators for numerous endogenous pruritogens released by skin-resident cells to provoke both histaminergic and non-histaminergic itch [7]. Furthermore, the TRPV1-expressing sensory fibers mediate skin inflammation through facilitating the function of dermal immune cells in a mouse model of psoriasis [8]. To investigate if TRPA1 and/or TRPV1 are also downstream mediators of hBD2-induced itch, we tested if genetic ablation of TRPA1 or TRPV1 function affects hBD2-induced scratching. Strikingly, the number of the hBD2-induced scratching bouts was markedly reduced in the *Trpv1*$^{-/-}$ but not the *Trpa1*$^{-/-}$ mice when compared with *wild-type* mice (Fig 1, *C-D*). One possibility for the involvement of TRPV1 in hBD2-induced itch is that hBD2 might promote excitability of cutaneous pruriceptors through directly activating TRPV1. To test this possibility, we examined the effect of hBD2 on DRG neurons isolated from *wild-type* mice using live-cell Ca^{2+} imaging. Surprisingly, no intracellular Ca^{2+} ($[Ca^{2+}]_i$) response was observed when 10 μM hBD2 was applied to *wild-type* DRG neurons (Fig 1, *F*). Consistent with the Ca^{2+} imaging data in DRG neurons, hBD2 did not activate membrane currents in HEK293 cells transfected with either mouse TRPV1 or human TRPA1 DNA construct (Fig 1, *G-H*). These results suggest that TRPV1 is a key downstream mediator of hBD2-induced itch but hBD2 does not directly activate TRPV1. This conclusion was further supported by the finding that TRPV1-deficiency abolished $[Ca^{2+}]_i$ response in DRG neurons elicited by applications of hBD2-treated *wild-type* skin superfusates (see Fig E2 in this article's Online Repository at www.jacionline.org).

Since mast cell-derived histamine is one of the best studied pruritogens, especially in allergic itch, and TRPV1 is the major downstream mediator of histaminergic itch, we asked if mast cells are involved in hBD2-elicited itch by measuring hBD2-induced scratching in mast cell-deficient *Kit*$^{W\text{-}sh}$ "*sash*" mice. Surprisingly, hBD2 evoked comparable scratching responses between the *sash* mice and the *wild-type* mice (Fig 1, *E*), suggesting that mast cells are dispensable and histamine might not play an essential role in hBD2-induced itch, which is consistent with clinical observations that psoriatic itch is refractory to oral anti-histamines in more than 80% patients [1,2].

Since hBD2 receptors CCR2 and CCR6 are abundantly expressed by skin-resident cells [5,6], we tested hBD2-elicited scratching in both *ccr2*$^{-/-}$ and *ccr6*$^{-/-}$ mice. To our surprise, hBD2 induced comparable scratching responses among *wild-type*, *ccr2*$^{-/-}$ and *ccr6*$^{-/-}$ mice (Fig 2, *A-B*), suggesting that neither CCR2 nor CCR6 mediates hBD2-induced itch. Since TLR4 mediates mBD2-induced activation of dendritic cells and mBD2 and hBD2 share structural and functional similarities [9], we tested hBD2-induced scratching in *Tlr4*$^{-/-}$ mice. Strikingly, the hBD2-induced scratching response was markedly reduced in the *Tlr4*$^{-/-}$ mice compared with *wild-type* mice (Fig 2, *C*), suggesting that hBD2-induced itch requires TLR4. To further test if TLR4-expressing skin-resident immune cells are involved in the hBD2-induced itch, we conditionally ablated TLR4 expression in the myeloid cell lineage by generating the *LysM*^{cre}; *Tlr4*$^{f/f}$ mice as we found that TLR4 was primarily expressed by CD11b^{+}/CD11c^{-} dermal macrophages besides a small percentage of dendritic cells and eosinophils (see Fig E3 in this article's Online Repository at www.jacionline.org). Indeed, the number of hBD2-induced scratching bouts in the *LysM*^{cre}; *Tlr4*$^{f/f}$ mice was substantially reduced when compared with their *wild-type* littermates (Fig 2, *D*). To assess if hBD2 directly activates TLR4 in skin-resident cells, we performed live-cell Ca^{2+} imaging on skin-resident cells freshly isolated from mouse ear skin preparations. Consistent with behavioral testing, 10 μM hBD2 elicited a robust $[Ca^{2+}]_i$ response in 4.8% of the skin-resident cells examined (Fig 2, *E*) and the $[Ca^{2+}]_i$ response was completely absent from the skin-resident cells isolated from the *Tlr4*$^{-/-}$ and *LysM*^{cre}; *Tlr4*$^{f/f}$ mice or *Tlr4*$^{-/-}$ DRG neurons (Fig 2, *F-H*). Moreover, hBD2 also activated human skin resident myeloid cells, which was nearly abolished by a selective TLR4 antagonist LPS-RS (see Fig E4 in this article's Online Repository at www.jacionline.org). Surprisingly, the classic TLR4 ligand LPS from either *E. coli* or *S. enterica* did not evoke significant scratching. Since it is known that itch is constitutively inhibited by pain, we thus tested the effects of intraplantar injections of hBD2 or LPS on mechanical threshold. Indeed, we found that LPS markedly reduced the mechanical threshold while hBD2 had no effect (see Fig E5 in this article's Online Repository at www.jacionline.org), suggesting that LPS signaling pathway is associated with the pain sensation while the stimulation of TLR4 by hBD2 primarily generate itch sensation without inducing pain responses. Taken together, these results provide strong evidence that TLR4 expressed by skin-resident immune cells but not DRG neurons mediates hBD2-induced itch in mice.

