

Targeting IL-4/IL-13 signaling to alleviate oral allergen-induced diarrhea

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Background: Intestinal anaphylaxis (manifested by acute diarrhea) is dependent on IgE and mast cells.

Objective: We aimed to define the respective roles of IL-4 and IL-13 and their receptors in disease pathogenesis.

Methods: Wild-type mice and mice deficient in IL-4, IL-13, and IL-13 receptor (IL-13R) $\alpha 1$ (part of the type 2 IL-4 receptor [IL-4R]) were sensitized with ovalbumin (OVA)/aluminum potassium sulfate and subsequently given repeated intragastric OVA exposures. The IL-4R α chain was targeted with anti-IL-4R α mAb before or after intragastric OVA exposures.

Results: IL-4^{-/-} (and IL-4/IL-13^{-/-}) mice produced almost no IgE and were highly resistant to OVA-induced diarrhea, whereas allergic diarrhea was only partially impaired in IL-13^{-/-} and IL-13R $\alpha 1$ ^{-/-} mice. IL-13R $\alpha 1$ -deficient mice had decreased IgE levels, despite having normal baseline IL-4 levels. Intestinal mast cell accumulation and activation also depended mainly on IL-4 and, to a lesser extent, on IL-13. Prophylactic anti-IL-4R α mAb treatment, which blocks all IL-4 and IL-13 signaling, suppressed development of allergic diarrhea. However, treatment with anti-IL-4R α mAb for 7 days only partially suppressed IgE and did not prevent intestinal diarrhea.

Conclusion: Endogenously produced IL-13 supplements the ability of IL-4 to induce allergic diarrhea by promoting oral allergen sensitization rather than the effector phase of intestinal anaphylaxis. (J Allergy Clin Immunol 2009;123:53-8.)

Key words: Allergy, anaphylaxis, IL-4, IL-13, IL-13R $\alpha 1$, intestine, mast cell

Currently, 2% to 6% of the US population has food allergy,¹ a disease characterized by increased total and antigen-specific IgE eosinophilia, mastocytosis, and gastrointestinal dysfunction (eg,

Abbreviations used

alum: Aluminum potassium sulfate
IL-4R: IL-4 receptor
IL-13R: IL-13 receptor
MMCP1: Mouse mast cell protease 1
OVA: Ovalbumin
STAT6: Signal transducer and activator of transcription 6

vomiting, diarrhea, and failure to thrive). The development of experimental models of gastrointestinal hypersensitivity has provided important insight into the immunologic mechanisms responsible for this disease.^{1,2}

Allergen-induced acute diarrhea, which develops in mice sensitized intraperitoneally with ovalbumin (OVA)/aluminum potassium sulfate (alum: $\text{AlK}(\text{SO}_4)_2 \cdot 12\text{H}_2\text{O}$; A-7210; Sigma-Aldrich, Milwaukee, Wis) followed by repeated intragastric OVA administration, is dependent on IgE, mast cells, and mast cell-generated vasoactive mediators.³ The mild systemic features observed in this murine model of oral allergen-induced gastrointestinal allergy led us to use the term intestinal anaphylaxis to describe the IgE-mediated mast cell degranulation that occurs in the small intestine and leads to increased intestinal permeability and acute diarrhea without shock.^{3,4}

