

T_H1-promoting DNA immunization against allergens modulates the ratio of IgG1/IgG2a but does not affect the anaphylactic potential of IgG1 antibodies: No evidence for the synthesis of nonanaphylactic IgG1

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Background: In mouse, IgG1 has been reported to make up 2 functionally distinct phenotypes that also differ in their induction requirements. One of these phenotypes lacks anaphylactic activity.

Objective: We hypothesized that nonanaphylactic IgG1 could modulate allergic reactions and investigated whether such antibodies are induced by DNA immunization.

Methods: Mice were immunized with allergen-encoding plasmid DNA or with recombinant allergens and alum. Sera were analyzed for IgG subclasses by ELISA for anaphylactic IgE by rat basophil degranulation, and after heat inactivation of IgE for anaphylactic IgG by passive cutaneous anaphylaxis assay. IFN- γ and IL-5 from in-vitro restimulated spleen cells were quantitated by ELISA.

Results: After protein immunization, mice produced IgG1 and IgE, whereas DNA immunization elicited IgG1 and IgG2a but no IgE. However, all sera were positive for non-IgE-mediated passive cutaneous anaphylaxis. In the presence of anaphylactic IgG1, the additional occurrence of nonanaphylactic IgG1 cannot be strictly ruled out. To circumvent this problem, we immunized IL-4 receptor-deficient mice against Bet v 1a, because anaphylactic but not nonanaphylactic IgG1 has been reported to depend on IL-4. These animals produced only low amounts of IgG1, but sera were again positive for non-IgE-mediated anaphylactic activity.

Conclusions: Our results revealed no evidence for the production of nonanaphylactic IgG1. Furthermore, our data indicate that the development of non-IgE-mediated anaphylaxis does not require IL-4 receptor signaling. (*J Allergy Clin Immunol* 2003;112:579-84.)

Key words: Allergy, anaphylaxis, mouse, nonanaphylactic IgG1, T_H1, T_H2, genetic immunization, birch pollen, celery

Abbreviations used

id: Intradermal
PCA: Passive cutaneous anaphylaxis
RBL: Rat basophil leukemia
wt: Wild type

The development of IgE-mediated allergic diseases seems intimately linked to a dysregulation in the balance of T_H1 and T_H2 lymphocytes.¹⁻⁷ Current approaches to immunotherapy of allergic diseases generally aim at either inducing tolerance or redirecting the immune response to a T_H1-biased reaction. For the latter, genetic immunization⁸ has yielded promising results. Injection of allergen-encoding eukaryotic expression plasmids has been shown to induce T_H1-biased immune responses with promotion of IFN- γ -producing CD4⁺ T cells, elevated synthesis of IgG2a, and inhibition of IgE production.⁹⁻¹³ By use of such protocols, protective and therapeutic effects have been successfully established in laboratory animals with model antigens, as well as clinically relevant allergens.^{10,13-18} However, the underlying mechanisms are not yet fully understood. One explanation focuses on the inhibitory action of T_H1 cytokines on T_H2 cells with subsequent downregulation of IgE synthesis and IL-5-mediated eosinophilia.¹⁹ Alternately (or synergistically), T_H1-associated IgG2a could promote the rapid clearance of the allergen from the organism or directly inhibit mast cell and basophil degranulation by triggering inhibitory signal transduction by means of Fc γ R2 receptors.²⁰⁻²³

In mouse, anaphylaxis can be induced not only by IgE but also by IgG1 immunocomplexes.^{24,25} The development of IgG1-secreting B cells, although not strictly dependent on IL-4,^{26,27} is greatly promoted by this cytokine.²⁸⁻³⁰ Recently, however, mouse IgG1 has been reported to be made up of 2 functionally distinct subclasses that differ in their requirement for IL-4 and in their anaphylactic potential.^{31,32} In these experiments, nonanaphylactic IgG1 was produced in wild-type (wt) as well as in IL-4-deficient mice, whereas the anaphylactic type was found only in wt mice. Furthermore, immu-

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nization with Freund's adjuvant yielded high amounts of nonanaphylactic IgG1, whereas with alum, this subtype was suppressed in favor of anaphylactic IgG1.

