

IgE Generation and Mast Cell Effector Function in Mice Deficient in IL-4 and IL-13

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IL-4 and IL-13 are potent cytokines that drive production of IgE, which is critical to the development of atopic disease. In this study, we directly compared IgE generation and IgE-dependent mast cell effector function in mouse strains lacking IL-4, IL-13, IL-4 + IL-13, or their common receptor component, IL-4R α . Although serum IgE was undetectable under resting conditions in most animals deficient in one or both cytokines, peritoneal mast cells from mice lacking IL-4 or IL-13 had only partial reductions in surface IgE level. In contrast, peritoneal mast cells from IL-4/IL-13^{-/-} and IL-4R α ^{-/-} animals were severely deficient in surface IgE, and showed no detectable degranulation following treatment with anti-IgE in vitro. Surprisingly, however, intradermal challenge with high concentrations of anti-IgE Ab induced an ear-swelling response in these strains, implying some capacity for IgE-mediated effector function in tissue mast cells. Furthermore, upon specific immunization with OVA, both IL-4/IL-13^{-/-} and IL-4R α ^{-/-} mice produced detectable levels of serum IgE and Ag-specific IgG1, and generated strong ear-swelling responses to intradermal administration of anti-IgE. These findings suggest that a mechanism for IgE production exists in vivo that is independent of IL-4 or IL-13. *The Journal of Immunology*, 2005, 174: 7716–7724.

Specific IgE is the underlying requirement for atopic disease, being necessary and sufficient to define atopy. This relationship is underscored by observations that genetic polymorphisms predisposing humans to high serum IgE levels are associated with the development of asthma (1–3). Several of these polymorphisms affect genes encoding IL-4, IL-13, or their receptor components, attesting to the critical importance of these cytokines in regulating IgE levels in vivo (4–13). IL-4 and IL-13 are the only agents to date described to induce Ig class switch recombination to IgE. Both cytokines interact with a receptor consisting of IL-4R α paired with IL-13R α 1. IL-4 may also act through IL-4R α complexed with the γ common chain. In either case, signal transduction through IL-4R α results in the phosphorylation, dimerization, and nuclear translocation of STAT-6, which binds to a specific response element in the C ϵ promoter region, providing an essential signal for IgE production (14, 15).

Despite their shared receptor and overlapping activities, IL-4 and IL-13 do not appear to be interchangeable in vivo. In studies of murine allergic asthma, IL-13, but not IL-4, was found to be necessary and sufficient for generation of the asthmatic phenotype (16, 17). Although both cytokines are produced by activated Th2 cells, mast cells, and basophils, it is clear that their generation and function can be regulated independently. One major distinction is that T cells lack IL-13R α 1 and thus can respond to IL-4, but not IL-13 (18). B cells respond to both cytokines, and both induce IgE production, but distinctions exist in vivo. Neither IL-4-deficient nor IL-13-deficient mice produce wild-type levels of IgE following immunization with OVA (19–21) or treatment with anti-IgD (22), suggesting that one cytokine does not fully compensate for lack of

the other. In contrast, infection with the murine AIDS (MAIDS)³ virus generates a full IgE response in IL-4-deficient mice (22), indicating that IL-13 preferentially drives IgE production during this infection. Even more intriguing are observations that suggest IgE can be made in the absence of both IL-4 and IL-13, or in the absence of their shared signaling elements. Infection with MAIDS induces IgE in STAT6-deficient mice (23), and IL-4R α -deficient mice generate a serum IgE response, albeit weak and delayed, following immunization with OVA (19). Based on these observations, it is conceivable that additional pathway(s) exists to generate this important effector molecule.

In addition to that found in the circulation, IgE can exist in tissues, bound to the surface of mast cells. Mast cell-bound IgE has a longer $t_{1/2}$ than the circulating Ab (24), and can be a more sensitive indicator of bioavailable IgE levels (25). Cross-linking mast cell-bound IgE triggers degranulation and downstream effector function, including anaphylactic reactions. Such responses have been described in mice lacking IL-4R α (19), indicating that IgE effector function could be elicited in animals that lack responsiveness to either IL-4 or IL-13. Nevertheless, the use of OVA rather than anti-IgE to trigger the response leaves some question as to its IgE dependence. Oettgen et al. (26) showed that anaphylactic responses to Ag can be IgE independent, as they persisted in mice containing a homozygous C ϵ null mutation. In contrast, anaphylaxis triggered by anti-IgE was absent. Finkelman and colleagues (27) confirmed these results, showing that in mice lacking IgE or those deficient in Fc ϵ RI, Ag-dependent anaphylaxis could still be seen, whereas anti-IgE-dependent anaphylaxis was abolished. Anti-IgE-induced anaphylactic responses were complement independent in both studies (26, 27). To confirm and extend these observations, we now report anti-IgE-induced in vitro and in vivo IgE effector function in mouse strains deficient in IL-4 or IL-13, both IL-4 and IL-13, or the shared IL-4R α chain.

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³Abbreviations used in this paper: MAIDS, murine AIDS; PCA, passive cutaneous anaphylaxis; SCF, stem cell factor.

Materials and Methods

Mice

BALB/c mice were obtained from The Jackson Laboratory. IL-13R α 2^{-/-} mice were derived by M. Grusby (Harvard School of Public Health Boston, MA) (28). Mice deficient in IL-13 (21) and those lacking both IL-4 and IL-13 (29) were licensed from the Medical Research Council (Cambridge, U.K.), and bred at Taconic Farms. Mice lacking IL-4 (30) were purchased from The Jackson Laboratory. IL-4R α -deficient mice (31) were obtained through the Taconic Emerging Models Program (Taconic Farms). All mutant strains were on the BALB/c background. Age- and sex-matched animals were used in all experiments. All animal experiments were reviewed and approved by the Institutional Animal Care and Use Committee at Wyeth Research.

ELISA for serum Abs

To assay IgE levels, ELISA plates (MaxiSorp; Nunc) were coated overnight at 4°C with rat anti-mouse IgE (BD Biosciences). Plates were blocked for 1 h at room temperature with 0.5% gelatin in PBS, washed in PBS containing 0.05% Tween 20 (PBS-Tween), and incubated 6 h, room temperature with purified mouse IgE (BD Biosciences) as standard or with serum dilutions. Binding was detected with biotinylated anti-mouse IgE (BD Biosciences) using mouse IgG (Sigma-Aldrich) as a blocker. Binding was detected with peroxidase-linked streptavidin (Southern Biotechnology Associates) and Sure Blue substrate (Kirkegaard & Perry Laboratories). For determination of total IgG1, plates were coated with goat anti-mouse IgG (Sigma-Aldrich), and bound serum Ab detected with biotinylated rat anti-mouse IgG1 (BD Biosciences), using purified mouse IgG1 (BD Biosciences) as standard. For determination of OVA-specific IgE or IgG1, plates were coated with OVA (Sigma-Aldrich). Bound IgE was detected with biotinylated rat anti-mouse IgE (BD Biosciences) in the presence of mouse IgG as a blocking agent, and bound IgG1 was detected with biotinylated rat anti-mouse IgG1 (BD Biosciences).