Although increased quantities of both IL-4 and IL-13 are produced in the small and large intestines in this model, the roles of these cytokines and their receptors in the pathogenesis of intestinal anaphylaxis have not been explored.^{3,5,6} IL-4 and IL-13 both signal through receptors that contain the IL-4 receptor (IL-4R) α chain and activate signal transducer and activator of transcription 6 (STAT6), but only IL-4 signals through the type 1 receptor, whereas both cytokines signal through the type 2 receptor (composed of the IL-4R α and IL-13 receptor [IL-13R] $\alpha 1$ polypeptides). The relative roles of these 2 receptors can be distinguished by means of genetic deletion of the IL-13R $\alpha 1$ chain because such genetically engineered mice have an intact type 1 IL-4R but lack the type 2 IL-4R.^{7,8} T-cell responses should not be directly affected by IL-13R $\alpha 1$ deletion because T cells lack the type 2 receptor.⁹ Most murine B cells also express little or no type 2 IL-4R⁹; however, IL-4 and IL-13 signaling through this receptor might potentially influence the sensitization phase of allergic diarrhea by affecting the function of macrophages and dendritic cells.^{4,10,11} Based on their role in expulsion of nematode parasites,⁴ IL-4 and IL-13 might also be involved in the effector phase of allergic diarrhea. Indeed, IL-4R α ⁺ non-bone marrow-derived cells have been implicated in parasite expulsion.¹² Subsequent work by Shea-Donohue and colleagues has demonstrated parasite-induced STAT6-dependent alterations in both intestinal epithelial cell function and smooth muscle contractility.^{13,14} Collectively, these studies suggest a role for IL-4 and IL-13 in the effector phase of the disease by increasing the

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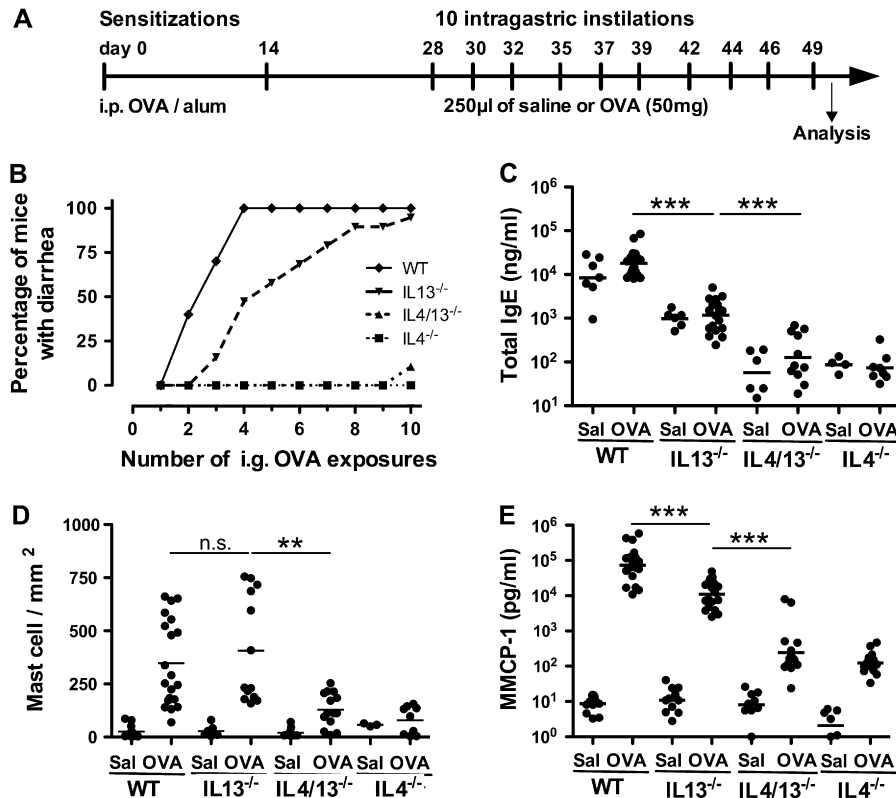


FIG 1. Ablated intestinal anaphylaxis in the absence of IL-4 and IL-13. **A**, Protocol timeline. **B**, Occurrence of diarrhea was assessed in OVA/alum-primed wild type, IL-13^{-/-}, IL-4/13^{-/-}, and IL-4^{-/-} mice (n = 9–14 mice per group) up to 60 minutes after each intragastric inoculation with 50 mg of OVA. **D**, Jejunum mast cell numbers were assessed by means of morphometric analysis of chloroacetate esterase-stained cells. **C** and **E**, IgE (Fig 1, C) and MMCP1 (Fig 1, E) levels were measured by means of ELISA in blood drawn 60 to 90 minutes after the last saline or OVA inoculation. Serum IgE and MMCP1 levels were 490 ± 204 ng/mL and 9.7 ± 7.8 pg/mL, respectively, in naive wild-type mice, which had 35 ± 25 mast cells/mm³ of jejunum. *i.p.*, Intraperitoneal; WT, wild-type; Sal, saline; n.s., not significant. ***P* < .01, ****P* < .001.