In conclusion, here we first report that hBD2, which is markedly up-regulated in differentiated keratinocytes of psoriatic patients, promotes itch sensation by activating TLR4-expressing cutaneous immune cells in mice. Our findings suggest that hBD2 could act as a potent endogenous pruritogen, which expands the roles of the antimicrobial beta-defensin family and may also provide new therapeutic targets against psoriatic itch.

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235 FIGURE LEGEND

236 **Fig 1** TRPV1 is the downstream mediator of hBD2-induced itch. **A**, Time course of the scratching
237 response induced by vehicle (circle), hBD2 at 2 (square) and 5 (triangle) µg/50 µl recorded for 30 min
238 after intradermal injection. **B**, Bar charts illustrate dose-dependent scratching response produced by
239 intradermal injections of hBD2. n=7 for vehicle, n=6 for 2 µg and n=9 for 5 µg hBD2. *p<0.05,
240 ****p<0.0001, ANOVA; **C-E**, hBD2-induced scratching was reduced in *Trpv1*$^{-/-}$ (**C**, n=5) but not *Trpa1*$^{-/-}$
241 (**D**, n=4) or mast cell-deficient *sash* mice (**E**, n=5). n=6 for *wild-type* mice in all groups. ****p<0.0001,
242 n.s, not significant, Student's t test; **F**, Representative traces showing $[Ca^{2+}]_i$ responses in individual DRG
243 neurons freshly isolated from *wild-type* mice in the presence of 10 µM hBD2. 100nM capsaicin and 100
244 mM KCl were used as positive controls. Each colored line represents an individual cell; **G-H**,
245 Representative I-V curves illustrate that 10 µM hBD2 did not activate HEK293 cells expressing mTRPV1
246 (**G**) or hTRPA1 (**H**) which were activated by 100 nM capsaicin or 100 µM AITC.

247 **Fig 2** TLR4 but not CCR2 or CCR6 mediates hBD2-induced itch. **A-D**, hBD2-induced acute itch was
248 severely attenuated in *Tlr4*$^{-/-}$ mice (**C**, n=7) and *LysM-cre*; *Tlr4*$^{f/f}$ conditional knockout mice (**D**, n=6) but
249 not *ccr2*$^{-/-}$ (**A**, n=6) or *ccr6*$^{-/-}$ (**B**, n=6) mice, compared with their control groups. **p<0.01, n.s, not
250 significant, Student's t test; **E-H**, Representative traces showing hBD2-evoked $[Ca^{2+}]_i$ response in skin-
251 resident cells freshly isolated from ear preparations of *wild-type* (**E**), *Tlr4*$^{-/-}$ (**F**), *Tlr4*^{CKO} (**G**) mice and in
252 acutely dissociated DRG neurons from the *Tlr4*$^{-/-}$ (**H**) mice (n=5 independent repeats). 100nM capsaicin,
253 100 mM KCl and 1 µM ionomycin were used as positive controls in relevant experiments. Each colored
254 line represents an individual cell.