Peritoneal mast cell degranulation

Peritoneal cavities of mice were lavaged with 5 ml of 25 mM PIPES, pH 7.2, containing 110 mM NaCl, 5 mM KCl, and 50 μ M EDTA. Peritoneal cells were pelleted and washed into DMEM containing 10 mM HEPES, 50 μ M 2-ME, 10% heat-inactivated FCS, 100 U/ml penicillin, 100 μ g/ml streptomycin, 2 mM L-glutamine, and 20 ng/ml mouse rIL-3 (R&D Systems), and incubated overnight at 37°C and 10% CO₂. Where indicated, 0.1 μ g/ml mouse IgE anti-DNP (Sigma-Aldrich) was added. For [³H]serotonin release, 1 μ Ci/ml [³H]5-hydroxytryptamine, [α , β -³H](N)-creatine sulfate (PerkinElmer Life and Analytical Sciences), was added to the overnight incubation. For the degranulation assay, cells were harvested, washed into pipes, albumin, calcium, magnesium buffer (25 mM PIPES, pH 7.2, containing 110 mM NaCl, 5 mM KCl, 5 mM CaCl₂, 2.5 mM MgCl₂, and 0.005% BSA), and challenged with the indicated concentration of rat anti-mouse IgE (BD Biosciences), compound 48/80 (Sigma-Aldrich), murine stem cell factor (SCF; R&D Systems), or ionomycin (Sigma-Aldrich) for 30 min at 37°C. Supernatants were collected and frozen. [³H]Serotonin release was determined by liquid scintillation counting. Histamine release was assayed by ELISA (Corixa). Total (maximum) serotonin or histamine was determined by lysing the cells with 1% Triton X-100. Spontaneous release was assayed by challenge with pipes, albumin, calcium, magnesium buffer instead of degranulating agent. The percentage of total release was calculated according to the formula: ((unknown - spontaneous)/(total - spontaneous)) $\times$ 100.

Flow cytometry

Peritoneal lavage was harvested, as described above. Cells were washed into PBS containing 1% BSA. In some cases, cells were preincubated for 60 min, 4°C with 5 μ g/ml mouse IgE anti-DNP (Sigma-Aldrich). Cells were stained with FITC-labeled Ab to mouse IgE and PE-labeled Ab to mouse c-kit (BD Biosciences) for 30 min on ice. Nonspecific binding was blocked by addition of Fc block (BD Biosciences) and rat IgG (Sigma-Aldrich). Cells were analyzed on a FACScan cytometer (BD Biosciences).

Passive cutaneous anaphylaxis

To induce mast cell degranulation, mice were challenged intradermally in the left ear with varying doses (0.02–100 ng in 20 μ l of PBS) of rat anti-mouse IgE (clone R35-72; BD Biosciences). As a control, rat IgG (Sigma-Aldrich) was injected intradermally into the right ear. Ear swelling was measured with an engineer's micrometer (Mitutoyo) at various time points postchallenge. Swelling was expressed as the change in ear thickness (Δ) from baseline preinjection values.

Active cutaneous anaphylaxis

Mice ($n = 10$ /group) were actively sensitized on days 0 and 14 with an i.p. injection of 20 μ g of OVA emulsified in 2.25 mg of alum (AlumImject; Pierce) for a total of 200 μ l. On day 28, mice were challenged intradermally in the left ear with 10 ng of anti-mouse IgE for a total volume of 20 μ l. As a control, mice received 10 ng of rat IgG (Sigma-Aldrich) into the right ear. Ear swelling was measured at various time points postchallenge, as described above, for the (PCA) studies. To assess serum IgE levels, blood was collected by retro-orbital puncture on days 0 and 28.

Tissue staining and confocal microscopy

Ears of mice were harvested, snap frozen in liquid nitrogen in OCT freezing medium (Tissue Tek), and stored at -80°C . Cryostat sections (4–5 μ m) were fixed in acetone, air dried, then incubated for 1 h at room temperature with PBS containing 1% BSA and PE-labeled Ab to c-kit (BD Biosciences). Rat IgG (Sigma-Aldrich) and Fc block (BD Biosciences) were included as blocking agents, and biotinylated anti-VCAM (BD Biosciences) and allophycocyanin-streptavidin (Molecular Probes) were used

