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IL-13Rα2 Has a Protective Role in a Mouse Model of Cutaneous Inflammation

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IL-13 is expressed in lesions of atopic dermatitis (AD) and has been associated with increased disease severity. IL-13 has two cog-nate receptors: IL-13Rα1 and IL-13Rα2. Although IL-13Rα2 expression is known to be induced in response to IL-13 in keratinocytes, its function in AD has never been evaluated. We characterized the loss of skin barrier function and the development of cutaneous inflammation in IL-13Rα2-null versus wild-type BALB/c mice following an epicutaneous allergen-sensitization/challenge model that shares similarities with human AD. Mice lacking IL-13Rα2 had significantly increased transepidermal water loss, cutaneous inflammation, peripheral eosinophilia, and IgG1 and IgE levels compared with wild-type mice. The rate of resolution of the cutaneous inflammation was not significantly altered in the IL-13Rα2-null mice. IL-13 induced expression of IL-13Rα2 in keratinocyte cell lines and primary human keratinocytes. Depletion of IL-13Rα2 in a keratinocyte cell line resulted in increased STAT6 signaling in response to IL-13. In conclusion, IL-13Rα2 serves a protective role in the pathogenesis of allergic inflammation and loss of skin barrier function in a mouse model of AD, suggesting that it may be an important endogenous regulator of IL-13-induced cutaneous inflammation in humans. The Journal of Immunology, 2010, 185: 6802–6808.

Abbreviations used in this paper: AD, atopic dermatitis; ASP, Aspergillus; HPR, hypoxanthine phosphoribosyltransferase; KO, knockout; MBP, major basic protein; siRNA, small interfering RNA; TEWL, transepidermal water loss; WT, wild-type.

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Materials and Methods

Mice

IL-13Rα2–deficient mice (27) and BALB/c mice (The Jackson Laboratory, Bar Harbor, ME) were kept in a specific pathogen-free environment. All procedures were performed in accordance with the ethical guidelines in the Guide for the Care and Use of Laboratory Animals of the Institutional Animal Care and Use Committee approved by the Veterinary Services Department of the Cincinnati Children’s Hospital Medical Center Research Foundation.
Epicutaneous Ag sensitization and challenge

The protocol followed was described previously (24), with the following changes. The mice were treated epicutaneously with 200 µg Aspergillus fumigatus extract (Greer Laboratories, Lenoir, NC) in 200 µl sterile saline or saline alone as control. The allergen was applied to a 2-cm2 gauze patch and taped to the back of the mice with Tegaderm, an adhesive bandage, and water-resistant tape. On day 7, the patch was removed, and eosinophil counts were measured by Discombe’s analysis using 5 µl blood obtained from the lateral tail vein, as previously described (28). Twenty-four hours after removing the patch, transdermal water loss (TEWL) was measured, and the patch was reapplied. This was repeated two more times. Twenty-four hours after the last patch, TEWL was measured, and the mice were euthanized following standard procedures.

To assess the resolution of inflammation, mice were subjected to the same protocol above. After the third patch was removed on day 21, TEWL was measured every other day for 8 d (days 23, 25, 27, and 29).

Measurement of TEWL

TEWL was measured using the Dermalab instrument DermaLab USB module (Cortex Technology, Hadsund, Denmark). The temperature and relative humidity of the rooms in which measurements were made did not differ significantly during the measurements. Measurements were recorded as g/m²/hr after the rate of TEWL had stabilized, usually after 60 s, when the SD became ≤0.2. TEWL was assessed on the back of the mice, over the surface where the patch containing allergen was applied. The probe was placed against the skin surface exposed to allergen, and the readings were recorded for 1 min. The probe was removed from the skin, replaced, and a second measurement was taken. An average of the two readings was used as the TEWL for each mouse. The same investigator measured TEWL on all of the mice for each of the experiments.

