IgG-blocking antibodies inhibit IgE-mediated anaphylaxis in vivo through both antigen interception and FcγRIIb cross-linking

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Although it has long been hypothesized that allergen immunotherapy inhibits allergy, in part, by inducing production of IgG Abs that intercept allergens before they can cross-link mast cell FcεRI-associated IgE, this blocking Ab hypothesis has never been tested in vivo. In addition, evidence that IgG-allergen interactions can induce anaphylaxis by activating macrophages through FcγRIII suggested that IgG Ab might not be able to inhibit IgE-mediated anaphylaxis without inducing anaphylaxis through this alternative pathway. We have studied active and passive immunization models in mice to approach these issues and to determine whether any inhibition of anaphylaxis observed was a direct effect of allergen neutralization by IgG Ab or an indirect effect of cross-linking of FcεRI to the inhibitory IgG receptor FcγRIIb. We demonstrate that IgG Ab produced during the course of an immune response or administered passively can completely suppress IgE-mediated anaphylaxis; that these IgG blocking Abs inhibit IgE-mediated anaphylaxis without inducing FcγRIII-mediated anaphylaxis only when IgG Ab concentration is high and challenge allergen dose is low; that allergen epitope density correlates inversely with the allergen dose required to induce both IgE- and FcγRIII-mediated anaphylaxis; and that both allergen interception and FcγRIIb-dependent inhibition contribute to in vivo blocking Ab activity.

We initiated such in vivo studies because of unexpected results that were obtained in an animal model of anaphylaxis in which mice were immunized with a goat Ab against mouse IgD (GtMD), which stimulates large IgG1, IgE, IL-4, and mast cell responses and a small IgG2a response, but little or no IgG3 or IgG2b production (refs. 15–19 and F.D. Finkelman, unpublished data) and challenged with 100 µg of the relevant Ag, goat IgG (GlgG) (20). Although GlgG challenge induced severe anaphylaxis, anaphylaxis was mediated by IgG, FcγRII, macrophages, and platelet-activating factor (PAF), rather than by IgE, FcεRI, mast cells, and histamine (20). In view of the strong IgE, IL-4, and mast cell responses that develop in GtMD-treated mice, it seemed unlikely that the failure of GlgG challenge to induce IgE-mediated anaphylaxis resulted from a lack of IgE or mast cells. Instead, the strong IgG anti–GlgG (IgGtGlgG) response that develops in these mice raised the possibility that IgGtGlgG blocked IgE-mediated anaphylaxis, either by intercepting GlgG before it could bind to IgE/FcεRI on mast cells or by cross-linking FcεRI to FcγRIIb. We have now performed in vivo studies to evaluate these possibilities. Our results show that allergen-specific IgG can block IgE-mediated anaphylaxis in vivo; define conditions under which blocking occurs without inducing FcγRIII-mediated anaphylaxis; and demonstrate the importance of both Ag interception and FcγRIIb-mediated inhibition as mechanisms of BA function.

Results

IgG BA inhibits IgE-mediated anaphylaxis in GtMD-immunized mice by intercepting Ag before it can cross-link mast cell–associated IgE. GtMD immunization induces marked increases in IgE and mastocytosis (ref. 17 and F.D. Finkelman, unpublished data). Despite this, challenging GtMD-immunized mice with 100 µg of the relevant Ag,
IgG, induces anaphylaxis that is independent of IgE, FcRI, and mast cells but requires IgG, FcγRII, and macrophages (20). Three mechanisms might inhibit IgE-mediated anaphylaxis in this system: (a) IgG Ab might intercept IgG before it could be bound by mast cell–associated IgE; (b) mouse IgG–anti-IgG complexes might inhibit mast cell FcεRII signaling by cross-linking FcεRI to FcγRIIb; and (c) “nonspecific” IgE produced by GtMD-immunized mice might displace IgE anti-IgG Ab from mast cell FcεRI.

We attempted to distinguish among these possibilities by increasing the dose of IgG used to challenge GtMD-immunized mice from 0.1 to 10 mg (Figure 1). Some GtMD-immunized mice were pretreated with anti–FcγRI/RII mAb 1 day before IgG challenge to block IgG-mediated anaphylaxis and FcγRIIB-associated inhibition of IgE-mediated anaphylaxis. Challenge with 0.1 or 10 mg of IgG induced anaphylaxis of similar severity, as measured by hypothermia (which reflects the development and degree of shock) and hemococoncentration (which reflects vascular leak), when mice were not pretreated with anti–FcγRI/RII mAb. However, only the 10-mg dose of IgG induced anaphylaxis in anti–FcγRI/RII mAb–treated mice (Figure 1, A and B). Increasing the dose of challenge Ag should saturate BA and allow Ag to cross-link mast cell–associated FcεRI but should not affect FcγRIIB-mediated inhibition of mast cell degranulation or competition between IgG-specific and nonspecific IgE for mast cell FcεRI. Thus, our observation supports the hypothesis that IgE-mediated anaphylaxis in GtMD-immunized mice is inhibited by IgG BA interception of the challenge Ag.