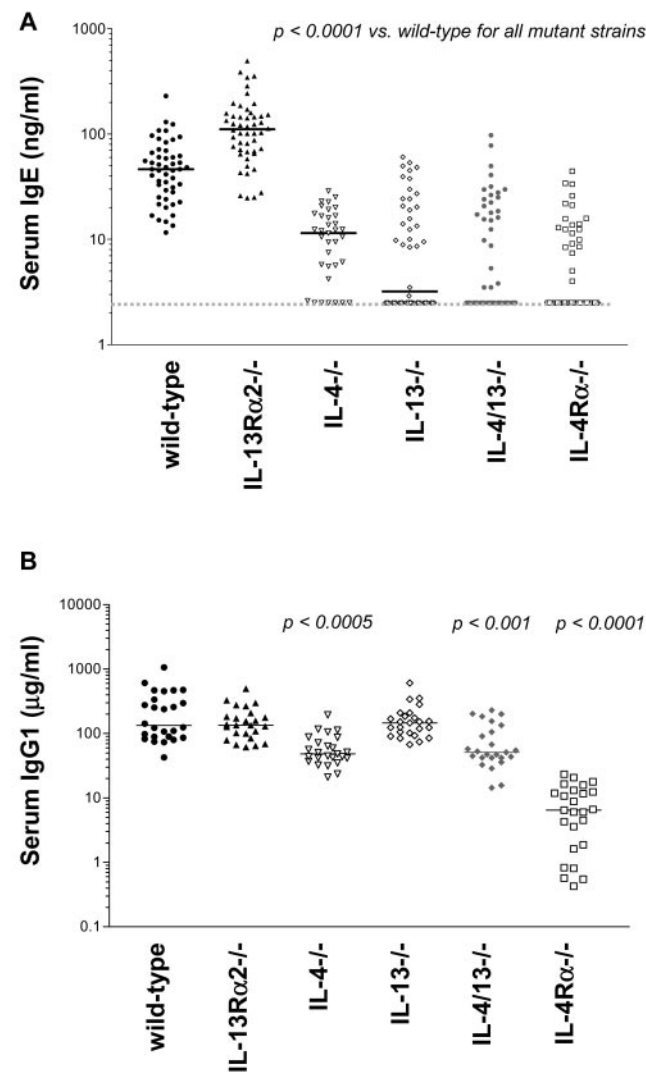


FIGURE 1. Mice lacking IL-4, IL-4/IL-13, or IL-4R have reduced serum IgE and IgG1 levels. Serum IgE and IgG1 concentrations were determined in naive BALB/c wild-type mice and in mutant strains lacking IL-4, IL-13, IL-4/IL-13, IL-4R α , and IL-13R α 2, as described in *Materials and Methods*. **A**, Serum IgE levels, determined from 35–50 mice of each strain. The broken line indicates the limit of assay sensitivity, 2.5 ng/ml. **B**, Total serum IgG1 levels, determined from 25 mice of each strain. Each point represents the mean of three separate determinations for an individual mouse. Horizontal bars represent the median value for each group. Values of p were determined by Student's t test.

as counterstains. Sections were fixed with 1% paraformaldehyde in PBS, and coverslips were mounted with ProLong antifade agent (Molecular Probes). Fluorescence was analyzed using a Nikon TE200 microscope with Radiance 2000 confocal system (Bio-Rad-Zeiss).

Results

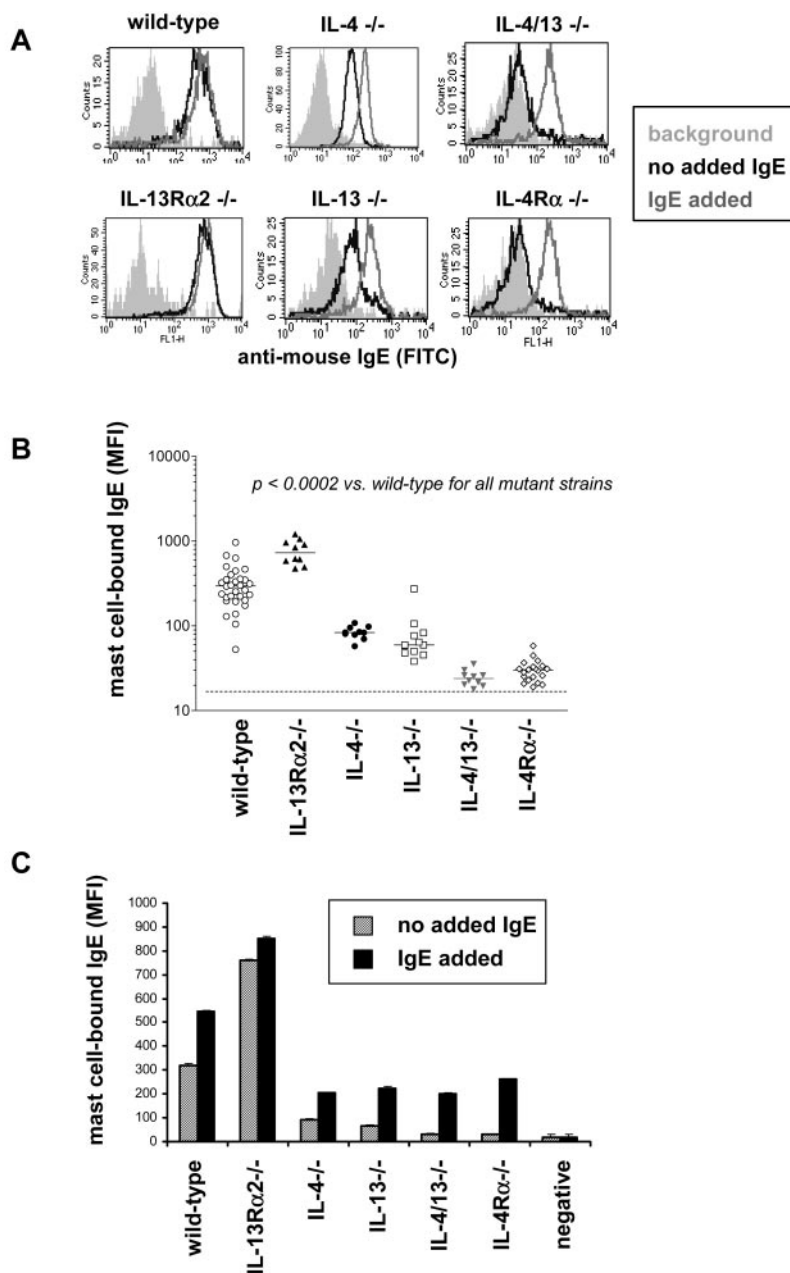
Mice deficient in IL-4 and/or IL-13 lack serum IgE

IL-4 and IL-13 are critical cytokines for IgE production in response to Ag challenge (14, 15). To investigate the requirement for these cytokines to support resting IgE levels in naive mice, serum was examined from wild-type mice and from mice genetically deficient in IL-4, IL-13, IL-4 + IL-13, IL-4R α , and IL-13R α 2. Mice deficient in IL-4, IL-13, both IL-4 and IL-13, or their common receptor component, IL-4R α , had greatly reduced or undetectable levels of serum IgE (Fig. 1) (21, 29, 32). For each of these strains, there was a distribution of serum IgE levels, such that some individual animals had detectable IgE, while the majority did not. In contrast, mice lacking the decoy receptor, IL-13R α 2 (18), displayed elevated levels of serum IgE, in agreement with previous studies (28).

In addition to IgE, IL-4 acts as a switch factor for IgG1 (33, 34), although the dependence on IL-4 is not as strict as for IgE (35). Therefore, we examined IgG1 levels in naive mice. Mice lacking IL-4, IL-4/IL-13, or IL-4R α were all deficient in circulating IgG1 levels (Fig. 1B). Interestingly, IL-13 $^{-/-}$ animals exhibited normal levels of serum IgG1 (Fig. 1B) combined with a profound deficiency in IgE (Fig. 1A), indicating that IL-13 may not fully share the IgG1 switch—promoting activity of IL-4.