sensitivity of intestinal tissue smooth muscle, epithelium, and vasculature to mediators released by mast cells.^{13–15}

Defining the specific involvement of IL-4 and IL-13 is particularly important because therapeutic agents that block these cytokines or their common receptor (IL-4R α) are being actively developed.^{16,17} These approaches are particularly timely because safety concerns have been raised by an anti-IgE clinical trial for peanut allergy.¹⁸

Using mice genetically deficient in IL-4, IL-13, or their receptors, we now demonstrate a central role for IL-4 in antigen-triggered intestinal mastocytosis and allergic diarrhea. Importantly, IL-13 and IL-13R α 1 are also shown to have a significant role.

METHODS

Animals

IL-4-deficient mice (BALB/c background) were obtained from Jackson Laboratory (Bar Harbor, Me). IL-13-deficient and IL-4/IL-13 double-deficient BALB/c background mice were originally obtained from Andrew McKenzie (Medical Research Council Laboratory of Molecular Biology, Cambridge, United Kingdom).¹⁹ However, the strategy used to disrupt the *IL13* gene had a cis effect on the nearby *IL4* gene, resulting in impaired IL-4 production.²⁰ IL-13R α 1-deficient mice were generated at Regeneron by Velocogene Technology, as recently reported,⁷ and backcrossed into the BALB/c background for at least 6 generations. Animals involved in these studies were housed under

specific pathogen-free conditions and treated in a humane manner according to institutional guidelines.

Intestinal anaphylaxis model

Mice were sensitized twice intraperitoneally, 2 weeks apart, with 50 μ g of OVA (grade V; A-5503; Sigma-Aldrich, St Louis, Mo) in the presence of 1 mg of alum adjuvant and then administered 10 or 50 mg of soluble OVA dissolved in 250 μ L of 0.9% sterile saline (Fig 1, A). Sensitized mice intragastrically inoculated with saline were used as control animals. Challenges were performed with intragastric feeding needles (22 gauge, 1.5 inches, 1.25-mm ball; 01-290-2B; Fisher Scientific Co, Pittsburgh, Pa). Diarrhea was assessed visually by closely monitoring mice for 1 hour after each oral allergen exposure.

In vivo cytokine capture assay

The *in vivo* cytokine capture assay was used to monitor *in vivo* production of IL-4, as previously described.²¹ Briefly, mice were injected intravenously with 200 μ L of biotinylated anti-mouse IL-4 (BVD4-1D11; 10 μ g) mAb. Mice were bled 24 hours later, and plasma levels of captured IL-4 were measured by means of ELISA (eBioscience, San Diego, Calif).

Intestinal mast cell quantification

The 5- μ m sections of 10% formalin-fixed jejunum tissue were stained for chloroacetate esterase–positive mucosal mast cells. Briefly, 50 μ L of New Fuchsin (1 g resuspended in 25 mL of HCl 2N, Sigma: N-0638) was mixed

with 50 μ L of sodium nitrate 4%. After hexazotized solution turned yellow, 900 μ L of Naphthol AS-D chloroacetate (100 mg resuspended in 50 mL of N-N dimethyl formamide and stored at -20°C) was added. Five hundred microliters was added to 10 mL of filtered phosphate buffer (13 mL of monobasic sodium phosphate [0.2 mol/L] + 87 mL of dibasic sodium phosphate [0.2 mol/L] + 100 mL of H_2O ; adjusted to pH 7.6). Freshly prepared pink solution was added to tissue sections for 5 to 10 minutes. Staining was stopped by washing slides in H_2O before lightly counterstaining with methyl green. At least 3 random sections per mouse were analyzed. Quantification of stained cells per square millimeter was performed by means of morphometric analysis with the Metamorph Imaging System (Universal Imaging Corp, West Chester, Pa).