On the basis of these findings, we investigated whether nonanaphylactic IgG1 might be generated in the course of T_H1-promoting genetic immunization and contribute to the antiallergic effects of such therapeutic approaches. With the major birch pollen allergen Bet v 1a and the celery allergen Api g1, mice were immunized with these proteins together with alum or by intradermal injection (id) of plasmid DNA encoding the respective allergen genes. Sera were analyzed for IgG1 and IgG2a levels by ELISA and for their anaphylactic activity by rat basophil degranulation and passive cutaneous anaphylaxis (PCA) assay.

METHODS

Mice

Female BALB/c mice purchased from the animal breeding facility at Himberg (Austria) or IL-4 receptor-deficient mice³³ maintained at the local animal facility, respectively, were used for experiments at 8 to 16 weeks of age.

Plasmid constructs

Eukaryotic expression vectors for the birch pollen allergen Bet v 1a (gb: X15877), pCMV-Bet, and, analogously, for the celery allergen Api g1 (gb: Z48967), pCMV-API, were constructed as previously described for pCMV-Bet.¹¹ Plasmid purification was performed with the Endo-Free Plasmid Giga kit (Qiagen, Germany). The endotoxin content of all preparations used for immunization was < 0.04 pg/μg DNA as measured by limulus assay (Pyroquant, Germany).

Recombinant allergens

Lyophilized recombinant Bet v 1a and Api g1 (BioMay, Austria) were reconstituted in pyrogen-free water and stored aliquoted at -20°C.

Immunization and serum collection

DNA immunization was performed by id injection of 100 μg plasmid DNA on the abdominal skin followed by 2 boosts at 2-week intervals and a final boost 4 weeks later. Immunization with alum was done in the same way with 5 μg of recombinant allergen adsorbed to the adjuvant gel (Alu-Gel-S, Serva, Germany). For some experiments, 5 μg of recombinant allergen was emulsified with CFA and injected id. Two boosts were given with incomplete Freund's adjuvant at 4-week intervals. Blood was collected from the tail vein 2 weeks after the last boost. Serum was prepared and stored at 4°C until analysis.

ELISA

Black High-Bind Immunoplates (Greiner, Austria) were coated with 100 ng/well of recombinant allergen. After blocking with 5 mg/mL BSA and 0.1% Tween-20 in PBS, serial dilutions of sera in blocking buffer were incubated for 1 hour. After being washed in PBS/0.1% Tween-20, bound antibodies were reacted with peroxidase-conjugated rat antimouse IgG1 (Serotec) or rat antimouse IgG2a (Pharmingen), respectively, and developed with BM chemiluminescence substrate (Boehringer Mannheim, Germany) measured on a Lucy-I microplate luminometer (Anthos Labtec, Austria). IgG1 and IgG2a concentrations were calculated from the serum dilution

that gave a signal of 10⁶ photon counts/second. Preceding calibration of the detection, antibodies revealed that this signal intensity represented 3.2 ng IgG2a and 1.6 ng IgG1, respectively.

Rat basophil leukemia (RBL) assay

Anaphylactic serum IgE was measured by degranulation of RBL cells (RBL-2H3, ATCC CRL-2256) as described.³⁴ In 96-well plates, 4 × 10⁴ cells/well were seeded overnight. The next day, murine sera were added at the indicated dilutions for 2 hours. After washing in Tyrode's buffer, degranulation was induced by adding 100 μL allergen (0.3 μg/mL) in Tyrode's buffer for 30 minutes at 37°C. Supernatants were analyzed for β-hexosaminidase by incubation with 80 μmol/L 4-methylumbelliferyl-*N*-acetyl-β-D-glucosaminide (Sigma) in citrate buffer (0.1 mol/L, pH 4.5) for 1 hour at 37°C. The reaction was stopped with 100 μL 0.2 mol/L glycine, 0.2 mol/L NaCl, pH 10.7, and fluorescence was measured at λ_{ex} = 360/λ_{em} = 465 nm on a microplate fluorescence reader (Spectrafluor, Tecan, Austria). Control wells without sera were included for spontaneous release, and total release was measured after the addition of 1% Triton X-100 to separate wells. Results were calculated as percent specific release (ie, 100 × [experimental - spontaneous]/(total - spontaneous)).