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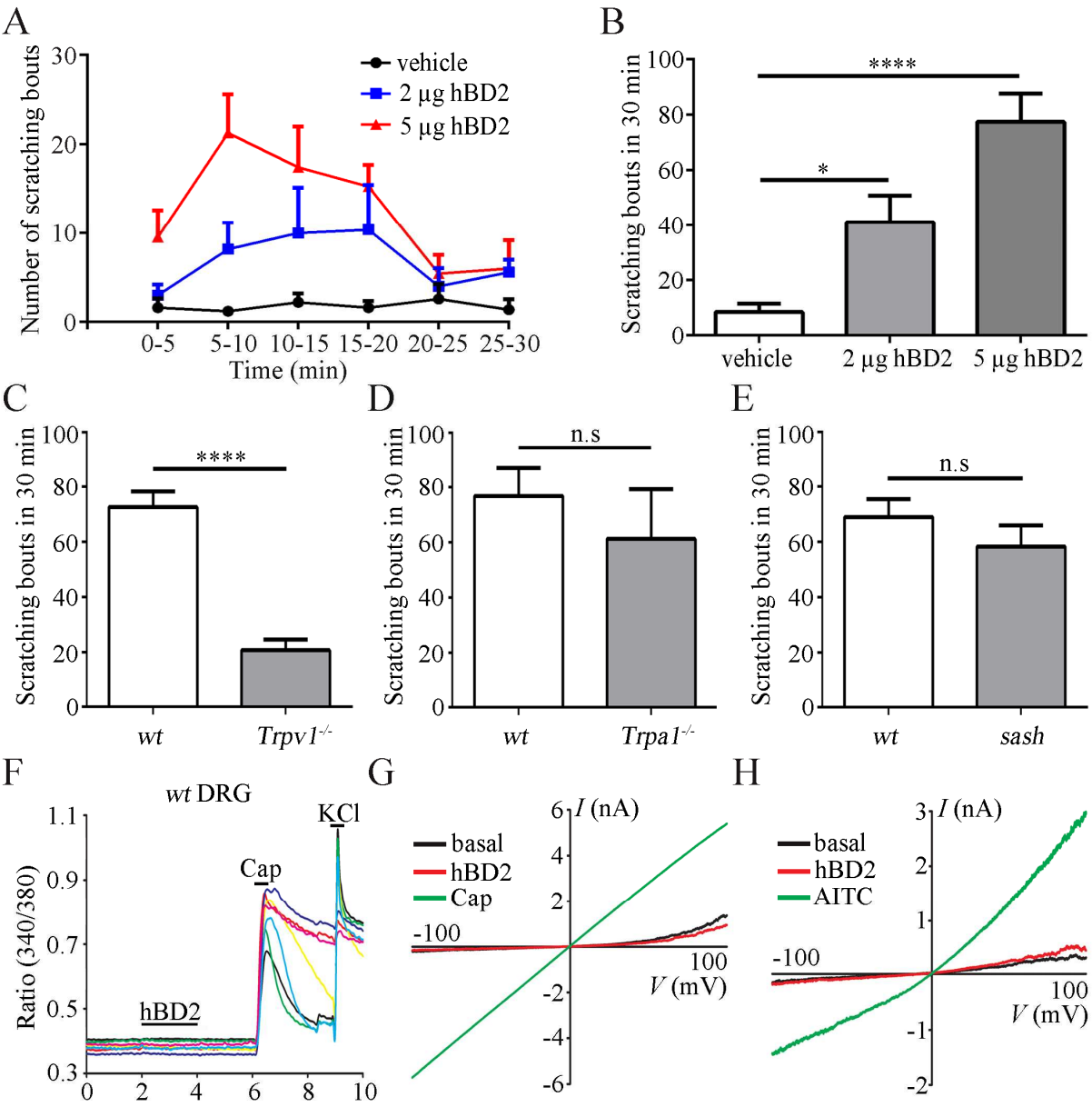