These results did not eliminate the possibility that IgG BA suppresses IgE-mediated anaphylaxis in GtMD-immunized mice by both intercepting Ag and cross-linking FcεRI to FcγRIIb. Anti–FcγRII/RII mAb blocks both the FcγRIIb-dependent, macrophage-dependent pathway of anaphylaxis and FcγRIIB-dependent inhibition of mast cell–mediated anaphylaxis, which makes it impossible to isolate FcγRIIB-dependent inhibition in WT mice. To isolate FcγRIIB inhibition, we compared the effects of anti–FcγRI/RII mAb on anaphylaxis induced by high-dose (10 mg) Ag challenge in GtMD-immunized WT and FcγRII-deficient mice. Anti–FcγRI/RII mAb had its expected inhibitory effect on anaphylaxis in WT mice, but little, if any, inhibitory or stimulatory effect in FcγRII-deficient mice (Figure 1C). Thus, Ag interception, rather than the cross-linking of FcεRI to FcγRIIb, accounts for most of the inhibition of IgE-mediated anaphylaxis in GtMD-immunized mice.

If IgG BA in GtMD-immunized mice inhibits IgE-mediated anaphylaxis by intercepting Ag, it should be possible to demonstrate IgG-Ag complexes in the blood of immunized, Ag-challenged mice and to directly show that serum IgG Ab blocks Ag binding to IgE. Experiments were performed to test each of these predictions. Because it is difficult to assay for the mouse IgG–IgG complexes that should be formed in GtMD-immune mice challenged with IgG, we instead used a system that takes advantage of the strong Ab response generated to molecules conjugated to GtMD but allows more sensitive and precise detection of the Ag-Ab complex. Mice primed with a conjugate of trinitrophenyl-GtMD (TNP-GtMD) develop a large IgG1 anti-TNP Ab response (21). TNP-OVA–mouse IgG complexes were easily detected in serum 5 minutes after TNP-GtMD–immunized mice were challenged with 1 mg of TNP-OVA (Figure 1D).

Figure 1
FcγRII–independent anaphylaxis in GtMD-primed mice requires challenge with a high dose of Ag. (A) BALB/c mice (5 per group) were primed i.c. with GtMD, then challenged i.v. 14 days later with 0.1 or 10 mg of IgG. Some mice were pretreated 24 hours before IgG challenge with 500 µg of anti–FcγRII/RII mAb to block IgG-mediated anaphylaxis. Rectal temperatures were followed for 2 hours after challenge. (B) Mice primed and challenged as in A had blood drawn before and 15 minutes after challenge. Hematocrit levels were determined. *P < 0.05 compared with other measured levels. (C) TNP-OVA-NIP was diluted in nonimmune serum or heat-inactivated serum pooled from mice immunized 10–12 days earlier with GtMD (εGtMD Asm) or TNP-GtMD (εTNP Asm). Binding of serum TNP-OVA-NIP by IgEcTNP was measured by ELISA. Means ± SEs are shown for all data in this and subsequent figures unless otherwise indicated.
Figure 2
IgE/FcεRII/mast cell–dependent anaphylaxis in GβδMD-primed mice requires challenge with a high dose of Ag. Mice (4–5 per group) were primed s.c. with 0.2 ml of GβδMD, then challenged i.v. 14 days later with GlgG. Temperature was followed for 2 hours after challenge, and the maximum temperature decrease was calculated. Mice were matched for genetic background in all experiments. (A) WT mice and mice deficient in FcεRII, IgE, or both were challenged as shown. (B) WT (+) and mast cell–deficient W/Wv (−) mice were treated as shown. (C) BALB/c mice were injected 15–30 minutes before challenge with 66 μg of C6209 (PAF antagonist), 0.2 mg of both tripolidine and cimetidine (H1 and H2 antagonists), all 3 antagonists, or no antagonist and challenged as shown. (D) BALB/c mice were injected i.v. with 1 mg of gadolinium (macrophage inhibitor) or saline 1 day before GlgG challenge. (E) BALB/c mice were injected s.c. with saline or 500 μg of anti–FcεRI/RII/RII mAb 1 day before GlgG challenge. Blood was drawn 2 hours after GlgG challenge, and MMCP-1 levels were determined. (F) BALB/c mice were injected s.c. with saline or 500 μg of anti–FcεRI/RII/RII mAb 1 day before GlgG challenge. Anticoagulated blood was obtained for histamine measurement 5 minutes after challenge. (G) BALB/c mice were bled 4 hours after challenge with the indicated dose of IgG, and IL-4 secretion was evaluated by in vivo cytokine capture assay (IVCCA) (51). *P < 0.05.