ELISA

Mouse mast cell protease 1 (MMCP1) and total IgE plasma levels were measured according to the manufacturers' instructions (respectively, Moredun Scientific, Midlothian, United Kingdom, and BD Biosciences Pharmingen, San Diego, Calif).

Antibody treatment

IL-4R α was blocked with 2 mg of an antibody (4-3 antibody, anti-IL-4R α hybrid IgG1 mAb) administered either intraperitoneally or intravenously, respectively, 24 or 3 hours before OVA exposure.

Statistical analysis

Data are expressed as the mean \pm SD. Statistical significance comparing different sets of mice was determined by using the Student unpaired *t* test or the nonparametric Mann-Whitney *U* test.

RESULTS

Allergen-induced diarrhea is mediated by IL-4 and potentially IL-13

To determine the respective importance of IL-4 and IL-13 in intestinal anaphylaxis, we sensitized mice deficient in IL-4, IL-13, or both cytokines intraperitoneally with OVA/alum and challenged them repeatedly intragastrically with OVA (Fig 1, A). IL-4 deficiency almost completely protected against allergic diarrhea; IL4/IL13 double-deficient mice had diarrhea on the 10th OVA exposure (Fig 1, B). Induction of allergic diarrhea required a significantly greater number of allergen challenges in IL13-deficient mice than in wild-type mice (Fig 1, B).

IgE and mast cell responses depend mainly on IL-4

Because IgE and mast cells are essential for the development of allergic diarrhea,³ we compared serum IgE levels, jejunal mast cell numbers, and serum levels of MMCP1 (an enzyme released by degranulating mast cells) in wild-type, IL-4-deficient, IL-13-deficient, and IL4/IL13-deficient mice that had been primed and challenged with OVA. Serum IgE levels were approximately 2 logs lower in IL-4- and IL4/IL13-deficient mice than in wild-type mice and approximately 1 log lower in IL-13-deficient mice than in wild-type mice (Fig 1, C). Jejunal mast cell numbers after OVA immunization were decreased 2- to 3-fold in IL-4- and IL4/IL13-deficient mice (Fig 1, D). Most importantly, MMCP1 responses in OVA-immunized IL-4- and IL4/IL13-deficient mice were 2 to 3 logs lower than MMCP1 responses in similarly treated wild-type mice (Fig 1, E), although OVA immunization stimulated an approximately 10-fold increase in MMCP1 levels, even in the absence of IL-4. Despite nonsignificantly altered intestinal mast cell levels, MMCP1 levels were significantly lower in IL-13-deficient mice than in wild-type mice (Fig 1, E).