Skin-scoring system

Mice were visually assessed for excoriations, erythema, and skin thickening in the 2-cm² area covered by the patch. Scores for erythema were 0 (no visible redness) or 1 (redness present). Skin thickening was scored as 0 (thickness comparable to wild-type [WT] mouse skin), 1 (slight thickening of skin), or 2 (significant thickening of the skin). Excoriations were scored as 0 (no scratches), 1 (up to three scratches), 2 (four to eight scratches), 3 (one third of the back), 4 (two thirds of the back), or 5 (the entire back). The measurements were made by two independent investigators, and the average of the scores was recorded for each parameter. The total score from the excoriations, redness, and thickening is presented as the skin score for each mouse.

Tissue processing, measurement of epidermal thickness, and eosinophil quantification

Skin tissues were fixed in 10% formalin immediately after mice were euthanized. Paraffin-embedded tissues were cut into 5-µm sections and stained with H&E, per the manufacturer’s protocol. Epidermal thickness was quantified using the video-assistant computer software program Image Pro Plus 4.1 (Media Cybernetics, Silver Spring, MD). Skin eosinophil numbers were measured by staining skin sections for MBP (major basic protein), as previously described (29). The MBP Ab was a kind gift of Dr. Jamie Lee (Mayo Clinic, Scottsdale, AZ).

IgG1 and IgE ELISAs

Plasma was diluted 1:100 for IgE and 1:10,000 for IgG1. ELISAs were performed using the appropriate kit from BD Biosciences (San Jose, CA). Briefly, ELISA wells were coated with 2 µg/ml anti-mouse IgE (or IgG1) mAb (BD Biosciences) overnight, blocked with 1% BSA for 1 h, washed with PBS/0.05% Tween-20, and then incubated with diluted plasma along with appropriate standards (BD Biosciences) for 1 h. Biotin-conjugated anti-mouse Ig mAb (2 µg/ml; BD Biosciences) was used for detection, followed by incubation with avidin-peroxidase (1 µg/ml; Pierce, Rockford, IL), and development with 2,2′-azino-di-(3-ethyl-benzthiazoline sulfonate) (Sigma-Aldrich, St. Louis, MO). Absorbance at 405 nm was measured within 30 min. For measurement of Aspergillus-specific IgE and IgG1 levels, wells were coated with 0.01% Aspergillus extract (Greer Laboratories) overnight, and the rest of the protocol was carried out as described above.

Cell culture

Neonatal and adult primary human keratinocytes were purchased from Cambrex (East Rutherford, NJ) and cultured in keratinocyte basal medium (KGM; Cambrex) supplemented with bovine pituitary extract, human epidermal growth factor, insulin, hydrocortisone, gentamicin, and amphotericin-B (SingleQuot; Cambrex) at 37°C in 5% CO2. HaCaT cells were kindly provided by Dr. T. Bowden (University of Arizona, Tucson, AZ) and cultured in DMEM medium supplemented with 10% FBS and 1% penicillin/streptomycin from Invitrogen (Carlsbad, CA). Cells were seeded onto 12-well culture plates or T-75 or T-175 flasks; after confluence reached 80%, they were treated with IL-4 (10 ng/ml), IL-13 (50 ng/ml), TNF-α (50 ng/ml), and IFN-γ (10 ng/ml), alone or in combination.

Supernatants and cell lysates

Cells were nonenzymatically detached with Versene (Invitrogen), washed twice in PBS, and resuspended at 5 million cells in 250 µl EMSA lysis buffer (10 mM HEPES [pH 7.9], 10 mM KCl, 0.1 mM EDTA, 1.5 mM MgCl2, 0.2% Nonidet P-40, 1.0 mM DTT; and 0.5 mM PMSF) for 10 min at 4°C, followed by centrifugation and collection of supernatants (cytoplasmic lysates) that were subsequently used for ELISA. STAT6 mRNA was performed as previously described (30).

IL-13Rα2 ELISA

Human IL-13Rα2 was detected by ELISA, as previously described (31), modified only by the use of polyclonal goat anti-human IL-13Rα2 (1 µg/ml; R&D Systems, Minneapolis, MN) for the capture Ab. The standard curve was linear from 100–800 pg/ml. Results are presented as IL-13Rα2 pg/ml of supernatant or pg/mg total protein for cell lysates (total protein determined by Bradford assay).

