Innate immune regulates cutaneous sensory IL-13 receptor alpha 2 to promote atopic dermatitis

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ARTICLE INFO

Keywords:
Atopic dermatitis
Innate immune
Toll-like receptor

ABSTRACT

The clinical significance and regulators of IL-13Rα2 in itch and atopic dermatitis (AD) remain unclear. To identify disease-driven regulatory circuits of IL-13Rα2, transcriptomic/pathological analysis was performed in skin from patients with AD, psoriasis, healthy subjects, and murine AD model. Functionality was investigated in sensory neurons, keratinocytes and animal model, by using knockdown (KD), calcium imaging, RNA-seq.

Abbreviations: AD, Atopic dermatitis; ANOVA, Analysis of variance; DAPI, 4′,6-diamidino-2-phenylindole; DEG, Dysregulated genes; EDNRA, Endothelin receptor type A; FCH, Fold change; FDR, False discovery rate; HC, Healthy control; IL-13Ra, IL-13 receptor alpha; IL-31OE, IL-31 overexpressing; KD, Knockdown; AL, Lesional AD; PL, Lesional psoriasis; mDRGs, Murine dorsal root ganglionic neurons; ANL, Non-lesional AD; PNL, Non-lesional psoriasis; phiKCs, Primary human keratinocytes; RPKM, Reads per kilobase per million; TLR, Toll-like receptors.

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https://doi.org/10.1016/j.bbi.2021.08.211
Received 9 April 2021; Received in revised form 5 August 2021; Accepted 6 August 2021
Available online 13 August 2021

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IL-13, Cytokine IL-13Rα1 and 2

1. Introduction

Atopic dermatitis (AD) is a chronic relapsing skin disease associated with increased risk of atopic comorbidities, skin infections and reduced quality of life (Bieber, 2008; Steinhoff et al., 2012; Weidinger et al., 2018). Neuro-immune circuits play essential roles in pathophysiology of pruritus and AD. Neuro-epithelial and neuro-immune interactions form an extensive network for cutaneous inflammatory and pruritic signals (Buhr et al., 2020; Cevikbas et al., 2007; Larkin et al., 2021; Meng et al., 2018; Meng and Steinhoff, 2016; Meng et al., 2019; Oetjen et al., 2017; Steinhoff et al., 2012; Steinhoff et al., 2003; Szollosi et al., 2019; Trier et al., 2019). Cytokine receptors on sensory nerves and keratinocytes critically contribute to the complex pro-inflammatory and pro-pruritic cascade in AD (Dati et al., 2021; Piccioni and Steinhoff, 2013; Trier et al., 2019). In addition, several innate immune receptor pathways have been implicated in the pathophysiology of AD including Toll-like receptors (Jin et al., 2009; Kaesler et al., 2014; Prescott et al., 2008; Skabytska et al., 2014; Volz et al., 2014; Vu et al., 2010). For example, impaired TLR2 function has been associated with the pathogenesis of AD (Jin et al., 2009; Niebuhr et al., 2009). Despite this, the pathogenicity of AD and itch modulated by innate immunity need to be defined in relation to the itch circuits. IL-13 has emerged as a prime target in AD (Guttman-Yassky et al., 2019; Scott et al., 2013). It is a key driver of type-2 T-helper (Th2) inflammation and is elevated in the lesional skin of AD patients (Leyva-Castillo et al., 2020; Meng et al., 2021). Upon release in peripheral skin, IL-13 activates neighbouring cells expressing its receptors, recruits inflammatory cells into the skin, and alters the skin microbiome. IL-13 also contributes to Th2-mediated inflammatory responses; (iii) the modulatory mechanism of innate immune inflammation to IL-13α1 transcriptional and functional alteration. This study sheds new light on the pathogenesis and progression of AD, in particular onto the neuro-immune circuits involved in this pruritic inflammatory disease condition, aiming at developing new targets to optimize current treatment options.

2. Methods

2.1. Animal rights

All animal procedures were performed in accordance with the Guidelines for Care and Use of Laboratory Animals of Henan University and approved by the Animal Ethics Committee of Henan University, China.

2.2. RNA-seq

Human skin original RNA-seq datasets and methods are available from our previous paper (Larkin et al., 2021), in which punch biopsies were taken from AD, both lesional (AL) and non-lesional AD (ANL), and psoriasis, both lesional (PL) and non-lesional (PNL), and healthy control (HC).

RNA-seq of cells and animal tissue was performed by BGI (Beijing Genomics institution). A >2-fold change (log2 < 1) in transcription was deemed significant in each comparison. FDR (false discovery rate) were determined and classified as ***FDR < 0.001, **0.001 < FDR < 0.01, *0.01 < FDR < 0.05, **FDR > 0.05.

2.3. Real-time reverse transcription PCR

Lesional and non-lesional skin of IL-31-overexpressing (OE) mice (Dillon et al., 2004) and age-matched wild-type control skin were quantified for mRNA level of TLR2. Primers were purchased from OriGene (China).

2.4. Culture of phKCs and murine primary sensory neurons

phKCs were cultured in KBM-Gold medium with KBM-Gold Single Quot KC supplement (Lonza) as described in (Meng et al., 2019). Cells were maintained in the above medium for 3 days before use.

Murine dorsal root ganglion were isolated from postnatal d5 C57BL/6 mice, and cultured for 7 days in vitro before use, as previously described (Meng et al., 2018).

