Specific β-Defensins Stimulate Pruritus through Activation of Sensory Neurons

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Pruritus is a common symptom of dermatological disorders and has a major negative impact on QOL. Previously, it was suggested that human β-defensin peptides elicit itch through the activation of mast cells. In this study, we investigated in more detail the mechanisms by which β-defensins induce itch by defining the receptors activated by these peptides in humans and mice, by establishing their action in vivo, and by examining their expression in inflammatory dermal diseases. We found that elevated expression of *DEFB103* is highly correlated with skin lesions in psoriasis and atopic dermatitis. We showed that the peptide encoded by this gene and related genes activate Mas-related G protein-coupled receptors with different potencies that are related to their charge density. Furthermore, we establish that although these peptides can activate mast cells, they also activate sensory neurons, with the former cells being dispensable for itch reactions in mice. Together, our studies highlight that specific β-defensins are likely endogenous pruritogens that can directly stimulate sensory neurons.

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INTRODUCTION

Itch is a common symptom in patients with inflammatory skin disorders, including atopic dermatitis (AD) and psoriasis (Leader et al., 2015; Lerner, 2018). This unpleasant sensation and the urge to scratch are ultimately triggered by the activation of neural circuits (Carstens and Akiyama, 2016). Even though it is long been known that the degranulation of mast cells and release of histamine evoke itch through the stimulation of sensory nerves, antihistamine treatment often fails to prevent chronic itch (Leslie et al., 2015). However, the identity of endogenous pruritic agents in these dermatological diseases is still unknown, although it has been speculated that various peptides are involved. These peptides include neuropeptides; somatostatin; vasoactive intestinal peptide; and substance P released by dermal nerve fibers as well as peptides released by immune cells, including eosinophil major basic protein (*PRG2*), and eosinophil major basic proteins (*RNASE 2* and *3*; and also the antimicrobial peptides, β-defensins and cathelicidin (*CAMP*)(Takahashi and Yamasaki, 2020). In addition, various other peptides such as PAMP-12 (*ADM*) and proteases, for instance, kallikreins and cathepsins that activate protease-activated receptors, have been suggested to be pruritogenic (Akiyama et al., 2015).

Defensins are short cationic peptides with net charges ranging from +2 to +11, which on the basis of their structures, have been divided into two groups: α-defensins and β-defensins (Niyonsaba et al., 2016). These peptides are antimicrobial, their expression is induced during skin inflammation (Dhople et al., 2006), and they are considered to be danger-associated molecular patterns causing inflammation (Niyonsaba et al., 2016).

It was shown that human β-defensin 2 and 3 can activate mast cells and cause mast cell degranulation (Subramanian et al., 2013; Zhang and McNeil, 2019) through Mas-related G protein-coupled receptors (MRGPRs) (Zhang and McNeil, 2019). However, whether β-defensins have roles in human itch has not been fully examined, and knowledge for how defensins act mechanistically is fragmentary. In this study, from dermatological samples sequencing data, we assessed whether defensin expression is altered in human diseases associated with itch. These analyses showed that the expression of the two β-defensins human β-defensin-2, encoded by *DEFB4A* and *DEFB4B*, and human β-defensin 3, encoded by *DEFB103A* and *DEFB103B*, is increased in psoriasis and AD. Although these peptides show elevated expression, we found that they are not equally potent activators of MRGPRs. Furthermore, we found that β-defensins preferentially activate sensory neurons over mast cells. Together, our studies provide additional insights into the action of β-defensins in pruritus, with β-defensin 3 (DEFB103) being the most potent pruritogen.

RESULTS

Analysis of human transcriptomic data reveals that β-defensins are candidate pruritogens in inflammatory skin diseases

To look for potential pruritogens in human skin diseases associated with chronic itch, we performed an unbiased analysis of publicly available RNA-sequencing data (GSE121212 and GSE54456) from skin biopsies of patients

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Abbreviations: AD, atopic dermatitis; DRG, dorsal root ganglion; EC50, half-maximal effective concentration; MRGPR, Mas-related G protein-coupled receptor

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with psoriasis and AD comparing them with data from healthy donors (Li et al., 2014; Tsoi et al., 2019), searching for genes that encode secreted peptides with increased expression. This analysis revealed *DEFB103* and *DEFB4* (encoding human β-defensin 3 and 2, respectively) as the most upregulated genes in both psoriasis and AD (Figure 1a). Previously, it was reported that mast cells can be activated by β-defensins and other basic secretagogues, including some peptides (McNeil et al., 2015; Subramanian et al., 2013). Therefore, we used MA plots to simultaneously examine the levels of upregulation and the expression levels of genes encoding these two β-defensins and other cationic peptides, including LL-37 (cathelicidin, encoded by *CAMP*), PAMP-12 (*ADM*), cortistatin-14 (*CORT*), substance P (*TAC1*), somatostatin (*SST*), vasoactive intestinal peptide (*VIP*), eosinophil major basic protein (*PRG2*), and eosinophil major basic proteins (*RNASE2* and *RNASE3*), or other pruritogenic agents, including kallikreins (*KLK5* and *KLK7*) and cathepsins (*CTSS* and *CTSG*) (Figure 1b and c). These analyses revealed that compared with other basic secretagogues, these two β-defensins are the most differentially and abundantly expressed genes in inflammatory skin samples. The $\log_2$ fold changes for *DEFB4* were 4.1−8.4 in AD and psoriasis, which respectively indicated 17−338-fold increased expression in these samples. Among the genes we examined, we noticed that *CAMP* (encoding LL-37) was also modestly upregulated in both AD and psoriasis ($\log_2$ fold change 2.3−3.4). However, the expression levels (counts per million) of *DEFB103* and *DEFB4* were 50−1,000-folds higher than those of *CAMP*. Some peptides (kallikreins, cathepsins, PAMP) showed high basal expressions, which were not appreciably upregulated in inflamed skin ($\log_2$ fold change $\approx$ 0). In contrast, expression of substance P was downregulated in both inflammatory skin samples. These analyses suggested that β-defensins may have a more predominant role than LL-37 or other known pruritogenic peptides in itch.

