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.

Introduction

The rationale for allergen immunotherapy for atopic disorders has changed with time. Initially, "allergy vaccines" were thought to induce the production of IgG blocking antibody (BA), which might neutralize allergen molecules before they could interact with what were later discovered to be IgE Abs bound to FcεRI on mast cells and basophils (1, 2). More recently, this BA concept has been supplemented by evidence that IgG Ab–allergen complexes may inhibit mast cell signaling by cross-linking the immunoreceptor tyrosine activation motif–containing activating receptor FcεRI to the immunoreceptor tyrosine inhibition motif–containing inhibitory receptor FcγRIIb (3), and that immunotherapy may instead inhibit allergy by immunomodulation: decreasing Th2 cytokine production, increasing Th1 cytokine production, and/or activating regulatory T cells (4–7). Surprisingly, despite the long history of allergen immunotherapy, positive correlations between IgG Ab levels and protection against allergen-induced disease in some but not all studies (8–12), and in vitro experiments that demonstrated IgG Ab inhibition of antigen-induced (Ag-induced) mast cell/basophil degranulation and other IgE-mediated effects (5, 13, 14), there has been no in vivo proof of the BA concept.

Nonstandard abbreviations used: Ag, antigen; Asm, antiserum; BA, blocking antibody; GIgG, goat IgG; αGIgG Asm, heat-inactivated mouse anti-GIgG antiserum; GαMD, goat anti–mouse IgD antiserum; IgEαTNP, IgE anti-TNP mAb; IgGαGIgG, IgG anti-GIgG; IgGαTNP, purified IgG fraction of αTNP Asm; IVCCA, in vivo cytokine capture assay; MMCP-1, mouse mast cell protease-1; NIP, 3-nitro-4-hydroxy-5-iodophenylacetyl; PAF, platelet-activating factor; TNP, trinitrophenyl; αTNP Asm, heat-inactivated mouse anti-TNP antiserum; TNP-GαMD, TNP conjugated to GαMD; TNP-OVA, TNP conjugated to OVA; TNP-OVA-NIP, NIP conjugated to TNP-OVA.

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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 (GαMD, 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 (GIgG) (20). Although GIgG challenge induced severe anaphylaxis, anaphylaxis was mediated by IgG, FcγRIII, 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 GαMD-treated mice, it seemed unlikely that the failure of GIgG challenge to induce IgE-mediated anaphylaxis resulted from a lack of IgE or mast cells. Instead, the strong IgG anti-GIgG (IgGαGIgG) response that develops in these mice raised the possibility that IgGαGIgG blocked IgE-mediated anaphylaxis, either by intercepting GIgG 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 GαMD-immunized mice by intercepting Ag before it can cross-link mast cell–associated IgE. GαMD immunization induces marked increases in IgE and mastocytosis (ref. 17 and F.D. Finkelman, unpublished data). Despite this, challenging GαMD-immunized mice with 100 μg of the relevant Ag,

