



HHS Public Access

Author manuscript

J Allergy Clin Immunol. Author manuscript; available in PMC 2021 June 21.

Published in final edited form as:

J Allergy Clin Immunol. 2018 April ; 141(4): 1373–1381.e5. doi:10.1016/j.jaci.2017.05.027.

Anti-Fc γ RIIB mAb suppresses murine IgG-dependent anaphylaxis by Fc domain targeting of Fc γ RIII

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Abstract

Background: The inhibitory receptor Fc γ RIIB is expressed on human and murine bone marrow-derived cells and limits inflammation by suppressing signaling through stimulatory receptors.

Objective: We sought to evaluate the effects of K9.361, a mouse IgG_{2a} alloantibody to mouse Fc γ RIIB, on murine anaphylaxis.

Methods: Wild-type and Fc γ R-deficient mice were used to study anaphylaxis, which was induced by injection of 2.4G2 (rat IgG_{2b} mAb that binds both Fc γ RIIB and the stimulatory receptor Fc γ RIII), by actively immunizing IgE-deficient mice and then challenging with the immunizing antigen, and by passive immunization with IgG or IgE anti-2,4,6-trinitrophenyl mAb, followed by injection of 2,4,6-trinitrophenyl-ovalbumin. Pretreatment with K9.361 was assessed for its ability to influence anaphylaxis.

Results: Unexpectedly, K9.361 injection induced mild anaphylaxis, which was both Fc γ RIIB and Fc γ RIII dependent and greatly enhanced by β -adrenergic blockade. K9.361 injection also decreased expression of stimulatory Fc γ receptors, especially Fc γ RIII, and strongly suppressed IgG-mediated anaphylaxis without strongly affecting IgE-mediated anaphylaxis. The F(ab')₂ fragment of K9.361 did not induce anaphylaxis, even after β -adrenergic blockade, and did not deplete Fc γ RIII or suppress IgG-mediated anaphylaxis but prevented intact K9.361-induced anaphylaxis without diminishing intact K9.361 suppression of IgG-mediated anaphylaxis.

Conclusion: Cross-linking Fc γ RIIB to stimulatory Fc γ Rs through the Fc domains of an anti-Fc γ RIIB mAb induces and then suppresses IgG-mediated anaphylaxis without affecting IgE-

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mediated anaphylaxis. Because IgG- and IgE-mediated anaphylaxis can be mediated by the same cell types, this suggests that desensitization acts at the receptor rather than cellular level. Sequential treatment with the F(ab')₂ fragment of anti-FcγRIIB mAb followed by intact anti-FcγRIIB safely prevents IgG-mediated anaphylaxis.

Keywords

Fc receptors; anaphylaxis; mouse; signaling; IgG; IgE

Anaphylaxis is an acute multisystem syndrome that results from rapid and diffuse release of vasoactive mediators, cytokines, and enzymes by myeloid cells minutes to hours after exposure to an inciting trigger. In mice anaphylaxis can be induced through both the classical and alternative pathways.¹⁻³ The classical pathway depends on IgE-mediated activation of the high-affinity FcεRI on mast cells and basophils, whereas the alternative pathway depends on IgG-mediated activation of multiple myeloid cell types through Fcγ receptors (FcγRs), particularly FcγRIII. The classical pathway is well-established in human subjects, whereas the importance of the alternative pathway in human subjects is supported by considerable but not conclusive evidence.⁴

FcγRs regulate innate and adaptive immune responses in a coordinated manner that involves positive signals that activate cells and cause proinflammatory responses, as well as negative signals that suppress cellular activation and inflammation. Dysregulation of FcγR-mediated signaling has been implicated in wide-ranging disease processes.⁵⁻⁹ Murine stimulatory FcγRs include FcγRI (expressed by dendritic cells and mononuclear cell subpopulations in peripheral blood), FcγRIII (expressed in varying degrees by all peripheral blood myeloid cells), and FcγRIV (expressed by neutrophils and peripheral blood monocytes and dendritic cells).^{6,10} Activating FcγRs contain the immunoreceptor tyrosine-based activation domain (ITAM) on the Fc receptor γ chain (FcRγ). On the other hand, FcγRIIB is inhibitory because it lacks FcRγ but contains a cytosolic immunoreceptor tyrosine-based inhibitory domain (ITIM) domain.^{6,9,11,12}

Given the central role of murine activating FcγRs in IgG-mediated anaphylaxis and in several autoimmune and inflammatory disorders,¹³⁻²² the identification of a therapeutic strategy to modulate cell stimulation through FcγRs would have potential wide-ranging therapeutic implications. We previously characterized a mouse model of IgG-mediated anaphylaxis triggered by 2.4G2, a rat IgG_{2b} mAb that directly binds to and blocks both FcγRIII and FcγRIIB but also inhibits stimulation of mouse myeloid cells through FcγRI and FcγRIV.²³⁻²⁵ Our data suggest that 2.4G2 inhibition of FcγRI and FcγRIV results from an interaction between 2.4G2's Fc domains and these receptors.

