A key role for IL-13 signaling via the type 2 IL-4 receptor in experimental atopic dermatitis

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IL-13 and IL-4 are potent mediators of type 2–associated inflammation such as those found in atopic dermatitis (AD). IL-4 shares overlapping biological functions with IL-13, a finding that is mainly explained by their ability to signal via the type 2 IL-4 receptor (R), which is composed of IL-4Rα in association with IL-13Rα1. Nonetheless, the role of the type 2 IL-4R in AD remains to be clearly defined. Induction of two distinct models of experimental AD in Il13ra1−/− mice, which lack the type 2 IL-4R, revealed that dermatitis, including ear and epidermal thickening, was dependent on type 2 IL-4R signaling. Expression of TNF-α was dependent on the type 2 IL-4R, whereas induction of IL-4, IgE, CCL24, and skin eosinophilia was dependent on the type 1 IL-4R. Neutralization of IL-4, IL-13, and TNF-α as well as studies in bone marrow–chimeric mice revealed that dermatitis, TNF-α, CXL1, and CCL11 expression were exclusively mediated by IL-13 signaling via the type 2 IL-4R expressed by nonhematopoietic cells. Conversely, induction of IL-4, CCL24, and eosinophilia was dependent on IL-4 signaling via the type 1 IL-4R expressed by hematopoietic cells. Last, we pharmacologically targeted IL-13Rα1 and established a proof of concept for therapeutic targeting of this pathway in AD. Our data provide mechanistic insight into the differential roles of IL-4, IL-13, and their receptor components in allergic skin and highlight type 2 IL-4R as a potential therapeutic target in AD and other allergic diseases such as asthma and eosinophilic esophagitis.

INTRODUCTION

Atopic dermatitis (AD) is a common chronic inflammatory skin disease affecting about 230 million people worldwide. Patients with AD are classified into two endotypes, namely, extrinsic and intrinsic AD (1). Whereas T helper 1 (TH1), TH17, and TH22 polarization differs between AD endotypes, a strong TH12 axis is associated with both extrinsic and intrinsic AD (2). In particular, interleukin-4 (IL-4) and IL-13 are produced at elevated levels in the lesional skin and are central regulators of many of the hallmark features of AD, including epidermal hyperplasia, skin barrier dysfunction, and production of eosinophil and T cell chemokines (3, 4). The importance of IL-4, IL-13, and their associated receptors in AD is best exemplified by the ongoing pursuit to pharmacologically target these cytokines and/or their signaling components in AD (5, 6). More recently, dupilumab, an IL-4 receptor α (IL-4Rα) antagonist antibody, was approved in 2017 to treat patients with moderate-to-severe AD whose disease is not adequately controlled with topical prescription therapies or in cases when those therapies are not advisable (7). Thus, defining the relative contribution of IL-4, IL-13, and their signaling receptor chains to the development of AD is of great importance.

IL-4 shares overlapping biological functions with IL-13 (8–11), and both cytokines drive cellular signals via a complex network of receptors. IL-4 mediates its effects through either the type 1 IL-4R (i.e., IL-4Rα in association with the common γ chain) or the type 2 IL-4R (i.e., IL-4Rα in association with IL-13Rα1). Similar to IL-4, IL-13 delivers IL-4Rα-dependent effects by binding IL-13Rα1 and initiating signals via the type 2 IL-4R (12). Nonetheless, IL-13 may use a signaling complex that does not require IL-4Rα (13). In addition, IL-13Rα2, which has been previously suggested to be an IL-13 decay receptor (14–16), has been recently reported to mediate IL-13–driven activation and to act as an IL-13 signaling component (17, 18). Because IL-4 and IL-13 are increased in AD and these two cytokines can activate a type 2 IL-4R–dependent response, the relative contribution of IL-4 signaling via the type 1 or type 2 IL-4R in AD remains to be defined. Adding to this complexity, previous studies have shown that IL-13 is necessary for induction of dermatitis and transgenic overexpression of IL-13 in the skin induces dermatitis and skin remodeling (19, 20). Nonetheless, whether this is driven by IL-13Rα1 or IL-13Rα2 needs to be better clarified.

A valuable way to distinguish the contribution of the type 2 IL-4R is by genetic deletion of the IL-13Rα1 chain. Such genetically engineered mice harbor a functional deletion of the type 2 IL-4R but have an intact type 1 IL-4R (21, 22). Using this approach, we have previously shown that the clinical features of experimental asthma (e.g., airway hypersensitivity, mucus production, and fibrosis) are driven by IL-4 and IL-13 signaling via the type 2 IL-4R (21, 23). Nonetheless, whether the development of AD relies on a similar requirement is unknown.

Here, we show that the type 2 IL-4R is required for the development of oxazolone (OX)– and 2,4-dinitrofluorobenzene (DNFB)–triggered dermatitis in mice. We demonstrate that dermal and epidermal thickening, as well as tumor necrosis factor–α (TNF-α), CXL1, and CCL11 expression are dependent on IL-13 signaling via the type 2 IL-4R, which is expressed on nonhematopoietic cells. Furthermore, we establish in vivo and in vitro proofs of concept for targeting IL-13Rα1 in atopic diseases. Our data provide mechanistic insight into the differential roles of IL-4, IL-13, and their receptor components in the allergic skin and highlight the type 2 IL-4R as a target for future therapeutic approaches.
RESULTS

IL-13Rα1 is differentially expressed by hematopoietic and nonhematopoietic cells in the skin

To determine the role of the type 2 IL-4R in AD, we used a chronic model of repetitive skin challenges with OX, which has been shown to induce a Th2-dominated inflammatory response that is similar to human AD (5, 24). First, we aimed to determine the cellular source for IL-13Rα1 expression in the skin. Under baseline conditions, among the hematopoietic lineage, IL-13Rα1 was highly expressed by monocytes and neutrophils and, to a lesser extent, by eosinophils and lymphocytes. Expression of IL-13Rα1 was observed on the surface of nonhematopoietic cells as well, with nearly no expression by fibroblasts [defined as CD45+/platelet-derived growth factor receptor α+ (PDGFRα+) cells], which may express type 2 IL-4R (25). Assessment of IL-13Rα1 expression in OX-challenged mice revealed that the expression of IL-13Rα1 was stable on most cells. Nonetheless, a marked reduction was observed on the surface of monocytes (fig. S1), whereas IL-13Rα1 was increased on the surface of macrophages (fig. S1).