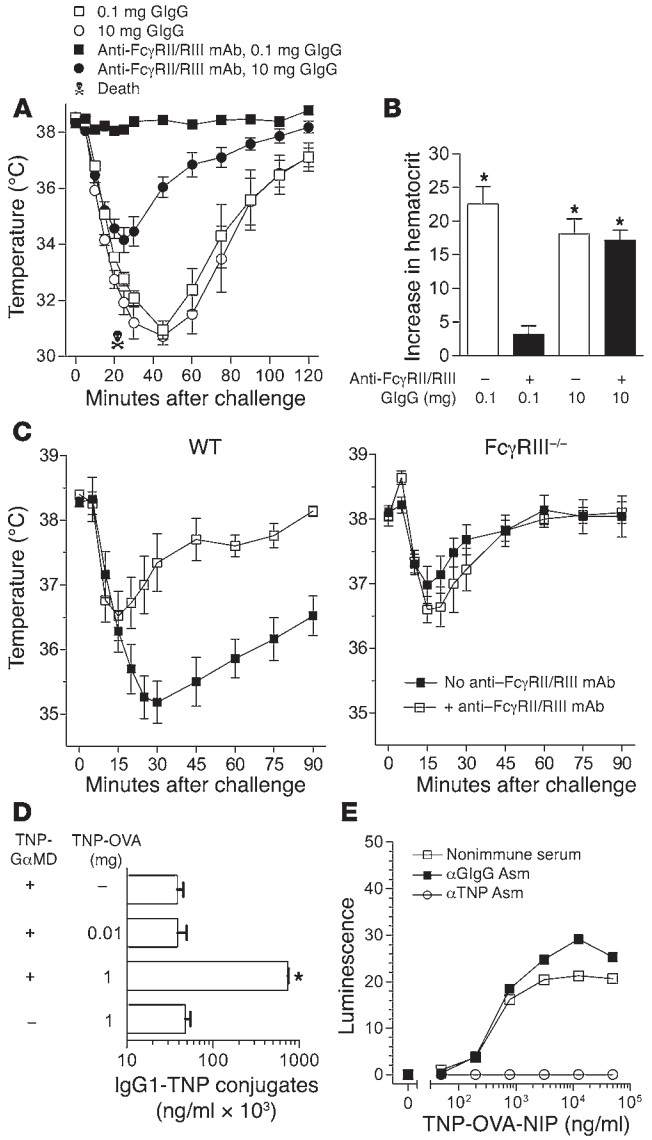

Figure 1
FcγRIII-independent anaphylaxis in GαMD-primed mice requires challenge with a high dose of Ag. (**A**) BALB/c mice (5 per group) were primed s.c. with GαMD, then challenged i.v. 14 days later with 0.1 or 10 mg of GIgG. Some mice were pretreated 24 hours before GIgG challenge with 500 μg of anti–FcγRII/RIII 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 mice treated with anti–FcγRII/RIII mAb and challenged with 0.1 mg of GIgG. (**C**) WT (left) and FcγRIII-deficient mice (right) were primed s.c. with GαMD, then challenged i.v. 14 days later with 10 mg of GIgG. Some mice were injected s.c. with 500 μg of anti–FcγRII/RIII mAb 24 hours before GIgG challenge. Rectal temperatures were followed for 90 minutes after challenge. (**D**) BALB/c mice were primed s.c. with TNP-GαMD or saline, then challenged 14 days later with 0, 0.01, or 1 mg of biotinylated TNP-OVA. Blood was drawn 5 minutes later, and IgG1–TNP-OVA complexes in serum were quantitated by ELISA. **P* < 0.05 compared with other measured levels. (**E**) TNP-OVA-NIP was diluted in nonimmune serum or heat-inactivated serum pooled from mice immunized 10–12 days earlier with GαMD (αGIgG Asm) or TNP-GαMD (αTNP Asm). Binding of serum TNP-OVA-NIP by IgEαTNP was measured by ELISA. Means ± SEMs are shown for all data in this and subsequent figures unless otherwise indicated.

GIgG, induces anaphylaxis that is independent of IgE, FcεRI, and mast cells but requires IgG, FcγRIII, and macrophages (20). Three mechanisms might inhibit IgE-mediated anaphylaxis in this system: (a) IgG Ab might intercept GIgG before it could be bound by mast cell–associated IgE; (b) mouse IgG–anti-GIgG complexes might inhibit mast cell FcεRI signaling by cross-linking FcεRI to FcγRIIb; and (c) "nonspecific" IgE produced by GαMD-immunized mice might displace IgE anti-GIgG Ab from mast cell FcεRI.