PCA

Anaphylactic activity of sera obtained from immunized mice was determined by PCA reactions in naive BALB/c mice as described, with slight modifications.³¹ Serum pools from each experimental group were incubated at 56°C for 3 hours to inactivate IgE. Inactivated sera were serially diluted in PBS, and 20 μL were injected id on the shaved back. After 2 hours, the animals were challenged intravenously with 100 μL of 0.5% Evan's blue and 50 μg recombinant allergen in PBS. Blue spots at the injection sites were inspected on the inner skin surface, and the PCA titer was determined as the reciprocal of the highest serum dilution that gave a positive result.

Production of cytokines by restimulated lymphocytes

Splenocytes were prepared 2 weeks after the last boost and, after lysis of erythrocytes, cultured with 500 μL/well of minimal essential medium with 5% FCS and antibiotics in 48-well tissue culture plates at 2 × 10⁶ cells/well. Wells were stimulated with 20 μg/mL allergen, or ovalbumin as an irrelevant antigen, for 48 hours. Supernatants were tested for IFN-γ and IL-5 by immunoassay on a Luminex-100 Multianalyte detection system (MiraiBio) following the manufacturer's instructions.

Statistical analysis

Significance between experimental groups was calculated by Mann-Whitney rank sum test or, if normality was given, by Student *t* test. Only *P* values < .05 were indicated in the figures.

RESULTS

Antibody and cytokine profiles are specifically modulated by different immunization strategies

The ratio of Ig subclasses elicited during an immune response is profoundly modulated by a multitude of microenvironmental factors such as specific T-cell subpopulations and their cytokines. In mouse, IgG1 and IgE are generally associated with T_H2-type reactions, whereas IgG2a is a T_H1-type Ig subclass. To gain insight into

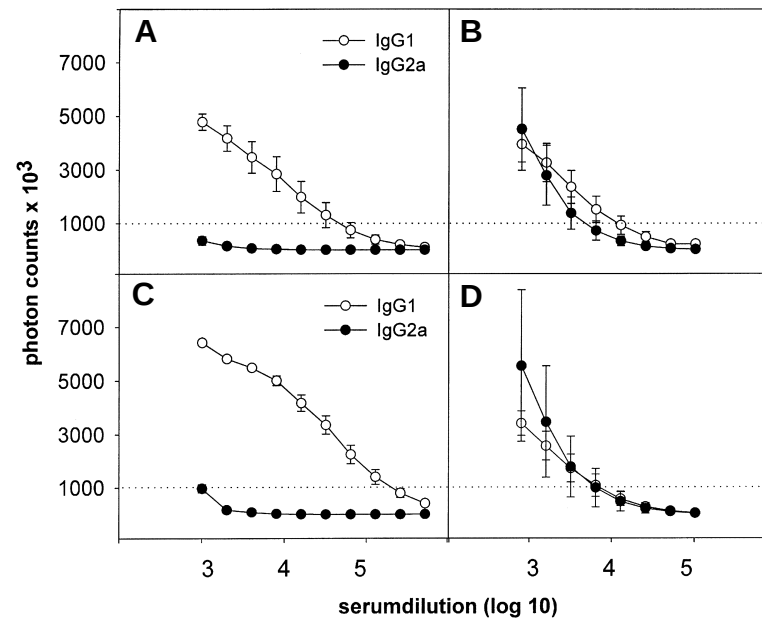


FIG 1. ELISA for IgG isotypes elicited against Bet v 1a (A, B) and Api g1 (C, D) after protein immunization with alum (A, C) or DNA immunization (B, D). Antibody concentrations, as listed in Table I, were calculated from the serum dilution that gave a signal of 10⁶ photon counts/second (dotted line). Data are shown as mean $\pm$ SEM of groups of 4 mice, each. A and C, Significance between IgG1 and IgG2a signals: $P = .029$ at all dilutions, as calculated by Mann-Whitney rank sum test.

the modulation of the antibody isotype profiles elicited by the different immunization strategies, we compared the production of IgG1 and IgG2a antibodies against the 2 plant allergens Bet v 1a and Api g1. BALB/c mice immunized with recombinant Bet v 1a and alum produced high levels of IgG1 but virtually no IgG2a. By contrast, genetic immunization yielded comparable concentrations of both IgG subclasses. Similar results were found with the celery allergen Api g1 (Fig 1, Table I).