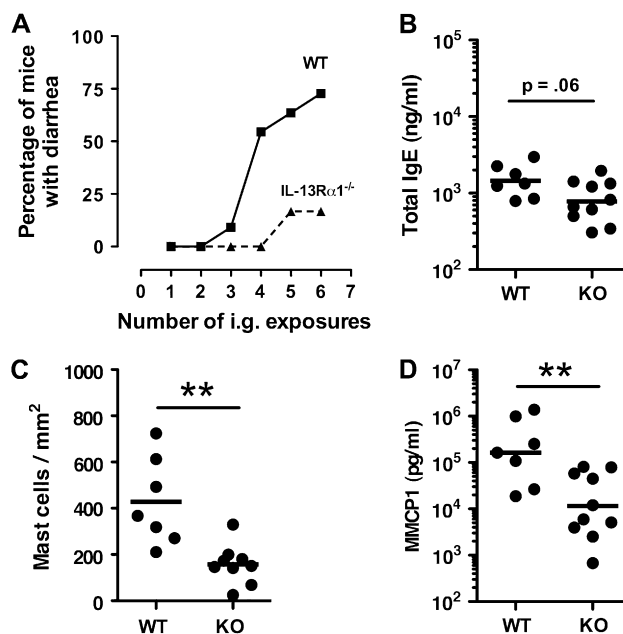


FIG 2. IL-13 contributes to IgE and mast cell allergic responses. **A**, Diarrhea occurrences in OVA/alum-primed wild-type and IL-13R α 1 $^{-/-}$ mice after 1 to 6 intragastric inoculations with 10 mg of OVA. **B-D**, Serum IgE levels (Fig 2, B), jejunal mast cell numbers (Fig 2, C), and serum MMCP1 levels (Fig 2, D) were compared in wild-type and IL-13R α 1-deficient mice (7-18 per group). WT, Wild-type; KO, knockout. ***P* < .01.

IL-13R α 1 $^{-/-}$ mice demonstrate a role for IL-13 in IgE- and mast cell-mediated allergic diarrhea

Because IL13-deficient mice produce subnormal amounts of IL-4,²⁰ the delayed development of intestinal anaphylaxis in IL13 $^{-/-}$ mice might result from diminished IL-4 production rather than the absence of IL-13. Indeed, we found that IL-4 production was reduced in naive IL13 $^{-/-}$ mice compared with that seen in wild-type mice, as determined by using the *in vivo* cytokine capture assay (66.1 ± 23.1 vs 115.1 ± 39.3 pg/mL, *P* < .01). This difference was not a direct effect of the absence of IL-13 signaling because mice with defective IL-13 signaling (IL13R α 1 $^{-/-}$) had comparable levels of IL-4 (187.9 ± 31.4 vs 170.7 ± 22.0 pg/mL for $-/-$ and $+/+$ mice, respectively). Consequently, we evaluated the ability of IL13R α 1-deficient mice to have allergic diarrhea and found that it was also significantly impaired (Fig 2, A).

The decrease in serum IgE levels in IL13R α 1-deficient mice was approximately 2-fold and did not quite reach statistical significance (Fig 2, B). These data suggest that IL-13 has a modest stimulatory effect on IgE production in OVA-immunized mice, resulting in impaired mast cell stimulation in the absence of IL-13 signaling, as shown by MMCP1 levels that were approximately 1 log lower in IL13R α 1-deficient mice than in wild-type mice (Fig 2, C and D).

Prophylactic targeting of IL-4R α alleviates allergic diarrhea

We used 4-3, an mAb to IL-4R α that blocks both IL-4 and IL-13 effects *in vitro* and in an *in vivo* mouse model of allergic airway disease (unpublished data), to inhibit the effects of IL-4 and IL-13 in experimental intestinal anaphylaxis. Initial experiments tested whether a single dose of 4-3, injected 1 day before the initiation of intragastric OVA administration, could inhibit the development of allergic diarrhea. This single dose strongly inhibited the