RT-PCR

Total RNA was isolated from cells using TRIzol reagent (Invitrogen), according to the manufacturer’s instructions, and were treated with DNase (Qiagen, Valencia, CA) before being reverse transcribed with a First-Strand Superscript Synthesis kit (Invitrogen). Conventional PCR analysis was performed using previously published primers for IL-13Rα2 (32) and the following primers: 5′-CCC TGG TGT TCT TCC TGA TAC TCT G-3′ forward and 5′-CAG TAC AGA GTG GTG TTC CTT G-3′ reverse for human IL-13Rα1 and 5′-AAG GTG AAG GTC GGA GTC AAC G-3′ forward and 5′-TGG AAG ATG ATG ATG GTA CTT G-3′ reverse for human GAPDH.

Quantitative analysis of human mRNA expression was done by real-time PCR using the LightCycler 480 and TaqMan Probes Master kit (both from Roche Applied Science, Indianapolis, IN). The primers and hydrolysis probes for IL-13Rα1 (forward 5′-GTC CCT TTA ACT TCC CGT GT-3′ and reverse 5′-CCC ATT GCA CAT ATG CAT C-3′; probe #12), IL-13Rα2 (forward 5′-GCA ATG CAC AAA TGG ATC AG-3′ and reverse 5′-GCC AAT CCT CTA CCA GAT CTT G-3′; probe #113), and hypoxanthine phosphoribosyltransferase (HPRT) (forward 5′-TGGAT TAG ATC TCC TAT GAC TGT AGG-3′ and reverse 5′-CGA ATC CTG ATT GAT TGA G-3′; probe #22) control were selected using the manufacturer’s online software (http://www.roche-applied-sciences/rtcpro/tpc/adp.jsp) and based on the published sequences of each mRNA (National Center for Biotechnology Information). Relative amounts of gene expression were normalized using HPRT expression.

Small interfering RNA-mediated knockdown of IL-13Rα2

The small interfering RNA (siRNA) sequence and protocol used were described previously (33). Briefly, 72 h after transfection with a control siRNA or IL-13Rα2 siRNA, HaCaT cells were treated for 30 min with 10 ng/ml IL-13 and harvested for RT-PCR (to measure the extent of knockdown) and EMSA (to measure STAT6 activity).

Statistical analysis

Reported values are expressed as mean ± SD. All statistical analysis was done using Prism software (GraphPad, San Diego, CA). For all studies, with the exception of that shown in Fig. 1B, statistical significance was assessed using one-way ANOVA, followed by the Tukey–Kramer posttest (for statistical significance between groups). Significance was set at a p value of 0.05. For Fig. 1B, because the score distribution was non-parametric, statistical significance was assessed using the Kruskal–Wallis test, followed by the Dunn posttest for comparison between groups.

Results

IL-13Rα2 protects against increased TEWL in mouse models of AD

Previous studies by our group and other investigators (34) established TEWL as a measure of skin barrier function. Increased
TEWL is associated with AD in children. We also observed an increase in TEWL in the skin of WT mice treated with *Aspergillus* compared with saline-treated mice, and this increase was exacerbated in the IL-13Rα2-null mice treated with *Aspergillus* after the third patch (Fig. 1A).

The skin was visually evaluated for AD-like phenotypes, including redness, thickening, and excoriations. WT *Aspergillus*-treated mice showed an increase in all three phenotypes compared with saline-treated mice. Although the average skin scores do not appear different between the WT and IL-13Rα2-null mice treated with *Aspergillus*, there was a modest increase in the number of mice with higher skin scores among the *Aspergillus*-treated IL-13Rα2-null mice (Fig. 1B). Together, the data indicate an increased disruption in the skin barrier following allergen treatment in the absence of IL-13Rα2.

**Increased sensitization to Aspergillus in the absence of IL-13Rα2**

We next tested whether sensitization to the allergen was affected by the absence of IL-13Rα2. Consistent with previous data (19), IL-13Rα2-null mice had elevated levels of total IgG1 and IgE at baseline. We did not observe a significant increase in IgG1 or IgE levels in the WT *Aspergillus*-treated mice (Fig. 2A, 2B). This could be due to the acute nature of our model; we predict that we will observe increased sensitization in more chronic models of AD, as demonstrated by other studies (24). Following *Aspergillus* treatment, we observed significant increases in total and *Aspergillus*-specific IgG1 and IgE levels in the IL-13Rα2-null mice, indicating that they are more easily sensitized than are WT BALB/c mice (Fig. 2).