To investigate the effect of both immunization methods at the T-cell level, we analyzed culture supernatants of antigen-restimulated splenocytes for IFN- γ and IL-5 as T_H1 and T_H2 indicator cytokines, respectively. IFN- γ was produced after DNA immunization but not after protein immunization with alum. IL-5 was found only at low concentrations but seemed clearly restricted to protein immunization with alum and was not detected after DNA immunization (Table I). The production of these cytokines was antigen specific, because they were not induced with the heterologous allergen, with ovalbumin, or without antigen (not shown). Again, similar results were obtained with both allergens.

Anaphylactic IgE is induced by protein immunization but not by DNA immunization

To determine the content of anaphylactic IgE, sera were tested for their ability to sensitize RBL cells (RBL-2H3) for antigen-induced release of β -hexosaminidase. Murine IgE, but not murine IgG, has been shown to mediate rat basophil degranulation and, hence, with murine sera, this assay is specific for IgE.^{34,35} In our

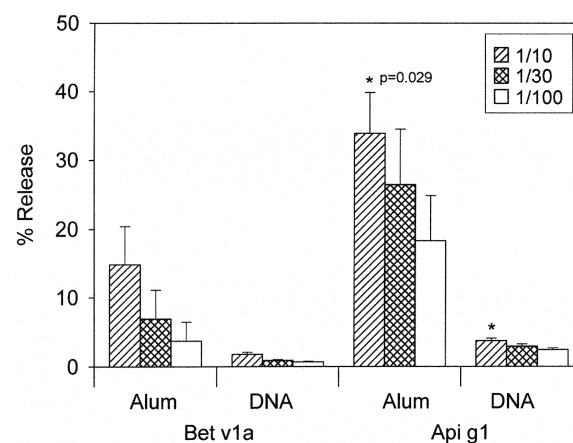


FIG 2. IgE-mediated allergen-specific degranulation of rat basophil leukemia-2H3 cells sensitized with the indicated sera at the indicated dilutions. Release without sensitization was 3%; sera of naive mice gave a specific release of less than 5% at a dilution of 1:10 (not shown). Results are reported as mean $\pm$ SEM of 4 mice. Significance was tested by Mann-Whitney rank sum test.

experiments, anaphylactic IgE was detected after protein immunization with alum but not after genetic immunization (Fig 2). All sera were negative after heat inactivation, confirming that the release was mediated by IgE. Degranulation was antigen specific, because no release was found with the heterologous antigen or with sera of naive mice (data not shown).

TABLE I. Cytokines, IgG subclasses, and specific anaphylactic activity of IgG1

	IFN- γ /IL5* (pg/mL)	IgG1/IgG2a (Ratio) $\dagger$ (μ g/mL)	PCA titer $\ddagger$	SAA $\S$ PCA/IgG1
Alum + rBetv1a	0/12.8	640/< 32 (>20)	80	0.125
pCMV-Bet v1a	3082/0	160/128 (=1.3)	20	0.125
Alum + rApi g1	4.7/22.3	1600/< 32 (>50)	480	0.300
pCMV-Api g1	2569/1.4	144/208 (=0.7)	80	0.556

PCA, Passive cutaneous anaphylaxis; SAA, specific anaphylactic activity.
*IFN- γ and IL-5 in culture supernatants, pooled from each experimental group, of spleen cells restimulated in vitro with the recombinant immunogen.
 $\dagger$ Antigen-specific IgG1 and IgG2a of serum pools from each group (by ELISA) and IgG1/2a ratio (in brackets).
 $\ddagger$ PCA titer: highest serum pool dilution that yielded skin blueing.
 $\S$ SSA of IgG1, SSA:PCA titer divided by the IgG1 concentration.

The specific anaphylactic potential of IgG1 is independent of the immunization method and of the cytokine profile

Although, both Ig subclass and cytokine profiles varied considerably with the immunization strategy, all sera contained high levels of IgG1. To assess the anaphylactic potential of these antibodies, serial dilutions of pooled sera from each experimental group were analyzed after heat inactivation of IgE by PCA assay. No PCA activity was detected with sera of naive mice. However, independently of the immunization method, and with both allergens, all sera contained significant amounts of anaphylactic IgG1. Moreover, normalization of the PCA titers for the respective serum IgG1 concentrations revealed that the specific anaphylactic activity of IgG1 induced by DNA immunization with pCMV-Bet was similar to that induced by protein immunization with rBet v 1a and alum. With Api g1, the specific anaphylactic activity was even higher after DNA immunization (Table I).