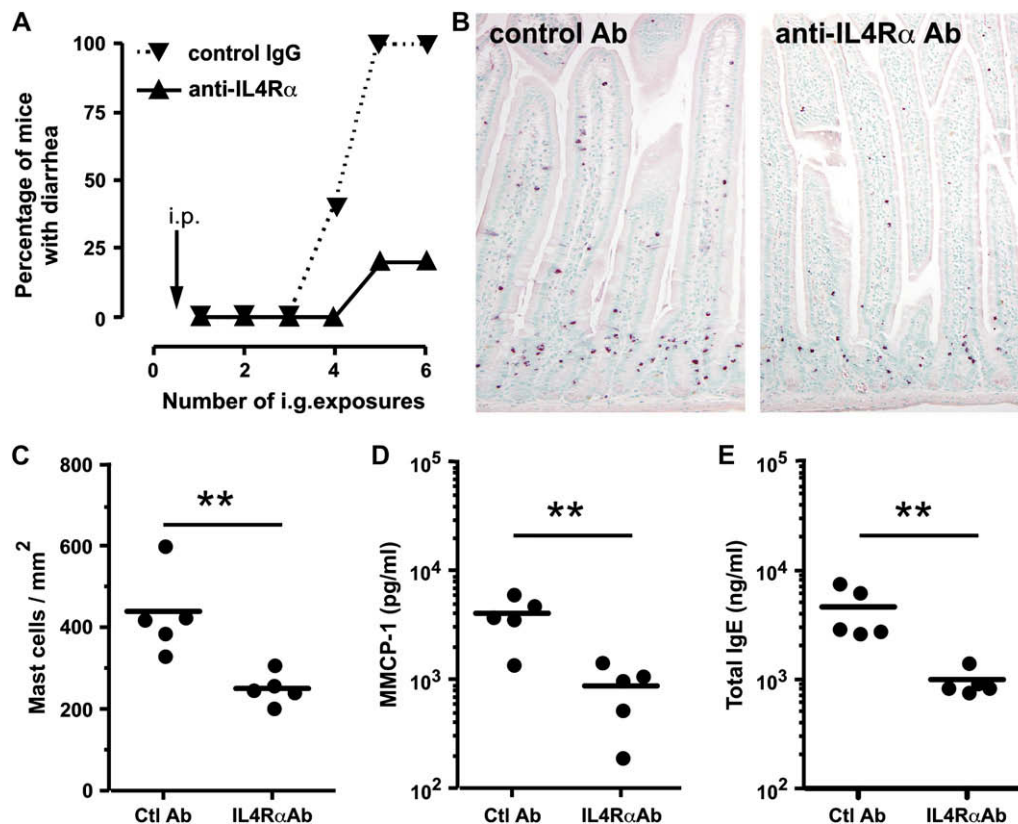


FIG 3. Prophylactic effects of anti-IL-4R α mAb. OVA/alum-primed BALB/c mice (5 per group) were injected intraperitoneally with either 2 mg of 4-3 anti-IL-4R α mAb or a control IgG1 mAb 1 day before the first intragastric inoculation with OVA. **A**, Development of diarrhea was assessed during the 60 minutes after each intragastric OVA inoculation. **B**, Representative jejunum section of antibody-treated mice stained with chloroacetate esterase. **C**, Intestinal mast cell numbers were assessed by means of morphometric analysis. **D** and **E**, Blood MMCP1 (Fig 3, **D**) and total IgE (Fig 3, **E**) levels were assessed 1 to 2 hours after the last allergen exposure. *i.g.*, Intragastric; *Ab*, antibody; *Ctl*, control. ***P* < .01.

development of allergic diarrhea after 4 to 6 intragastric doses of OVA (Fig 3, **A**). This delay in the development of allergic diarrhea was associated with impaired intestinal mast cell accumulation (Fig 3, **B** and **C**), decreased MMCP1 plasma levels (Fig 3, **D**), and decreased plasma IgE levels (Fig 3, **E**).

IL-4 and IL-13 are not required for the effector phase of allergic diarrhea

The ability of IL-4 and IL-13 to enhance smooth muscle contractility and epithelial permeability and secretion and the ability of these cytokines to increase sensitivity to mediators released by activated mast cells suggested that IL-4 and IL-13 might be contributing to the effector, as well as the sensitization, phases of intestinal anaphylaxis.¹³⁻¹⁵ However, administration of up to 3 doses of anti-IL-4R α mAb over a 7-day period (on days 37, 39, and 41) starting after OVA-immunized mice already had allergic diarrhea, failed to decrease the incidence of diarrhea after high-dose OVA challenge (Fig 4, **A**), although it decreased total IgE levels (Fig 4, **B**), intestinal mast cell numbers (Fig 4, **C**), and MMCP1 blood levels (Fig 4, **D**).

DISCUSSION

Taken together, our observations demonstrate that not only IL-4 but also IL-13 has a significant role in intestinal anaphylaxis.