Mice deficient in IL-4 or IL-13 display mast cell-bound IgE

Mast cells isolated from the peritoneal cavity of wild-type mice have high levels of cell surface IgE, most likely bound to Fc ϵ RI, and detected by staining the cells with anti-IgE Ab. They also express some level of unoccupied Fc ϵ RI, which can be assayed by incubating the mast cells with exogenous IgE. Comparison of



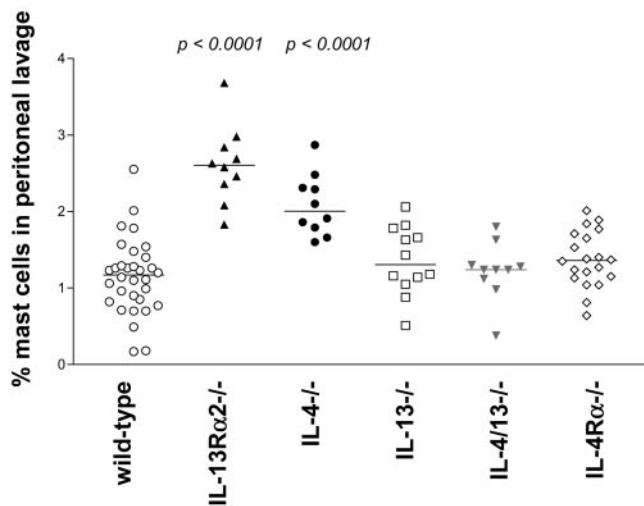


FIGURE 3. IL-13Rα2^{-/-} and IL-4^{-/-} strains have increased numbers of mast cells in peritoneal lavage. Peritoneal lavage was collected from BALB/c wild-type mice and from mutant strains lacking IL-4, IL-13, IL-4/13, IL-4Rα, and IL-13Rα2. Cells were stained with PE-labeled Ab to *c-kit*. Mast cells were identified by forward and side scatter profile in combination with high intensity staining for *c-kit*, and enumerated by flow cytometry as a percentage of total cells in peritoneal lavage. Each point represents an individual mouse. Values of *p* were determined by Student's *t* test.

anti-IgE staining on mast cells in the presence or absence of exogenous IgE indicates the presence of free IgE receptors that are available to be occupied (Fig. 2A). A large body of data has shown that both the proportion of occupied receptors and the overall level of receptor expression can be modulated by IgE exposure (36–39), and thus will reflect mast cell accessibility to IgE in vivo.

Peritoneal mast cells from wild-type and IL-13Rα2^{-/-} mice were found to have a high density of surface IgE receptor, which was almost fully occupied with endogenous IgE (Fig. 2, A and B). Peritoneal mast cells from IL-4^{-/-} and IL-13^{-/-} mice had intermediate levels of mast cell-bound IgE, and could capture addi-

tional exogenous IgE. Peritoneal mast cells from IL-4/13^{-/-} and IL-4Rα^{-/-} mice had little, if any, surface IgE staining above the negative control level, and a large proportion of empty FcεRI, indicated by their capacity to bind exogenous IgE (Fig. 2, A and B). Interestingly, for mice lacking IL-4, IL-13, IL-4/13, or IL-4Rα, total binding capacity following loading with exogenous IgE was highly comparable (Fig. 2C), indicating that the total IgE receptor density does not fall below a minimum level, even with low IgE exposure.

Mice deficient in IL-4 or IL-13 have normal numbers of peritoneal mast cells

In addition to IgE receptor expression, it has been proposed that the level of circulating IgE modulates mast cell number in vivo by affecting cell survival (40, 41). Mast cells were quantitated as a percentage of total peritoneal leukocytes in wild-type and mutant mice. Total cell numbers recovered in peritoneal lavage, as well as the percentages of lymphocytes and monocytes, were comparable for all groups (data not shown). IL-13Rα2^{-/-} mice had an increased percentage of mast cells in the peritoneal lavage (Fig. 3). IL-4^{-/-} mice also had a higher percentage of peritoneal mast cells (Fig. 3), in agreement with findings reported by Chen et al. (42). IL-13^{-/-}, IL-4/13^{-/-}, and IL-4Rα^{-/-} strains all had peritoneal mast cell frequency comparable with wild type (Fig. 3). Thus, a compensatory increase in mast cell number was not a general feature of those strains that had impaired IgE responses.

Peritoneal mast cells from mice deficient in IL-4 or IL-13 show IgE-dependent degranulation in vitro

Peritoneal mast cells can be degranulated in vitro by treatment with anti-IgE to cross-link IgE bound to FcεRI. As histamine releasability has been shown to correlate with the number of IgE molecules on mast cells (43), this response was compared with or without exposure of the cells to exogenous IgE in vitro. Maximum release was quantitated following solubilization of the cell membranes with Triton X-100. Wild-type mast cells achieved 22% maximum degranulation with 1000 ng/ml anti-IgE, which increased to 30% when the cells were preloaded with IgE (Fig. 4).

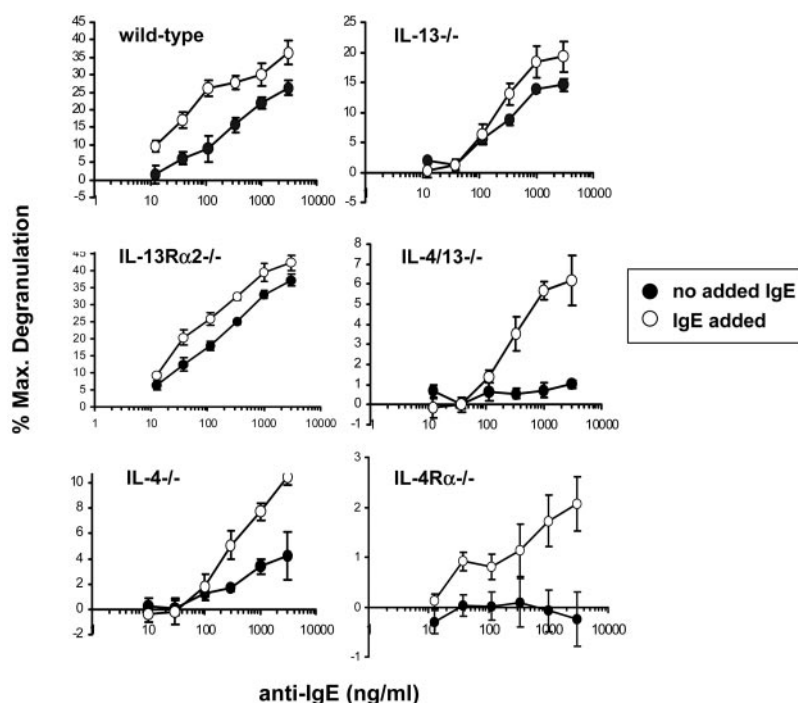


FIGURE 4. Peritoneal mast cells from mice lacking IL-4 or IL-13 are deficient in degranulation with anti-IgE. Peritoneal lavage was collected from BALB/c wild-type mice and from mutant strains lacking IL-4, IL-13, IL-4/13, IL-4Rα, and IL-13Rα2. Lavage from four to eight individual mice was pooled for each strain. Untreated cells (●) or cells loaded with exogenous IgE (○) were challenged with the indicated concentration of anti-mouse IgE. Degranulation was measured, as described in *Materials and Methods*. Data are shown as mean ± SD for four replicate challenge cultures per point.