Induction of anaphylactic IgG does not depend on IL-4R signaling

The preceding experiments have not revealed evidence for the presence of nonanaphylactic IgG1 after DNA immunization. However, it has been shown previously that the induction of anaphylactic IgG1 depends on IL-4, whereas nonanaphylactic IgG1 was promoted by T_H1 cytokines and immunization with Freund's adjuvant.^{31,32} Therefore, on attempting to establish a method for the production of nonanaphylactic IgG1 for further studies, we tested whether immunization of IL-4 receptor-deficient (IL-4R^{-/-}) mice with Bet v 1a in CFA would induce nonanaphylactic sera. As shown in Fig 3, A, knockout mice produced high levels of IgG2a but only little amounts of IgG1. Antigen-restimulated spleen cells from IL-4R^{-/-} and wt BALB/c mice produced IFN- γ (417 and 246 pg/mL, respectively), as well as IL-5 (213 and 317 pg/mL, respectively). Furthermore, anaphylactic IgE was found in the sera of wt but not IL-4R^{-/-} mice (Fig 3, B). Nevertheless, heat-inactivated sera from wt and knockout mice still showed high anaphylactic potential (Fig 3, C). This was confirmed to be mediated by

IgG1, because depletion of sera, by immunoaffinity chromatography, from IgG1 but not IgG2a or IgG2b abolished the PCA activity (not shown).

DISCUSSION

In this study, we have explored whether nonanaphylactic IgG1 is induced in response to genetic immunization against allergens. Injection (id) of plasmid DNA induces immune reactions with a significant contribution of T_H1 reactions as evidenced by the appearance of IgG2a and IFN- γ . Therefore, analogous to previous reports having shown that nonanaphylactic IgG1 was promoted by T_H1 environments,³² we expected that genetic immunization, also, would provide suitable conditions for that. We hypothesized that such antibodies could be involved in the prophylactic and therapeutic effects of genetic immunization on allergic diseases.

To test this hypothesis we compared the immune response to id injection of allergen-encoding eukaryotic expression plasmids with that elicited by protein immunization with recombinant allergens and alum as a T_H2-promoting adjuvant.

As expected, protein immunization with alum yielded a typical T_H2 response with high levels of IgG1, with anaphylactic IgE and IL-5, but virtually no IgG2a and no IFN- γ . By contrast, genetic immunization induced substantial amounts of IFN- γ and serum IgG2a but no IgE and no IL-5. Hence, in the face of the reported cytokine requirements, DNA immunization should favor the synthesis of nonanaphylactic IgG1. Therefore, if such antibodies were indeed present in DNA immune sera at reasonable concentrations, then, even together with anaphylactic IgG1 in the same sample, the specific anaphylactic IgG1 activity (ie, the PCA titer normalized for the IgG1 concentration) should be significantly lower than in sera of mice immunized with protein and alum. However, in our experiments, this value was identical (for Bet v 1a) and even higher (for Api g1) with DNA immune sera, indicating that the immune response induced by DNA immunization was not dominated by nonanaphylactic IgG1. It remains to be clarified why, although apparently providing a suitable cytokine profile, DNA immunization failed to induce