Because the human genome encodes 67 defensin genes, we also examined whether other defensins might be involved in itch by assessing their abundance in skin samples from healthy donors as well as the samples from lesional sites and nonlesional sites of psoriasis and AD (Figure 1d). This analysis showed that *DEFB1* is constitutively expressed in healthy, lesional, and nonlesional skin and that *DEFB4* is highly expressed in psoriatic and AD skin; however, in some patients, *DEFB4* is also highly expressed in nonlesional skin. By contrast, *DEFB103* is selectively expressed in lesional skin but not in nonlesioned skin. Interestingly, analysis of a third dataset, which was recently reported for a very large cohort of patients with psoriasis, showed that *DEFB103* is again prominently expressed (Reynolds et al., 2021). In addition, because this report provided single-cell sequencing of dermal cells, we examined the cells that express *DEFB103*. This revealed that keratinocytes and not immune cells express *DEF103B* in psoriasis (Figure 1e) (Reynolds et al., 2021). Together, our analyses showed that *DEFB4* and *DEFB103* are candidate basic secretagogues in psoriasis and AD and, with regard to inflammation, that *DEFB103* is the most relevant.

Mouse β-defensins exhibit distinct proinflammatory features in vivo

To examine the activation potencies of DEFB103 and DEFB4 on mast cells, we performed experiments with LAD2 cells, an immortalized human mast cell line (Kirshenbaum et al., 2003). Interestingly, calcium imaging revealed that activation by DEFB103 is very different from that by DEFB4 (Figure 2a and b), with DEFB103 being about 10-fold more potent (Figure 2c) (half-maximal effective concentrations [EC50s] of 0.74 ± 0.23 μM and 7.0 ± 6.3 μM for DEFB103 and DEFB4, respectively), and these recorded potencies likely reflect the extent of degranulation (Subramanian et al., 2013). To investigate whether the different in vitro potencies of these peptides produce similar effects in vivo, we next tested them in mice. Differences in the naming and the low sequence identity of defensins across species make it challenging to easily define the mouse orthologs of human defensins. Mouse *Defb14* has been suggested to be the mouse ortholog of human *DEFB103* (Hinrichsen et al., 2008; Röhrl et al., 2008) with 75.6% sequence identity, and *Defb4* has been suggested to be the mouse ortholog of human *DEFB4* (Jia et al., 2000), but its structure is less well-conserved with 48.7% identity (Figure 2d and e). On the basis of this, we decided to test the effects of mouse Defb14 and Defb4 in vivo on mast cells using Evans blue to measure the extent of mast cell–induced extravasation on injection of peptide into the footpad. In agreement with our results from LAD2 cells, recombinant Defb14, the ortholog of human DEFB103, caused extensive extravasation (Figure 2f). By contrast, Defb4 caused only minimal extravasation, a level that was not statistically different from that of saline injection (Figure 2g). Together, these results show that human DEFB103 and mouse Defb14 are effective mast cell activators.

β-Defensins evoke itch by stimulating MRGPRA3-sensory neurons

Given the difference in the ability of β-defensin peptides to activate mast cells, we wondered whether these peptides also exhibit different pruritogenic potencies. We reanalyzed RNA-sequencing data of mouse skin samples from the MC903-induced mouse model of atopic-like dermatitis (GSE90883) (Oetjen et al., 2017) and data from the imiquimod-induced model of psoriasiform dermatitis (GSE161084) to determine whether β-defensins contribute to inflammatory itch in these mouse models and which ones might be involved. In both models, *Defb1* and *Defb6* are constitutively highly expressed, whereas the expression of *Defb14*, *Defb4*, and *Defb3* is significantly upregulated (Figure 3a and b). These results confirm our analysis of human transcriptomic data by showing that in mouse models of chronic itch, such as in human dermal skin diseases, the expression of select defensins is upregulated. Because *Defb3* is also upregulated in inflamed skin and shares moderate sequence identity (41.9%) with human *DEFB4* (Figure 2e), we examined the itch responses evoked by Defb14, Defb4, Defb1, as well as Defb3. These peptides induced scratching, but there were major differences in evoked itch responses. Defb14 (1 mM, 10 μl) evoked the most intense itch phenotype, whereas Defb4 and Defb1 peptides induced effects that were not significantly