OX- and DNFB-induced dermatitis are mediated via the type 2 IL-4R

To define the role of the type 2 IL-4R in atopic skin disease, wild-type (WT) and Il13ra1−/− mice were challenged with OX. OX-challenged WT mice displayed increased ear thickening (Fig. 1A and fig S2), but ear thickness in OX-challenged Il13ra1−/− mice was comparable to control (acetone-treated) mice (Fig. 1A). Histopathological assessment of the skin obtained from OX-challenged WT mice revealed hyperkeratosis, increased numbers of nuclei, and epidermal thickening (Fig. 1, B and C). OX-challenged Il13ra1−/− mice displayed decreased numbers of nuclei and decreased epidermal thickening (Fig. 1, B and C).

To further establish the role of the type 2 IL-4R in dermatitis, WT and Il13ra1−/− mice were treated with DNFB (26). DNFB-challenged Il13ra1−/− mice displayed decreased dermatitis as observed by decreased ear and epidermal thickening in comparison with DNFB-treated WT mice (fig. S3).

Next, we were interested in determining the role of the type 2 IL-4R in the regulation of proinflammatory cytokines such as TNF-α, which has been shown to be involved in AD (27). Skin expression of TNF-α after OX and DNFB challenge was dependent on the type 2 IL-4R (Fig. 1D and fig S3). IL-4 is a key cytokine mediating the induction of immunoglobulin E (IgE), which serves as an important biomarker and effector of Th2 immunity and atopy (28). Assessment of skin IL-4 levels revealed that although IL-4 was decreased in OX-challenged Il13ra1−/− mice (Fig. 1E), an IL-13Rα1–independent pathway exists for induction of IL-4. Serum IgE levels in WT mice were elevated after OX treatment (Fig. 1F). OX-challenged Il13ra1−/− mice exhibited slightly higher serum IgE levels in comparison with OX-challenged WT mice (Fig. 1F). IL-4 was induced to a similar extent in DNFB-treated WT and Il13ra1−/− mice (fig. S3). Serum IgE was slightly (but statistically significantly) decreased in DNFB-treated Il13ra1−/− mice (fig. S3).

Skin chemokine production and subsequent cellular migration is differentially regulated by the type 2 IL-4R

The inflammatory milieu in AD is characterized by the induction of various chemokines promoting cellular migration (29). Therefore, we were interested to determine whether the type 2 IL-4R regulates chemokine production and subsequent cellular influx into the skin. OX-challenged WT mice displayed increased expression of CXCL1, CCL11, and CCL24 (Fig. 1, G to I). Induction of CXCL1 and CCL11 in response to OX challenge was dependent on the type 2 IL-4R (Fig. 1, G and H), whereas OX-induced CCL24 expression was independent of the type 2 IL-4R (Fig. 1I). CCL17 expression was modestly increased by OX treatment, and this increase was also dependent on the type 2 IL-4R (Fig. 1I). Consistent with these findings, cellular infiltration of leukocytes was differentially regulated by the type 2 IL-4R. OX-challenged WT mice displayed increased accumulation of various CD45+ cells predominantly consisting of neutrophils (defined as CD11b+/Ly6G+Ly6Cmed cells), eosinophils (defined as CD11b+/Ly6G−/Ly6C−/SiglecF+ cells), as well as T cells (defined as SScd4+/SScnd4−/CD11b−/CD3+ cells) and macrophages (defined as CD11b+/Ly6G−/Ly6C−/SiglecF−/CD11c+ cells) (Fig. 1K).

Whereas neutrophil accumulation was dependent on the type 2 IL-4R, accumulation of T cells and macrophages was regulated to a lesser extent and eosinophil accumulation was independent of the type 2 IL-4R (Fig. 1K).

Similar to our findings in the OX model, the expression of CCL11 and CCL24 (but not CCL24) was decreased in the ears of DNFB-treated Il13ra1−/− mice in comparison with WT mice (fig. S3). Consistently, infiltration of neutrophils and T cells into the skin of DNFB-treated mice was reduced in the absence of Il13ra1, whereas infiltration of eosinophils was not affected by the lack of Il13ra1 (fig. S3). Together, these data demonstrate that the type 2 IL-4R differentially mediates accumulation of immune cells in the skin and highlight a type 2 IL-4R–independent mechanism for eosinophil recruitment into the skin in AD.

OX-induced dermatitis is mediated by IL-13 and IL-4 via the type 2 IL-4R

The type 2 IL-4R mediates signals from both IL-4 and IL-13 (3). Thus, we were interested to determine the cellular source for IL-4 and IL-13 in OX-challenged mice. To this end, IL-4 and IL-13 reporter mice (4Get and Il13smarm, respectively) were treated with OX. In this experimental setting, cells that have the capacity to secrete IL-4 are marked by enhanced green fluorescent protein (eGFP) (30), and cells that express endogenous IL-13 are marked by the expression of the human CD4 surface marker (31). Although we could not detect IL-13 expression in skin homogenates by enzyme-linked immunosorbent assay (ELISA), ~25% of all skin CD4+ T cells stained positive with anti-human CD4 (fig. S4). Human CD4 was also detected on the surface of 1.47% of γδ T cells and 2.07% of natural killer (NK) T cells. CD8+ T cells were negative for human CD4 expression (fig. S4). Assessment of eGFP+ T cells in the skin of IL-4 reporter mice revealed that the cellular sources for IL-4 expression after OX challenge were NKT cells (~12.5%), CD4+ T cells (~8.5%), and a small proportion of CD8+ T cells (~3.5%) (fig. S4).