These observations raised questions about the likely effects of injecting mice with an mAb that only binds to FcγRIIB, such as the mouse IgG_{2a} allo-mAb K9.361.²⁶ Theoretically, this mAb can inactivate myeloid cells by directly transmitting negative signals through FcγRIIB, although most evidence suggests that FcγRIIB prevents signaling through stimulatory receptors to which it has been cross-linked rather than by directly making cells refractory to other signals.²⁷ Alternatively, K9.361 might increase the sensitivity of myeloid cells to subsequent ligation of their stimulatory receptors by blocking IgG-associated ligand cross-

linking of the stimulatory receptors to Fc γ RIIB. A third possibility is that K9.361 might bind to stimulatory Fc γ Rs through its Fc domains and affect the expression, responsiveness, or both of those receptors.

Distinguishing among these possibilities would provide insight into the mechanisms involved in inhibitory receptor function and the interactions among stimulatory and inhibitory receptors and might also suggest strategies for suppressing anaphylaxis and other inflammatory disorders. Consequently, we investigated the effects of injecting mice with K9.361 and its F(ab')₂ fragment, which should be capable of binding to and cross-linking Fc γ RIIB but lacks the Fc domains required to interact with other Fc γ Rs. Our results indicate that K9.361 indirectly induces mild anaphylaxis through an Fc-dependent interaction with Fc γ RIII and then blocks active and passive IgG-mediated but not IgE-mediated anaphylaxis. In addition, treatment with K9.361 and its F(ab')₂ fragment provides an approach to safely inhibit IgG-mediated anaphylaxis.

METHODS

Mice

Male and female BALB/c and C57BL/6 mice were purchased from Taconic (Hudson, NY) and used at 7 to 15 weeks of age. Some experiments also used BALB/c mice that were bred in house. Fc γ RI- and Fc γ RIII-deficient mice on a C57BL/6 background were originally obtained from Jeffrey Ravetch (Rockefeller University, New York, NY); these mice were bred to each other to generate mice deficient in both Fc γ RI and Fc γ RIII. F2 offspring were typed by using PCR to identify double-deficient offspring. Fc γ RIIB-deficient mice and FcR γ chain-deficient mice on a BALB/c background were purchased from Jackson Laboratories (Bar Harbor, Me). IgE-deficient mice on a BALB/c background were a gift of Hans Oettgen (Boston Children's Hospital, Boston, Mass). All transgenic mice were bred in house.

BALB/c female mice, 6 to 8 weeks old, were treated intratracheally 3 times per week for 3 weeks with chicken egg white (EW) protein and assessed for airway hypersensitivity by using Buxco with gradient methacholine challenge. EW-sensitized mice were subsequently challenged by means of oral gavage with 100 mg of protein and assessed for temperature decrease and diarrhea and then grouped according to the severity of sensitization.

Antibodies, antigens, and pharmaceuticals

Monoclonal antibodies or the hybridomas that produce them were obtained as follows: K9.361 (mouse IgG_{2a} anti-mouse Fc γ RIIB mAb; a gift of Ulrich Hammerling, Sloan-Kettering, New York, NY), 2.4G2 (rat IgG_{2b} anti-mouse Fc γ RII/RIII mAb; ATCC, Rockville, Md), EM95 (rat IgG_{2a} anti-mouse IgE mAb; a gift of Zelig Eshhar, Rehovot, Israel), CBPC-101 (mouse IgG_{2a} anti-myeloma protein; John Abrams, DNAX, Palo Alto, Calif), SF1-1.1.10 (mouse IgG_{2a} anti-H-2K^d mAb; ATCC), M1/70 (rat IgG_{2b} anti-mouse CD11b mAb; ATCC), J1.2 (rat IgG_{2b} anti-4-hydroxy-3-nitrophenylacetyl; a gift from John Abrams, DNAX), 1B7.11 (mouse IgG₁ anti-2,4,6-trinitrophenyl [TNP] mAb; ATCC), HY1.2 (mouse IgG_{2a} anti-TNP mAb; Shozo Izui, University of Geneva, Geneva,

Switzerland; mouse IgE anti-TNP, ATCC). Monoclonal antibodies produced as ascites in pristane-primed athymic nude mice were purified, as previously described.²⁸ A goat antiserum to mouse IgD was produced, as previously described.²⁸ Ovalbumin (OVA) was purchased from Sigma (St Louis, Mo). OVA was labeled with 2,4,6-trinitrophenyl- ϵ -aminocaproyl-O-succinamide (TNP-OSu; Biosearch Technologies, Petaluma, Calif). Biotinylation was achieved by alkalizing stock mAb in NaHCO₃ at a pH of 8.0 and then mixing mAb with EZ Link biotin (Thermo Scientific, Waltham, Mass) at a 10:1 weight ratio for 2 hours at room temperature, followed by dialysis against normal saline. Propranolol was obtained from Hikma Pharmaceuticals (London, United Kingdom). Histamine ELISA kits were purchased from IBL-America (Minneapolis, Minn).