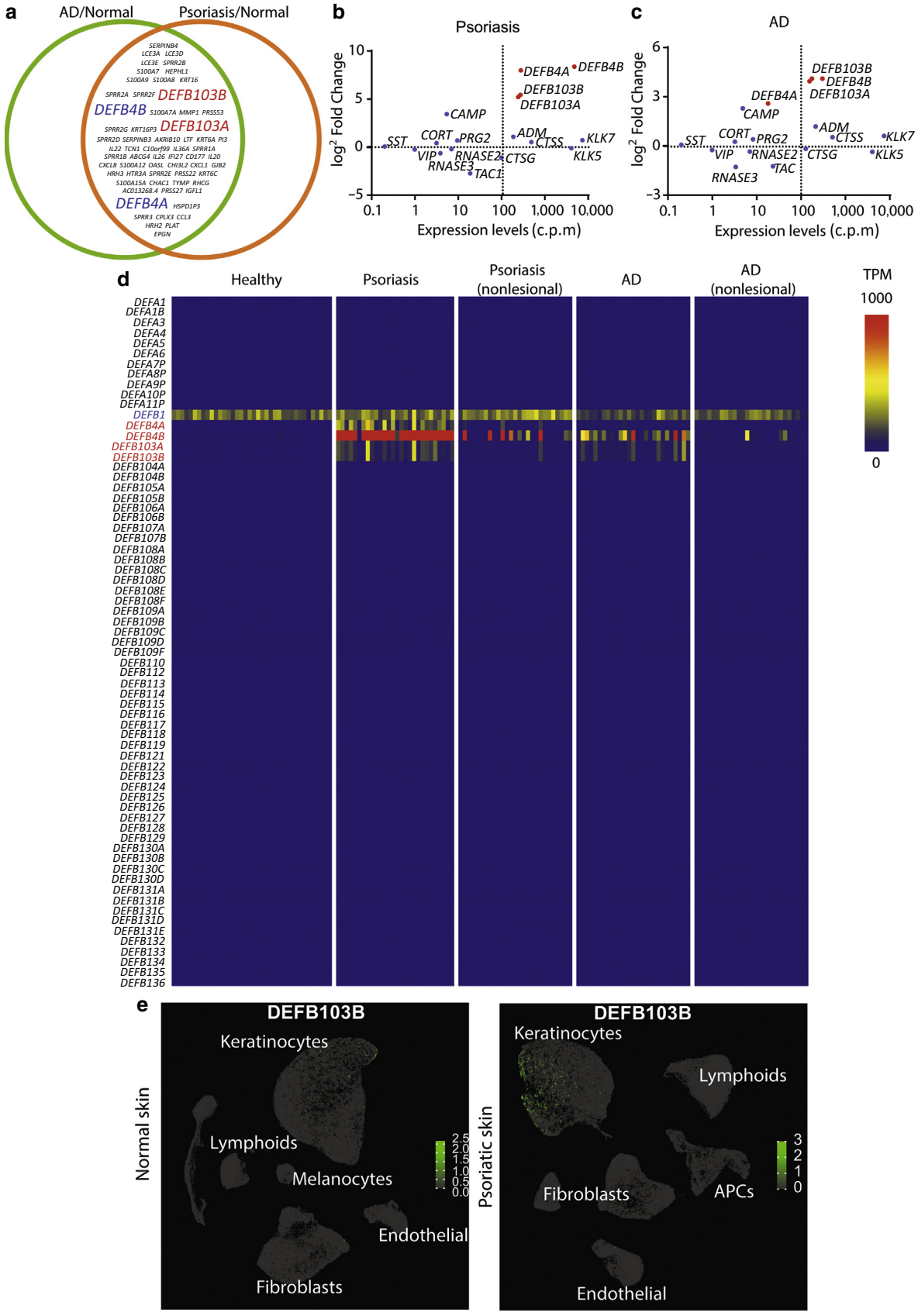

Figure 1. Identification of *DEFB4* and *DEFB103* as potential pruritogens. (**a**) Schematic of the top 100 differentially upregulated genes in skin biopsies from patients with AD and psoriasis. MA plot of defensins in skin samples from patients with (**b**) psoriasis and (**c**) AD (GSE121212). (**d**) Heatmap of gene expression levels (as TPM) of all defensin genes in healthy, lesional and nonlesional psoriasis, and lesional and nonlesional AD skin samples (GSE121212, n = 38, 28, 27, 27, and 27, respectively). (**e**) Single-cell RNA-sequencing analysis (UMAP) of normal (66,243 cells from five donors) and psoriatic (71,077 cells from three