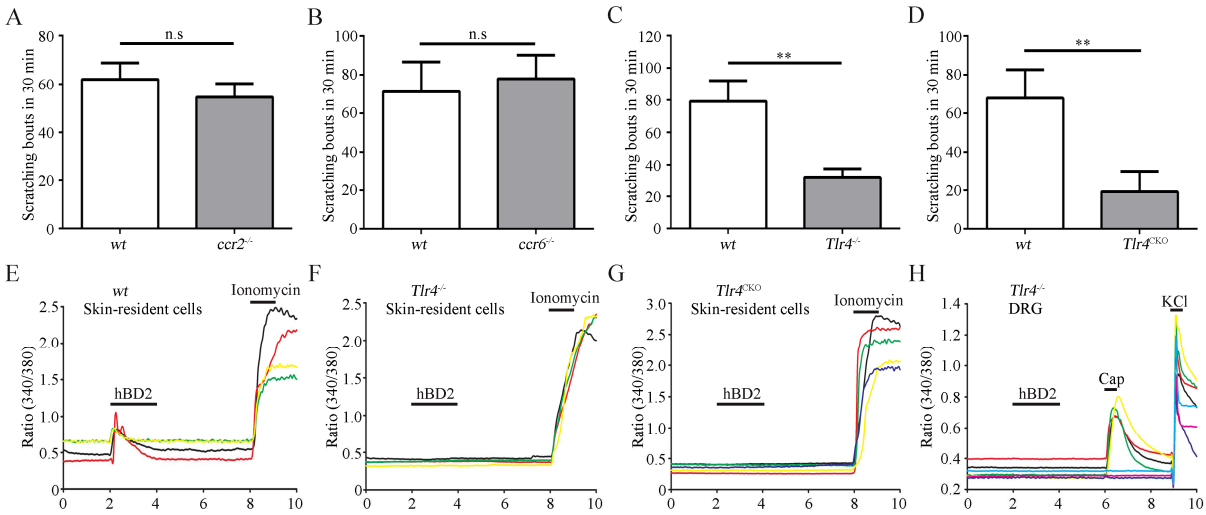
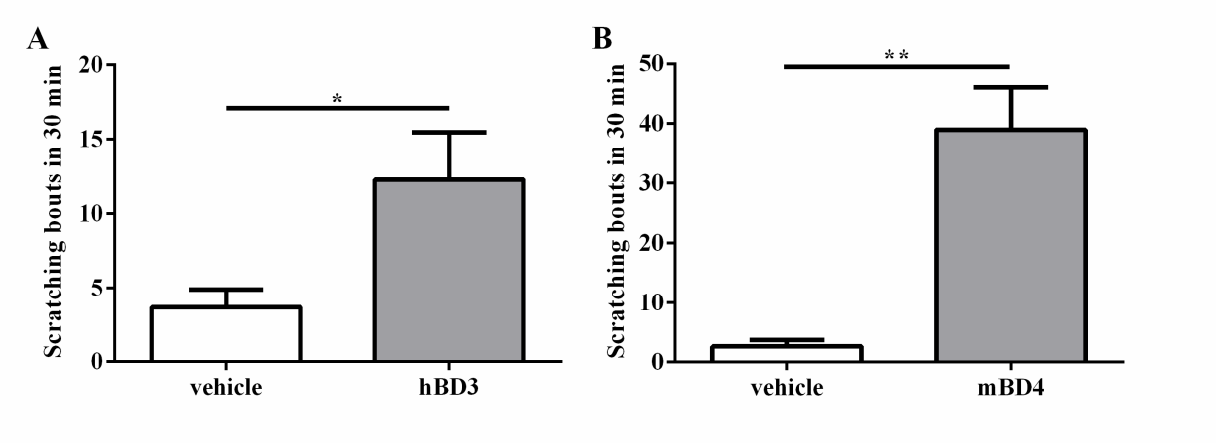
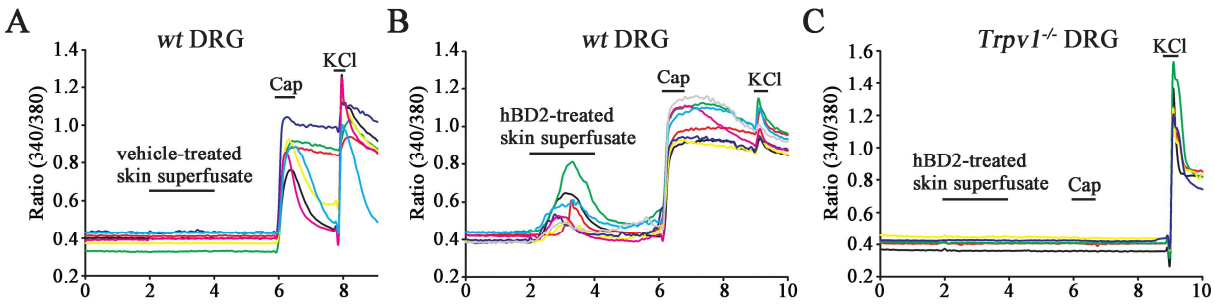