We attempted to distinguish among these possibilities by increasing the dose of GIgG used to challenge GαMD-immunized mice from 0.1 to 10 mg (Figure 1). Some GαMD-immunized mice were pretreated with anti–FcγRII/RIII mAb 1 day before GIgG challenge to block IgG-mediated anaphylaxis and FcγRIIb-associated inhibition of IgE-mediated anaphylaxis. Challenge with 0.1 or 10 mg of GIgG induced anaphylaxis of similar severity, as measured by hypothermia (which reflects the development and degree of shock) and hemoconcentration (which reflects vascular leak), when mice were not pretreated with anti–FcγRII/RIII mAb. However, only the 10-mg dose of GIgG induced anaphylaxis in anti–FcγRII/RIII 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 GIgG-specific and nonspecific IgE for mast cell FcεRI. Thus, our observation supports the hypothesis that IgE-mediated anaphylaxis in GαMD-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 GαMD-immunized mice by both intercepting Ag and cross-linking FcεRI to FcγRIIb. Anti–FcγRII/RIII mAb blocks both the FcγRIII-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–FcγRII/RIII mAb on anaphylaxis induced by high-dose (10 mg) Ag challenge in GαMD-immunized WT and FcγRIII-deficient mice. Anti–FcγRII/RIII mAb had its expected inhibitory effect on anaphylaxis in WT mice, but little, if any, inhibitory or stimulatory effect in FcγRIII-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 GαMD-immunized mice.

If IgG BA in GαMD-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–GIgG complexes that should be formed in GαMD-immune mice challenged with GIgG, we instead used a system that takes advantage of the strong Ab response generated to molecules conjugated to GαMD but allows more sensitive and precise detection of the Ag-Ab complex. Mice primed with a conjugate of trinitrophenyl-GαMD (TNP-GαMD) develop a large IgG1 anti-TNP Ab response (21). TNP-OVA–mouse IgG complexes were easily detected in serum 5 minutes after TNP-GαMD–immunized mice were challenged with 1 mg of TNP-OVA (Figure 1D).