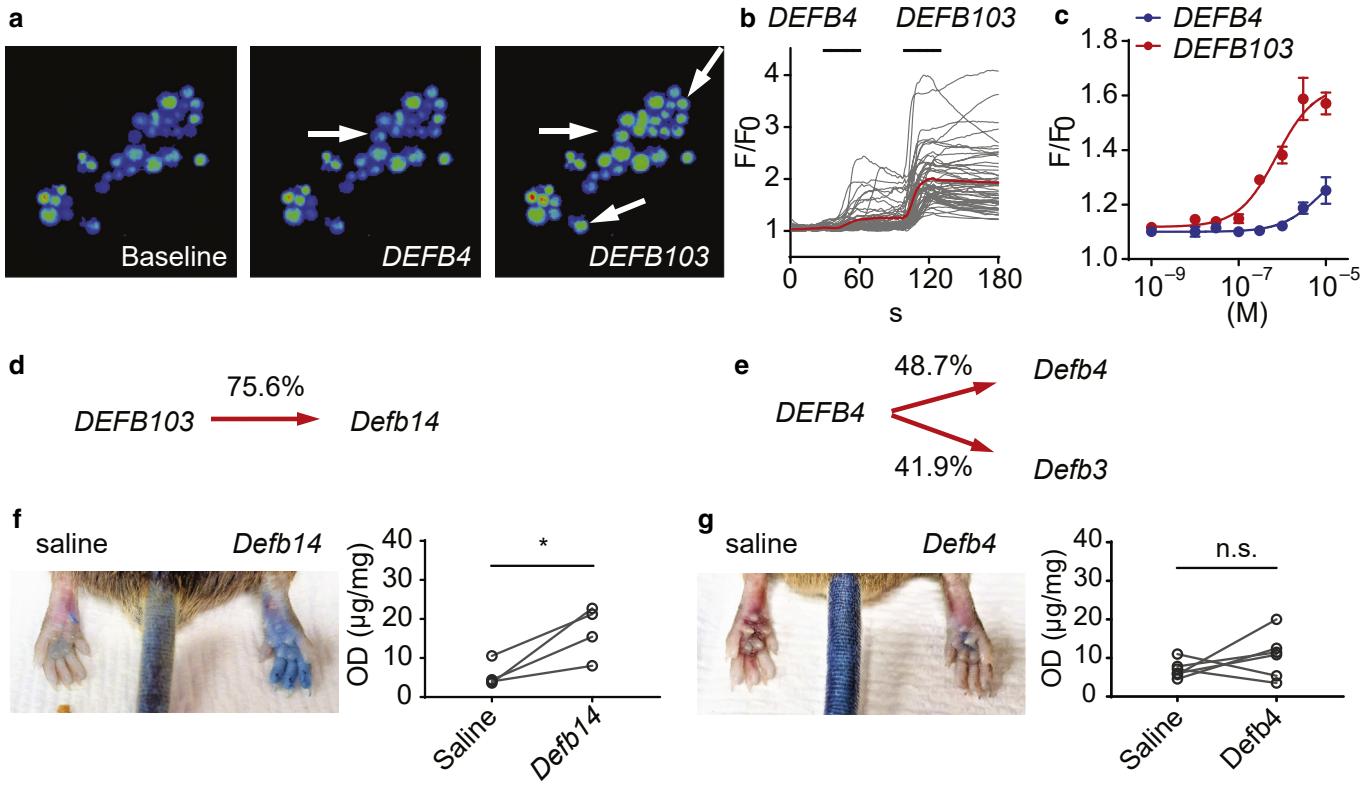

Figure 2. β-Defensins exhibit mixed capability for activation of mast cells. (**a**) Calcium imaging of fields of LAD2 mast cells treated with recombinant *DEFB4* and *DEFB103* peptides (1 μM). DEFB103 activated more LAD2 cells and evoked a stronger calcium influx than DEFB4 (indicated by arrows). (**b**) Individual cellular (black lines) and averaged calcium responses (red traces), evoked by β-defensins, in LAD2 cells. (**c**) Dose-dependent responses of calcium influx evoked by β-defensin peptides in LAD2 cells. (**d, e**) Primary sequence alignments of human and mouse β-defensins. (**f, g**) Evans blue extravasation assay for evaluating mast cell activation in vivo. (**e**) Plantar subcutaneous injection of recombinant Defb14 (1 mM, 10 μl) triggered significant extravasation (*$P = 0.036$, paired *t*-test, n = 4). (**f**) In contrast, extravasation evoked by Defb4 was not statistically different from that evoked by saline injection ($P = 0.29$, paired *t*-test, n = 6). n.s., not significant; OD, optical density; s, second.

different from those of saline injection. Intriguingly, Defb3 peptide also evoked a strong itch phenotype despite the fact that it shares a similar sequence to Defb4, which had negligible effects (Figure 3c). The latter result suggests that there are likely factors that contribute to pruritogenic potency, which are not solely determined by the degree of primary sequence homology.

To assess whether the pruritogenic effects of Defb14 are due to mast cell activation as previously suggested (Subramanian et al., 2013; Zhang and McNeil, 2019), we tested mice in which mast cells are depleted by expression of diphtheria toxin fragment A under the control of a mast cell–specific protease 5 gene, *Mcpt5*-Cre::Rosa-stop-DTA mice (Peschke et al., 2015). Surprisingly, for a peptide thought to elicit itch behavior through the activation of mast cells, the ablation of mast cells did not attenuate Defb14-induced scratching (Figure 3d). This suggests that Defb14 might stimulate itch sensory neurons. To test this hypothesis, we performed calcium imaging on dissociated dorsal root ganglion (DRG) neurons expressing calcium indicator GCaMP6f (Mishra et al., 2011; Solinski et al., 2019b). As postulated, Defb14-induced calcium increased in DRG neurons. Interestingly, the subset of DRG neurons that were Defb14 responsive also responded to chloroquine (and histamine and capsaicin) (Figure 3e and f). The specific receptor for chloroquine is MRGPRA3 (Liu et al., 2009; Xing et al., 2020), and the neurons expressing this receptor coexpress receptors for histamine and capsaicin, showing that Defb14 specifically stimulates MRGPRA3 neurons. To test this further, we performed patch-clamp recordings on dissociated genetically marked *Mrgpra3*-Cre-GFP neurons. As expected, applying Defb14 peptide (1 μM) (and chloroquine) triggered action potentials discharges in these cells (Figure 3g). In addition, corroborating that MRGPRA3 neurons are required for Defb14-induced itch, the ablation of MRGPRA3 neurons (*Mrgpra3*-Cre::Rosa-stop-DTA mice) significantly attenuated the scratching responses evoked to Defb14 (1 mM) (Figure 3h).

MRGPRs are activated by specific β-defensins

Previously, it was reported that human DEFB103 and DEFB4 as well as mouse Defb14 and Defb3 can activate human MRGPRX1 and MRGPRX2 and mouse MRGPRA3 and MRGPRC11, respectively (Subramanian et al., 2013; Zhang

donors) skin samples. The color bar indicates the expression levels (TPM) at $\log_{10}$ scale. AD, atopic dermatitis; APC, antigen-presenting cell; c.p.m., count per million; TPM, transcript per million; UMAP, uniform manifold approximation and projection.