To prepare samples for RNA-seq, mDRGs and phKCs were incubated with Pam3CSK4 (1 µg/ml, Sigma) or FSL-1 (1 µg/ml, Sigma) or vehicle for 6 h before cells were harvested for RNA-seq.
2.5. Knockdown of IL-13Rα1 and IL-13Rα2 and cytokine array

Cells were incubated in culture medium containing shRNA lentiviral particles that specifically target IL-13Rα1, IL-13Rα2 (OBio Technology Corp.), or non-targeted scrambled lentiviral particles, as previously described (Meng et al., 2018). Cells were lysed in LDS sample buffer for Western blotting to confirm the knockdown effect. Rabbit anti-IL-13Rα1 (Abcam) or goat anti-IL-13Rα2 (R&D Systems) and mouse monoclonal SNAP-25 (BioLeged) or SNAP-23 (Synaptic Systems) were used for immunoblot.

For cytokine array, cells incubated with or without Pam3CSK4 (1 μg/ml) or vehicle for 6 h at 37 °C were washed and stimulated by IL-13 (100 ng/ml, Abcam) at 37 °C for 24 h. Cell-culture supernatants were analysed using proteome profiler mouse XL cytokine array kit (R&D Systems) before being analysed with ImageJ software. Resultant densitometry values from treated samples were calculated relative to nontreated control values to determine the fold change.

2.6. Behavioural experiments

To establish a prolonged model of AD, MC903 (2 nmol/20 μl in ethanol, Sigma) or vehicle (ethanol) was topically applied to the left ear of C57BL/6 female mice. This was repeated daily for 12 consecutive days. The ear was harvested, paraffinized and embedded before being sectioned for immunohistochemical staining (Meng et al., 2018).

To analyse the influence of TLR2 heterodimer activation on IL-13-induced itch-like behaviour, C57 BL/6 mice were pre-administered with Pam3CSK4 (20 μg/100 μl sterilized water) by gavage every three days for 3 times (Tunis et al., 2015). In another experiment, FSL-1 (0.3 mg/kg, 60 mg/ml, Abcam) at 37 °C were washed and stimulated by IL-13 injection. Control mice were given sterilized water only. All of these mice were then intradermally injected with IL-13 (1 μg/10 μl) or vehicle into the left cheek. All the mice were video-recorded for 1 h for the analysis of scratching bouts. Data presented as means ± SEMs (n = 8 mice/group); *P < 0.05, **P < 0.01, and ***P < 0.001, 2-way analysis of variance (ANOVA).

2.7. Intracellular Ca2+ measurements

mDRGs incubated with Pam3CSK4 (1 μg/ml) or vehicle for 6 h were loaded with Fluo-4 AM. Cells were then video-imaged with ImageXpress Micro 4 Automated Cell Imaging System (Molecular Devices) using Matexpress6 software at 8 s intervals. After a baseline period, IL-13 (100 ng/ml) was applied. For each cell, intracellular calcium increases were normalised to F/F0, with F denoting the fluorescence and F0 the baseline fluorescence, and graphed relative to time. Responding cells were analysed and presented as % total cells (Steinhoff et al., 2000). The chi-square statistic with Yates correction is applied for p-value significance evaluation.

2.8. Immunofluorescence staining

Paraffin sections were deparaffinised, rehydrated before permeabilization and incubation in block reagent, as described previously (Meng et al., 2018); cultured phKCs were permeabilized and blocked. Specimens were then incubated with rabbit anti-IL-13Rα1 or goat anti-IL-13Rα2 in blocking solution (4 °C, overnight). The specimens were washed in PBS and incubated with donkey anti-rabbit Alexa 594 and donkey anti-goat Alexa 488. After the final wash of the secondary antibody, specimens were mounted onto slides using anti-fade reagents containing DAPI (4′,6-diamidino-2-phenylindole). Images were taken by IX73 Olympus inverted microscope using CellSens Dimension Imaging software (Larkin et al., 2021).

3. Statistical analysis

For RNA-seq, average fold change for genes upregulated were plotted. Differentially expressed gene (DEG) are defined based on thresholds: (log2FCH ≥ 1.0 and the adjusted p-value FDR < 0.05). FDRs were indicated as ***FDR < 0.001, **0.001 < FDR < 0.01, *0.01 < FDR < 0.05, **FDR > 0.05. For genes not reaching these thresholds, although FDR < 0.05, these are not defined as DEGs.

Other data are presented as mean ± SEM with dot plots or bar graphs. We made two-group comparisons with a two-tailed Student’s t-test followed by Welch’s correction, except that datasets in Fig. 4J, K and Fig. 5C-F were analyzed using 2-way ANOVA followed by Bonferroni’s post hoc analysis to determine where those differences occurred among multiple groups. The criterion for statistical significance is *P < 0.05, **P < 0.05; **P < 0.01; ***P < 0.001 (Prism 7, GraphPad Software, La Jolla, CA, USA).

4. Results

4.1. Transcription of IL-13Rα2 in skin is correlated with disease activity of AD, but not psoriasis

To examine the clinical relevance of IL-13Rα2 with respect to disease activity in human AD and psoriasis, we analysed skin samples from lesional AD (AL), non-lesional AD (ANL), lesional psoriasis (PL) and non-lesional psoriasis (PNL), and compared to healthy controls (HC). IL-13Rα2 mRNA levels were found to be upregulated in AL vs. HC (Fig. 1A) and in AL vs. ANL (Fig. 1B). IL-13 mRNA levels were also increased in AL vs. HC (Fig. 1A), and in AL vs. ANL (Fig. 1B). In contrast, IL-13Rα1 mRNA levels were not altered in AL vs. HC or in AL vs. ANL.