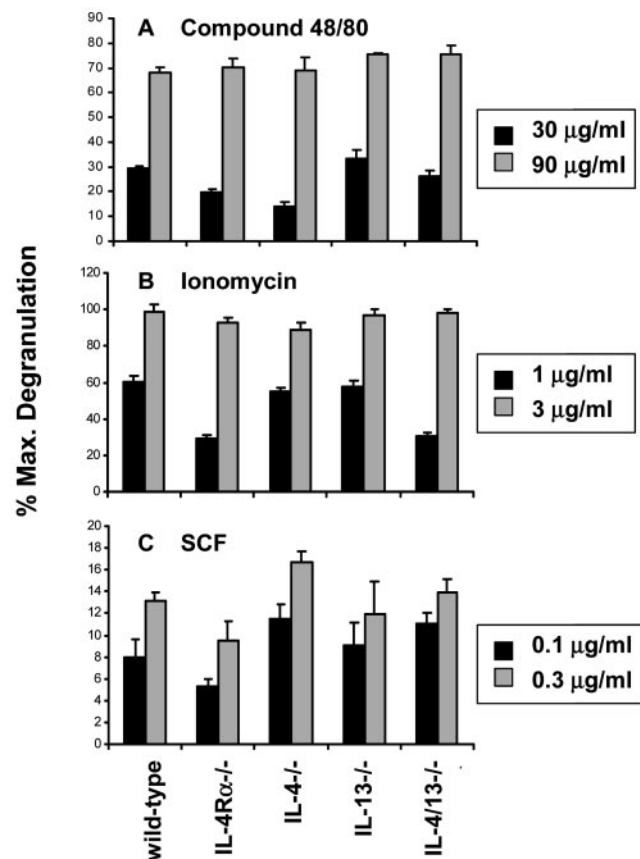


FIGURE 5. Peritoneal mast cells of all mutant strains show full degranulation responses to IgE-independent agents. Peritoneal lavage was collected from BALB/c wild-type mice and from mutant strains lacking IL-4, IL-13, IL-4/13, IL-4Rα, and IL-13Rα2. Lavage from four to eight individual mice was pooled for each strain. Cells were challenged with the indicated concentration of: *A*, compound 48/80; *B*, ionomycin; and *C*, SCF. Data are shown as mean ± SD for four replicate challenge cultures per treatment.

Consistent with their higher levels of bound IgE (Fig. 2), mast cells from IL-13Rα2^{-/-} mice reached 30% maximum degranulation with 1000 ng/ml anti-IgE, which increased to 40% upon loading with exogenous IgE (Fig. 4). In contrast, mice lacking either IL-4 or IL-13 had reduced degranulation responses, achieving only 8 and 18% maximum degranulation, respectively, after challenge with 1000 ng/ml anti-IgE, even after exposure to exogenous IgE (Fig. 4). Peritoneal mast cells isolated from mice lacking both IL-4 and IL-13, or those lacking IL-4Rα, degranulated only after IgE

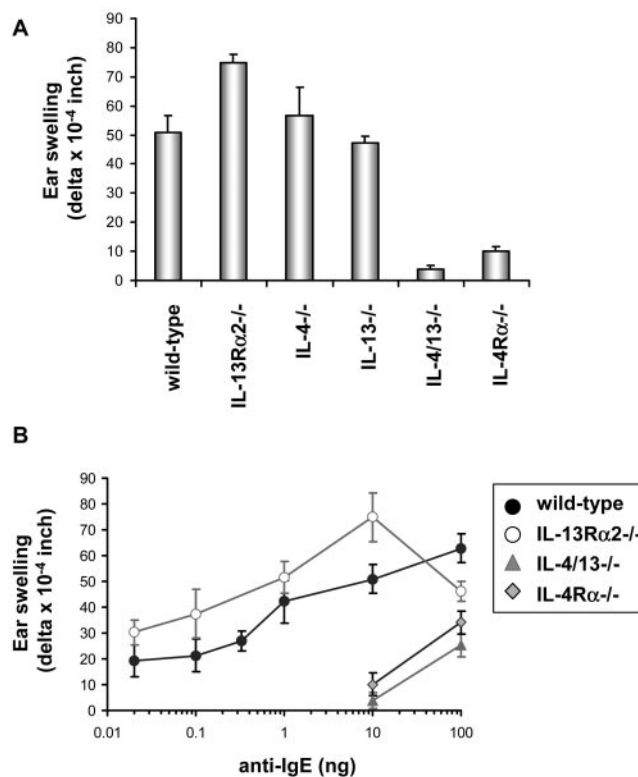


FIGURE 6. IL-4/13^{-/-} and IL-4Rα^{-/-} mice are deficient in anti-IgE-induced PCA. Naive mice from the indicated strain were challenged intradermally in the right ear with anti-mouse IgE Ab (rat IgG1), and ear thickness was determined 1 h later. Mice were challenged in the left ear with a control rat IgG1 of irrelevant specificity, which produced no detectable ear swelling. *A*, Ear thickness at 1 h postchallenge with 10 ng of anti-IgE, with prechallenge values subtracted. *B*, Anti-IgE dose response for PCA of naive mice from the indicated strain. Values shown are mean ± SD for five animals per group.

was added exogenously to fill empty receptors (Fig. 4), reaching 6 and 2% maximum degranulation, respectively, with 1000 ng/ml anti-IgE. Even though they showed a low level of degranulation in response to anti-IgE, peritoneal mast cells from IL-4/IL-13- or IL-4Rα-deficient mice did not have a general defect in degranulation, as they displayed normal responses to the IgE-independent stimuli compound 48/80, ionomycin, and SCF (Fig. 5). Table I shows that the levels of total and spontaneous degranulation did not differ significantly between wild-type and cytokine-deficient strains.