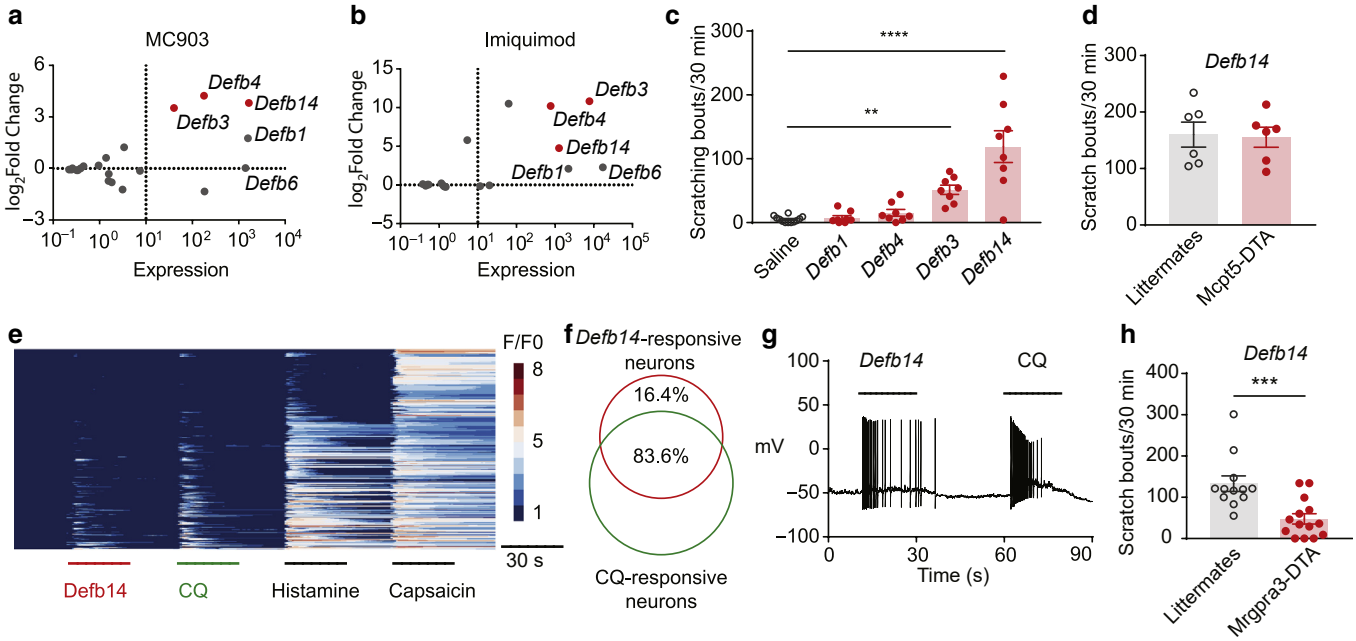

Figure 3. β-Defensins directly activate sensory neurons. MA plot of defensins in skin samples from (**a**) MC903-treated (GSE90883) and (**b**) imiquimod-treated mice (GSE161084). (**c**) Scratching bouts elicited by intradermal injection of 1 mM of the indicated recombinant β-defensins and saline (n = 8–12). Defb3- and Defb14-induced scratching was significantly greater than that for saline (**$P = 0.003$ and ****$P < 0.0001$, respectively; ANOVA Fisher's LSD posthoc analysis). (**d**) Mast cells–deficient mice (*Mcpt5*-DTA) and wild-type controls exhibited similar responses to Defb14 (1 mM, $P = 0.87$, two-tailed *t*-test). (**e**) Calcium imaging of 352 cultured DRG neurons transfected with AAV9-GCaMP6f. (**f**) A total of 46 of 55 Defb14-responsive neurons responded to CQ. (**g**) Whole-cell patch-clamp recording of *Mrgpra3*-Cre-GFP neurons revealed that Defb14 (1 μM) and CQ (100 μM) evoked action potential discharges (n = 8/8). (**h**) Scratching bouts of *Mrgpra3*-DTA mice (n = 14) were significantly reduced compared with those of the controls (n = 12, ***$P = 0.007$, two-tailed *t*-test). CQ, chloroquine; DRG, dorsal root ganglion; LSD, least significant difference; min, minute; s, second.

and McNeil, 2019). However, whether all these β-defensins share similar potencies across different MRGPRs is unclear. To determine the activation thresholds for the receptors, we performed fluorescence imaging plate reader calcium imaging on the eight human MRGPRs. We tested recombinant DEFB103 and DEFB4 on human embryonic kidney 293 cells transiently expressing MRGPRs together with Gα15 subunit and the calcium indicator GCaMP6s. Our results showed that DEFB103 exhibits higher potency on MRGPRX1 than MRGPRX2 with EC50s of 0.2 μM and 0.7 μM, respectively (Figure 4a and Table 1) and with other receptors responding at higher concentrations (>10 μM). In contrast, DEFB4 poorly activated MRGPRX2 with an EC50 of 17.5 μM and negligibly stimulated other MRGPRs (Figure 4b and Table 1). These results establish that human β-defensins relatively selectively activate MRGPRX1 and MRGPRX2 and that DEFB103 is considerably more potent at activating these receptors than DEFB4. Next, we performed a similar pharmacological assessment of mouse MRGPRs. We found that Defb14 activated MRGPRB2 as well as MRGPRA1 and MRGPRA3 with EC50s of 1.7 μM, 0.6 μM, and 1.0 μM, respectively (Figure 4c). Defb14, with lower affinity, also activated other receptors, including MRGPRA2B and MRGPRC11 (Table 2). These results show that β-defensins are capable of activating MRGPRB2 expressed in mast cells and can potently stimulate MRGPRs expressed in sensory neurons, including several previously unappreciated receptors shown previously to be responsible for chronic itch (Zhu et al., 2017).

It has been suggested that the bactericidal activity of β-defensins is related to their charge density (Bai et al., 2009; Klüver et al., 2005). To see whether the charge density of β-defensins is also related to their potencies in activating MRGPRs, we calculated the net charge values (Moore, 1985) of the β-defensins we studied and found that the peptides with the greatest net positive charge had the greatest potency for activation of MRGPRs. The β-defensins with net charge values higher than +10 (from +10.6 to +11.6) exhibited higher potency than those with net charge values around +6 (from +5.7 to +6.6) (Figure 4d–f). These results provide a rationale for the relative higher potency of Defb3 than of Defb4 to elicit scratching in mice (Figure 3a) and to activate mast cells (Figure 2) and suggest that the net charge of β-defensins is a major determinant for inducing pruritus.