We examined the relative contribution of IL-4 and IL-13 via the type 2 IL-4R to OX-induced pathology and neutralized IL-4 using an anti–IL-4 antibody in OX-challenged WT and Il13ra1−/− mice (32). In this experimental setting, comparison between WT and Il13ra1−/− mice in the isotype control–treated mice reflects the contribution of the type 2 IL-4R to disease pathology, similar to the experimental setting that was shown in Fig. 1 (Fig. 2A). Comparison between WT mice treated with isotype control and WT mice treated with anti–IL-4 revealed the relative contribution of IL-4 to OX-induced pathology because these mice can still mediate IL-13 signaling via the type 2 IL-4R (Fig. 2A). Comparison between Il13ra1−/− mice
treated with isotype control and \(\text{Il13ra1}^{-/-}\) mice treated with anti–IL-4 will reveal the relative contribution of IL-4 via the type 1 IL-4R to OX-induced pathology (Fig. 2A). Last, comparing OX-induced responses in WT and \(\text{Il13ra1}^{-/-}\) mice that have been treated with anti–IL-4 will reveal the relative contribution of IL-13 via the type 2 IL-4R to OX-induced pathology (Fig. 2A).

Consistent with the results shown in the previous section (Fig. 1), isotype control–treated \(\text{Il13ra1}^{-/-}\) mice displayed decreased OX-induced dermatitis as observed by ear and epidermal thickness measurements (Fig. 2, B to D). Neutralization of IL-4 did not further protect OX-challenged \(\text{Il13ra1}^{-/-}\) mice from ear thickening (Fig. 2C). Histopathological analysis of the skin demonstrated a statistically significant but minor effect for IL-4 neutralization on OX-induced epidermal thickening in WT mice (Fig. 2, B and D). Nonetheless, these mice still displayed a thicker epidermis than OX-challenged \(\text{Il13ra1}^{-/-}\) mice, which were treated with either isotype control or anti–IL-4 antibody (Fig. 2D). The finding that anti–IL-4 treatment did not reduce epidermal thickness in OX-challenged \(\text{Il13ra1}^{-/-}\) mice demonstrates that epidermal thickening is not mediated by the type 1 IL-4R and is likely driven by IL-4 and IL-13 signaling via the type 2 IL-4R.

TNF-\(\alpha\) levels were decreased in OX-challenged \(\text{Il13ra1}^{-/-}\) mice regardless of anti–IL-4 antibody treatment and remained up-regulated in OX-challenged WT mice despite anti–IL-4 treatment (Fig. 2E). Thus, proinflammatory cytokine production in OX-induced dermatitis is exclusively mediated by IL-13 signaling via the type 2 IL-4R.

IL-4 levels in the skin were abolished in mice treated with the IL-4–neutralizing monoclonal antibody (mAb), thus validating the efficiency of the anti–IL-4 treatment (Fig. 2F). Reduced expression of IL-4 was not due to competition of the neutralizing antibody with our ELISA kits (21). Consistent with our previous observation (Fig. 1F), IgE levels in isotype-treated, OX-challenged \(\text{Il13ra1}^{-/-}\)
mice were slightly higher (albeit not statistically significant) than those in isotype control–treated, OX-challenged WT mice (Fig. 2G). Neutralizing IL-4 in OX-challenged WT and Il13ra1−/− mice caused a marked reduction in serum IgE levels (Fig. 2G). Thus, IgE production after OX challenge is exclusively mediated by IL-4 signaling through the type 1 IL-4R.

Immune cell infiltration in OX-induced dermatitis is synergistically mediated by the type 1 and type 2 IL-4Rs

To further understand the roles of IL-4, IL-13, and the type 2 IL-4R in OX-induced dermatitis, we assessed the expression of various chemokines in OX-challenged WT and Il13ra1−/− mice that were treated with an IL-4–neutralizing mAb. Similar to our previous findings (Fig. 1), OX-challenged Il13ra1−/− mice that were treated with isotype control displayed significant reductions in CXCL1, CCL11, and CCL17, but not CCL24 (Fig. 2, H to K). Expression of CXCL1, CCL11, and CCL17 in OX-challenged Il13ra1−/− mice was dependent on IL-13 signaling via the type 2 IL-4R because no further reduction was observed in the levels of these chemokines in anti–IL-4–treated, OX-challenged WT or Il13ra1−/− mice (Fig. 2, H, I, and K). Anti–IL-4–treated, OX-challenged WT and Il13ra1−/− mice displayed a marked reduction in CCL24 levels, which was reduced to the same
extent in these mice after anti–IL-4 treatment (Fig. 2J). Thus, OX-induced CCL24 expression is primarily dependent on IL-4 signaling via the type 1 IL-4R.

Immunophenotyping of OX-challenged skin after anti–IL-4 antibody treatment revealed differential contribution of IL-4 and IL-13 via the type 1 and type 2 receptors to infiltrating cells into the skin, as observed by assessment of total CD45+ cells (Fig. 2L). Infiltration of macrophages and T cells, which were decreased in OX-challenged, isotype-treated Il13ra1−/− mice, was not further reduced after anti–IL-4 treatment (Fig. 2, M and N). Neutrophil and monocyte accumulation, which was decreased in isotype control–treated IL-4 treatment (Fig. 2, M and N). Neutrophil and monocyte accumulation, which was decreased in isotype control–treated Il13ra1−/− mice after OX challenge, was further reduced after anti–IL-4 treatment (Fig. 2, O and P). Eosinophilic infiltration was not altered in OX-challenged, isotype control–treated Il13ra1−/− mice but was reduced after anti–IL-4 treatment (Fig. 2Q). Collectively, these data demonstrate that in OX-challenged mice, IL-13 signaling via the type 2 IL-4R regulates T cell and macrophage accumulation, whereas neutrophil and monocyte accumulation is dependent on both IL-13 signaling via the type 2 IL-4R as well as IL-4 interacting with the type 1 IL-4R (Fig. 2, M to P). Eosinophilic infiltration was dependent on IL-4 signaling via the type 1 IL-4R (Fig. 2Q).