In contrast to AD, in psoriasis IL-13x1 showed small but significant increment in PL vs. HC (Fig. 1C), based on the thresholds: (log2FCH ≥ 1.0 and FDR < 0.05). However, neither IL-13 nor IL-13Rα2 transcription was altered in PL vs. PNL (Fig. 1D). Thus, IL-13Rα2 mRNA levels are not changed in skin lesions of human psoriasis compared to controls. Moreover, when AL is compared directly to PL, both IL-13 mRNA and IL-13Rα2 mRNA levels were significantly upregulated (Fig. 1E). The close association of IL-13Rα2 expression with disease activity of AD, rather than psoriasis, may represent an important disease-specific role of IL-13 Rα2 in AD, but not in psoriasis, for inflammation and itch.

Further, we analysed IL-13 and IL-13Rα1/2 expression in skin from a MC903-induced murine AD model (Larkin et al., 2021). RNA-seq revealed that the transcription levels of IL-13 and IL-13Rα2 (but not IL-13Rα1) were increased in ears of MC903-treated, when compared to vehicle-treated mice (Fig. 1F), based on log2FCH ≥ 1.0 and FDR < 0.05. Thus, similar to human AD, IL-13Rα2 represents an AD disease-regulated gene in mice.

Immunohistochemically, the protein levels of IL-13Rα1 and IL-13Rα2 within the epidermis in MC903-treated ears are increased when compared to controls (Fig. 1G, H). MC903 induced ear skin model has been deemed to display key features associated with AD, including inflammation, skin thickening, transdermal water loss, hypervascularization, spongiosis, strong immune cell infiltration, epidermal hyperplasia and intensive pruritic behaviours (Li et al., 2006; Oetjen et al., 2017). But the MC903 mouse model still scratches normally in the post hoc analysis to determine where those differences occurred among multiple groups. The criterion for statistical significance is **P < 0.05, **P < 0.05; **P < 0.01; ***P < 0.001 (Prism 7, GraphPad Software, La Jolla, CA, USA).

4.2. IL-13 Upregulates IL-13Rα2 transcription levels in Keratinocytes, and contributes to modulation of neuro-immune circuits

To address the underlying mechanism for the disease-driven
upregulation of IL-13Rα2 in the skin including epidermis, we investigated its location in cultured human primary keratinocytes (phKCs). Robust expression levels of IL-13Rα1 and IL-13Rα2 in phKCs was confirmed, showing IL-13Rα1 resided closer to the cell surface than IL-13Rα2 (Fig. 2A). Live cell calcium imaging revealed that a fraction (22.8%) of phKCs responded to 100 ng/ml IL-13 (Fig. 2B). RNA-seq revealed that transcription of IL-13Rα2, but not IL-13Rα1, was upregulated when treated with IL-13 (Fig. 2C), which is in consistent with previous report (Furue et al., 2020b). To gain insight into the influence of IL-13Rα1 and IL-13Rα2 on each other, knockdown (KD) using specific shRNA against each receptor was performed, and compared to scrambled shRNA-treated control cells (Fig. 2D, E). Western blot analysis confirmed that KD of IL-13Rα2 expression to ~56% was achieved in comparison to scrambled shRNA-treated cells (Fig. 2D). Subsequently, in a separate experiment, the IL-13Rα2KD phKCs were treated with or without IL-13 before RNA-seq. Sequencing results revealed that in IL-13Rα2KD cells IL-13Rα1 expression was not altered regardless of IL-13 treatment (Fig. 2F), suggesting IL-13Rα2 has no impact on IL-13Rα1 expression. In a similar experiment, knockdown of IL-13Rα1 by its specific shRNA achieved ~70% (Fig. 2E). Interestingly, IL-13Rα1KD significantly reduced IL-13Rα2 transcription when treated with IL-13 (Fig. 2G). This effect was not observed without IL-13 treatment (Fig. 2G). This result suggests that IL-13Rα1 influences IL-13Rα2 transcription in the presence of IL-13.

To study the influence of KD of these receptors on the IL-13-induced alterations of genes in phKCs, we then analyzed RNA-seq on each KD cells in comparison to the scrambled shRNA-treated controls. In the scrambled-shRNA treated phKCs, IL-13 upregulated transcription levels of IL-31RA, EDNR (endothelin receptor type A), IL-7R, IL-1R2, as well as NRPI (Fig. 2H) and numerous inflammatory and itch mediator genes (Fig. 2I and J). IL-13Rα1KD and IL-13Rα2KD differentially affected these gene transcription levels, with IL-13Rα1KD being more effective in downregulation than IL-13Rα2KD (Fig. 2H-I). Interestingly, IL-13Rα1KD downregulated majorities of the genes in IL-13-treated IL-13Rα1KD cells vs. IL-13-treated scrambled shRNA control cells (Fig. 2H-J). In contrast, IL-13Rα2KD reduced some of them, to a lesser extent, and these include EDNR, NRPI, CCL20, CCL26, and SERPIN family members etc. (Fig. 2H-J).

Taken together, IL-13-driven upregulation of IL-13Rα2 in keratinocytes may explain the observed enhanced level of epidermal IL-13Rα2 in human AD skin. IL-13Rα2 independently participated in regulation of certain genes (i.e. EDNR, CCL20, CCL26, CXCL6, SERPIN family, as well as TNF family members).

Subsequently, we analyzed if IL-13-induced genes (c.f. Fig. 2H-J) were affected by knockdown of each receptors in phKCs under basal (vehicle-treated) condition. Among IL-13 induced genes, IL-36G, S100A7, SERPINB3 and SERPINB4 transcripts were reduced in IL-13Rα1KD phKCs even without IL-13 treatment (Fig. 3A), and SERPINB3 and SERPINB4 transcripts were downregulated in IL-13Rα2KD phKCs (Fig. 3B).