Coexpression of MRGPRX1 and histamine receptor in human sensory neurons

Because we found that DEFB103 is a potent ligand for MRGPRX1 and because it is found at high levels in the skin of patients with AD and psoriasis, this suggests that it is a strong candidate pruritogen in humans. It has also been reported that *MRGPRX1* is expressed in human DRG (Flegel et al., 2015; Ray et al., 2018; Solinski et al., 2019a) and in neurons that express the peripheral itch neurotransmitter NPPB. To more fully characterize the expression of *MRGPRX1* in sensory neurons, we examined its coexpression with *HRH1* using in situ hybridization. As shown in Figure 5, all *MRGPRX1s* coexpress *HRH1* (122 of 122 neurons, three

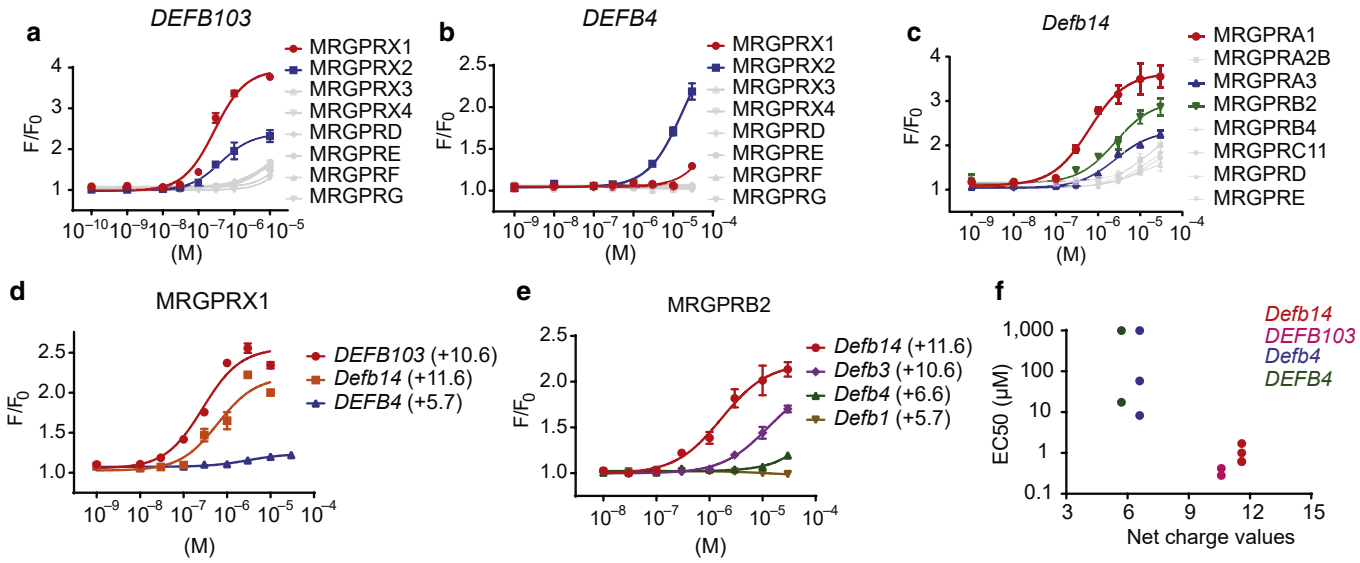

Figure 4. Characterization of MRGPRs activated by β-defensins. FLIPR screening of HEK-293 cells heterologously expressing human MRG PRs revealed that (**a**) DEFB103 potently activated human MRGPRX1 and MRGPRX2, (**b**) DEFB4 weakly activated MRGPRX2, and (**c**) Defb14 potently stimulated mouse MRGPRA1, MRGPRA3, and MRGPRB2. (**d**–**f**) The potency for activation of MRGPRs by defensins is related to the net charge values of peptides. (**d**) Human MRGPRX1 displayed a higher affinity for DEFB103 (+10.6) and Defb14 (+11.6) than for DEFB4 (+5.7). (**e**) Mouse MRGPRA1 exhibited a higher affinity for Defb14 and Defb3 (+10.6) than for Defb4 (+6.6) and Defb1 (+5.7). All results are averages from 3–4 replicates ± SEM. (**f**) DEFB103 and Defb14 exhibited higher potency (≤1 μM) in activating MGPRX1, MRGPRX2, MRGPRA1, MRGPRA3, and MRGPRB2. By contrast, DEFB4 and Defb4 exhibited low potency (≥10 μM). EC50, half-maximal effective concentration; FLIPR, fluorescence imaging plate reader; HEK-293, human embryonic kidney 293; MRGPR, Mas-related G protein-coupled receptor.

donors). Not all *HRH1*-positive neurons coexpress *MRGPRX1* (122 of 168 neurons).

DISCUSSION

The skin is the first barrier protecting our bodies from environmental pathogens, and the production of antimicrobial peptides is a primary method of defense. In addition, some of the antimicrobial peptides are danger-associated molecular patterns (Seong and Matzinger, 2004). In this study, we investigated the potential mechanisms of action of the danger-associated molecular pattern, β-defensins, and uncovered several unappreciated and critical features of the way these molecules work. Unexpectedly, we exposed that at least in mouse models of chronic itch, they act predominantly on sensory neurons. We further showed that β-defensins are not equally pruritogenic by examining the properties of several defensins that were previously not fully studied. Specifically, we show that DEFB103 and the mouse homolog Defb14 are the most pertinent with regard to itch-inducing effects, with other β-defensins, such as Defb1 and Defb4, being much less effective. In addition, we showed that net charge is an important determinant in predicting the itch potency of these peptides. Finally and importantly, we provide evidence that in human conditions associated with chronic itch, β-defensins expression is elevated, and we show that these peptides can activate both sensory neurons expressing MRGPRX1 (where it is coexpressed with histamine receptor) and mast cells expressing MRGPRX2, providing strong evidence that DEFB103 is likely an endogenous pruritogen in AD and psoriasis.