**IL-13 is required for OX-induced dermatitis, TNF-α, CCL11, CCL17, and neutrophilic infiltration into the skin**

To better establish the respective roles of IL-13 and IL-4, we undertook a pharmacological approach whereby IL-13 was neutralized in OX-treated WT mice using a commercial rat anti-mouse IL-13 IgG1 antibody. Neutralization of IL-13 was confirmed by complete reduction in soluble IL-13Ra2 (sIL-13Ra2) expression in anti–IL-13–treated mice (Fig. 3A). Similar to our findings in Il13ra1−/− mice (Fig. 1), neutralization of IL-13 resulted in a significant reduction in ear thickness, epidermal thickness, and TNF-α expression in the skin (Fig. 3, B to D). Furthermore, neutralization of IL-13 caused a marked reduction in the expression of CCL11 and CCL17 but had no effect on CCL24 expression (Fig. 3E). Consistent with our findings in OX-challenged Il13ra1−/− mice (Fig. 1), neutralization of IL-13 resulted in decreased infiltration of neutrophils to the skin, whereas eosinophilic levels were not affected (Fig. 3, F and G).

Because TNF-α levels were reduced in OX-challenged Il13ra1−/− mice and were also reduced after neutralization of IL-13, we were interested to define whether decreased neutrophilia was due to decreased TNF-α expression. To this end, TNF-α was neutralized in acetone- and OX-treated WT mice. Neutralization of TNF-α had no effect on ear thickness (Fig. 3H), CCL24 expression (Fig. 3I), or infiltration of neutrophils into the skin (Fig. 3J).

**OX-induced dermatitis is mediated by the type 2 IL-4R expressed on nonhematopoietic cells**

IL-13Rα1 is expressed by hematopoietic and nonhematopoietic cells (fig. S1). Thus, we aimed to determine the relative contribution of these lineages to the type 2 IL-4R–dependent response. To this end, bone marrow–chimeric mice were generated by transferring bone marrows that were obtained from either C57BL6 CD45.1 mice (herein WT) or C57BL6 Il13ra1−/− mice (expressing the CD45.2 variant) into WT or Il13ra1−/− mice. Engraftment efficiency was determined in the peripheral blood by flow cytometry using CD45.1 and CD45.2 antibodies. All mice exhibited nearly complete leukocyte engraftment (fig. S5).

In comparison with WT recipient mice, and irrespectively of the donor mice, Il13ra1−/− recipient mice displayed decreased OX-induced ear thickness (Fig. 4A) and decreased epidermal thickness (Fig. 4, B and C). Il13ra1−/− mice that received WT bone marrow cells were protected from OX-induced ear thickening exactly to the same extent as
the control Il13ra1−/− mice (P = 0.9999; Fig. 4A). These data demonstrate that expression of the type 2 IL-4R on nonhematopoietic cells is necessary for the development of OX-induced dermatitis. Similarly, skin TNF-α levels were decreased in Il13ra1−/− recipient mice, regardless of the genotype of the graft bone marrow (Fig. 4D).

Skin IL-4 levels were increased in OX-challenged, Il13ra1−/− recipient mice irrespective of the donor bone marrow (Fig. 4E). Consistent with this finding, serum IgE levels in OX-challenged, Il13ra1−/− recipient mice were slightly higher than those of the OX-challenged WT mice (Fig. 4F). This finding suggests a suppressive pathway for IgE production, which is mediated by the type 2 IL-4R that is expressed by nonhematopoietic cells.

Expression of CXCL1 and CCL11 was dependent on the type 2 IL-4R, which is expressed by nonhematopoietic cells, as their expression was decreased in OX-challenged, Il13ra1−/− recipient mice, regardless of genotype of the bone marrow donor (Fig. 4, G and H). Although CCL24 levels were comparable among WT mice and Il13ra1−/− mice, which received Il13ra1−/− bone marrow cells (Fig. 4I), CCL24 was markedly increased in Il13ra1−/− mice that received bone marrow cells from WT mice (Fig. 4I).

Immunophenotyping of the cellular infiltrate in OX-challenged mice after bone marrow transfer revealed that accumulation of neutrophils and macrophages (Fig. 4, J to L) in OX-challenged skin was markedly suppressed in recipient Il13ra1−/− mice irrespective of bone marrow donor. Monocyte and eosinophil numbers in the skin were comparable in all groups (Fig. 4N). These data demonstrate that expression of CXCL1 and accumulation of neutrophils after OX challenges are mediated by nonhematopoietic cells via the type 2 IL-4R.

**Generation of a mouse IL-13Rα1–neutralizing mAb**

Our data suggested that neutralization of the type 2 IL-4R, i.e., by blocking IL-13Rα1, may be a beneficial approach for treating AD. Thus, we aimed to generate an IL-13Rα1–neutralizing mAb. To this end, Il13ra1−/− mice were immunized and boosted with the extracellular domain (ECD) of *Mus musculus* IL-13Rα1 in the presence of adjuvant (see Supplementary Methods). Thereafter, mice were bled,
and their antisera were tested for the ability to bind plate-immobilized IL-13Rα1 ECD (Fig. S6, A and B). Subsequently, antisera were examined for receptor neutralizing activity using IL-13–stimulated MC38 cells (Fig. S6C). Serum from mouse #6, which also displayed the highest antibody titer (Fig. S6A), was capable of inhibiting IL-13–induced responses in vitro, suggesting the presence of neutralizing antibodies. Subsequently, bone marrow cells were obtained from mouse #6, plasma cells were sorted (CD138+), and then antibody genes coding for variable heavy (VH) and variable light (VL) fragments were cloned as single-chain variable fragments (scFvs) into a yeast display vector. Yeast display was applied to enrich mouse IL-13Rα1 (mIL-13Rα1) ECD-specific clones (see illustration in fig. S7). After several enrichment steps, a mIL-13Rα1 ECD-specific scFv was isolated, which was then reformatted to a mouse IgG1 anti–IL-13Rα1 mAb termed “MS8.”