IL-13 activates intra-epidermal nerve terminals of sensory neurons to propagate itch, and these terminals are abundantly present in skin (Feld et al., 2016). Thus, it is necessary to assay sensory neuronal IL-13Rα2 transcription. TLR innate immune response has been associated with the pathogenesis of AD (Jin et al., 2009; Niebuhr et al., 2009). Therefore, attention was then paid to dysfunctional TLR in the regulation of IL-13 receptors.

Indeed, our data confirmed an increased TLR2 transcription in ears from MC903- vs. vehicle-treated mice by RNA-seq (Fig. 4A). In skin from IL-31-overexpressing AD model mice (Meng et al., 2018), increased TLR2 transcription were also detected when compared with their age matched wild-type mice (Fig. 4B). We thus used TLR2 heterodimer agonists Pam3CSK4 (1 μg/ml) or FSL-1 (1 μg/ml) to treat dorsal root ganglion neurons (mDRGs) and phKCs for 6 h before RNA-seq. Interestingly, this revealed an upregulated transcription of IL-13Rα2 in mDRGs, but not IL-13Rα1 (Fig. 4C and D). Moreover, these agonists did not significantly influence IL-13Rα1 and Rα2 in phKCs (Fig. 4E and F). The different results between these cell types suggest a tissue-specific regulation of IL-13Rα2. Our findings highlight disease driven-TLR2 activation in AD may promote neuronal IL-13Rα2 transcription.

As a cutaneous regulator, IL-13 activates sensory neurons and participates in the initiation of AD and itch (Erickson et al., 2021). Hence, we investigated whether TLR2 facilitates IL-13 signalling in sensory neurons. Cultured mDRGs were then pre-incubated with Pam3CSK4 (1 μg/ml) or vehicle for 6 h before washing, and application of IL-13 (100 ng/ml) for measurement of calcium imaging. In comparison to IL-13 alone that elicited calcium spikes in a small fraction (3%) of neurons (Fig. 4G), Pam3CSK4 increased IL-13-elicited calcium influx in 5.67% neurons (Fig. 4H). The increment in the number of IL-13 responders is significant. (For G and H, the chi-square statistic with Yates correction is 5.1983. The p-value is 0.022609). The changes of the area under the curve (AUC) also confirmed the enhanced intracellular calcium by Pam3CSK4 almost doubled that of IL-13 alone (Fig. 4I).

To assay the biological consequence of TLR2-enhanced IL-13 signaling in itch, we used mouse cheek model. Pam3CSK4 (20 μg/100 μl) or vehicle (sterilized water) was orally administrated to mice three times by gavage for over 7 days. Next day after the final gavage, IL-13 (1 μg) or vehicle was intradermally injected into one cheek. IL-13 alone did not induce a significant increase in scratching bouts compared to a vehicle injection, and this is consistent with previous findings (Oetjen et al., 2017). In mice pre-administered with Pam3CSK4, IL-13 injection caused about a doubling of scratching compared to vehicle injection (Fig. 4J).

To reaffirm if activation of TLR2 impact on IL-13 mediated itch signaling, we also used FSL-1 to activate TLR2 before IL-13 cheek...
Fig. 2. IL-13 upregulates IL-13Rα2 in cultured phKCs; knockdown IL-13Rα1 and –2 differentially regulate itch receptor transcription and inflammatory/itch mediator synthesis. A. Immunofluorescence staining for IL-13Rα1 and 2 in cultured phKCs; B. calcium transients elicited by IL-13 in phKCs; C. IL-13Rα1/2 transcription changes analysed by RNA-seq in phKCs after treatment with IL-13 vs. control; immunoblots for KD of IL-13Rα2 (D) and IL-13Rα1 (E) in phKCs; RNA-seq for transcription of IL-13Rα1 (F) and IL-13Rα2 (G) in IL-13Rα2KD and IL-13Rα1KD phKCs with or without IL-13 treatment. (H, I, J) IL-13-induced gene expression changes in IL-13Rα1KD and IL-13Rα2KD phKCs relative to scrambled shRNA-treated control. ***FDR < 0.001, **0.001 < FDR < 0.01, *0.01 < FDR < 0.05, ns FDR > 0.05.
epidermal barrier genes after treatment with IL-13 (6 h) vs. vehicle in scram
13Ra2 in cultured phKCs without IL-13 treatment. C. Transcriptional changes of
attributed to enhanced IL-13R
moted the itch-like response of mice to IL-13, an outcome likely to be
induce a significant increase in scratching bouts compared to injection
- injection. Because FSL-1 is more toxic than Pam3CSK4 when it was given
bled control shRNA-treated phKCs, and in phKCs after IL-13R
regulation of IL-13-induced genes after knockdown of (A) IL-13Ra1 and (B) IL-
Fig. 3.

injection. Askef. B. RNA-seq showing down-regulation of IL-13-induced genes after knockdown of (A) IL-13Ra1 and (B) IL-
13Ra2 in cultured phKCs without IL-13 treatment. C. Transcriptional changes of epidermal barrier genes after treatment with IL-13 (6 h) vs. vehicle in scrambled control shRNA-treated phKCs, and in phKCs after IL-13Ra1KD or IL-
13Ro2KD. DEG threshold: 2-fold up- or down-regulation with FDR < 0.05. ***FDR < 0.001, **0.001 < FDR < 0.01, *0.01 < FDR < 0.05, **FDR > 0.05.

injection. Because FSL-1 is more toxic than Pam3CSK4 when it was given to mice through oral route (Tunis et al., 2015), we chose to administer FSL-1 through the route of intraperitoneal injection (i.p.). In our result, mice received i.p. injection of FSL-1 (0.3 mg/kg) 14 h before IL-13 cheek injection showed a greater level of scratching bouts, in contrast to the intraperitoneal injection of vehicle (sterilized water) (Fig. 4K). Moreover, mice received IL-13 or intraperitoneal injection of FSL-1 did not induce a significant increase in scratching bouts compared to injection of vehicle(s).