Table I. Levels of total and spontaneous [³H]serotonin release from peritoneal cells^a

Peritoneal Cells after Overnight Incubation with No Added IgE			Peritoneal Cells after Overnight Incubation with Exogenous IgE		
Cells	Total ^b	Spontaneous ^c	Cells	Total ^b	Spontaneous ^c
Wild-type	25,755 ± 2,133	3,273 ± 75	Wild-type	20,822 ± 698	3,287 ± 621
IL-4 ^{-/-}	28,052 ± 1,401	2,197 ± 154	IL-4 ^{-/-}	23,044 ± 930	4,258 ± 73
IL-13 ^{-/-}	25,429 ± 1,412	3,403 ± 143	IL-13 ^{-/-}	19,345 ± 1,172	3,918 ± 93
IL4/13 ^{-/-}	28,509 ± 4,176	2,125 ± 106	IL4/13 ^{-/-}	22,886 ± 820	3,042 ± 109
IL-4Rα ^{-/-}	28,327 ± 1,379	2,076 ± 135	IL-4Rα ^{-/-}	22,786 ± 1,256	2,656 ± 87

^a Peritoneal cells from the indicated strain were incubated overnight with [³H]serotonin in the presence of absence of 0.1 µg/ml mouse IgE. Degranulation was determined as cpm of [³H]serotonin released in a 30 min assay (see *Materials and Methods*). Data shown are mean cpm ± SD of five replicate determinations.

^b Total release was determined following lysis of cells with 0.1% Triton X-100.

^c Spontaneous release was determined by treatment of cells with buffer alone.

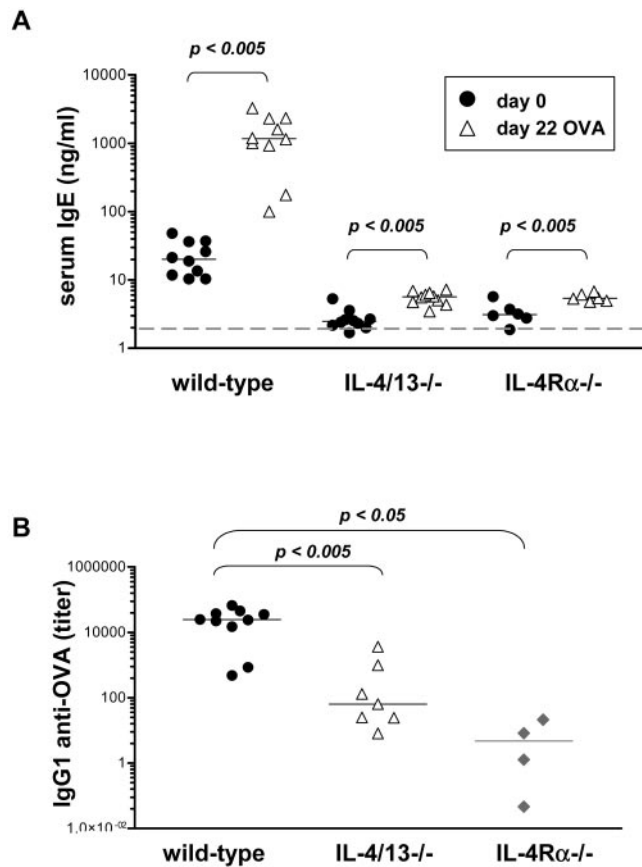


FIGURE 7. OVA immunization induces serum IgE and OVA-specific IgG1 in IL-4/13^{-/-} and IL-4Rα^{-/-} mice. BALB/c wild-type, IL-4/13^{-/-}, and IL-4Rα^{-/-} mice were immunized i.p. with OVA in alum on day 0, then boosted on day 14. Sera were collected on day 22. **A**, Total serum IgE levels, in ng/ml. **B**, OVA-specific IgG1 levels, expressed as serum dilution required to reach OD of 0.5 in a specific ELISA. The dashed line represents the limit of assay sensitivity. Each point represents the mean of three separate determinations for an individual mouse. Horizontal bars represent the median value for each group. Values of p were determined by Student's t test.

Mice deficient in IL-4/IL-13 or IL-4Rα exhibit IgE-dependent PCA

To examine the influence of IL-4 and IL-13 on IgE effector function in vivo, PCA was induced in wild-type and mutant mice. Injection of anti-IgE, but not control Ab, into the ears of naive wild-type mice induced a swelling response that was strong at 1 h, and persisted for over 6 h. The induction of this response with anti-IgE ensured that a mast cell-dependent event was being studied. Compared with wild-type controls, IL-13Rα2^{-/-} mice displayed enhanced PCA responses (Fig. 6A), consistent with their high levels of serum IgE (Fig. 1), mast cell-bound IgE (Fig. 2), and IgE-mediated mast cell degranulation (Fig. 4). A dose titration revealed that a ~10-fold lower concentration of anti-IgE was required to elicit a comparable ear-swelling response in IL-13Rα2^{-/-} mice as compared with wild-type controls (Fig. 6B).

Surprisingly, despite their lack of serum IgE and low levels of peritoneal mast cell-bound IgE, mice lacking either IL-4 or IL-13 displayed full ear-swelling responses as compared with wild-type controls, using a 10 ng/ear challenge dose of anti-IgE (Fig. 6A). These observations suggest that the ear-swelling response is induced by very low levels of mast cell-bound IgE. Animals deficient in both IL-4 and IL-13, however, failed to show a PCA response at the 10 ng/ear dose and required 100 ng/ear anti-IgE to

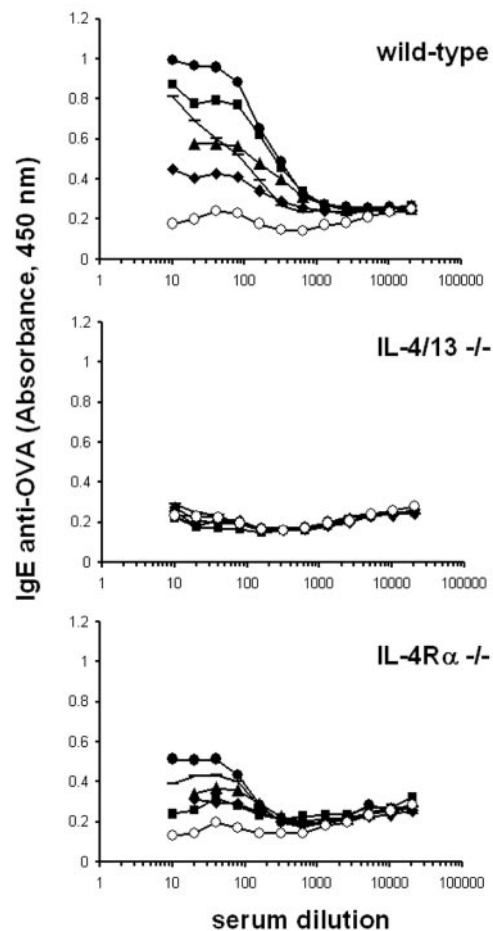


FIGURE 8. OVA immunization induces OVA-specific IgE in sera of wild-type and IL-4Rα^{-/-} mice. BALB/c wild-type, IL-4/13^{-/-}, and IL-4Rα^{-/-} mice were immunized i.p. with OVA in alum on day 0, then boosted on days 14 and 28. Sera were collected on day 34 and tested for the presence of OVA-specific IgE by ELISA. Filled symbols, sera from individual immune mice. Open symbols, average values for sera of same mice before immunization.