In vitro, MS8 was capable of binding mIL-13Rα1 [median effective concentration (EC50) = 1.46 nM, by ELISA] but not human IL-13Rα1 (hIL-13Rα1) or streptavidin, which was used as a control antigen (Fig. 5A). Furthermore, MS8 dose-dependently inhibited IL-13–induced secretion of sIL-13Rα2 by MC38 cells (Fig. 5B). MS8 was capable of partially inhibiting IL-4–induced effects in MC38 cells as well (Fig. 5C). MS8 did not impair TNF-α–induced IL-6 production by MC38 cells (Fig. 5D), demonstrating a specific inhibitory effect on IL-13 and IL-4 through the type 2 IL-4R. Furthermore, MS8 was capable of binding spleen monocytes (CD11b+Ly6GmedLy6C high) from WT mice. Yet, it did not show any binding to spleen monocytes obtained from Il13ra1−−/− mice (Fig. 5E). To determine whether MS8 can be used pharmacologically in vivo, we tested its capacity to neutralize IL-13 in an acute model of IL-13–induced eosinophilic lung inflammation where mice are intratracheally challenged with IL-13 (33). Consistent with its capability to neutralize IL-13 in vitro, IL-13–challenged mice, which were intraperitoneally treated with MS8, exhibited reduced levels of CCL24 in their bronchoalveolar lavage fluid (BALF) compared with isotype control–treated mice (Fig. 5F). Decreased CCL24 expression was accompanied with a marked reduction in eosinophil BALF percentage and total cell counts (Fig. 5G and H). These results demonstrate that MS8 is a pharmacologically functional mIL-13Rα1 antagonist.

**Antibody-mediated neutralization of the type 2 IL-4R protects mice from OX-induced dermatitis**

Next, we sought to determine whether in vivo neutralization of the type 2 IL-4R via targeting IL-13Rα1 may protect mice from OX-induced dermatitis. Accordingly, WT mice were challenged with OX and treated with either isotype control or MS8. OX-challenged mice that were treated with MS8 displayed a marked decrease in ear thickness (Fig. 6A). Histopathological assessment of the skin obtained from MS8–treated mice revealed decreased numbers of nuclei and decreased epidermal thickening (Fig. 6B and C).

Furthermore, mice treated with MS8 displayed significantly decreased
expression of TNF-α (Fig. 6D). Neutralization of IL-13Rα1 resulted in slightly increased expression of IL-4 (Fig. 6E). Nonetheless, no differences in serum IgE were observed between MS8 and isotype-treated mice (P = 0.065; Fig. 6F).

**Antibody-mediated neutralization of the type 2 IL-4R decreases OX-induced chemokine production and subsequent cellular migration**

Similar to the results that were obtained for OX- and DNFB-challenged Il13ra1−/− mice (Fig. 1), mice treated with MS8 displayed decreased expression of CXCL1 and CCL11 in response to OX challenge (Fig. 6, G and H). Although CCL24 expression was slightly reduced by MS8 treatment, this did not reach statistical significance (Fig. 6I). Subsequently, cellular infiltration of leukocytes after MS8 treatment was also assessed. OX-challenged, MS8-treated mice displayed decreased total leukocyte infiltration (Fig. 6I). Decreased cellular infiltration in response to MS8 treatment was evident in T cells, macrophages, and neutrophils (Fig. 6J). Despite our finding that eosinophilic infiltration after OX challenge was independent of IL-13Rα1, MS8 treatment resulted in significantly decreased eosinophilia as well (Fig. 6J). Collectively, these data suggest that targeting IL-13Rα1 in AD may have beneficial therapeutic value.

**Generation of a hIL-13Rα1–neutralizing mAb**

Prompted by the ability of anti-mouse IL-13Rα1 mAbs to alleviate experimental AD, we aimed to generate anti-human IL-13Rα1 mAbs. To this end, Il13ra1−/− mice were immunized and boosted with the ECD of hIL-13Rα1 in the presence of adjuvant. Thereafter, mice were bled and their antisera were examined for the ability to bind plate-immobilized hIL-13Rα1 ECD (fig. S8, A and B), and the ability of the antisera to neutralize hIL-13Rα1–dependent receptor activation was assessed using IL-13–stimulated A549 cells (fig. S8C). Serum from mouse #2 was capable of inhibiting IL-13–induced responses in vitro, suggesting the presence of neutralizing antibodies. Subsequently,
bone marrow cells were obtained from mouse #2, antigen-specific B cells were sorted, and then genes of VH and VL antibody fragments were cloned as scFvs into a yeast display vector. Yeast display was applied to enrich hIL-13Rα1 ECD-specific clones. After several enrichment steps, a chimeric antibody (i.e., human IgG1 domains fused to mouse V genes) that neutralizes hIL-13Rα1 termed “2HA6” was generated. 2HA6 was capable of binding hIL-13Rα1 (EC50 = 0.67 nM, by ELISA) but not mIL-13Rα1 or streptavidin (Fig. 7A). 2HA6 dose-dependently inhibited IL-13–induced CCL26 secretion (Fig. 7B) but not IL-4– or TNF-α–mediated secretion of CCL26 or IL-6, respectively. Thus, 2HA6 was capable of specifically suppressing IL-13 through the human type 2 IL-4R. Neutralizing antibodies toward receptors can block their activity by direct competition with their ligand on the ligand-binding site or by other means, such as preventing receptor dimerization. To determine whether 2HA6 directly competes with IL-13 on its respective binding site, we conducted a competitive ELISA testing the ability of IL-13 and 2HA6 to bind the ECD of hIL-13Rα1 (Fig. 7E). Plates were coated with the ECD of hIL-13Rα1 ECD, and IL-13 was added with no antibody treatment, with an isotype control antibody, or with 2HA6. A horseradish peroxidase–conjugated anti–IL-13 detection antibody was added, followed by 3,3′,5,5′-tetramethylbenzidine (TMB) and optical density absorbance was determined. As expected, in the absence of any competing antibody or in the presence of an isotype control antibody, IL-13 was capable of binding the hIL-13Rα1 ECD (Fig. 7E). IL-13 was capable of binding the ECD of hIL-13Rα1 even in the presence of neutralizing concentrations of 2HA6 (Fig. 7E). Collectively, these data suggest that 2HA6 neutralized IL-13 activity by a mechanism that does not involve direct competition with IL-13.