Thus, these results strengthen the notion that TLR2 activation promoted the itch-like response of mice to IL-13, an outcome likely to be attributed to enhanced IL-13Ra2 activity in sensory neurons.

4.5. TLR2-regulated IL-13 signalling orchestrates specific cytokine and chemokine release from sensory neurons through IL-13Ra2-dependent pathway

To investigate the mechanism underlying the Pam3CSK4 caused itch sensitization to IL-13, the functional contribution of IL-13Ra2 to TLR2 regulated IL-13 response in sensory neurons was evaluated. KD of each IL-13Ra was carried out in cultured mDRGs. Western blot analysis confirmed that expression levels of IL-13Ra1 and IL-13Ra2 were reduced by their respective shRNA compared to the scrambled shRNA treatment (Fig. 5A, B). Cytokine array demonstrated that in mDRGs treated with scrambled shRNA, Pam3CSK4 pre-treatment (for 6 h, followed by washing) enhanced IL-13-mediated release of cytokines including chemerin, TNFα, CCL17 and CCL20 (Fig. 5C-F). These cytokines are known to be related to neurogenic inflammation and AD (Deftu et al., 2018; Stojek, 2016). Importantly, Pam3CSK4-enhanced IL-13-elicted release of cytokines chemerin (Fig. 5C), CCL17 (Fig. 5E) and CCL20 (Fig. 5F) was significantly reduced in the IL-13Ra2KD mDRGs compared to the scrambled shRNA-treated cells. In contrast, TNFα was not affected by IL-13Ra2KD (Fig. 5D). Thus, these results highlight that TLR2 modulates IL-13Ra2 downstream mediators involved in human dermatitis and neurogenic inflammation.

This finding provides a previously unknown immune-modulatory mechanism of pruritic signaling in AD by revealing an innate TLR2 immune-based exaggeration of IL-13-mediated itch. IL-13-induced IL-
13Ro2 signaling in keratinocytes and TLR2 upregulated peripheral IL-
13Ro2 signalling can drive itching sensation and facilitate neurogenic inflammation (Fig. 6). Taken together, peripheral TLR2-IL13Ra2 might be a new therapeutic target to control AD and chronic itch.

5. Discussion

Our study highlights the clinical relevance of the IL13Ra2 signaling pathway in controlling itch responses, inflammatory cascades, and neurogenic inflammation in AD. Cutaneous IL-13Ra2 upregulation is observed in lesional AD, but not psoriasis, and is functionally confirmed in AD murine models. In AD, this upregulation occurs in a disease activity-dependent manner.

Previous findings suggested IL-13, IL-4, and TNFα enhance expression of IL-13Ra2 in human keratinocytes (Furue et al., 2020a; Sivaprasad et al., 2010). These studies indicate that IL-13Ra2 acts as the decoy receptor to antagonize and inhibit IL-13Ra1 function. Our findings confirmed that IL-13Ra1KD reduced IL-13-induced IL-13Ra2 levels in keratinocytes, a feature not observed under normal condition. Thus, we add on new information that IL-13Ra2 expression is dependent on IL-
13Ro1 (Bitton et al., 2020), but only if IL-13 is present.

The observed influence of IL-13Ra1KD on IL-13-induced IL-13Ro2 transcription somehow masked dissecting IL-13Ra2 function. To overcome this, we reduced the IL-13Ra2 expression in keratinocytes using shRNA thereby down-regulating IL-13-induced transcriptomic changes of certain itch receptors and inflammatory mediators (c.f. Fig. 2H-J). These include EDNRA, CCL20, CCL26, CXCL6 and SERPINs. Thus, we conclude that keratinocyte-derived IL-13Ra2 may mediate IL-13-induced signaling independently from IL-13Ro1. Our study revealed a direct disease-driven modulation and engagement of IL-13Ro2 in AD condition. Our findings also argue against IL-13Ro2 being solely a decoy receptor in keratinocytes because IL13Ro2 actively participates in signal transduction pathways. In fact, in other biological process such as the malignant melanoma and glioblastoma mutiforme, IL-13Ro2 is implicated in inducing angiogenesis and promoting signal transduction (Fichtner-Feigl et al., 2007; Fichtner-Feigl et al., 2006; Okamoto et al., 2019).

Apart from epidermal keratinocytes, IL-13 directly activates sensory neurons to promote itch sensation enriched in AD skin. However, IL13Ro2 function in sensory nerves has been ignored long-time. Here, for the first time we demonstrate that IL13Ro2 is upregulated in TLR2 agonist-treated peripheral sensory neurons. Disturbances of innate and adaptive immune responses were implicated in pathogenesis of AD (Boguniewicz and Leung, 2011; Weidinger et al., 2018). Innate TLR2 signaling not only promotes itch and pain but convert transient TH2 cell-mediated dermatitis into persistent inflammation which is linked to chronic human AD (Kaesler et al., 2014; Liu et al., 2012; Niebuhr et al., 2009; Wang et al., 2020). Thus, our findings herein provide an
Fig. 4. TLR2 activation upregulates IL-13Rα2 in cultured mDRGs, but not in pKCs; TLR2 agonist, Pam3CSK4 enhanced IL-13-evoked calcium influx in mDRGs, and elevated IL-13-elicited scratching behaviour in mice. A. RNA-seq based heatmap of TLR transcription in ear skin of MC903- vs. vehicle-treated mice. B. Real-time reverse transcription PCR for TLR2 in lesional and non-lesional skin of IL-31-overexpressing (OE) mouse vs. age matched wild-type mice. RNA-seq of IL-13Rα1/2 in Pam3CSK4-treated or FSL-1-treated vs. vehicle-treated mDRGs (C, D) and pKCs (E, F). Representative traces for calcium spikes elicited by IL-13 in mDRGs without (G) or with (H) Pam3CSK4 pre-treatment for 6 h; Pie chart showing the fraction of IL-13 responders. I) Area under curve of IL-13 elicted calcium spikes in mDRGs pretreated with or without Pam3CSK4. J) Orally administrated Pam3CSK4 or (K) intraperitoneal injection of FSL-1 enhanced IL-13-induced scratch bouts in a mouse cheek model (2-way ANOVA followed by Bonferroni’s post hoc analysis). Data are presented as mean ± SEM; n ≥ 3. For B and I, Student 2-tailed t-test: *P < 0.05.