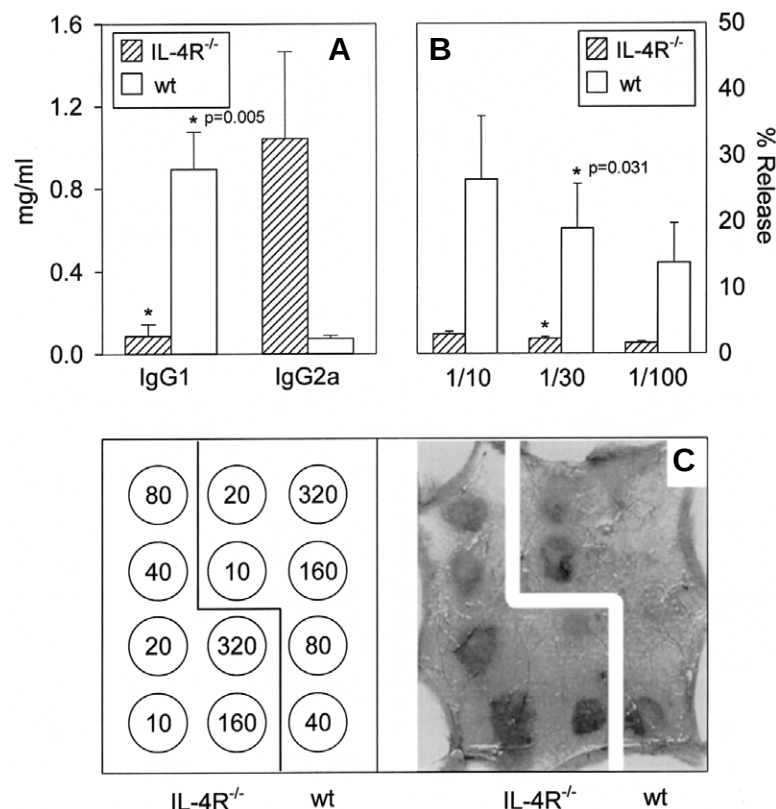


FIG 3. IL-4R^{-/-} or wild-type BALB/c mice (n = 4 in each group) were immunized with rBet v 1a and CFA. **A**, Bet v 1a-specific serum antibody isotypes (ELISA). **B**, IgE-mediated antigen-specific degranulation of rat basophil leukemia-2H3 cells sensitized with serial serum dilutions as indicated. Significance was tested by Student *t* test. **C**, *Right*, heat-resistant passive cutaneous anaphylaxis, induced with serial log2 dilutions (starting with 1:10) of pooled sera from each group; *left*, positioning scheme and dilution factors of applied serum pools.

nonanaphylactic IgG1. The appearance of anaphylactic antibodies is frequently discussed in the context of T_H1/2 cytokine balance. Furthermore, with respect to genetic immunization, additional factors such as the induction of CD8⁺ T cells or special activation mechanisms of antigen presenting cells could possibly influence the characteristics of immune reactions.

Despite the well-documented inhibitory effect of DNA immunization on IgE production, concomitant induction of anaphylactic IgG1 has been found with selected mouse strains but not with others.³⁶ This might at least partially explain why, in some model systems, residual systemic anaphylaxis was observed after such treatment, although IgE levels were dramatically reduced.¹⁴

Although anaphylactic IgG1 has been reported to depend on IL-4,³² we were unable to raise nonanaphylactic IgG1 in IL-4R knockout mice and, not shown in this article, in IL-4 knockout mice. Such mice produced only low levels of IgG1, but these antibodies again proved anaphylactic in PCA assays. In the course of this study, we have also tested protein immunization with Freund's adjuvant and gene gun immunization (ie, epicutaneous particle bombardment with DNA-coated gold particles). However, although differing in IgG subclass

levels, all these sera were positive for heat-resistant PCA activity (not shown).

This provoked the question whether there is, indeed, a separate IgG1 phenotype existing in terms of Fc-γ receptor triggering. In this study, we were interested in IgG1-mediated anaphylaxis as such and, hence, although an intriguing question, the molecular nature of this activity was not our primary focus. It might be noteworthy that, in our experiments and those of others, PCA activity is a phenomenologic parameter and does not discriminate IgG1 subtypes at a molecular level. For example, with Bet v 1a and CFA, we have obtained sera with a specific anaphylactic potential of only about 20% of that achieved with alum (not shown). Principally, this could reflect a mixture of 2 types of IgG1 that differ in their capability to trigger mast cell surface receptors. Alternately, it could equally well be due to a lower efficiency for antigen cross-linking (eg, caused by lower antigen affinity or a too limited epitope variety). Although some older studies have demonstrated IgG1 with differences in complement fixation³⁷ or protein A binding,³⁸ more biochemical research will be required to clarify such questions. Generally, however, with a multitude of different antigens tested in different mouse strains and with differ-

ent immunization protocols, until now we have never obtained sera with substantial amounts of IgG1 that lacked heat-resistant anaphylactic activity.

In conclusion, our data did not reveal any evidence for the induction of nonanaphylactic IgG1. Furthermore, it was clearly demonstrated that IgG1-mediated anaphylactic activity does not strictly depend on IL-4 receptor signaling and is not even restricted to T_H2 polarized immune reactions.

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