**DISCUSSION**

Over the past decade, pathological effects driven by IL-4 and IL-13 have drawn considerable attention to various allergic diseases including AD (34). Although agents that target these cytokines (3), their receptors, and/or subsequent signaling intermediates are being developed for treatment of AD, the signals that are mediated by IL-4 and IL-13 via the type 2 IL-4R are not fully understood. One of the important aspects of our study was the ability to dissect the relative contribution of IL-4 and IL-13 to diverse responses that were mediated by the type 2 IL-4R. This analysis is of great importance because recent data highlight unique activities for IL-4, IL-13, and their respective receptor chains, but the exact contribution of each cytokine/receptor is currently unknown (35–39). For example, IL-4 can induce mucosal pathology even in the absence of IL-13, and treatment with an IL-13 antagonist does not inhibit the effects of IL-4 (35). Yet, it is currently unknown whether the IL-13–independent effects of IL-4 are mediated via the type 1 or type 2 IL-4R, especially in the skin. Moreover, previous data demonstrated that IL-13 is capable of exerting specific and IL-4–independent signals (16, 37). IL-13 has been shown to mediate ovalbumin-induced dermatitis via signals that are independent of type 2 IL-4R (40). Last, IL-13 signaling via IL-13Rα2 has been reported to induce lung and gastrointestinal pathologies (18, 41). Using a neutralizing antibody toward IL-4 and IL-13, we demonstrated that OX-induced epidermal thickening was exclusively mediated by the type 2 IL-4R and predominantly driven by IL-13. Furthermore, CXCL1, CCL17, and CCL11 production were dependent on IL-13 signaling via the type 2 IL-4R. Although these data strongly imply IL-13Rα1 as the main signaling receptor for IL-13 in the skin of OX-challenged mice, this interpretation should be made with caution. We previously observed decreased expression of IL-13Rα2 in the lungs of IL13RA1−/− mice (42), and consistent with that report, in this study, we observed at least 50% reduction in skin IL-13Rα2 levels. Because the expression of IL-13Rα2 is partially dependent on IL-13Rα1, it is still possible that in the absence of IL-13Rα1, IL-13 signaling via IL-13Rα2 is decreased as well. The precise contribution of IL-13Rα2 to IL-13 signaling in skin pathologies remains to be defined. IL-4 and IL-13 may influence each other in a feedback loop. Although we were able to identify the cellular source for IL-4– and IL-13– and, in the skin, we could not detect IL-4 or IL-13 in the serum of mice with experimental AD and could not detect IL-13 in the skin. The inability to detect serum IL-4 or IL-13 may be due to low levels of secretion and short half-life of cytokines in vivo. Thus, one limitation of our experimental design is the fact that we cannot rule out any cross talk between these two cytokines and cannot offer information regarding their co-regulation and subsequent bioactivity.

We report that IL-4 signaling via the type 1 IL-4R was critical for OX-dependent induction of IgE, CCL24, and eosinophilia. Thus, the expression of the eosinophil-homing chemokines CCL11 and CCL24 were differentially regulated by IL-13 signaling via the type 2 IL-4R and IL-4 signaling via the type 1 IL-4R, respectively. These data suggest compartmentalized expression for CCL11 and CCL24 in...
the skin, where CCL11 is produced by nonhematopoietic cells that express high levels of the type 2 IL-4R. CCL24 is likely produced by nonhematopoietic cells, which predominantly express the type 1 IL-4R and respond primarily to IL-4. These data are consistent with previous reports in allergic lungs showing that CCL24 was localized to macrophages (43). Our data demonstrate that increased expression of CCL24 is sufficient for maximal eosinophil recruitment, at least in OX- and DNB-induced AD. The distinct dependence of CCL11 and CCL24 on the type 2 and type 1 IL-4Rs, respectively, in the skin is in marked contrast to the restricted requirement of the type 2 IL-4R for induction of both chemokines during allergic airway disease (21, 23). An additional difference is noted with regard to antigen-induced IgE production. After intranasal sensitization using two distinct allergen preparations (i.e., house dust mite and Aspergillus fumigatus), induction of IgE was independent of the type 2 IL-4R (23). Nonetheless, IgE production induced by subcutaneous challenge with Schistosoma mansoni egg antigen after intraperitoneal sensitization with ovalbumin and alum was at least partially dependent on the type 2 IL-4R (22). Our data demonstrate that serum IgE levels were increased in the absence of Il13ra1 after repetitive skin challenges with OX and decreased in the absence of Il13ra1 after repetitive skin challenges with DNB. The opposing regulation of IgE by the type 2 IL-4R likely reflects that IgE production is dependent on IL-4 signaling via the type 1 IL-4R and IL-13Rα1 as well as additional factors that determine the role of IL-13Rα1 in a specific model. OX and DNBF are haptons that elicit an immune response upon binding to larger proteins that render them immunogenic. Thus, it is possible that the different responses of OX and DNBF may be elicited upon binding of these haptons to different proteins. These data also indicate that the type 2 IL-4R (likely expressed by non-IgE-secreting cells) may compete under specific settings with the type 1 IL-4R on IL-4 signaling, thus reducing IgE production. This further suggests that the differential requirement for type 2 IL-4R in induction of IgE and chemokines is dependent on the route of sensitization, the nature of the antigen, the affected tissue, and the relative induction of IL-4 in comparison with IL-13. Note that our conclusions regarding the relative contribution of IL-4, IL-13, and their signaling chains should be confined to OX- and DNB-induced disease. This is specifically important because experimental AD can be induced by various models including different haptons (e.g., trinitrochlorobenzene), allergens (e.g., house dust mite and ovalbumin), and genetically engineered models (e.g., Il14tg, Il13tg, Tslp tg, and Il18tg), which may induce different levels of IL-4/IL-13, and thus, their dependency on the type 2 IL-4R may be different. Furthermore, OX-challenged Il13ra1−/− mice and OX-challenged mice that were treated with IL-13Rα1-neutralizing antibodies did not show ear and epidermal thickness restored to baseline levels. Thus, additional factors beyond IL-13Rα1, which may be dependent on the experimental model, may still have a role.