To directly determine whether Ag immunization can inhibit Ag binding to IgE, we immunized mice with GβδMD or TNP-GβδMD and evaluated the ability of their serum to block TNP-OVA binding by IgE anti-TNP mAb (IgE/anti-TNP). This was done by mixture of immune or nonimmune serum with a doubly haptenated Ag (TNP-OVA–3-nitro-4-hydroxy-5-iodophenylacetyl [TNP-OVA-NIP]), capture of this Ag onto microtiter plate wells with anti-NIP mAb, and then determination of whether captured TNP-OVA-NIP could be bound by IgE/anti-TNP. This assay detected IgE anti-TNP binding to as little as 2 × 10^3 ng of TNP-OVA-NIP per milliliter in serum from nonimmune or GβδMD-immune mice which lack anti-TNP Ab but did not detect IgE anti-TNP binding to the highest concentration of TNP-OVA tested (5 × 10^4 ng/ml) in serum from TNP-GβδMD–immunized mice (Figure 1E). Thus, immune serum specifically inhibits IgE binding to Ag by a factor of more than 250.

Characterization of anaphylaxis induced by low and high doses of challenge Ag in GβδMD-immunized mice. To provide additional evidence that induction of IgE-mediated anaphylaxis in GβδMD-immune mice requires high-dose Ag challenge, we characterized IgE, FcεRII, cell type, and mediator requirements for anaphylaxis in GβδMD-immune mice challenged with either low-dose (0.1–0.25 mg) or high-dose (10 mg) GlgG. FcεRII-deficient, Igε-deficient, and FcεRII/IgE–double-deficient mice were used to evaluate the importance of the IgG/FcεRII and IgE/FcεRII anaphylaxis pathways in these experiments. With low-dose Ag challenge, anaphylaxis was FcεRII-dependent and IgE-independent, while high-dose challenge induced anaphylaxis through both pathways (Figure 2A). Double-deficient mice failed to develop anaphylaxis when challenged with either a high or a low Ag dose. Consistent results were observed when neither anaphylaxis pathway was operative because FcεRII-deficient mice were pretreated with anti-IgE mAb to neutralize IgE and desensitize mast cells, or IgE-deficient mice were treated with the anti–FcεRII/RII mAb to block FcεRII/RII and desensitize macrophages (not shown). Studies with mast cell–deficient, W/Wv mice were also consistent. Although blocking FcεRII with anti–FcεRII/RII mAb abolished the anaphylactic response to low-dose, but not high-dose, Ag challenge in WT mice, anti–FcεRII/RII mAb blocked this response to both low- and high-dose Ag challenge in W/Wv mice (Figure 2B). Furthermore, consistent with observations that FcεRII-mediated anaphylaxis is predominantly PAF-dependent while IgE-mediated anaphylaxis is predominantly histamine-dependent (20), responses to low-dose Ag challenge were inhibited more by a PAF antagonist than by antihistamine, while the opposite sensitivity to mediator antagonists was seen for high-dose Ag challenge (Figure 2C). Similarly, gadolinium, which inhibits macrophage, but not mast cell, function (22–24), suppressed the response to low-dose, but not high-dose, Ag challenge (Figure 2D). Finally, studies performed to directly evaluate IgE-mediated mast cell activation revealed 50-fold higher serum levels of mouse mast cell protease-1 (MMCP-1) and 10-fold higher serum levels of histamine (both markers of mast cell degranulation) in mice challenged with high- rather than low-dose Ag (Figure 2, E and F), and these responses were not substantially inhibited by anti–FcεRII/RII mAb. In con-
by high-dose Ag in G, as we have hypothesized. However, experiments with actively immunized mice did not rule out an alternative possibility that IgE-dependent anaphylaxis is induced by low-dose Ag challenge, whether this putative inhibitory factor is Ag-specific, or whether it is an IgG Ab. Investigation of each issue required studies in which IgE-dependent anaphylaxis could be studied in the absence of IgG BA and concentrations of IgE and IgG Abs could be precisely defined and flexibly adjusted.

To develop such a system, mice were primed with IgE/αTNP and challenged 1 day later with TNP-OVA. In contrast to the more than 250-µg dose of Ag required to induce IgE-mediated anaphylaxis in the GαMD system, anaphylaxis in IgE/αTNP-primed mice was induced by as little as 10 ng of TNP-OVA, and a plateau in severity was approached at approximately 1 µg (Figure 3A). When mice were instead primed with heat-inactivated mouse anti-TNP antisera (αTNP Asm), which contains IgG but not IgE antibodies to TNP, more than 10 µg of TNP-OVA was required to induce anaphylaxis, and anaphylaxis was more severe in mice challenged with 500 µg of TNP-OVA than in mice challenged with 100 µg (Figure 3B). Mice primed with either IgE/αTNP or αTNP Asm did not respond to i.v. OVA that was not TNP-conjugated (data not shown). The approximately 1,000-fold difference in the doses of Ag required to induce anaphylaxis in mice primed with IgE/αTNP versus αTNP Asm suggested that αTNP Asm might be able to block anaphylaxis in IgE/αTNP-primed mice without inducing IgG-mediated anaphylaxis, if the dose of challenge Ag were less than that required to induce anaphylaxis by the FcyRII-dependent pathway.