Fig. 5. Effect of TLR2 on IL-13-induced cytokine release after IL-13Rα1/2 knockdown in cultured mDRGs. IL-13Rα1 (A) and IL-13Rα2 (B) proteins were reduced by their specific shRNA compared to each scrambled shRNA-treated control. C-F, Effect of IL-13Rα1/2 knockdown on IL-13-induced chemerin (C), TNFα (D), CCL17 (E), CCL22 (F) release from mDRGs (2-way ANOVA followed by Bonferroni’s post hoc analysis). Pam3CSK4 pre-treatment increased IL-13 induced release. Data are presented as mean ± SEM; n ≥ 3. For A and B, Student 2-tailed t-test: ***P < 0.001.
important insight into IL-13-promoted neuro-immune itch circuits, and may help to understand the function of IL-13Ra2 upregulated during certain types of infections associated with AD.

Previous finding has shown that activation of TLR2/1 heterodimers by Pam3CSK4 mediates pain and itch, whereas activation of TLR2/6 heterodimers by lipoteichoic acid (LTA) or zymosan drives itch (Wang et al., 2020). Together, TLR2 is required for both histamine-dependent, histamine acute itch, dry skin itch as well as chronic allergic contact dermatitis (ACD) itch (Wang et al., 2020). However, the underlying mechanism still remains unclear. In our study, upregulation of sensory neuronal IL13Ra2 by TLR2/1 agonist Pam3CSK4 and TLR2/6 by FSL-1 also resulted in an enhanced itch-like behaviour when compared to IL-13-injection alone in mice. KD experiments in sensory neurons confirmed that IL13Ra2 promotes itch and inflammatory cytokine release from keratinocytes. The enhanced inflammatory mediator release mediated by IL-13Ra2 through TLR2 activation can be independent from IL13Ra1. Posttranscriptional mediators induced by IL-13Ra2 include chemerin, and CCL17, CCL22, known to be implicated in skin lesion or AD (Gutschman et al., 2017; Homey et al., 2006; Renert-Yuval and Guttman-Yassky, 2020). Thus, this brand new pathway that employs innate immune TLR2 to potentiate IL-13Ra2 effects in itch underlies a disease-driven innate immune-boosted neuronal IL-13 signalling mechanism in pruritic AD.

Here, we report tissue-specific mechanisms involved in regulation of IL-13Ra2 in keratinocytes and sensory neurons in AD, highlighting differential immune-neuronal modulatory mechanisms of IL-13 signalling in skin inflammation and itch in AD pathogenesis, strengthening the concept that the innate and adaptive immune system both represent major itch regulators by modulating itch, scratching, cutaneous sensation, and sensory perception beside their role in inflammation and skin barrier (Steinhoff et al., 2018).

Indeed, there is an ongoing debate about the role of IL-13 in inducing itch in mice. One previous report showed IL-13 intradermal injection could not directly induce scratching behaviours in mouse cheek, instead, it potentiates other pruritogens to induce itch (Oetjen et al., 2017). A later report showed IL-13 was able to directly induce cheek scratching at a certain window of concentration (Campion et al., 2019). Moreover, the IL-13 transgenic mice display TRPA1-dependent itch-like behaviour (Oh et al., 2013). Thus, fine-tuned concentration-dependent mechanisms may lead to different effects on receptors and subsequent signalling systems, as known for many receptors (Green et al., 2006; Green and Dong, 2016). In this study, IL-13 alone induced a slight increment in scratching bouts in mice received vehicle for Pam3CSK4, but this effect did not reach statistical significance. However, oral administration of Pam3CSK4 or intraperitoneal injection of FSL-1 significantly induced itch-like behaviours when compared with IL-13 alone. Because these agonists of TLR2 upregulate IL-13Ra2 in sensory neurons and the direct activation by IL-13 is dose-dependent, we conclude that IL-13Ra2 functionally activated by Pam3CSK4 or FSL-1 contributed to this elevated itch response. However, the possibility of TLR2-independent events induced by Pam3CSK or FSL-1 stimulation cannot be completely excluded from regulation of IL-13Ra2 expression in sensory neurons.

Overall, our findings reveal a new mechanism how IL13Ra2 contributes to neuroimmune circuits by releasing mediators from sensory nerves involved in immune regulation and skin barrier function, and from keratinocytes involved in itch and inflammation, thereby pinpointing a distinct regulatory mechanism of IL-13Ra2 in AD. We propose that the TLR2-IL-13Ra2 pathway represents a new target for the treatment of chronic itch and neuroinflammation in AD.
References

Akdin, C.A., Arkwright, P.D., Brugi, M.C., Busse, W., Gadina, M., Guttmann-Yasky, E., Kashiwakura, K., Mitamura, Y., Viam, L., Wu, J., Palamour, O., 2020. Type 2 immunity in the skin and lungs. Allergy 75 (7), 1582–1605.