Our data are consistent with the current paradigm that the type 2 IL-4R is predominantly expressed by nonhematopoietic cells, whereas the type 1 IL-4R is generally restricted to leukocytes (3, 44). Thus, expression of IL-4, IgE, CCL24, and subsequent eosinophilia is mediated by hematopoietic cells in experimental AD. IgE and CCL24 levels were both increased to a greater extent in WT mice that received Il13ra1−/− bone marrow cells. This suggests that a subset of leukocytes that express the type 2 IL-4R reside adjacent to IgE-producing B cells, which express the type 1 IL-4R. These two cells are sufficiently proximate to each other so that both cells compete on IL-4 signaling/uptake. CCL24 can be produced by IL-13Rα1+ hematopoietic cells such as macrophages, which express both the type 1 and type 2 IL-4Rs (45, 46). We hypothesize that in the absence of Il13ra1, macrophages produce CCL24 in response to IL-4 via the type 1 IL-4R.

The key roles of IL-4 and IL-13 in allergic diseases designated them as key targets for therapy. Certainly, over a dozen different therapeutic entities including mutated ligands, soluble receptors, and antibodies have been developed in the past years aiming to target IL-4, IL-13, or IL-4Rα (3). With the exception of tralokinumab, an anti–IL-13 antibody, which showed clinical efficacy in AD (5), most studies evaluating the individual targeting of IL-4 or of IL-13 showed disappointing results, mainly due to the lack of efficacy. In contrast, dual IL-4/IL-13 neutralization by targeting IL-4Rα (i.e., dupilumab) was efficacious and has recently been approved by the U.S. Food and Drug Administration for the treatment of AD (47). Our experimental data imply a greater role for IL-13 in mediating the pathology of AD in comparison with IL-4. Nonetheless, we cannot rule out a role for IL-4 in human AD because blocking IL-4Rα by dupilumab has proven clinically efficacious. Furthermore, although IL-13 inhibitors such as lebrikizumab and tralokinumab induced clinical improvement in moderate to severe AD (5, 48), their actual efficacy needs to be confirmed because patients enrolled in these trials were concomitantly treated with corticosteroids. Together, new strategies for dual blockade of IL-4 and IL-13 are required to manage clinically significant aspects of allergic diseases such as AD. One such strategy could be achieved by neutralizing IL-13Rα1. Using this approach, we isolated and generated a new antibody capable of neutralizing mIL-13Rα1 termed MS8. MS8-treated mice displayed only a 30% reduction in OX-induced ear thickness. This is likely due to the fact that we used a “single-dose” experimental regime and did not achieve a complete inhibitory effect in vivo. We predict that additional dose-response experiments will achieve better inhibition. In addition, MS8-treated mice manifested a trend toward decreased serum IgE levels (P = 0.065) and a minor (1.25-fold) increase of skin IL-4 levels. Most notable, MS8 alleviated skin eosinophilia, whereas eosinophil levels were not altered in OX-challenged Il13ra1−/− mice. A plausible explanation for this phenomenon could be that MS8 was capable of partially suppressing IL-4–induced responses in vitro. Thus, infiltration of eosinophils, which is driven by IL-4, may be suppressed by MS8. An additional explanation may be that MS8 affects the survival, but not migration, of eosinophils, which can be regulated by IL-4/IL-13 via the type 2 IL-4R (49, 50).

Furthermore, we generated a human mAb capable of neutralizing hIL-13–induced effects by targeting hIL-13Rα1 termed 2HA6. Although 2HA6 did not block IL-4–induced signaling via IL-13Rα1, our biochemical analyses showed that the light chain of 2HA6 did not contribute to its binding or biological activities, and the mode of action is by preventing the complex formation of the type 2 IL-4R heterodimer after IL-13 stimulation. Given this mode of action, we predict that by light chain shuffling and/or point mutations, 2HA6 may be modified to serve as a dual IL-4/IL-13 blocker in the future.

In summary, our results establish that the critical role of the type 2 IL-4R in AD pathogenesis is primarily mediated by its interactions with IL-13. Furthermore, we dissected the contribution of the different IL-4Rs mediating IL-4– and IL-13–induced skin pathology. Last, we show that pharmacological targeting of the type 2 IL-4R via blockade of IL-13Rα1 may serve as a potent target for the treatment of AD and perhaps additional allergic diseases.
Materials and Methods

Study design

The objective of this study was to examine the role of the type 2 IL-4R in AD and to pharmacologically target this pathway to alleviate AD. To this end, we used a combination of in vivo and in vitro assays primarily using mice. We designed and performed the experiments mainly in the fields of cellular immunology and molecular biology. The number of replicates for each experiment is indicated in the figure legends.

Mice

Male and female Il13ra1−/− mice (backcrossed >F9 to C57BL/6) were generated as previously described (21). C57BL/6 WT mice were obtained from Harlan Laboratories (Rehovot, Israel). IL-4/GFP-enhanced transcript (4Get) and smart13 knock-in mice (Il13ra1smart) were obtained from The Jackson Laboratory (JAX mice). All experiments were reviewed and approved by the Animal Care Committee of Tel Aviv University and were performed in accordance with its regulations and guidelines regarding the care and use of animals for experimental procedures. All of the experiments were conducted in the specific pathogen–free facilities of the Tel Aviv University. In all experiments, age-, weight-, and sex-matched mice were used.

OX- and DNFB-induced dermatitis

Mice (6- to 8-week-old male and female) were sensitized on day 0 by the application of 15 μl of 1% OX (Sigma-Aldrich, Rehovot, Israel) in acetone on days 0 and 3 by the application of 10 μl of 0.25% DNFB (Sigma-Aldrich, Rehovot, Israel) in acetone and olive oil (4:1 ratio, respectively) on both ear flanks (60 μl per mouse). On day 7, the mice were challenged in the same manner with 0.5% OX or 0.1% DNFB (three times per week, for up to 10 challenges in OX-induced AD or up to 7 challenges in DNFB-induced AD). Ear thickness was measured by a digital caliper, and total ear tissues were taken for further analyses.