To test this possibility, unprimed or IgE/αTNP-primed mice were injected with saline, αTNP Asm, or, as a control, heat-inactivated mouse anti-GIgG antisera (αGIgG Asm; produced by mice immunized with GαMD), then challenged with 1 µg of TNP-OVA.
Significant hypothermia developed in mice that initially received IgE-TNP with or without αGIgG Asm but did not develop in mice that initially received both IgE-TNP and αTNP Asm (Figure 3C). Thus, a constituent of serum from TNP-GtMD–immunized, but not GtMD-immunized, mice can block IgE-mediated anaphylaxis in vivo without mediating FcγRIII-dependent anaphylaxis when mice are challenged with a relatively low dose of Ag.

To demonstrate that IgG is the TNP-GtMD immune serum constituent that blocks IgE-mediated anaphylaxis, we purified the IgG fraction of αTNP Asm (IgGtMD-TNP) from this serum and tested its ability to block IgE-mediated anaphylaxis. Concentrations of the αTNP Asm and its IgG fraction were adjusted to similar anti-TNP Ab titers, as determined by ELISA (not shown). Anaphylaxis was inhibited by the IgG fraction at least as well as by the unfractionated antisera (Figure 3D). To determine whether IgGtMD-TNP Ab could also mediate anaphylaxis, presumably through the FcγRIII-dependent mechanism, in mice challenged with a higher dose of Ag, mice primed with purified IgGtMD-TNP were challenged with 70 ng or 500 μg of TNP-OVA. Anaphylaxis developed in mice challenged with the high, but not the low, TNP-OVA dose (Figure 3E).

Finally, to prove the FcγRIII-dependence of anaphylaxis in mice primed with αTNP Asm and challenged with Ag and demonstrate the ability of high-dose Ag to overcome IgG blocking of IgE-mediated anaphylaxis, as in our active anaphylaxis model, we primed anti-TNP Ab–primed mice with a TNP-OVA preparation that averaged 10.4 TNP moieties per OVA molecule (TNP<sub>10.4</sub>-OVA). Because not all allergens have so many identical determinants (epitopes) on a single Ag molecule and high epitope density should increase the ability of an allergen to cross-link IgE/FcRII on mast cells and make it more difficult to block IgE/FcRI cross-linking with an IgG BA, we investigated the influence of Ag epitope density on IgE- and FcγRIII-mediated anaphylaxis and on IgG BA inhibition of IgE-mediated anaphylaxis (Figure 4). As expected, the quantity of TNP-OVA required to induce anaphylaxis in mice primed with a fixed dose of IgE-TNP or αTNP Asm increased as the molar TNP/OVA ratio decreased, although the increase was less marked for IgE-mediated anaphylaxis than for IgG-mediated anaphylaxis (Figure 4A, left and right panels, respectively).

To determine whether the quantity of αTNP Asm required to inhibit IgE-mediated anaphylaxis or IgE-mediated basophil IL-4 production is affected by challenge Ag epitope density, mice were primed with 10 μg of IgE-TNP, then challenged with doses of TNP<sub>10.4</sub>-OVA, TNP<sub>4.7</sub>-OVA, TNP<sub>1.3</sub>-OVA, or TNP<sub>0.4</sub>-OVA that induce similar degrees of mast cell–dependent hypothermia and basophil-dependent IL-4 production but are too low to induce FcγRIII-dependent anaphylaxis. Results of these studies demonstrate that the quantity of αTNP Asm required to block hypothermia and IL-4 production is relatively constant when differences in challenge Ag epitope density are compensated for by adjustment of challenge Ag dose and that more αTNP Asm is required to inhibit IL-4 production than to block the development of hypothermia (Figure 4B). Because the amount of IgG Ab required to block IgE/FcRII–mediated anaphylaxis is not affected by decreas-
IgG BA inhibits IgE-mediated anaphylaxis mechanisms. Our active anaphylaxis studies suggested that IgG BA suppresses IgE-mediated anaphylaxis by Ag interception rather than by cross-linking FcεRI to FcγRIIb. It remained possible, however, that Ag interception and FcεRI-FcγRIIb cross-linking are redundant inhibitory mechanisms. If so, the inhibitory effect of FcεRI-FcγRIIb cross-linking might only become apparent when concentrations of IgG BA are limiting. To evaluate this possibility, we compared the ability of αTNP Asm to (a) inhibit IgE-mediated anaphylaxis and IgE induction of basophil IL-4 secretion in WT versus FcγRIIb-deficient mice (Figure 5A) and (b) inhibit the same phenomena in FcγRII- deficient mice that had been treated with anti–FcγRII/RIII mAb, to selectively block FcγRIIb signaling, or with an isotype-matched control mAb (Figure 5B). Inhibition of FcγRIIb signaling did not affect IgE-mediated anaphylaxis but substantially decreased the basophil IL-4 response, in the absence of αTNP Asm, in both sets of experiments. Addition of αTNP Asm inhibited IgE-mediated anaphylaxis and basophil IL-4 secretion in all experiments, even when FcγRIIb was absent or blocked. However, 2- to 4-fold more αTNP Asm was required to suppress IgE-mediated anaphylaxis, and more than 4-fold more αTNP Asm was required to suppress basophil IL-4 secretion to the same extent in mice in which FcγRIIb was absent or blocked as in mice in which FcγRIIb was present and functional. Thus, IgG BA inhibits IgE-mediated anaphylaxis by both intercepting Ag molecules and cross-linking FcεRI to FcγRIIb. FcεRI-FcγRIIb cross-linking is not required to inhibit IgE-mediated anaphylaxis or IL-4 production when IgG BA is present in excess, but it amplifies the inhibitory effect of limiting concentrations of IgG BA.