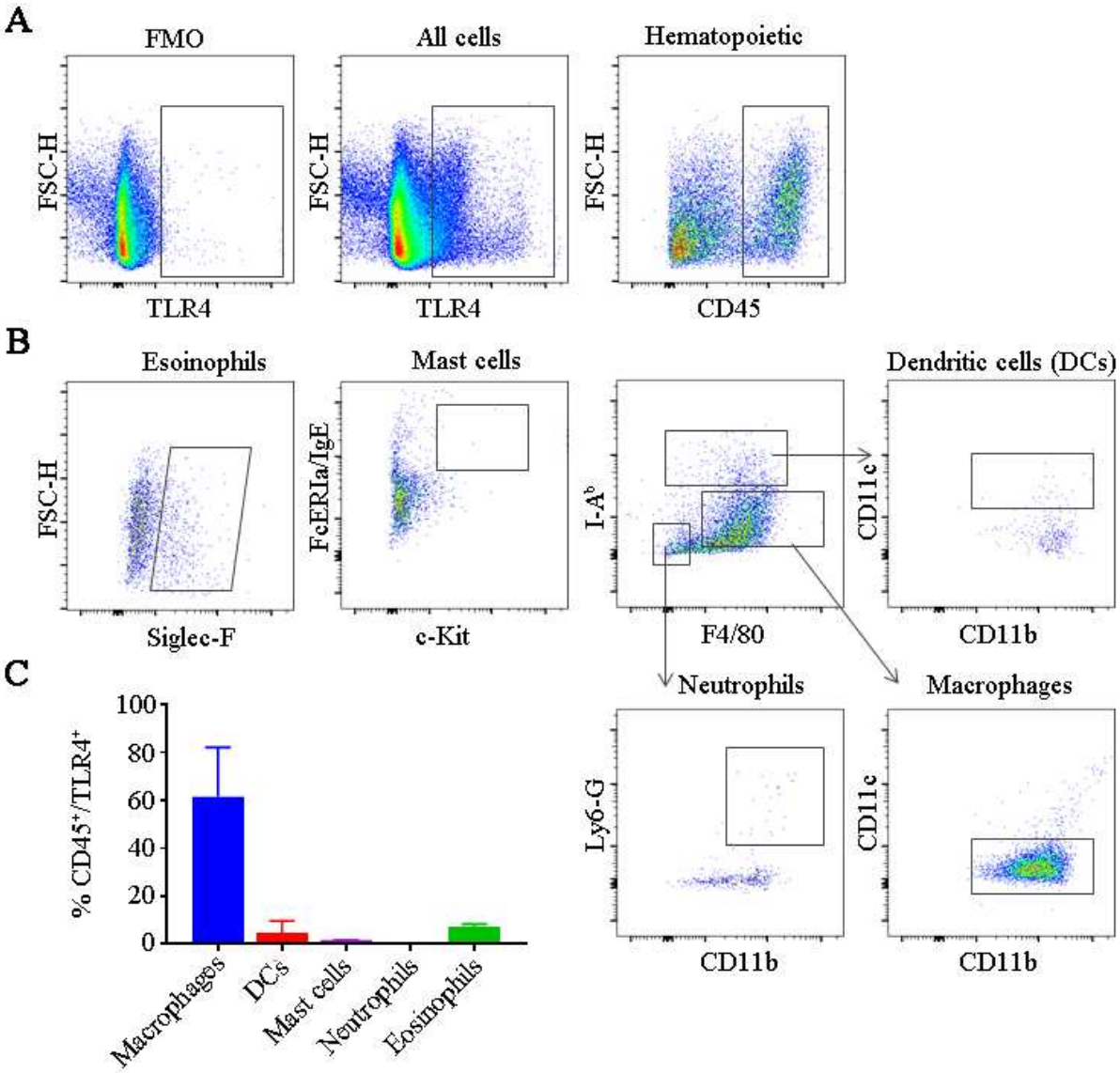
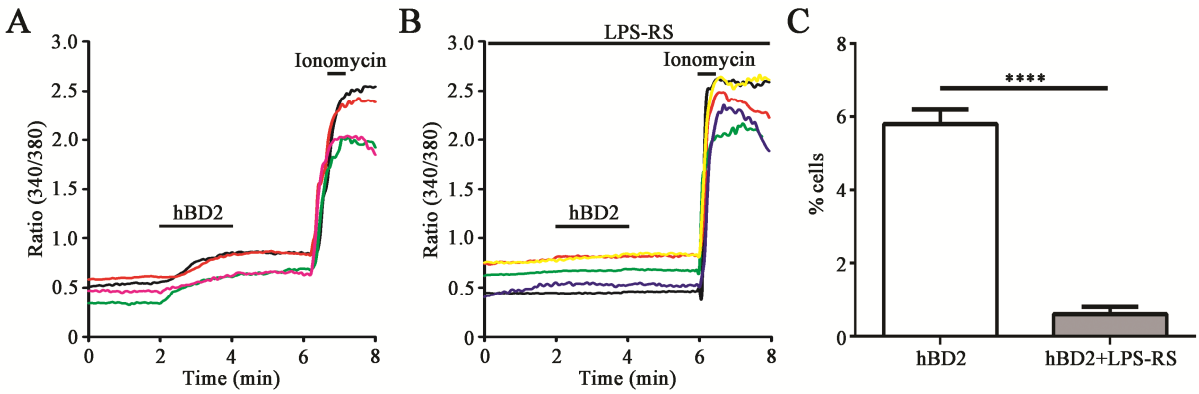