In neutralization experiments, mice were intraperitoneally treated twice a week with rat IgG1 anti-IL-4 (120 μg per mouse, clone 11B11, BioXCell, Lebanon, NH), rat anti-IL-13 IgG1 (150 μg per mouse, clone eBio1316H, eBioscience, San Diego, CA, USA) or rat IgG1 isotype control (clone HPRN, BioXCell, Lebanon, NH), rat anti-TNF-α IgG1 (150 μg per mouse, clone XT3.11, BioXCell, Lebanon, NH), or rat IgG1 isotype control (clone HPRN or TNP6A7, BioXCell, Lebanon, NH). In the IL-13Ra1 neutralization experiments, mice were intraperitoneally treated three times a week with anti-IL-13Ra1 clone MS8, produced in house, 200 μg per mouse) or mouse IgG1 isotype control (clone MOPC-21, BioXCell, Lebanon, NH).

Generation of bone marrow–chimeric mice

C57BL6 WT mice (on the CD45.1 background) and Il13ra1−/− mice (on the CD45.2 background) were irradiated with 9-Gy total body irradiation (TBI) using the BIOBEAM Gamma Irradiator (Gamma-Service Medical GmbH, Germany). Thereafter, mice received ciprofloxacin (20 μg/ml; Teva Pharmaceuticals, Petah Tikva, Israel) in drinking water for 14 days. Twenty-four hours after TBI, recipient mice were intravenously injected with 5 × 10^6 bone marrow cells (in saline) that were obtained from donor mice (CD45.1 or Il13ra1−/−). Mice were challenged with OX 10 weeks after TBI.

Histology

Paraffin-embedded sections (5 μm) were processed and stained with hematoxylin and eosin (Patho-Lab Diagnostics, Israel), and independent images were captured at x20 magnification from each slide. Ten epidermal thickness measurements per slide were made, and average thickness was calculated per image (ImageJ, National Institutes of Health).

Ear lyse preparation

Ears were placed in ice-cold IP Lysis Buffer (750 μl, Pierce #87778, Thermo Fisher Scientific, Waltham, MA) supplemented with a protease inhibitor cocktail (Sigma-Aldrich, Rehovot, Israel). Thereafter, the ears were homogenized and centrifuged (14,000g, 10 min, 4°C), and supernatants were collected, aliquoted, and stored at −20°C.

Enzyme-linked immunosorbent assay

Cytokines and IgE levels were measured by ELISA according to the manufacturer’s instructions. The following kits for mouse proteins were used: IL-4 (BioLegend, CA, USA); IL-6, CCL17, CCL24, and IL-13Ra2 (R&D Systems, Minneapolis, MN, USA); TNF-α, CCL2, CCL11, and CXCL1 (PeproTech, Rehovot, Israel); and IgE (BD Biosciences, San Jose, CA, USA). The following kits for human proteins were used: CCL26 (R&D Systems, Minneapolis, MN, USA) and IL-6 (PeproTech, Rehovot, Israel).

Flow cytometry

Single-cell suspensions of enzymatically digested ears were stained using the following antibodies: CD45-APC (allophycocyanin), CD11b-PerCP (peridinin chlorophyll protein)/Cy5.5, CD11c-FITC (fluorescein isothiocyanate), CD8α-PE (phycoerythrin) anti-human CD4-FITC, and CD45-APC-Cy7, anti–GALCerCD1d complex–PE, CD4-PerCP/Cy5.5, T cell receptor γδ–APC, and CD8α-BV510 (Brilliant Violet 510) (BioLegend, San Diego, CA, USA); Ly6C-PE/Cy7, F4/80-AF700 (Alexa Fluor 700), CD4-AF488, CD3e–PE/Cy7, anti–GALCerCD1d complex–PE, CD4-PerCP/Cy5.5, T cell receptor γδ–APC, and CD8α-BV510 (Brilliant Violet 510) (BioLegend, San Diego, CA, USA); and Ly6G-APC/Cy7 and B220-PerCP/Cy5.5 (BioGems, Westlake Village, CA, USA). 4′,6-Diamidino-2-phenylindole (DAPI) (Sigma-Aldrich, Rehovot, Israel) was used as a marker for cell death.

Cell counts were measured using Flow-Count Fluorospheres (Beckman Coulter, Brea, CA) according to the manufacturer’s instructions. Events were acquired using a Gallios flow cytometer, and data were analyzed using Kaluza software (Beckman Coulter, Brea, CA, USA).

Statistical analysis

P values of mouse datasets were determined by one-way analysis of variance (ANOVA) and unpaired two-tailed Student’s t test with 95% confidence interval. All statistical tests were performed with GraphPad Prism V8 software. Data are shown as means ± SEM. *P < 0.05; **P < 0.01; ***P < 0.001.

Supplementary Materials

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Table S1. Primers for amplification of mouse V genes.

Fig S1. Expression of IL-13Ra1 in the skin.

Fig S2. IL-13Ra1 regulates OX-induced dermatitis.

Fig S3. The type 2 IL-4R is required for DNFB-induced AD.

Fig S4. Assessing the cellular source for IL-4 and IL-13 in OX-induced dermatitis.

Fig S5. Engraftment efficiency of following adoptive transfer of WT and Il13ra1−/− bone marrow cells.

Fig S6. IL-13Ra1 antisera inhibit IL-13–induced IL-13Ra2 secretion by MC38 cells.

Fig S7. Schematic representation of the yeast display system used in this study to isolate IL-13Ra1–binding antibodies.

Fig S8. IL-13Ra1–specific antisera inhibit IL-13–induced CCL26 secretion by A549 cells.

Fig S9. Gating strategy for identification of immune cells in the ears.

View/request a protocol for this paper from Bio-protocol.
REFERENCES AND NOTES


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A key role for IL-13 signaling via the type 2 IL-4 receptor in experimental atopic dermatitis

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Dermatitis details
Type 2 inflammation mediated by IL-4 and IL-13 has key roles in atopic dermatitis (AD). IL-4 and IL-13 signal via the type 2 IL-4 receptor (R), but the contribution of this receptor to AD is not well understood. Bitton et al. now show that dermatitis symptoms and expression of TNF-α, CXCL1, and CCL11 were dependent on IL-13 signaling via the type 2 IL-4R in nonhematopoietic cells, and these responses could be inhibited by pharmacological blockade of IL-13Rα1. In contrast, IL-4 signaling via the type 1 IL-4R was required for IL-4 and CCL24 expression and eosinophilia. These results provide insight into the role of type 2 IL-4R in AD and how it may be potentially targeted for other allergic diseases.