**Discussion**

Our studies provide direct in vivo evidence that allergen-specific IgG BA can protect against IgE-mediated immunopathology. This evidence was obtained in 2 in vivo systems: a relatively natural model (active immunization) and a model that is more artificial but also more precise and flexible (passive immunization). Priming in the active immunization model was achieved by immunization with GtxMD, which induces large GliG-specific IgE and IgG responses (15, 16). Using this model, IgE/FcεRI/mast cell–mediated anaphylaxis could only be induced by a high dose of Ag, while a lower Ag dose could induce IgG/FcγRII/ macrophage–dependent anaphylaxis. This combination of a large IgG response to immunization and the need for high-dose Ag challenge to induce IgE-mediated anaphylaxis suggested that the IgG was intercepting challenge Ag before it could reach the IgE. This possibility was supported by direct evidence that IgG Abs in serum form complexes with injected Ag and inhibit Ag binding to IgE.

This interpretation was confirmed in a system in which Ab transfer was used both to prime mice for IgE-mediated anaphylaxis

es in Ag epitope density that are compensated for by increases in Ag dose while decreases in Ag epitope density increase the Ag dose required to induce IgG/FcγRII- mediated anaphylaxis more than the dose required to induce IgE/FcεRI-mediated anaphylaxis, the ability of IgG Ab to block IgE/FcεRI-mediated anaphylaxis without permitting FcγRII-mediated anaphylaxis increases as Ag epitope density decreases.

**IgG BA inhibits anaphylaxis by 2 mechanisms.** Our active anaphylaxis studies suggested that IgG BA suppresses IgE-mediated anaphylaxis by Ag interception rather than by cross-linking FcεRI to FcγRIIb. It remained possible, however, that Ag interception and FcεRI-FcγRIIb cross-linking are redundant inhibitory mechanisms. If so, the inhibitory effect of FcεRI-FcγRIIb cross-linking might only become apparent when concentrations of IgG BA are limiting. To evaluate this possibility, we compared the ability of αTNP Asm to (a) inhibit IgE-mediated anaphylaxis and IgE induction of basophil IL-4 secretion in WT versus FcγRIIb-deficient mice (Figure 5A) and (b) inhibit the same phenomena in FcγRII-deficient mice that had been treated with anti–FcγRII/RIII mAb, to selectively block FcγRIIb signaling, or with an isotype-matched control mAb (Figure 5B). Inhibition of FcγRIIb signaling did not affect IgE-mediated anaphylaxis but substantially decreased the basophil IL-4 response, in the absence of αTNP Asm, in both sets of experiments. Addition of αTNP Asm inhibited IgE-mediated anaphylaxis and basophil IL-4 secretion in all experiments, even when FcγRIIb was absent or blocked. However, 2- to 4-fold more αTNP Asm was required to suppress IgE-mediated anaphylaxis, and more than 4-fold more αTNP Asm was required to suppress basophil IL-4 secretion to the same extent in mice in which FcγRIIb was absent or blocked as in mice in which FcγRIIb was present and functional. Thus, IgG BA inhibits IgE-mediated anaphylaxis by both intercepting Ag molecules and cross-linking FcεRI to FcγRIIb. FcεRI-FcγRIIb cross-linking is not required to inhibit IgE-mediated anaphylaxis or IL-4 production when IgG BA is present in excess, but it amplifies the inhibitory effect of limiting concentrations of IgG BA.