elicit detectable ear swelling above background (Fig. 6B). We estimate that >100-fold higher concentration of anti-IgE was required to elicit ear-swelling responses in IL-4/13^{-/-} or IL-4Rα^{-/-} mice comparable to those seen in wild-type controls (Fig. 6B). Staining of ear tissue sections with PE-labeled Ab to c-kit revealed that tissue mast cell content was comparable in wild-type, IL-4/13^{-/-}, and IL-4Rα^{-/-} mice (21.1 ± 4.2 , 23.6 ± 6.7 , and 22.5 ± 5.8 mast cells per field, respectively, at $\times 20$ magnification; $n = 12$ fields/strain), arguing against the possibility that low responses were due to decreased mast cell numbers in the IL-4/13^{-/-} or IL-4Rα^{-/-} strains. Because the response of IL-4/13^{-/-} and IL-4Rα^{-/-} mice required such high doses of anti-IgE injected into the ear, however, we cannot rule out that the ear swelling was due to mast cell degranulation resulting from complement activation, as opposed to an IgE receptor-mediated mechanism. Nevertheless, ear swelling above background was seen in each of four separate experiments with these strains.

Mice deficient in IL-4/IL-13 or IL-4Rα have enhanced IgE responses following immunization

To examine IgE responses following Ag exposure in wild-type, IL-4/13^{-/-}, and IL-4Rα^{-/-} mice, animals were immunized and boosted with OVA. Immunization increased total IgE levels in

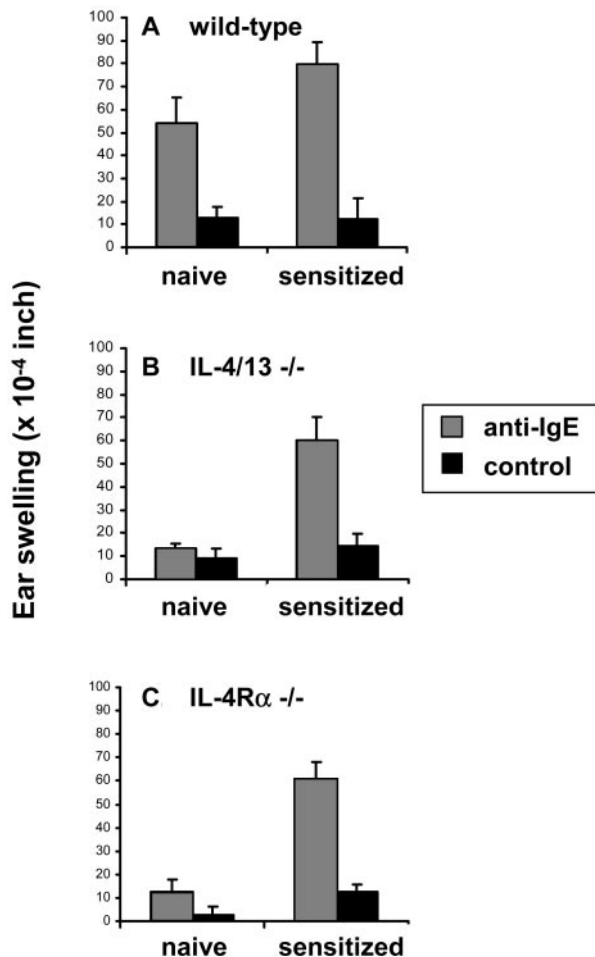


FIGURE 9. OVA immunization boosts IgE-dependent PCA reactions in IL-4/13^{-/-} and IL-4Rα^{-/-} mice. BALB/c wild-type (A), IL-4/13^{-/-} (B), and IL-4Rα^{-/-} (C) mice were immunized i.p. with OVA in alum on day 0, then boosted on day 14. On day 28, mice were challenged in the right ear with 10 ng of anti-mouse IgE Ab (rat IgG1) and in the left ear with a control rat IgG1 of irrelevant specificity. Ear thickness was measured 1 h later, and prechallenge values were subtracted. Values shown are mean ± SEM for five animals per group.

wild-type mice ~100-fold (Fig. 7A). Serum IgE levels in IL-4/13^{-/-} and IL-4Rα^{-/-} strains also increased, to detectable levels in all animals (Fig. 7A). Furthermore, OVA-specific IgG1 was detectable in both mutant strains (Fig. 7B), confirming their Ag responsiveness. OVA-specific IgE could also be found in sera from wild-type and IL-4Rα^{-/-} mice following immunization, although it was undetectable in sera of IL-4/13^{-/-} animals (Fig. 8). Because detection of IgE activity in the *in vivo* PCA response proved to be much more sensitive than serum IgE measurements by ELISA, we also examined effects of OVA immunization on the anti-IgE-induced ear swelling. Immunization with OVA induced a striking increase in ear-swelling responses to anti-IgE in IL-4/13^{-/-} and IL-4Rα^{-/-} mice, such that challenge with 10 ng of Ab elicited a strong PCA reaction (Fig. 9). Thus, a modest increase in serum IgE levels was associated with a strong boost in IgE-dependent ear-swelling responses in IL-4/13^{-/-} and IL-4Rα^{-/-} mice.

Discussion

The cytokines IL-4 and IL-13 are key regulators of B cell switch recombination to IgE. Thus, mice deficient in either IL-4 or IL-13 are impaired in serum IgE production following OVA immuniza-

tion (20, 21), *Nippostrongylus brasiliensis* infection (21), infection with *Plasmodium chabaudi* (44), or treatment with anti-IgD (22), indicating that both cytokines normally contribute to IgE production *in vivo*. Mice deficient in both IL-4 and IL-13, those lacking IL-4Rα, and those deficient in STAT6 would be expected to lack IgE completely, and most studies investigating serum IgE levels support this prediction (29, 45–47). Surprisingly, however, IgE has been detected in IL-4Rα knockout mice following immunization with OVA (19) and in STAT6-deficient animals infected with MAIDS (23), suggesting that under some conditions, IgE could be produced independently of IL-4 and IL-13.