**IL-13Ra2 mice have increased eosinophils in the blood but not in the skin**

On histological examination, WT and IL-13Rα2-null mice exhibited epidermal thickening and dermal infiltration of inflammatory cells (Fig. 3A). We measured the increase in epidermal thickness. Although there was a trend toward an increase in the thickness in *Aspergillus*-treated WT mice compared with the saline group, the increase was statistically significant in the *Aspergillus*-treated null mice compared with the saline group (Fig. 3B). To address the possibility that the skin from the IL-13Rα2-null mice had a weakened epidermal barrier, we measured the epidermal thickness in naive WT and IL-13Rα2-null mice. The epidermal thickness for the naive WT and KO mice was 17 ± 6.2 µm and 16.3 ± 2.7 µm, respectively; it was 26.1 ± 9.5 µm and 25.9 ± 5 µm for the saline-treated WT and KO mice, respectively. Although the saline patch caused an increase in the epidermal thickness, there was no difference between the two genotypes, indicating that the absence of IL-13Rα2 does not affect the epidermal barrier in the absence of allergen.

Because we observed an increase in inflammatory cells in the skin, we measured eosinophil numbers in the blood and skin.
Aspergillus treatment. Eosinophil numbers were increased in the blood in WT and IL-13Rα2–null mice following Aspergillus treatment, with a much greater magnitude of increase in the null mice (Fig. 3C). Eosinophil numbers were also elevated in the skin following Aspergillus treatment (not statistically significant by ANOVA). However, the increase in eosinophil numbers in the skin was comparable in both groups of Aspergillus-treated mice (Fig. 3D). Together, the data suggest that the eosinophilic component of the local inflammatory response was not affected by the absence of IL-13Rα2 but that IL-13Rα2 does play a critical role in the epidermal barrier function.

We also measured the number of CD3+ cells in the skin sections and did not observe a significant difference in the WT and IL-13Rα2–null mice (30.8 ± 7.3 and 40.3 ± 17.4 cells/field for WT + Aspergillus and KO + Aspergillus mice, respectively).

IL-13Rα2 is not involved in resolution of changes to the skin barrier

Taken together, the above experiments indicate a clear role for IL-13Rα2 in attenuating the induction of the immune response and skin-barrier function in response to A. fumigatus extract. We next asked whether the resolution of skin-barrier disruption would be different in the IL-13Rα2–null mice. We measured TEWL every other day for 8 d following removal of the third allergen patch applied to the skin. In the WT and IL-13Rα2–null mice, the TEWL returned to baseline by day 8 after removal of allergen, suggesting that the absence of IL-13Rα2 does not result in a sustained inflammatory response (Fig. 4). Levels of IgG1 and IgE remained elevated in the IL-13Rα2–null mice after 8 d (data not shown), suggesting that the increase in sensitization is the consequence, not the cause, of impaired skin-barrier function.

Expression of IL-13Rs is induced in primary neonatal keratinocytes and transformed keratinocytes in response to IL-13