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This interpretation was confirmed in a system in which Ab transfer was used both to prime mice for IgE-mediated anaphylaxis...
and to inhibit IgE-mediated anaphylaxis. Studies with this passive transfer system demonstrated that IgE-mediated anaphylaxis can be inhibited by transfer of purified Ag-specific IgG Ab. This transfer system also allowed differentiation of Ag dose requirements for IgE- versus IgG-mediated anaphylaxis and definition of the circumstances in which IgG Ab can protect against IgE-mediated anaphylaxis without inducing anaphylaxis through the IgG/FcγRII/macrophage pathway. The most critical differentiating factor for the induction of IgE- versus IgG-mediated anaphylaxis was the amount of challenge Ag. In the absence of IgG BA, IgE-mediated anaphylaxis could be induced by less than 50 ng of TNP-OVA, while induction of IgG-mediated anaphylaxis required more than 1 μg of the same Ag. In contrast, in the presence of BA, the quantity of Ag required to trigger IgE-mediated anaphylaxis increased substantially, until considerably more Ag was required to induce IgE-mediated anaphylaxis than IgG/FcγRII–mediated anaphylaxis, as seen in our active anaphylaxis system. Thus, IgG BA has a purely protective effect when the quantity of challenge Ag is less than that required to trigger IgG-mediated anaphylaxis. This protective effect is lost, however, as the amount of challenge Ag dose is increased. This results both from insufficient interception of challenge Ag before it can cross-link IgE/FcγRI on mast cells and from the generation of enough Ag–IgG Ab complexes to activate FcγRII-dependent mediator production by macrophages. Thus, IgG BA should be more protective in people challenged with a low dose of allergen (for example, an insect sting) than in people challenged with a high dose of allergen (for example, infusion of an antibiotic).

IgE-mediated anaphylaxis in mice primed with IgE and TNP and challenged with TNP-OVA was suppressed when mice were also injected with heat-inactivated serum pooled from mice immunized with TNP-OVA, which contained IgG anti-TNP and IgG anti-GlGG Ab, but not when mice were injected with heat-inactivated serum pooled from GlGG-immunized mice, which contained anti-GlGG but not anti-TNP Ab. Therefore, IgG inhibition of IgE-mediated anaphylaxis is Ag-specific.

Transfer of IgE and IgG Ab allowed comparison of the effects of varying the epitope density of the challenge Ag on IgE- versus IgG-mediated anaphylaxis and on the consequent ability of IgG Ab to protect against IgE-mediated anaphylaxis without mediating FcγRII-dependent anaphylaxis. Increasing the hapten density of TNP-OVA reduced the quantity of TNP-OVA required to induce IgG-mediated anaphylaxis more than it reduced the quantity of TNP-OVA required to induce IgE-mediated anaphylaxis, and, as a result, decreased the relative ability of IgG Ab to inhibit IgE-mediated anaphylaxis without inducing FcγRII-dependent anaphylaxis. These observations suggest that immune complexes that contain several IgG molecules may be required to efficiently cross-link FcγRII (a low-affinity receptor) and activate macrophages, while more limited cross-linking of mast cell FcRI by a high-affinity interaction between Ag and FcRI-associated IgE can efficiently induce mast cell degranulation.

Finally, studies with both active and passive immunization models defined and quantitated the importance of FcRI-FcγRIIb interactions in BA inhibition of anaphylaxis. Interactions between the stimulatory and inhibitory receptors were not required for BA suppression of IgE-mediated anaphylaxis: suppression was seen in both the active and the passive anaphylaxis models in FcγRIIb-deficient mice and in WT and FcγRII-deficient mice in which FcγRIIb function was blocked by anti–FcγRII/RII mAb. Furthermore, IgE-mediated anaphylaxis, in the absence of BA, did not differ in severity between WT and FcγRIIb-deficient mice or between anti–FcγRII/RII mAb–treated and control mAb–treated FcγRII-deficient mice. This suggests that a direct IgE-FcγRIIb interaction did not inhibit IgE-mediated anaphylaxis in our model, although such inhibition has been observed in another study (26). However, our data suggest inhibition of IgE-mediated basophil IL-4 production by an IgE/FcγRIIb interaction: IgE-mediated IL-4 responses were 2- to 3-fold higher in FcγRIIb-deficient mice than in WT mice, and in WT mice treated with anti–FcγRII/RII mAb than in WT mice treated with a control mAb. Furthermore, experiments in our passive anaphylaxis model confirmed the previously reported importance of IgG-FcγRIIb interactions in the regulation of anaphylaxis (26, 27). Two- to 4-fold more IgG BA was required to inhibit IgE-mediated anaphylaxis in FcγRIIb-deficient mice than in WT mice, and in anti–FcγRII/RII mAb–treated FcγRII-deficient mice than in mice of the same strain that were treated with a control mAb. Thus, IgG BA inhibits IgE-mediated anaphylaxis through 2 mechanisms: it intercepts Ag before it can cross-link mast cell FcRI-associated IgE, and it cross-links FcRI to FcγRIIb. FcRI-FcγRIIb cross-linking appears to contribute importantly to BA function when BA levels are limiting but is redundant when BA concentrations are high relative to concentrations of Ag. Our demonstration that FcRI-FcγRIIb cross-linking can suppress IgE-dependent anaphylaxis is consistent with evidence that IgG-IgE Fc fusion proteins suppress mast cell degranulation (28, 29).