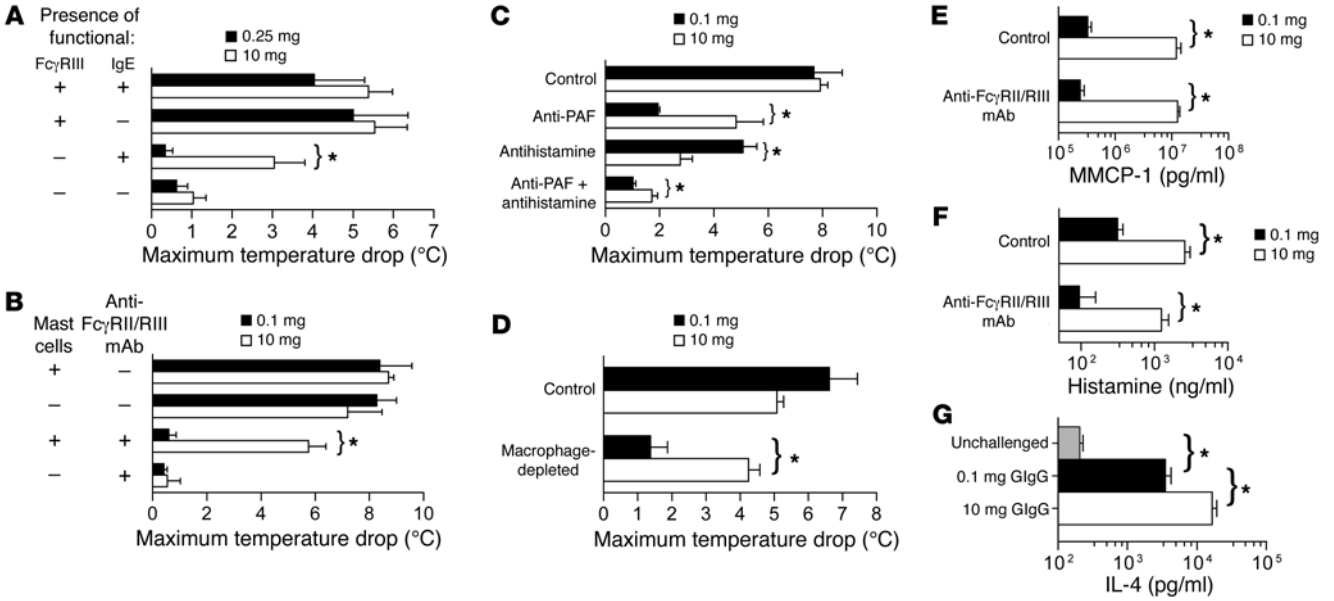

Figure 2

IgE/FcεRI/mast cell–dependent anaphylaxis in GαMD-primed mice requires challenge with a high dose of Ag. Mice (4–5 per group) were primed s.c. with 0.2 ml of GαMD, then challenged i.v. 14 days later with GIgG. 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 FcγRIII, IgE, or both were challenged as shown. (**B**) WT (+) and mast cell–deficient W/W^v (–) mice were treated as shown. (**C**) BALB/c mice were injected 15–30 minutes before challenge with 66 μg of CV6209 (PAF antagonist), 0.2 mg of both triprolidine 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 GIgG challenge. (**E**) BALB/c mice were injected s.c. with saline or 500 μg of anti–FcγRII/RIII mAb 1 day before GIgG challenge. Blood was drawn 2 hours after GIgG challenge, and MMCP-1 levels were determined. (**F**) BALB/c mice were injected s.c. with saline or 500 μg of anti–FcγRII/RIII mAb 1 day before GIgG 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 GIgG, 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 GαMD or TNP-GαMD and evaluated the ability of their serum to block TNP-OVA binding by IgE anti-TNP mAb (IgEαTNP). 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 IgEαTNP. This assay detected IgE anti-TNP binding to as little as 2×10^2 ng of TNP-OVA-NIP per milliliter in serum from nonimmune or GαMD-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-GαMD–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 GαMD-immunized mice. To provide additional evidence that induction of IgE-mediated anaphylaxis in GαMD-immune mice requires high-dose Ag challenge, we characterized IgE, FcR, cell type, and mediator requirements for anaphylaxis in GαMD-immunized mice challenged with either low-dose (0.1–0.25 mg) or high-dose (10 mg) GIgG. FcγRIII-deficient, IgE-deficient, and FcγRIII/IgE–double-deficient mice were used to evaluate the importance of the IgG/FcγRIII and IgE/FcεRI anaphylaxis pathways in these experiments. With low-dose Ag challenge, anaphylaxis was FcγRIII-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 FcγRIII-deficient mice were pretreated with anti-IgE mAb to neutralize IgE and desensitize mast cells, or IgE-deficient mice were treated with the anti–FcγRII/RIII mAb to block FcγRII/RIII and desensitize macrophages (not shown). Studies with mast cell–deficient, W/W^v mice were also consistent. Although blocking FcγRIII with anti–FcγRII/RIII mAb abolished the anaphylactic response to low-dose, but not high-dose, Ag challenge in WT mice, anti–FcγRII/RIII mAb blocked this response to both low- and high-dose Ag challenge in W/W^v mice (Figure 2B). Furthermore, consistent with observations that FcγRIII-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–FcγRII/RIII mAb. In con-