To evaluate a role for IL-13α2 in the skin, we focused our attention on keratinocytes, the critical cell type in the skin that produces proinflammatory cytokines and chemokines (1, 25). Because AD is most prevalent in early childhood, we examined IL-13Rα1 and IL-13Rα2 expression in human primary neonatal keratinocytes following Aspergillus treatment. Eosinophil numbers were increased in the blood in WT and IL-13Rα2–null mice following Aspergillus treatment, with a much greater magnitude of increase in the null mice (Fig. 3C). Eosinophil numbers were also elevated in the skin following Aspergillus treatment (not statistically significant by ANOVA). However, the increase in eosinophil numbers in the skin was comparable in both groups of Aspergillus-treated mice (Fig. 3D). Together, the data suggest that the eosinophilic component of the local inflammatory response was not affected by the absence of IL-13Rα2 but that IL-13Rα2 does play a critical role in the epidermal barrier function.
Cells were treated with IL-4, IL-13, TNF-α, or IFN-γ, individually or in combination, and IL-13Rα1 and IL-13Rα2 mRNA expression was determined by quantitative PCR. Consistent with studies by Purwar et al. (35), expression of IL-13Rα2, but not IL-13Rα1, was dependent upon the cytokine milieu (Fig. 5A, 5B) in the HPKs. IL-4, IL-13, and TNF-α, but not IFN-γ, led to significant increases in IL-13Rα2 mRNA (Fig. 5A). TNF-α acted synergistically with IL-4 and IL-13. Strikingly, IL-4 and TNF-α together induced a 25-fold greater IL-13Rα2 message compared with that of untreated neonatal keratinocytes (p < 0.001). Similar results were seen in the HaCaT cells (Fig. 6A) and primary adult keratinocytes (data not shown).

We also examined the kinetics of IL-13Rα1 and IL-13Rα2 induction in neonatal keratinocytes (Fig. 5C). IL-13Rα2 mRNA was induced by 4 h of stimulation with IL-4 or IL-13, individually or in combination with TNF-α. By 12 h, the combination of IL-4 or IL-13 plus TNF-α resulted in greater IL-13Rα2 expression compared with the individual cytokines alone. Incubation with TNF-α alone also led to slightly elevated expression, but only at 24 h. There was no effect observed with IFN-γ alone (data not shown). Similar trends were observed for adult keratinocytes, and there was no significant effect on IL-13Rα1 expression at any time point (data not shown).

We confirmed that IL-13Rα2 protein was induced on the surface of HaCaT cells after IL-4 and IL-13 stimulation by flow cytometry (Fig. 6B). IL-13 stimulation increased surface expression compared with IL-4. TNF-α synergized with IL-4 and IL-13 to increase IL-13Rα2 surface expression. Cytokine stimulation with IL-4 or IL-13, alone or in combination with TNF-α, did not alter surface expression of IL-13Rα1 (data not shown).

Having demonstrated IL-13Rα2 surface induction on HaCaT cells after cytokine stimulation, we confirmed that this was due to an increase in total IL-13Rα2 protein and was not simply the result of increased mobilization from intracellular stores to the surface. To this end, we measured total IL-13Rα2 protein in HaCaT cell lysates after incubation with or without cytokines. As depicted in Fig. 6C, IL-4 and IL-13, individually or in combination with TNF-α, increased total IL-13Rα2 protein.

Depletion of IL-13Rα2 results in increased STAT6 activation following IL-13 treatment

To evaluate the role of increased IL-13 Rα2 expression in keratinocytes, we assayed STAT6 activity following IL-13 treatment in HaCaT cells in which IL-13Rα2 had been depleted using siRNA. We verified the decrease in mRNA levels by RT-PCR (Fig. 7A). We observed an increase in STAT6 activation in the control
siRNA-treated cells 30 min following addition of IL-13 and a further increase in the absence of IL-13Rα2, supporting its role as a decoy receptor in the skin (Fig. 7B, 7C).

Discussion
AD results in significant morbidity for patients and families, with associated health care costs ranging from $0.9–3.8 billion (36). Current therapeutics broadly target allergic inflammatory cascades, but it would be advantageous to develop agents specifically targeting the key mediators in AD. IL-13 plays a central role in allergic inflammation and has been implicated in AD pathogenesis, yet little is known regarding the regulation of IL-13 responses in the skin. This gap in understanding has hindered the development of novel therapeutic interventions for this disease targeting IL-13 or its signaling pathways. In this study, we demonstrated that Th2 cytokines, as well as allergic cutaneous inflammation in vivo, result in a preferential induction in the expression and distribution of IL-13Rα2 compared with IL-13Rα1. Because IL-13Rα2 binds IL-13 with high affinity and can impact IL-13 responses, even small variations in expression or distribution are likely to have biological significance. Elucidating the mechanisms that govern these changes in IL-13Rα2 in the skin may yield new insights into the regulation of Th2 inflammation in AD and potentially lead to new pharmacotherapeutic options.