Because IgG BA may be present in limiting amounts in allergy patients who have received immunotherapy, the inhibitory effect of cross-linking FcRI to FcγRIIb is likely to have an important role in controlling IgE-mediated anaphylaxis. As a result, the efficacy of immunotherapy may be affected by FcγRIIb polymorphisms: BA and immunotherapy that induces BA production may most effectively suppress IgE-mediated anaphylaxis in people who have allelic forms of the FcγRIIb gene that are associated with the most potent inhibitory FcγRIIb function (30, 31).

Two reservations must be considered about the relevance of our predictions to human disease and therapy. First, FcγRII-dependent anaphylaxis, as demonstrated in our mouse model, has never been demonstrated in humans. This may result from the difficulty of detecting this phenomenon rather than from its absence. Because humans, like mice, have macrophages that express FcγRII and that can be induced by IgG-Ag complexes to secrete inflammatory mediators (32), there is no a priori reason to believe that mice and humans differ in this regard. More likely, the quantities of allergen-specific IgG Ab and allergen that are required to induce FcγRII-dependent anaphylaxis may rarely be achieved in humans. The occurrence of Ag-mediated anaphylaxis in the absence of detectable IgE specific for the relevant Ag (33), however, suggests that IgG-mediated anaphylaxis may be a human, as well as a mouse, phenomenon. Furthermore, more aggressive allergen immunization, made possible by blocking of IgE-mediated anaphylaxis with a human IgG anti-IgE mAb (34) and potentially with other chimeric proteins (28, 35), may raise quantities of allergen-specific IgG Ab to the level required to induce IgG-mediated anaphylaxis.

Secondly, it is not clear that IgG blocking of IgE-mediated anaphylaxis, which we demonstrated in a model in which mice are challenged i.v. with allergen, will occur when allergen challenge occurs through mucosal routes. Because IgG levels are low in the gastrointestinal tract and mast cells that can bind allergen-specific IgE are located in intestinal villi, it seems doubtful that IgG Abs inhibit the induction of intestinal mast cell degranulation by
ingested allergens. Results of preliminary studies, however, support the possibility that other isoforms, such as IgA, inhibit IgE-mediated mucosal allergy; lower doses of Ag are required to induce IgE/mast cell–mediated allergic diarrhea in J chain–deficient mice, which have approximately 10% of normal intestinal IgA levels, than in WT mice of the same background strain (R.T. Strait et al., unpublished data). It is also possible that ingested Ags only induce systemic anaphylaxis if they are absorbed from the gut and bind to mast cells associated with the circulation. If so, IgG BA would be expected to have a major role in limiting systemic anaphylaxis even when Ag is ingested. Consequently, it seems likely that immunotherapy suppresses anaphylactic and other IgE-mediated allergic disorders, including allergic disorders that predominantly affect mucosal organs, by inducing BA, as well as through distinct mechanisms that decrease IgE secretion, suppress Th2 responses, and stimulate Th1 and regulatory T cell responses (36–42).

Methods
Mice. BALB/c mice were purchased from the National Cancer Institute. Mast cell–deficient WBB6F1-Kit<sup>-/-</sup> (W/W<sup>–</sup>) mice and WBB6F1-Kit<sup>+/+</sup> x WBB6F1<sup>–/–</sup> F<sub>1</sub> (W/+) mice (which have a normal phenotype) (43) along with FcγRIIb-deficient (27) and C57BL/6 FcγRIIb-sufficient mice were purchased from Jackson Laboratory. IgE-deficient mice (44) were a gift from Phillip Leder (Harvard University, Cambridge, Massachusetts, USA), and FcγRIIb-deficient mice (26) were a gift from Jeffrey Ravetch (Rockefeller University, New York, New York, USA). All experimental procedures were performed with approval from the Institutional Animal Care and Use Committees of the Cincinnati Children's Hospital Research Foundation and the Department of Veterans Affairs Medical Center (Cincinnati, Ohio, USA).