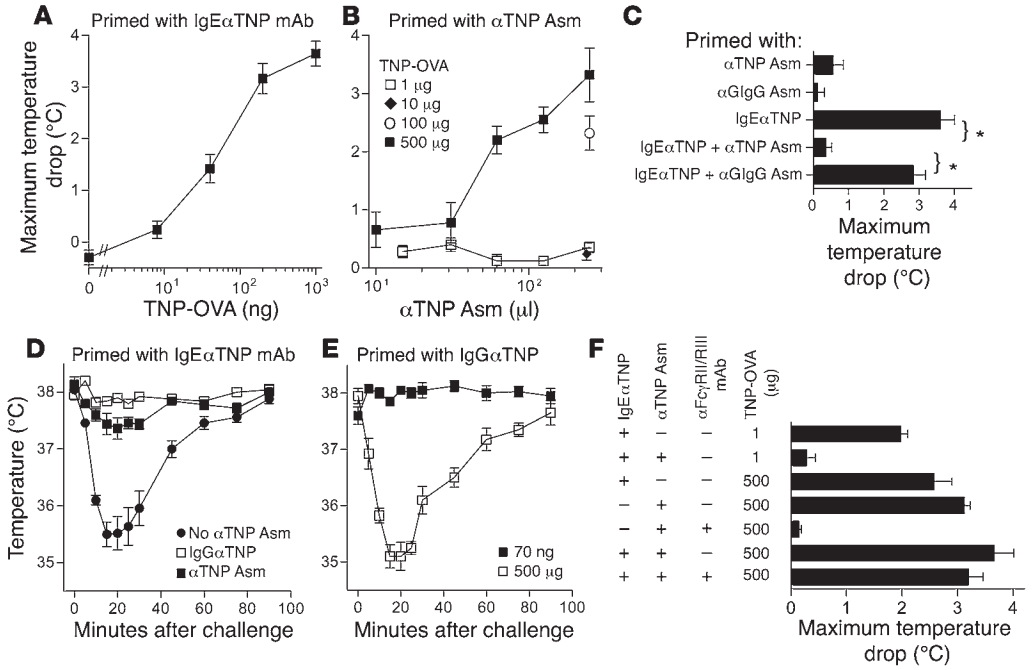

Figure 3

Identification of the serum factor that blocks IgE-mediated anaphylaxis as Ag-specific IgG. (**A**) BALB/c mice (5 per group) were primed with 10 μg of IgEαTNP i.v., then challenged i.v. 24 hours later with the doses of TNP-OVA shown on the abscissa. Maximum temperature decreases during the 90 minutes after challenge were calculated for this and all subsequent panels. (**B**) BALB/c mice (5 per group) were primed i.v. with the doses of αTNP Asm shown on the abscissa and challenged i.v. 24 hours later with the indicated doses of TNP-OVA. (**C**) BALB/c mice (5 per group) were primed i.v. with 10 μg of IgEαTNP, 250 μl of αGIgG Asm, and/or 250 μl of αTNP Asm as indicated, and challenged i.v. 24 hours later with 1 μg of TNP-OVA. *$P < 0.05$. (**D**) BALB/c mice (5 per group) were primed i.v. with 10 μg of IgEαTNP plus saline, 250 μl of IgGαTNP, or 125 μl of αTNP Asm, then challenged i.v. 24 hours later with 70 ng of TNP-OVA. (**E**) BALB/c mice (5 per group) were primed i.v. with 250 μl of IgGαTNP, then challenged i.v. 24 hours later with 70 ng or 500 μg of TNP-OVA. (**F**) BALB/c mice (5 per group) were primed i.v. with either 10 μg of IgEαTNP or 250 μl of αTNP Asm or both and treated with saline or 500 μg of anti–FcγRII/RIII mAb. Mice were challenged i.v. 24 hours later with 1 or 500 μg of TNP-OVA.

trast, large IL-4 responses were generated in response to even low-dose Ag challenge, although high-dose challenge further increased the response approximately 6-fold (Figure 2G). Ag-induced IL-4 responses in this system are generated predominantly by basophils in response to IgE cross-linking and are approximately 10-fold more sensitive than mast cell MMCP-1 and histamine responses to IgE cross-linking (25). Taken together, these observations demonstrate that the IgG/FcγRIII/macrophage/PAF pathway of anaphylaxis is induced at least as strongly by low-dose as by high-dose Ag in GαMD-immunized mice, while high-dose Ag challenge is required to induce the IgE/FcεRI/mast cell/histamine pathway in these mice.

IgE-dependent anaphylaxis is induced by very low doses of Ag in the absence of BA but is inhibited by Ag-specific IgG BA. The greater quantity of Ag required to induce IgE-mediated than to induce FcγRIII-mediated anaphylaxis in GαMD-immunized mice might reflect IgG BA interception of Ag, as we have hypothesized. However, experiments with actively immunized mice did not rule out an alternative possibility: more Ag might be required to activate mast cells, even in the absence of BA, than to activate macrophages. Nor could active immunization experiments directly determine whether immune serum contains a factor that inhibits IgE-mediated anaphylaxis 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 IgEαTNP 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 IgEαTNP-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 antiserum (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 IgEαTNP 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 IgEαTNP versus αTNP Asm suggested that αTNP Asm might be able to block anaphylaxis in IgEαTNP-primed mice without inducing IgG-mediated anaphylaxis, if the dose of challenge Ag were less than that required to induce anaphylaxis by the FcγRIII-dependent pathway.

To test this possibility, unprimed or IgEαTNP-primed mice were injected with saline, αTNP Asm, or, as a control, heat-inactivated mouse anti-GIgG antiserum (αGIgG Asm; produced by mice immunized with GαMD), then challenged with 1 μg of TNP-OVA.