Previously, it was suggested that β-defensins can activate human mast cells; however, whether β-defensins activate rodent mast cells is inconclusive (Chen et al., 2007; Niyonsaba et al., 2001; Subramanian et al., 2013). We speculate that these inconsistent results could be partly due to different β-defensins that were tested.

In addition, we found that mast cells are unnecessary to trigger scratching in mice. This may account for why antihistaminergic treatments are largely ineffective at alleviating itch in many dermal diseases. However, we note that basophils and eosinophils, cells not eliminated in our studies, are reported to express MRGPRX2 (Wedi et al., 2020). These cell types might also contribute to itch in the mouse model we used and may be active in certain itch exacerbations (Wang et al., 2021). In addition, activation of MRGPRB2 in mast cells was shown to elicit itch through

Table 1. Summary of EC50s of Human MRGPRs (Shown in μM) of β-Defensin

MRGPR/Peptide	X1	X2	X3	X4	D	E	F	G
DEFB103	0.28 ± 0.05	0.42 ± 0.11	7.4 ± 1.9	ND	ND	ND	ND	ND
DEFB4	58.3 ± 18	17.5 ± 3.8	ND	ND	ND	ND	ND	ND
Defb14	0.61 ± 0.17							

Abbreviations: EC50, half-maximal effective concentration; MRGPR, Mas-related G protein-coupled receptor; ND, not determined.
ND indicates an EC50 that could not be estimated from the fitted data.

Table 2. Summary of EC50s of Mouse MRGPRS (Shown in μM) of Mouse β-Defensin

MRGPR/Peptide	A1	A3	B2	A2b	C11	B4	D	E
Defb14	0.62 ± 0.16	1.0 ± 0.18	1.7 ± 0.45	19.4 ± 8.6	21.6 ± 11.7	ND	ND	ND
Defb3	2.7 ± 0.6	17.5 ± 3.8	11.5 ± 3.5					
Defb4	ND	58 ± 12	>999					
Defb1	ND	ND	ND					

Abbreviations: EC50, half-maximal effective concentration; MRGPR, Mas-related G protein-coupled receptor; ND, not determined.
ND indicates an EC50 that could not be estimated from the fitted data.

nonhistaminergic processes (Meixiong et al., 2019). Interestingly, it was recently reported that tick-secreted β-defensins can activate MRGPRs on sensory neurons to induce itch. The EC50 of the described defensin for human MRGPRX1 is 4.32 μM (Li et al., 2021), a potency about 15-folds lower than that of DEFB103. In agreement with the idea that charge ratio is a critical determinant in potency, the net charge value for tick defensin is +5.8 versus +10.6 for DEFB103. Therefore, overall, our studies and the findings of others show that β-defensins directly activate sensory neurons but may also stimulate various immune cells to indirectly induce itch. Our findings provide clarification of the mechanisms involved in β-defensin–induced pruritus and offer further confirmation that they are endogenous pruritogens.

MATERIALS AND METHODS

Transcriptome analyses

RNA sequence reads downloaded from Gene Expression Omnibus (https://www.ncbi.nlm.nih.gov/geo/) by SRA-Toolkit with accession numbers indicated in the text. Sequence reads were mapped to Ensembl reference transcriptomes GRCh38.v98 (*Homo sapiens*) and GRCm38.v98 (*Mus musculus*) and counted by kallisto (Bray et al., 2016). Raw counts were imported into R and RStudio by tximport (Soneson et al., 2016) for differential gene expression analysis by DESeq2 (Love et al., 2014), and differentially expressed genes were plotted using http://jvenn.toulouse.inra.fr/app/index.html.

Animals

Experiments using mice followed National Institutes of Health (Bethesda, MD) guidelines and were approved by the National Institute of Dental and Craniofacial Research Animal Care and Use Committee. Male and female mice aged 6–12 weeks were used: C57BL/6N (Envigo, Indianapolis, IN), ROSA-stop-DTA (Jackson Laboratory, Bar Harbor, ME) (Ivanova et al., 2005), *Mrgpra3*-Cre-eGFP (Han et al., 2013), and *Mcpt5*-Cre (Peschke et al., 2015).

Extravasation

Hind paws were injected subcutaneously with 10 μl β-defensin peptides (GenScript Biotech, Piscataway, NJ and Biomatik, Wilmington, DE), and an equal volume of saline was injected into the contralateral paw. Evans blue was used to assay extravasation as described (Zhang and McNeil, 2019).

Itch behavior

Behavioral assessment of scratching was conducted as described (Solinski et al., 2019b). Bouts were defined as scratching events directed toward the site of injection from lifting the hind leg from the ground to returning it.

Mast cell culture and imaging

LAD2 cells were maintained in StemPro-34 (Gibco, Waltham, MA) supplemented with recombinant human stem cell factor (100 ng/ml)