Reagents. GmMD (15, 45); GgG; rat IgG2b anti–mouse FcγRI/RII mAb (24G2) (46) from ATCC; rat IgG2b anti–4-hydroxy-3-nitrophenylacetyl mAb (J1.2), a gift from John Abrams (DNAX Research Inc., Palo Alto, California, USA); rat IgG2a anti–mouse IgE mAb (EM-95) (47), a gift from Zelig Eshhar (Weizmann Institute, Rehovot, Israel); and mouse IgExTNP (IGEL 2a) (48) from ATCC were prepared as described (20, 49). TNP-labeled GmMD was prepared by mixture of 20 ml of GmMD in 1 ml of 0.1 M NaHCO<sub>3</sub> buffer, pH 9.6, with 25 mg of TNP-succinyl-Osu (Biosearch Technologies Inc.) dissolved in 1 ml of DMSO and incubation of the mixture overnight at room temperature. The incubated solution was dialyzed against 5 changes of 0.15 M NaCl/0.01 M NaHCO<sub>3</sub>, pH 8.0. TNP-OVA was similarly produced by mixture of 50 mg of OVA in 5 ml of bicarbonate buffer with serial 4-fold dilutions of TNP-succinyl-Osu (starting concentration, 25 mg/ml) in DMSO. TNP-OVA-NIP was produced by mixture of NIP-succinyl-Osu (Biosearch Technologies Inc.) with TNP<sub>0</sub>-OVA at a 1:2 weight ratio in DMSO and dialyzing as above. TNP-OVA was biotinylated with E-Z Link sulfo-NHS-biotin (Pierce) at a 10:1 weight ratio in DMSO. eTNP Asm was produced by injection of BALB/c mice i.e.p. with 0.2 ml of TNP-GmMD. Mice were bled 10–12 days after immunization, and sera were pooled. The pooled serum was heated to 56°C for 30 minutes to inactivate complement and IgE. The IgG fraction of eTNP Asm was purified by ammonium sulfate fractionation (25–50% saturated cut) followed by DEAE-cellulose (DE-52; Whatman International Ltd.) ion exchange chromatography. Fractions were tested for the presence of mouse IgG1 and non-Ig proteins by gel double diffusion, and appropriate fractions were pooled. The PAF antagonist CV6209 was purchased from Moredun.

Measurement of IL-4, histamine, and MMCP-1. Mice were injected with biotinylated anti–IL-4 mAb (BVD4-1D11) (50) at the time of TNP-OVA challenge. Serum was collected 2 hours later, and IL-4 was measured by in vivo cytokine capture assay (IVCCA) (51). Blood drawn 5 minutes after Ag challenge and placed immediately on ice had histamine content measured by ELISA with a kit purchased from IBL. Serum levels of MMCP-1 were measured in blood drawn 2 hours after Ag challenge with an ELISA kit purchased from Moredun.

ELISA. IgG1 anti-TNP activity was quantitated with ELISA plate wells coated with TNP<sub>0</sub>-OVA and blocked with SuperBlock (Pierce). Serial dilutions of sera and fraction sera were added to wells, followed sequentially by affinity-purified rabbit anti–mouse J1 Ab (15), alkaline phosphatase–labeled goat anti–rabbit Ab (15), and Tris-based buffer with p-nitrophenyl phosphate substrate (Calbiochem). IgG1–TNP-OVA-biotin complexes in mouse serum were captured onto ELISA plate wells coated with streptavidin and were detected with rabbit anti–mouse IgG1 Ab (Zymed Laboratories Inc.), followed by alkaline phosphatase–labeled goat anti–rabbit Ig (15) and substrate (p-nitrophenyl phosphate; Calbiochem). The ability of IgExTNP to bind to TNP in the presence of IgG anti-TNP was determined by addition of serum containing TNP-OVA-NIP with or without IgG anti-TNP Ab to ELISA plate wells coated with J1.2, a rat IgG2b anti–4-hydroxy-3-nitrophenylacetyl mAb that cross-reacts with NIP, and then addition of biotin-labeled IgExTNP, followed by HRP-streptavidin and SuperSignal ELISA substrate (Pierce Biotechnology). ELISA plates were read for absorbance with a Multiskan MCC/340 ELISA reader (Thermo Electron Corp.) or for luminescence with a Fluoroskan Ascent FL reader (Thermo Electron Corp.).

Active anaphylaxis model. Mice (5 per group except where noted otherwise) were primed with 0.2 ml GmMD or TNP-GmMD s.c., then challenged 14 days later i.v. with GgG or TNP-OVA. All experiments were repeated at least once.

Passive anaphylaxis model. Mice were primed i.v. with different combinations of 10 μg of IgExTNP and variable amounts of 0.6GgG Asm, eTNP Asm, or IgGGeG, then challenged i.v. 24 hours later with TNP-OVA or OVA.

Anaphylaxis. The severity of the anaphylactic shock was assessed by change in temperature, activity level, and/or hematocrit, as previously described (20, 52).

Treatment with inhibitors. FcγRIIb/RII, histamine, PAF, and macrophage function was inhibited as described (20, 53).

Evaluation of TNP-OVA molar ratio. The absorbance of TNP-OVA conjugates was measured at wavelengths of 280 and 340 μM with a Spectronic GENESYS Spectrophotometer (Spectronic Instruments), and TNP/OVA molar ratio was determined as described (54).

Statistics. Differences in temperature, hematocrit, and concentrations of histamine, MMCP-1, and IL-4 between groups of mice were compared using the Mann-Whitney t test (GraphPad Prism 4.0; GraphPad software). A P value less than 0.05 was considered significant.

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