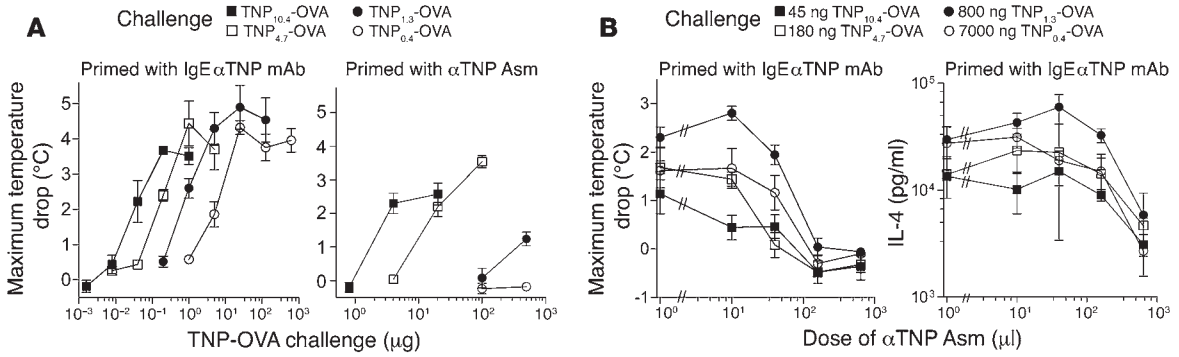

Figure 4

Effects of Ag epitope density on IgE- and FcγRIII-mediated anaphylaxis and IgG BA inhibition of IgE-mediated anaphylaxis. (**A**) BALB/c mice (5 per group) were primed i.v. with either 10 μg of IgEαTNP (left) or 40 μl of αTNP Asm (right), then challenged i.v. 24 hours later with TNP-OVA. Doses of TNP-OVA conjugates are indicated on graph abscissas; molar TNP/OVA ratios of the different conjugates tested are indicated in the figure. Maximum temperature decreases during the 90 minutes after challenge were determined. (**B**) BALB/c mice (5 per group) were primed i.v. with 10 μg of IgEαTNP and injected i.v. with the quantities of αTNP Asm indicated on the graph abscissas. Mice were injected i.v. 24 hours later with 10 μg of biotin–anti–IL-4 mAb and challenged i.v. with the indicated doses of the TNP-OVA conjugates. Maximum temperature decreases during the 90 minutes after challenge were determined (left). Blood was drawn 2 hours after challenge, and IL-4 secretion was evaluated by IVCCA (right) (51).

Significant hypothermia developed in mice that initially received IgEαTNP with or without αGIgG Asm but did not develop in mice that initially received both IgEαTNP and αTNP Asm (Figure 3C). Thus, a constituent of serum from TNP-GαMD–immunized, but not GαMD-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-GαMD immune serum constituent that blocks IgE-mediated anaphylaxis, we purified the IgG fraction of αTNP Asm (IgGαTNP) 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 antiserum (Figure 3D). To determine whether IgGαTNP Ab could also mediate anaphylaxis, presumably through the FcγRIII-dependent mechanism, in mice challenged with a higher dose of Ag, mice primed with purified IgGαTNP 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 mice with IgEαTNP, αTNP Asm, or both, blocked FcγRIII-mediated anaphylaxis with anti–FcγRII/RIII mAb in some mice, and challenged mice with 1 or 500 μg of TNP-OVA. IgE-dependent anaphylaxis was induced by challenge with 1 μg of TNP-OVA in mice primed only with IgEαTNP but blocked in mice that also received αTNP Asm. This blocking was overcome when the dose of challenge Ag was increased to 500 μg (Figure 3F). The 500-μg dose of Ag also induced FcγRIII-mediated anaphylaxis (it induced anaphylaxis in mice pretreated with only αTNP Asm but not in mice pretreated with both αTNP Asm and anti–FcγRII/RIII mAb). Taken together, these results demonstrate that (a) IgE-dependent anaphylaxis requires less Ag than FcγRIII-dependent anaphylaxis in the absence of IgG BA; (b) Ag-specific IgG BA increases the dose of Ag required to induce IgE-mediated anaphylaxis and, if the Ag dose is sufficiently high, allows the development of FcγRIII-dependent anaphylaxis; and (c) the inhibitory effect of IgG BA on IgE-mediated anaphylaxis can be overcome by an increase in the dose of challenge Ag. These results are consistent with observations in our active immunization anaphylaxis model, in which the high concentrations of mouse IgGαGIgG induced by GαMD immunization support FcγRIII-mediated anaphylaxis when mice are challenged with 100 μg of GIgG but block IgE-mediated anaphylaxis unless the dose of challenge Ag is increased substantially.