Our finding that IL-4-deficient mice are protected from OVA-induced diarrhea is supported by an earlier study using a different model, which showed that mice pretreated with an anti-IL-4 antibody before sensitization did not have diarrhea.^{3,5,6} Confirmation of the delayed development of allergic diarrhea observed in IL13-deficient mice with studies in IL13R α 1-deficient mice was important because it could result from their decreased production of IL-4,²⁰ which would be expected to decrease IgE and mast cell responses.²² In contrast, IL-4 production appears to be normal in IL13R α 1-deficient mice. Although it could be argued that the delayed development of diarrhea in these mice might reflect the lack of IL-4, rather than IL-13, signaling through the type 2 IL-4R, this seems unlikely given the greater production of IL-13 than IL-4⁵ and the more potent signaling of IL-13 than IL-4 through the type 2 receptor.²³ In addition, this explanation cannot account for the accelerated development of allergic diarrhea in IL13R α 2-deficient mice (data not shown).

Surprisingly, in contrast to observations made in allergic airway disease models,²⁴ IL-13 is important in the sensitization and propagation phases rather than the effector phase of intestinal anaphylaxis. This point is of importance because the only available data thus far in the gastrointestinal tract suggested that IL-13 and IL-4 had a significant effect on effector functions (eg, parasite expulsion).⁴ Furthermore, IL-4 is able to influence murine mast cell development under certain conditions.²⁵ However,

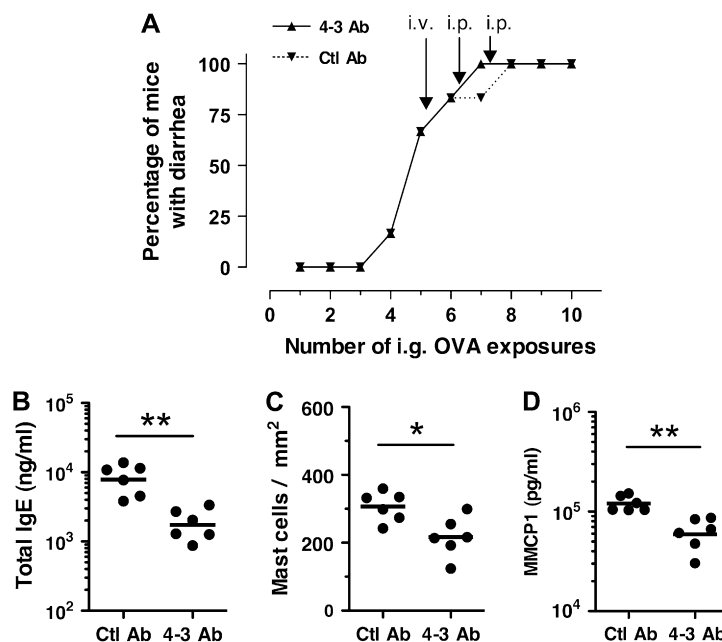


FIG 4. IL-4 and IL-13 are not required during the effector phase of allergic diarrhea. **A**, Once diarrhea had developed (after 4 intragastric challenges with OVA), BALB/c mice (6 per group) were injected with 2 mg of 4-3 or control IgG1 mAb before the fifth, sixth, and seventh OVA inoculations ($n = 6$ mice per group). **B**, One week and 3 OVA inoculations later, mice were killed, and plasma IgE levels were determined. **C** and **D**, Jejunum mast cell numbers (Fig 4, C) and MMCP1 blood levels (Fig 4, D) were assessed 1 to 2 hours after the last OVA inoculation. Ab, Antibody; Ctl, control; i.v., intravenous; i.p., intraperitoneal; i.g., intragastric. * $P < .05$, ** $P < .01$.

blocking both IL-4 and IL-13 signaling with a high dose of a potent anti-IL-4R α mAb for as long as 7 days had little effect on allergic diarrhea induced by a high dose of allergen. It is unlikely that this negative result reflected inadequate IL-4R α blockade because even a single dose of the 4-3 mAb suppressed allergic diarrhea for more than 2 weeks in the prophylactic model. Although intravenous injections of large doses of IL-4 or IL-13 are able to exacerbate systemic anaphylaxis, IL-4 and IL-13 blood levels after repeated oral allergen exposures were 100-fold lower (data not shown) and most likely insufficient to have a major effect on the effector phase of the disease.²⁶