A
Number of scratching bouts
30
20
10
0
vehicle
2 µg hBD2
5 µg hBD2
0-5
5-10
10-15
15-20
20-25
25-30
Time (min)
B
Scratching bouts in 30 min
100
80
60
40
20
0
****
*
vehicle
2 µg hBD2
5 µg hBD2
C
Scratching bouts in 30 min
****
wt
Trpv1-/-
D
Scratching bouts in 30 min
n.s
wt
Trpa1-/-
E
Scratching bouts in 30 min
n.s
wt
sash
F
wt DRG
Ratio (340/380)
1.1
0.9
0.7
0.5
0.3
KCl
Cap
hBD2
0
2
4
6
8
10
G
I (nA)
6
4
2
-2
-4
-6
basal
hBD2
Cap
-100
100
V (mV)
H
I (nA)
3
2
1
-1
-2
basal
hBD2
AITC
-100
100
V (mV)

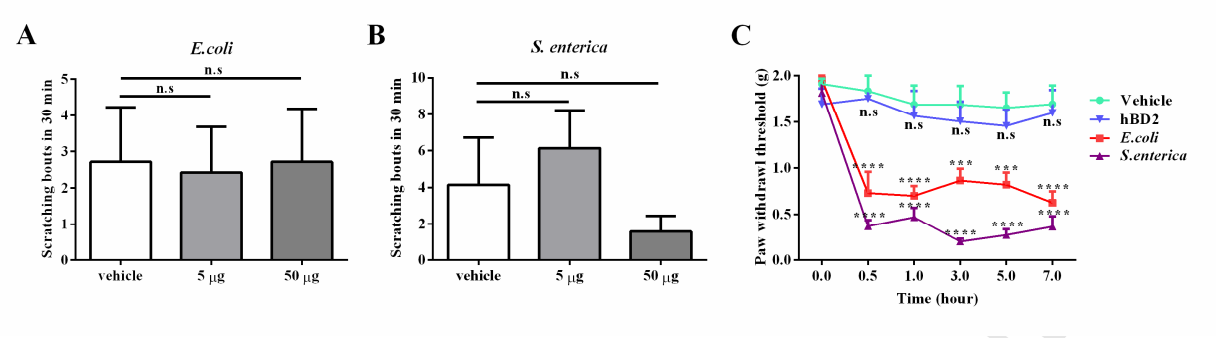












METHODS

Animals. C57BL/6J, *ccr2*$^{-/-}$, *ccr6*$^{-/-}$, *Tlr4*$^{-/-}$, *LysM*^{cre}, *Tlr4*$^{f/f}$, and mast cell-deficient *Kit*$^{W\text{-}sh}$ "sash" mice were obtained from Jackson Laboratories (Bar Harbor, ME, USA). *Trpv1*$^{+/+}$ and congenic *Trpv1*$^{-/-}$ mice on the C57BL/6J background were obtained from Jackson Laboratory (Bar Harbor, ME, USA). The *Trpa1*$^{+/+}$ and congenic *Trpa1*$^{-/-}$ mice on the C57BL/6J background were described previously [E1]. All transgenic mice were extensively backcrossed to C57BL/6J for 10 or more generations. Conditional knockout TLR4 in the myeloid cell lineage was generated by mating the *LysM-cre* mice with *Tlr4*$^{f/f}$ mice and wild-type littermates were used as the control in the behavior testing. All mice were housed under a 12 h light/dark cycle with food and water provided ad libitum. All behavioral tests were videotaped from a side angle, and behavioral assessments were done by observers blind to the treatments or genotypes of animals. All experiments were performed in accordance with the guidelines of the National Institutes of Health and the International Association for the Study of Pain, and were approved by the Animal Studies Committee at Washington University School of Medicine.