Andrews, A.L., Nasir, T., Baccigualdi, F., Holloway, J.W., Nordin, B., Davies, D.E., 2006. Type 2 IL-13 signaling via the IL-4Rα receptor in experimental atopic dermatitis. Sci. Immunol. 5 (44), 201938. https://doi.org/10.1126/sciimmunol.201938

Bittel, A., Avias, S., Reichman, H., Iman, M., Karo-Aatar, D., Arozza, N.P., Rosenberg, P., Dreesendroux, V., Nahary, L., Rothenberg, M.E., Benham, I., Muntz, A., 2020. A key role for IL-13 in the pathogenesis of oral lichen planus. J. Invest. Dermatol. 144 (6), 1358–1368.

Buhl, T., Ikoma, A., Kempkes, C., Cevikbas, F., Sulk, M., Buddenkotte, J., Akiyama, T., Furue, M., Ulzii, D., Nakahara, T., Tsuji, G., Furue, K., Hashimoto-Hachiya, A., Kido-Furue, K., 2020. Novel insights into the TRPV3-mediated itch in atopic dermatitis. J. Allergy Clin. Immunol. 147 (3), 1110–1114.e5.

Castilho, J.M., Galon, C., Mashiko, S., Bosonnette, R., McGurk, A., Ziegler, S.F., Dong, C., McKenzie, A.N.J., Sarfati, M., Geha, R.S., 2020. IL2C activation by keratinocyte-derived IL-25 drives IL-13 production at sites of allergic skin inflammation. J. Allergy Clin. Immunol. 145 (6), 1606–1614.e4.

Li, Z., Heng, Z., Zhan, L., Sun, W., Metzger, D., Chambon, P., 2006. Topical vitamin D3 and low-calcemic analogs alleviate atopic dermatitis in mouse models. J. Invest. Dermatol. 127 (1), 172–178.

Larkin, C., Chen, W., Sahbi, L.L., Shan, C., Dajono, Z., Sreerad, A., Bulh, T., Fan, Y., O’Neill, J., Wang, S., Wang, J., 2021. Novel insights into the TRPV3-mediated itch in atopic dermatitis. J. Allergy Clin. Immunol. 147 (3), 1110–1114.e5.

Mishima, T., Muki, F., Kusunose, S., Sano, Y., Nakamura, M., Hirano, T., 2014. Sensory neurons co-opt classical immune signaling pathways to mediate atopic dermatitis. J. Allergy Clin. Immunol. 134 (1), 157–165.

Nakahara, M., 2020b. Implications of IL-13Ralpha2 in atopic skin inflammation. J. Dermatol. 47 (9), 979–984.

Oh, M.-H., Oh, S.Y., Lu, J., Lou, H., Myers, A.C., Zhu, Z., Zheng, T., 2013. TRPAIL-dependent pruritus in IL-13-induced chronic atopic dermatitis. J. Immunol. 191 (11), 5371–5382.

Ohkoma, H., Yoshimatusu, Y., Tomizawa, T., Kunita, A., Takayama, R., Morikawa, T., Komura, D., Takahashi, K., Oshima, T., Sato, M., Komai, K., Podyama-Inoue, K.A., Uchiha, H., Hamada, H., Fujii, K., Ishikawa, S., Fukayama, M., Fukuhara, T., Wazab, T., 2019. Interleukin 13 receptor alpha2 in a novel marker and potential therapeutic target for human melanoma. Sci. Rep. 9, 1281.

Pincelli, C., Heinzel, P., 2013. Recapitulating atopic dermatitis in three dimensions: cross talk between keratinocytes and nerve fibers. J. Invest. Dermatol. 133 (6), 1425–1435.

Prescott, S.L., Noakes, P., Chow, B.W.Y., Brehler, L., Thornton, C.A., Hollams, E.M., Ali, M., van den Bigelaar, A.J.H., Tulic, M.K., 2008. Presymptomatic differences in Toll-like receptor function in infants who have atopy. J. Allergy Clin. Immunol. 122 (2), 391–399.e5.

Renert-Yuval, Y., Guttman-Yassky, E., 2020. New treatments for atopic dermatitis targeting beyond IL-4/IL-13 cytokines. Ann. Allergy Asthma Immunol. 124 (1), 28–35.

Sivaprasad, U., Warrier, M.R., Gibson, A.M., Chen, W., Tabata, Y., Bass, S.A., Grueny, E., Demircioglu, D., Kempf, W., Wang, P.L., Schaller, M., Rocken, M., Gotz, F., Biedermann, T., 2014. 2014. New treatment for pruritus in IL. J. Allergy Clin. Immunol. 134 (1), 1051–1061.e7.

Steinhoff, M., Neisius, U., Puri, R.K., Kawakami, K., Strober, W., 2007. Induction of IL-13 triggers TGF-beta1-dependent tissue fibrosis in chronic 2,4,6-trinitrobenzene sulfonic acid colitis. J. Immunol. 178, 5859–5870.

Sugiyama, Y., Saito, M., Sato, T., Fujita, T., Nakata, T., Ishii, A., Nakane, Y., Ushikusa, Y., 2020. Implication of il-13 receptor in patients with atopic dermatitis. J. Allergy Clin. Immunol. 147 (4), 979–988.

Sugiyama, Y., Tanaka, Y., Ito, T., Tsuji, G., Kido-Nakakura, M., Nakahara, T., Furue, M., 2020a. Genetic and environmental implication of atopic dermatitis. J. Allergy Clin. Immunol. 147 (4), 979–988.