Because mast cell-bound IgE could be a more sensitive indicator of IgE production *in vivo* than serum IgE levels (25), we investigated mast cell-mediated IgE effector function in mice lacking IL-4 and/or IL-13, or those lacking the shared IL-4Rα chain. Although the presence of either IL-4 or IL-13 alone was not sufficient to maintain wild-type levels of serum IgE, peritoneal mast cells recovered from IL-4^{-/-} or IL-13^{-/-} animals had detectable IgE bound to the cell surface. In contrast, mice deficient in both IL-4 and IL-13, or in IL-4Rα, had no detectable peritoneal mast cell-bound IgE. If challenged with high levels of anti-IgE in the ear, however, both IL-4/13^{-/-} and IL-4Rα^{-/-} mice exhibited a PCA response. Thus, PCA was the most sensitive indicator of IgE responsiveness, and implied that some level of IgE exists, even in the absence of IL-4 or IL-13 function.

Analysis of peritoneal mast cells from IL-4^{-/-} and IL-13^{-/-} animals revealed that IgE could be bound to the cell surface even when no IgE was detectable in serum, as has been noted previously (25). In serum, the *t*_{1/2} of IgE is only 8–12 h (48, 49), but in tissues, it extends to ~6 days (24). IgE bound to peritoneal mast cells was readily detectable in IL-4^{-/-} or IL-13^{-/-} mice, but at levels clearly lower than those found on wild-type mast cells. Although others have failed to detect IgE bound to mast cells of IL-4^{-/-} mice (42, 50), our demonstration of mast cell-bound IgE by flow cytometry is supported by the ability of peritoneal mast cells from IL-4^{-/-} and IL-13^{-/-} animals to degranulate in response to anti-IgE *in vitro*, and by the induction of a PCA reaction following administration of anti-IgE in these strains.

By adding IgE back to peritoneal mast cells, the surface IgE receptor can be saturated (50). Although this method is not quantitative, it gives an indication of the total surface receptor density and relative levels of occupied vs empty receptors. IgE receptors were almost completely occupied on peritoneal mast cells of wild-type mice. In contrast, for mice lacking IL-4, IL-13, or both, the presence of empty receptors could be demonstrated. Along with decreased receptor occupancy, peritoneal mast cells from these strains had an apparent decrease in IgE receptor expression, as inferred by their reduced capacity to bind exogenous IgE. Thus, low serum IgE levels were associated with low IgE receptor expression. In contrast, the high levels of serum IgE in IL-13Rα2^{-/-} mice were associated with increased mast cell IgE-binding capacity. These findings support the model that exposure to IgE regulates mast cell surface IgE receptor expression (36–39).

Mice lacking both IL-4 and IL-13, and those lacking IL-4Rα, did not have detectable IgE in the serum or bound to peritoneal mast cells, and showed no peritoneal mast cell degranulation *in vitro* when challenged with anti-IgE. Therefore, we were surprised to find that anti-IgE elicited a mild PCA reaction even in naive IL-4/13^{-/-} and IL-4Rα^{-/-} mice, although this required >100× higher anti-IgE concentration than that which produced ear swelling in wild-type mice. Thus, it appears that anti-IgE could trigger activation of skin mast cells, but not peritoneal mast cells in these strains. One explanation for these findings is that *in vitro* determination of histamine or serotonin release following peritoneal mast

cell degranulation is simply less sensitive than the in vivo PCA response. It is also possible that tissue mast cells have a lower activation threshold in terms of IgE receptor cross-linking than peritoneal mast cells, or that more IgE is bound to mast cells in skin than to those in the peritoneum. A reliable quantitative comparison could not be made using current methods. Nevertheless, in tissues, mast cells effectively localize and concentrate IgE bound to the high affinity receptor (51, 52), and in this way could maximize effector responses even when low levels of IgE are available.

Furthermore, mast cells in tissues could be exposed to higher levels of IgE than those in the peritoneum, and could derive IgE from sources outside the circulation. It has been suggested that tissue mast cells may acquire IgE directly from lymphatics rather than from the serum (51). In addition, local production of IgE at mucosal and tissue sites has been demonstrated conclusively by the detection of switch circles, which are obligatory intermediates in the switch recombination process (53). Switch recombination occurring at local tissue sites may be driven in part by IL-4 and IL-13 produced by the mast cells themselves (54–57). IgE produced in a local tissue environment rich in mast cells would most likely bind to those mast cells without ever being released into the circulation. Consistent with this, mast cells in tissues demonstrate IgE-mediated effector function even in the absence of detectable serum IgE (58), and levels of cell-bound IgE are more responsive to allergen challenge or parasite infection than serum IgE (59–61).

Upon immunization with OVA, total serum IgE became readily detectable in IL-4/13^{-/-} and IL-4Rα^{-/-} mice, and PCA responses were greatly enhanced. The relatively strong induction of anti-IgE-mediated PCA responses in association with only marginal increases in serum IgE following immunization is consistent with observations that even low level IgE receptor occupancy can support the degranulation of basophils (62, 63) or mast cells (43). The increased IgE levels and effector responses following immunization confirm and extend previous reports of OVA-induced anaphylactic responses in IL-4Rα^{-/-} mice (19), by indicating that neither IL-4 nor IL-13 is absolutely required for IgE production or for IgE-dependent mast cell activation in vivo. These observations suggest the existence of an additional pathway to IgE, the exact nature of which remains to be elucidated. IL-4 or IL-13 interacts with a receptor form containing the IL-4Rα chain, triggering the phosphorylation, dimerization, and nuclear translocation of STAT6, which promotes Cε gene expression and isotype switch recombination to IgE (14, 15). It is conceivable that IgE could be produced in the absence of IL-4Rα if another receptor form could interact with STAT6. Such a model has been proposed by Mattes et al. (64), who reported that IL-13 can promote airways hyperactivity in mice lacking IL-4Rα, but not in those lacking STAT6. In addition, the finding that IL-15 can trigger STAT6 phosphorylation in mast cells (65) raises the possibility that cytokines other than IL-4 or IL-13 could use STAT6 to drive IgE production. Others have shown that mice lacking STAT6 exhibit various IL-13-mediated allergic-type responses (66) and can even produce IgE under certain activation conditions (23), raising the alternative possibility of a STAT6-independent pathway leading to IgE in mice lacking IL-4 and IL-13. Finally, even though IL-4 and IL-13 may facilitate IgE switch recombination, the event may occur at some low rate even in the absence of these agents.

These findings have important implications for the treatment of IgE-mediated atopic diseases. IL-4 and IL-13 are clearly the most critical inducers of IgE in vivo, and numerous approaches targeting these cytokines are being developed as therapies for asthma and allergy (67). The present results suggest that even in the absence of IL-4 and IL-13, alternative pathways to IgE may exist, enhanced by immunization, and resulting in IgE effector responses in vivo.

Additional investigation is required to fully understand the nature of these pathways and their contribution to atopic reactions.

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Disclosures

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