HEK293T cell culture and transfection. HEK293T cells were grown as a monolayer maintained in DMEM (Life Technologies, Carlsbad, CA, USA), supplemented with 10% FBS (Life Technologies, Carlsbad, CA, USA), 100 units·mL^{-1} penicillin, and 100 μg·mL^{-1} streptomycin in a humidified incubator at 37°C with 5% CO_2. The cells were transiently transfected with cDNAs for mouse TRPV1 (mTRPV1) or human TRPA1 (hTRPA1) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Following transfection, the cells were maintained in DMEM at 37°C for 24 hours before use.

Fresh isolation of mouse skin-resident cells. Fresh mouse ear skin preparations were cut and separated using forceps and digested in 0.25 mg/ml Liberase TL (Roche, Risch-Rotkreuz, Switzerland) in DMEM media for 90 minutes at 37°C as described [E2]. Samples were mashed through 70 μm cell strainers and washed with DMEM media supplemented 10% FBS (Life Technologies, Carlsbad, CA, USA), 100 units/ml penicillin, and 100 μg·mL^{-1} streptomycin. Single-cell suspensions were used for subsequent Ca^{2+} imaging assays.

Isolation and short-term culture of mouse DRG neurons. Mouse spinal columns were removed and placed in ice-cold HBSS; neurons were acutely dissociated and maintained as described [E3,4]. In brief, laminectomies were performed and bilateral DRG were dissected out. After removal of connective tissues, DRG were transferred to a 1 mL Ca^{2+}/Mg^{2+}-free HBSS containing 2 μL saturated $NaHCO_3$, 0.35 mg l-cysteine and 20 U papain (Worthington, Lakewood, NJ, USA), and incubated at 37°C for 10 min. The suspension of DRG was centrifuged, the supernatant was removed, 1 mL Ca^{2+}/Mg^{2+}-free HBSS containing 4 mg collagenase type II and 1.25 mg dispase type II (Worthington) was added and incubated at 37°C for 10 min. After digestion, neurons were pelleted, suspended in neurobasal medium containing 2% B-27 supplement, 1% L-glutamine, 100 U·mL^{-1} penicillin plus 100 μg·mL^{-1} streptomycin, and 50 ng·mL^{-1} nerve growth factor, plated on a 12 mm coverslip coated with poly-L-lysine (10 μg·mL^{-1}) and cultured under a humidified atmosphere of 5% CO_2/95% air at 37°C for 18–24 hr before use.

Live-cell Ca^{2+} imaging on freshly isolated skin-resident cells and DRG neurons. Fura-2-based ratiometric measurement of $[Ca^{2+}]_i$ was performed as described previously [E5]. Freshly isolated skin-resident cells and cultured DRG neurons were loaded with 4 μM Fura-2 AM (Life Technologies, Carlsbad, CA, USA) in culture medium at 37°C for 60 min. Cells were then washed three times and incubated in HBSS at room temperature for 30 min before use. Fluorescence at 340 and 380 nm excitation wavelengths was recorded on an inverted Nikon Ti-E microscope equipped with 340, 360 and 380 nm excitation filter wheels using NIS-Elements imaging software (Nikon Instruments Inc., Melville, NY, USA). Fura-2 ratios (F340/F380) reflecting changes in intracellular Ca^{2+} upon stimulation were recorded. Values were obtained from 100–250 cells in time-lapse images from each coverslip. Threshold of activation was defined as 3 standard deviations above the average (~ 20% above the baseline).

Whole-cell patch-clamp recordings. Whole-cell patch-clamp recordings were performed using an multiclamp 700B amplifier (Molecular Devices, Sunnyvale, CA, USA) at room temperature (22–24 °C) on the stage of an inverted phase-contrast microscope equipped with a filter set for green fluorescence protein visualization (Nikon Instruments Inc., Melville, NY, USA). Pipettes pulled from borosilicate glass (BF 150-86-10; Sutter Instrument Company, Novato, CA, USA) with a Sutter P-97 pipette puller had resistances of 2–4 MΩ when filled with pipette solution containing 140 mM CsCl, 1 mM $MgCl_2$, 0.5 mM EGTA, and 10 mM HEPES with pH 7.3 and 315 mOsm·L^{-1}. A Ca^{2+}-free extracellular solution was used for whole-cell recording to avoid Ca^{2+}-dependent desensitization of TRPV1 or TRPA1 containing 140 mM NaCl, 5 mM KCl, 0.5 mM EGTA, 1 mM $MgCl_2$, 10 mM glucose, and 10 mM HEPES (pH was adjusted to 7.4 with NaOH, and the osmolarity was adjusted to ≈ 340 mOsm/l with sucrose). The whole-cell membrane currents were recorded using voltage ramp from −100 to +100 mV for 500 ms at holding potential of 0 mV. Data were acquired using or Clampex 10 (Molecular Devices, Sunnyvale, CA, USA). Currents were filtered at 2 kHz and digitized at 10 kHz.