Influence of Ag epitope density on the inhibition of anaphylaxis by blocking Ab. Our conclusions about BA function were drawn from studies in which anti-TNP Ab–primed mice were challenged with a TNP-OVA preparation that averaged 10.4 TNP moieties per OVA molecule ($TNP_{10.4}$-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/FcεRI on mast cells and make it more difficult to block IgE/FcεRI 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 IgEαTNP 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 IgEαTNP, then challenged with doses of $TNP_{10.4}$-OVA, $TNP_{4.7}$-OVA, $TNP_{1.3}$-OVA, or $TNP_{0.4}$-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/FcεRI–mediated anaphylaxis is not affected by decreas-

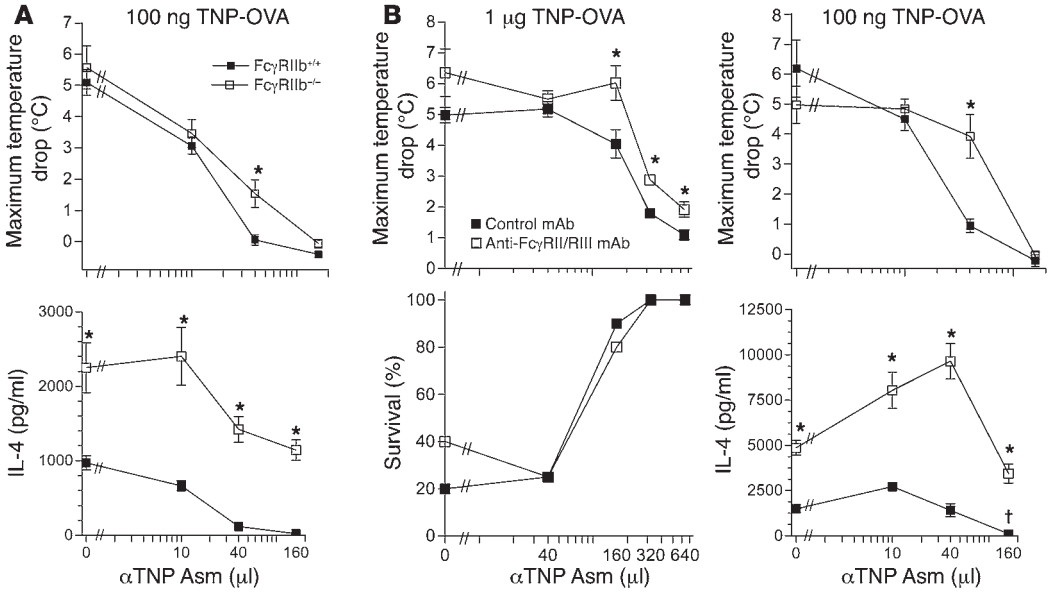

Figure 5

IgG BA inhibits IgE-mediated anaphylaxis through both FcγRIIb-dependent and -independent mechanisms. (**A**) WT and FcγRIIb-deficient mice (8–10 per group) were primed i.v. with 10 μg of IgEαTNP and treated i.v. with the quantities of αTNP Asm indicated on the graph abscissas. Mice were injected i.v. 24 hours later with 10 μg of biotin–anti–IL-4 mAb and challenged i.v. with 100 ng of TNP-OVA. Maximum temperature decreases during the 90 minutes after challenge were determined. Blood was drawn 2 hours after challenge, and IL-4 secretion was determined by IVCCA. All mice survived. (**B**) FcγRIII-deficient mice (5 per group) were primed i.v. with 10 μg of IgEαTNP and treated i.v. with the quantities of αTNP Asm indicated on the graph abscissas and s.c. with 500 μg of either anti–FcγRII/RIII mAb or isotype-matched control mAb. Mice were injected i.v. 24 hours later with 10 μg of biotin–anti–IL-4 mAb and challenged i.v. with 1 μg or 100 ng of TNP-OVA. Maximum temperature decreases during the 90 minutes after challenge were determined. Survival was 100% for all mice challenged with 100 ng of TNP-OVA and as indicated for mice challenged with 1 μg of TNP-OVA. Blood was drawn 2 hours after challenge, and IL-4 secretions were determined by IVCCA for mice challenged with 100 ng TNP-OVA. $^{*}P < 0.05$. $^{\dagger}P < 0.05$ compared with control mAb–treated mice that received no αTNP Asm.

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/FcγRIII–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 FcγRIII-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 FcγRIII-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 GαMD, which induces large GIgG-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/FcγRIII/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

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/FcγRIII/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/FcγRIII–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/FcεRI on mast cells and from the generation of enough Ag–IgG Ab complexes to activate FcγRIII-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 IgEαTNP and challenged with TNP-OVA was suppressed when mice were also injected with heat-inactivated serum pooled from mice immunized with TNP-GαMD, which contained IgG anti-TNP and IgG anti-GIgG Ab, but not when mice were injected with heat-inactivated serum pooled from GαMD-immunized mice, which contained anti-GIgG 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 FcγRIII-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 FcγRIII-dependent anaphylaxis. These observations suggest that immune complexes that contain several IgG molecules may be required to efficiently cross-link FcγRIII (a low-affinity receptor) and activate macrophages, while more limited cross-linking of mast cell FcεRI by a high-affinity interaction between Ag and FcεRI-associated IgE can efficiently induce mast cell degranulation.