To examine the role of IL-13Rs in vivo, we adapted a well-described murine model of epicutaneous sensitization and challenge that shares many characteristics with AD, including gross cutaneous inflammation, epidermal thickening, dermal inflammatory cell infiltrate, and peripheral eosinophilia. In this article, we demonstrate that in the absence of IL-13Rα2, each of the characteristics of AD described above is exacerbated, supporting a role for IL-13Rα2 as a negative regulator of cutaneous allergic inflammation. Consistent with this mechanism, STAT6 activation by IL-13 is significantly enhanced in keratinocytes in which IL-13Rα2 is depleted. Our findings are consistent with a recent report that IL-13Rα2 deletion exacerbates the effect of IL-13 overexpression on the development of experimental eosinophilic esophagitis, a disease that shares several features with AD, including hyperproliferation and fibrosis of squamous epithelium (37).

HaCaT keratinocytes and HPKs express functional IL-13Rs, and we observed that IL-4 and IL-13 increase IL-13Rα2 without altering IL-13Rα1 expression. These findings support previous studies in HaCaT cells by David et al. (25, 38, 39) but contrast those of Wongpiyabovorn et al. (40) in HPKs. The latter group reported that only IL-4 combined with IFN-γ, but not IL-4 or IL-13 alone, upregulated IL-13Rα2 mRNA. Additionally, they observed that IFN-γ and IL-13 could upregulate the mRNA expression of IL-13Rα1 in HPKs. Potential reasons for the disparate results could be the investigators’ use of conventional PCR and measurements taken at only one time point versus our use of quantitative real-time PCR and multiple time points. Furthermore, the increase in IL-13Rα2 mRNA in our experiments correlated with induction of IL-13Rα2 protein in keratinocytes. These other studies did not examine IL-13Rα2 protein levels.

Recent studies indicate that IL-13Rα2 may have a dual role in the pathogenesis of Th2 inflammation. First, IL-13Rα2 serves as a decoy receptor through its high IL-13–binding affinity, short cytoplasmic tail, and lack of a definitive signaling motif, as well as its existence as a soluble receptor. This inhibitory role is supported by in vitro studies demonstrating that IL-13Rα2 overexpression leads to decreased IL-13 signaling and in vivo studies using IL-13Rα2 gene-targeted mice (19–21). Because IL-13 can induce IL-13Rα2, the receptor functions as part of a self-regulatory feedback loop designed to attenuate IL-13 responses. Second, IL-13Rα2 may contribute to inflammation through TGF-β, as demonstrated recently by Fichtner-Feigl et al. (22) in murine models of oxalazzone-induced colitis and bleomycin-induced lung fibrosis. These opposing roles of IL-13Rα2 may hinge on tissue specificity, because the mechanisms by which IL-13 mediates its own effects were shown to differ between tissues. For example, IL-13–mediated lung fibrosis is TGF-β dependent (41), whereas IL-13–mediated liver fibrosis following Shistosoma mansoni infection is TGF-β independent (42).

Further evidence for the complex role of IL-13Rα2 in regulating Th2 inflammatory responses comes from studies demonstrating that the membrane-bound receptor can inhibit IL-4 signaling in glioblastoma cells and primary human fibroblasts (43, 44). The relative distribution of IL-13Rα2 within the subcellular compartments of various tissues may also be a factor in determining whether the cumulative effect of the receptor is proinflammatory or anti-inflammatory. Alternative splicing of IL-13Rα2 in mice leads to distinct membrane-bound and soluble IL-13Rα2, and we demonstrated differential regulation of these two transcripts in vivo in an experimental allergic asthma model (45). These alternatively spliced variants of IL-13Rα2 may have distinct biologic functions. In conclusion, our research demonstrates that IL-4 and IL-13 can induce total IL-13Rα2 in skin cells, and this receptor modulates IL-13 signaling pathways in keratinocytes. Although further studies are warranted to delineate the role of this receptor in cutaneous Th2 inflammation, our data suggest that IL-13Rα2 may be a useful biomarker of AD and likely has a key role in other allergic disorders.

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