These observations raise the question of how IL-13 contributes to the sensitization phase of intestinal anaphylaxis. Notably, murine T cells do not directly respond to IL-13 because they lack IL-13R α 1. Furthermore, IL-4 is much more potent than IL-13 at inducing isotype switching by B cells.^{10,27} One possibility is that IL-13 directly stimulates isotype switching by a small but important B-cell subset.²⁸ Indeed, in the absence of IL-13 signaling, baseline IgE levels are significantly decreased in IL13R α 1-deficient mice.⁷ This is consistent with earlier observations of increased IgE levels in mice with no soluble IL-13 decoy receptor (IL-13R α 2) and in mice that were engineered to overproduce IL-13.^{29,30} However, the ability of IL13-deficient mice to mount an antibody response to OVA is only partially affected because OVA-specific IgG1 levels were not significantly lower in IL13-deficient mice compared with those seen in wild-type mice (data not shown). Alternatively, the ability of IL-13 to influence dendritic cell maturation and ability to regulate T_H2 cytokine production might indirectly promote IgE production and mastocytosis.¹¹

The observation that 10% of IL4/IL13 double-deficient mice had diarrhea by the 10th allergen exposure indicates that the IL-4

requirement for induction of allergic diarrhea is not absolute. Detectable IgE serum levels have been observed in naive IL-4R α - and IL4/IL13-deficient mice.²² Furthermore, OVA-specific IgE levels were observed in IL-4- and IL-4R α -deficient mice after OVA sensitization either through the intraperitoneal route or through repeated intranasal instillations.³¹ This would support our findings that IgE is present in IL-4- and IL4/IL13-deficient mice after sensitization, albeit at barely detectable levels. Although we did not measure a significant induction of total plasma IgE levels after repeated intestinal allergen exposures, mast cell-bound OVA-specific IgE levels might have increased without a detectable increase in circulating levels of IgE in multiply immunized IL-4- and IL4/IL13-deficient mice. We observed a 2-fold increase in mast cell accumulation associated with a log increase in MMCP1 plasma levels in these mice, indicating that repeated immunization could induce mast cell activation through an IL-4/IL-13-independent pathway. Similar results were observed with STAT6-deficient mice (data not shown). Interestingly, intestinal mastocytosis is actually increased more in STAT6-deficient mice than in wild-type mice after infection with some nematodes.³²

Finally, one would expect that blocking IL-4R α should (1) normalize smooth muscle contractions and epithelial cell functions, (2) reduce T_H2-related antibody responses (IgE and IgG1), (3) decrease intestinal inflammation, and (4) impair mast cell degranulation with release of MMCP1, serotonin, and platelet-activating factor in allergen-immunized mice. Indeed, our findings demonstrate that a prophylactic approach blocks diarrhea development. Although treatment with anti-IL-4R α for 7 days failed to block the diarrheal response to allergen challenge, it significantly decreased IgE, mast cell, and MMCP1 responses, despite continuing allergen administration. This suggests that a longer

period of treatment with this mAb might suppress allergic diarrhea. Although murine models of food allergy do not completely mimic human disease, the ability of anti-IL-4R α to decrease IgE production might make it particularly effective as a treatment for food allergy when paired with a second biologic agent, such as a nonactivating anti-IgE mAb.

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Key messages

- Using mice genetically deficient in IL-4 and IL-13 signaling, we demonstrated a central role for IL-4 in antigen-triggered intestinal mastocytosis and allergic diarrhea; however, IL-4 and IL-13 are not absolutely required for the development of intestinal anaphylaxis.
- IL-13 contributes to allergic diarrhea and does so by promoting oral allergen sensitization rather than the effector phase of intestinal anaphylaxis.
- Short-term treatment of established allergic diarrhea with an inhibitor of both IL-4 and IL-13 is partially effective.

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