Finally, studies with both active and passive immunization models defined and quantitated the importance of FcεRI-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 FcγRIII-deficient mice in which FcγRIIb function was blocked by anti–FcγRII/RIII 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/RIII mAb–treated and control mAb–treated FcγRIII-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/RIII 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/RIII mAb–treated FcγRIII-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 FcεRI-associated IgE, and it cross-links FcεRI to FcγRIIb. FcεRI-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 FcεRI-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 FcεRI 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, FcγRIII-mediated 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 FcγRIII 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 FcγRIII-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 isotypes, 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^{W}/Kit^{W-v} (W/W^{v}) mice and (WBB6F1-Kit^{W}/Kit^{W-v} × WBB6F1$^{+/+}$)F_1 (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 FcγRIII-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. GαMD (15, 45); GIgG; rat IgG2b anti–mouse FcγRII/RIII 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 IgEαTNP (IGEL 2a) (48) from ATCC were prepared as described (20, 49). TNP-labeled GαMD was prepared by mixture of 20 ml of GαMD in 1 ml of 0.1 M $NaHCO_3$ 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 $NaHCO_3$, 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 $TNP_{0.4}$-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. αTNP Asm was produced by injection of BALB/c mice i.p. with 0.2 ml of TNP-GαMD. 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 αTNP 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 BIOMOL. The H1 receptor antagonist triprolidine and the macrophage inhibitor gadolinium were purchased from Sigma-Aldrich. The H2 receptor antagonist cimetidine was purchased from Tocris. Abs for measurement of in vivo IL-4 secretion were obtained from BD.

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.

ELISAs. IgG1 anti-TNP activity was quantitated with ELISA plate wells coated with $TNP_{10.4}$-OVA and blocked with SuperBlock (Pierce). Serial dilutions of sera and serum fractions were added to wells, followed sequentially by affinity-purified rabbit anti–mouse γ1 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 IgEαTNP 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 IgEαTNP, 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 GαMD or TNP-GαMD s.c., then challenged 14 days later i.v. with GIgG 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 IgEαTNP and variable amounts of αGIgG Asm, αTNP Asm, or IgGαGIgG, 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/RIII, 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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