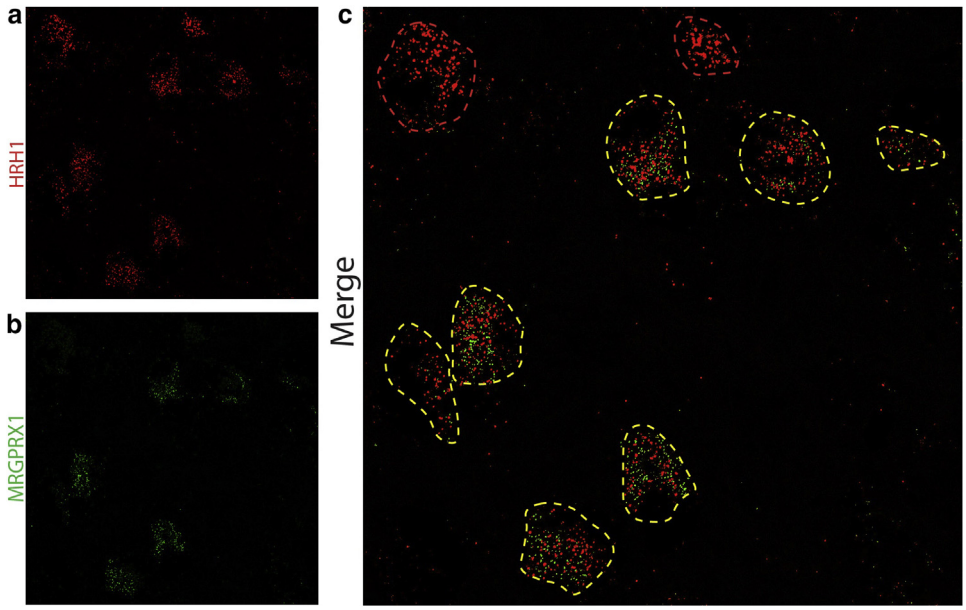

Figure 5. *MRGPRX1* is coexpressed with histamine receptor in DRG neurons. (**a**) ISH of human DRG revealed that *MRGPRX1*-positive neurons coexpress (**b**) *HRH1* as shown in the (**c**) merged image. Yellow outlines indicate double-positive neurons, and red profiles the *HRH1*-alone neurons. DRG, dorsal root ganglion; ISH, in situ hybridization.

and penicillin/streptomycin and as described previously (Kirshenbaum et al., 2003). LAD2 cells were loaded with Fluo-8 AM (Abcam, Waltham, MA) for 30 minutes at 37 °C, washed twice in Hanks' balanced salt solution (Gibco), and incubated for 30 minutes at room temperature.

DRG neuron culture and imaging

Primary cultures of DRG neurons were generated from C57BL/6N mice as described previously (Li et al., 2017). Briefly, DRGs were incubated in 5 mg/ml collagenase/dispase (10269638001, Millipore-Sigma, Burlington, MA) for 30 minutes; cells were mechanically dissociated, seeded on poly-D lysine–coated coverslips, and cultured for 48 hours (DMEM/F12, 10% fetal bovine serum, penicillin/streptomycin, 100 ng/ml nerve GF, 50 ng/ml Glial cell line–derived neurotrophic factor, Millipore-Sigma). For calcium imaging, DRG neurons were transfected with AAV9-Syn-GCaMP6.

Electrophysiology

Whole-cell current and voltage clamp were performed on *Mrgrpa3*-Cre-GFP neurons as described (Tseng et al., 2019). The extracellular solution contained 140 mM sodium chloride, 4 mM potassium chloride, 2 mM calcium chloride, 1 mM magnesium chloride, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, and 10 mM glucose at pH of 7.4 and osmolality of 310 mOsm/kg. The pipette solution contained 140 mM potassium chloride, 1 mM magnesium chloride, 1 mM EGTA, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 3 mM adenosine triphosphate, and 0.5 mM guanosine triphosphate at pH of 7.4 and osmolality of 300 mOsm/kg.

Fluorescence imaging plate reader screening

Human embryonic kidney K293 cells were cultured in DMEM/F12 supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. For transient expression, 8×10^5 cells were seeded, cultured for 24 hours, and transfected using TransIT-293 (Mirus Bio, Madison, WI). Cells were transfected with GCaMP6s, Gα15 subunit, together with individual MRGPRs. Expression vectors for *MRGPRX1*, *MRGPRX2*, *MRGPRX3*, *MRGPRX4*, *MRGPRD*, *MRGPRE*, *MRGPRF*, and MRGPRG were obtained from Addgene (Watertown, MA). Mouse *Mrgpr* expression vectors for *Mrgpra1*, *Mrgpra2b*, *Mrgpra3*, *Mrgprb2*, *Mrgprb4*, *Mrgprc11*, and *Mrgprd* were obtained from Xinzhong Dong at Johns Hopkins University (Dong et al., 2001) and for *Mrgpre* (GenScript Biotech). After 48 hours, cells were plated at 20,000 cells per well in 96-well plates with Hanks' balanced salt solution (with calcium and magnesium), and measurements were made.

Peptide net charge value calculation

The net charge Z of a peptide at a certain pH can be estimated by calculating with the following formula:

$$z = \sum_i N_i \frac{10^{pKi}}{10^{pH} + 10^{pKi}} - \sum_j N_j \frac{10^{pH}}{10^{pH} + 10^{pKj}}$$

where N_i is the number, and pK_i is the pKa values of the *N*-terminus and the side chains of arginine, lysine, and histidine. The *j*-index pertains to the *C*-terminus and the aspartate, glutamate, cysteine, and tyrosine (Moore, 1985).

In situ hybridization

Multilabeling in situ hybridization was performed using the RNAscope technology (Advanced Cell Diagnostics, Newark, CA) according to the manufacturer's instructions on human DRG. Images were collected on an Eclipse Ti (Nikon, Melville, NY) confocal laser-scanning microscope. Multiple sections were imaged and counted from three donors.

Statistical analysis

GraphPad Prism, version 7.0 (GraphPad Software, San Diego, CA), was used for statistical analyses and the generation of figures. Differences were considered significant for $^*P < 0.05$. Exact *P*-values and definitions and the number of replicates as well as the definitions of center and dispersion are given in the respective figure legends. The sample sizes used were similar to those generally used in the field. Heatmaps were generated with the R package pheatmap.

Data availability statement

Dataset related to this article can be found at https://www.ncbi.nlm.nih.gov/geo/ accession numbers GSE121212, GSE54456, GSE90883, and GSE161084 and at https://developmentcellatlas.ncl.ac.uk/datasets/hca_skin_portal.

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CONFLICT OF INTEREST

The authors state no conflict of interest.

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AUTHOR CONTRIBUTIONS

Conceptualization: PYT, MAH; Investigation: PYT; Supervision: MAH; Original Draft Preparation: PYT, MAH; Reiview and Editing: PYT, MAH

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