Acute itch behavior. Mice were shaved on the nape of the neck two days before assay. On the day of experiment, mice were acclimated for 1 hr by placing each of them individually in the recording chamber followed by intradermal injection of hBD2 to the nape of the neck (50 μl per site). Immediately after the injection, mice were videotaped for 30 min without any person in the recording room. After the recording, the videotapes were played back and the number of scratching bouts towards the injection site was counted by an investigator blinded to the treatment.

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SUPPLEMENTARY FIGURE LEGEND

Fig E1 Both hBD3 and mBD4 produce scratching responses when injected intradermally. **A**, intradermal injections of hBD3 (5 µg/50 ul) elicited a scratching response in *wild-type* mice. n=7 for each group. **p*<0.05, Student's t test; **B**, intradermal injection of mBD4 (5 µg/50 ul) induced a scratching response in *wild-type* mice. n=6 for each group. ***p*<0.01, Student's t test.

Fig E2 hBD2-treated skin superfusates evoked a robust $[Ca^{2+}]_i$ response in *wild-typet* but not *Trpv1*$^{-/-}$ DRG neurons. A, Representative traces showing that vehicle-treated skin superfusate did not evoke a $[Ca^{2+}]_i$ response in the *wild-type* DRG neurons (n=5 independent repeats); B, Representative traces showing that 10 µM hBD2-treated skin superfusate evoke a $[Ca^{2+}]_i$ response in the *wild-type* DRG neurons (n=5 independent repeats); C, The $[Ca^{2+}]_i$ response evoked by hBD2-treated skin superfusate was not present in the *Trpv1*$^{-/-}$ DRG neurons (n=5 independent repeats).

Fig E3 TLR4 is expressed primarily by dermal macrophages. **A**, Representative FACS plots of TLR4-positive cells in the skin and fluorescence minus one (FMO) negative control. TLR4$^+$ cells are ~45% CD45$^+$; **B**, Macrophages were defined as I-A$^{b\text{-}lo/-}$ F4/80$^+$ CD11b$^+$ CD11c$^-$, dendritic cells were defined as I-A$^{b\text{-}hi}$ F4/80$^{+/-}$ CD11b$^{+/-}$ CD11c$^+$, mast cells were defined as c-Kit$^+$ FcERIa/IgE$^+$, eosinophils were defined as Siglec-F$^+$, neutrophils were defined as CD11b$^+$ Ly6-G$^+$ I-A^{b-} F4/80$^-$; **C**, Bar chart showing the percentages of cells found in each of the specified gates. Data are representative of three independent experiments.

Fig E4 hBD2 stimulates human skin resident myeloid derived cells via TLR4. **A**, Representative traces showing that 10 µM hBD2-evoked a $[Ca^{2+}]_i$ response in myeloid derived cells freshly isolated from human forearm skin (n=5 independent repeats); **B**, The selective TLR4 antagonist LPS-RS (2 µg/ml) nearly abolished the hBD2-induced $[Ca^{2+}]_i$ responses in the human skin-resident myeloid derived cells (n=5 independent repeats); C, Bar charts illustrated that percentage of human skin-resident myeloid derived cells responded to hBD2, hBD2 plus LPS-RS. *****p*<0.0001, Student's t test.

Fig E5 Injection of LPS, a TLR4 ligand, evoked acute pain but not itch sensation. **A-B**, Intradermal injections of LPS (5 µg and 50 µg) from *E. coli* and *S. enterica* into *wild-type* mice didn't caused significantly scratching responses compared with vehicle control. n=6 for each group. n.s, not significant, ANOVA; **C**, Time course of changes in Paw withdrawal thresholds in response to von Frey filaments before and at several time points after intraplantar injections of 5 µg hBD2 or LPS from *E. coli* and *S. enterica*. n=6 for each group. ****p*<0.001, *****p*<0.0001, n.s, not significant, ANOVA.