Sugiyama, Y., Tsuchida, T., Furue, M., 2015. Role of IL-31 receptor alpha2 in health and disease. Immunol Rev 202 (1), 191

Sugiyama, Y., Tsuchida, T., Furue, M., 2015. Role of IL-31 receptor alpha2 in health and disease. Immunol Rev 202 (1), 191

Tsuchida, T., Furue, M., 2015. New mechanism underlying IL-31-induced atopic dermatitis. J. Allergy Clin. Immunol. 134 (1), 1051–1061.e7.

Uchida, H., Hamada, H., Fujiu, K., Ishikawa, S., Fukayama, M., Fukuhara, T., Uchida, H., Hamada, H., Fujii, K., Ishikawa, S., Fukayama, M., Fukuhara, T., Wazab, T., 2019. Interleukin 13 receptor alpha2 in a novel marker and potential therapeutic target for human melanoma. Sci. Rep. 9, 1281.

Wu, X., Rosenkilde, S., Strober, W., 2006. IL-13 signaling through the IL-13alpha2 receptor is involved in induction of TGF-beta1 production and fibrosis in chronic 2,4,6-trinitrobenzene sulfonic acid colitis. J. Immunol. 178, 5859–5870.

Xiao, S., 2010. IL-13 receptor alpha 2: a regulator of IL-13 and IL-4 signal transduction in primary human fibroblasts. J. Allergy Clin. Immunol. 126 (1), 13–20.

Yancopoulos, G.D., D. Hamilton, J.D., 2019. Dupilumab progressively improves dermatitis and skin barrier function in patients with atopic dermatitis. J. Allergy Clin. Immunol. 143 (1), 155–172.

Yuki, A., Akiyama, T., Furue, M., Ulzii, D., Nakahara, T., Tsuji, G., Furue, K., Hashimoto-Hachiya, A., Kido-Furue, K., 2020. Novel insights into the TRPV3-mediated itch in atopic dermatitis. J. Allergy Clin. Immunol. 147 (3), 1110–1114.e5.

Zee, J.M., Galon, C., Mashiko, S., Bosonnette, R., McGurk, A., Ziegler, S.F., Dong, C., McKenzie, A.N.J., Sarfati, M., Geha, R.S., 2020. IL2C activation by keratinocyte-derived IL-25 drives IL-13 production at sites of allergic skin inflammation. J. Allergy Clin. Immunol. 145 (6), 1606–1614.e4.

Zhou, Y., Li, X., Sun, W., Metzger, D., Chambon, P., 2006. Topical vitamin D3 and low-calcemic analogs alleviate atopic dermatitis in mouse models. J. Invest. Dermatol. 127 (1), 172–178.

Zhou, Y., Li, X., Sun, W., Metzger, D., Chambon, P., 2006. Topical vitamin D3 and low-calcemic analogs alleviate atopic dermatitis in mouse models. J. Invest. Dermatol. 127 (1), 172–178.

Zhou, Y., Li, X., Sun, W., Metzger, D., Chambon, P., 2006. Topical vitamin D3 and low-calcemic analogs alleviate atopic dermatitis in mouse models. J. Invest. Dermatol. 127 (1), 172–178.
Stott, B., Lavender, P., Lehmann, S., Pennino, D., Durham, S., Schmidt-Weber, C.B., 2013. Human IL-31 is induced by IL-4 and promotes TH2-driven inflammation. J. Allergy Clin. Immunol. 132 (2), 446–454.e5.

Szollosi, A.G., McDonald, I., Szabo, I.L., Meng, J., van den Bogaard, E., Steinhoff, M., 2019. TLR3 in Chronic human itch: a keratinocyte-associated mechanism of peripheral itch sensitization. J. Invest Dermatol. 139 (2393–2396), e2396.

Trier, A.M., Kim, B.S., 2019. Sensory neurons drive anticipatory immunity. Cell 178 (4), 771–773.

Tunis, M.C., Dawod, B., Carson, K.R., Veinotte, L.L., Marshall, J.S., 2015. Toll-like receptor 2 activators modulate oral tolerance in mice. Clin. Exp. Allergy 45 (11), 1690–1702.

Ulzii, D., Kido-Nakahara, M., Nakahara, T., Tsuji, G., Furue, K., Hashimoto-Hachiya, A., Furue, M., 2019. Scratching counteracts IL-13 signaling by upregulating the decoy receptor IL-13Ralpha2 in keratinocytes. Int. J. Mol. Sci. 20.

Vu, A.T., Baba, T., Chen, X., Le, T.A., Kinoshita, H., Xie, Y., Kamijo, S., Hiramatsu, K., Ikeda, S., Ogawa, H., Okumura, K., Takai, T., 2010. Staphylococcus aureus membrane and diacylated lipopeptide induce thymic stromal lymphopoietin in keratinocytes through the Toll-like receptor 2-Toll-like receptor 6 pathway. J. Allergy Clin. Immunol. 126, 985–993, 993.e981–983.

Wang, T.-T., Xu, X.-Y., Lin, W., Hu, D.-D., Shi, W.u., Jin, X., Wang, H., Song, N.-J., Zhang, Y.-Q., Zhang, L., 2020. Activation of different heterodimers of TLR2 distinctively mediates pain and itch. Neuroscience 429, 245–255.

Weidinger, S., Beck, L.A., Bieber, T., Kabašima, K., Irvine, A.D., 2018. Atopic dermatitis. Nat. Rev. Dis. Primers 4 (1). https://doi.org/10.1038/s41572-018-0001-z.

Zurawski, S.M., Vega, F., Huyghe, B., Zurawski, G., 1993. Receptors for interleukin-13 and interleukin-4 are complex and share a novel component that functions in signal transduction. EMBO J. 12 (7), 2663–2670.