Preparation of F(ab')₂ fragments

K9.361 and 2.4G2 mAbs were digested for 7.5 hours at 37°C with immobilized pepsin (Thermo Scientific) while being rotated and then purified with the NAb Protein A Plus column (Thermo Scientific) and concentrated with centrifugal filter devices (Amicon, EMD Millipore, Norwood, Ohio). Aliquots were compared with undigested intact mAb under nonreducing conditions by using SDS-PAGE with 7% polyacrylamide gels (NuPAGE Tris-Acetate; Life Technologies, Grand Island, NY) and lacked detectable intact IgG (see Fig E1 in this article's Online Repository at www.jacionline.org).²⁵

Detection of systemic anaphylaxis

Mice were challenged intravenously with the appropriate triggering antibody or antigen, after which serial body temperatures were determined every 5 to 15 minutes for at least 1 hour with a rectal probe to follow and quantify hypothermia, a sign of shock.¹³ Similar results were seen in male and female mice.

Preparation of nucleated cells

Blood obtained by means of tail vein incision was collected in microtainers that contained K₂EDTA (BD Biosciences, San Jose, Calif). Red blood cells were lysed with ammonium-chloride-potassium lysis buffer; cells were washed twice with ice-cold HN (filtered Hanks' buffer containing 10% newborn calf serum), maintained at 4°C, and resuspended in HNA (HN plus 0.2% NaN₃). Single-splenocyte suspensions were prepared, as previously described.²⁹

Immunofluorescence staining

Peripheral blood cells (1×10^6) or splenocytes (2×10^6) in 0.1 mL of HNA were stained with 0.25 to 2 μ g each of fluorochrome-labeled mAbs. Neutrophils were identified as Ly6G⁺ cells (BioLegend, San Diego, Calif) with relatively high forward scatter (FSC) and side scatter (SSC). Mononuclear cells were identified as B220⁻CD3⁻CD11b⁺ cells with relatively low FSC and SSC. Mononuclear cell subtypes were identified by using Ly6C and CD11c staining. Basophils were identified as IgE⁺CD117⁻B220⁻CD3⁻ cells that had slightly higher FSC and SSC than monocytes. Alexa Fluor 594 and Alexa Fluor 647 were used, respectively, to label K9.361 and 9E9 (hamster IgG anti-mouse Fc γ RIV mAb; a gift of Jeffrey Ravetch, Rockefeller University, New York, NY).³⁰ bv421-labeled mouse IgG₁ anti-

mouse CD64 (anti-Fc γ RI) was purchased from BioLegend. Fluorescence isothiocyanate–labeled rat IgG_{2a} anti-Fc γ RIII was purchased from R&D Systems (Minneapolis, Minn). The fluorochrome-labeled isotype antibodies bv421-labeled mouse IgG₁ (BioLegend), Alexa Fluor 594–labeled CBPC101, fluorescein isothiocyanate–labeled rat IgG_{2a} (BioLegend), and Alexa Fluor 647–labeled Hamster IgG₁ (BioLegend) were used as negative controls.

In some experiments cells were incubated at 4°C with 10 μ g of unlabeled K9.361 before staining as an additional negative control. Cells were washed twice after staining, fixed with 2% to 4% paraformaldehyde, and analyzed with an LSR II (BD Biosciences), and data were analyzed by using FACSDiva software. Similar results were generated by using cells obtained from male and female mice.

Statistics

One-tailed Mann-Whitney *U* tests or 1-way ANOVA with Bonferroni multiple comparison posttesting were calculated to test for statistical significance.

RESULTS

Anti-Fc γ RIIB mAb induces Fc γ RIII-dependent anaphylaxis

Our previous studies demonstrated that 2.4G2 (an mAb that binds to both the stimulatory Fc γ RIII and the inhibitory Fc γ RIIB) induces Fc γ RIII-dependent anaphylaxis that is manifested as shock-associated hypothermia. In contrast, we expected that K9.361, a mouse IgG_{2a} allo-mAb that only binds directly to Fc γ RIIB, would block this receptor and possibly transmit a negative signal to Fc γ RIIB-expressing cells without inducing anaphylaxis. Surprisingly, a small temperature decrease rapidly developed in BALB/c mice injected with this mAb (Fig 1, A). Because the temperature decrease was not always greater than that observed in mice injected with saline or isotype-control mAbs, we pretreated mice with the β -adrenergic receptor antagonist propranolol, which prevents compensatory mechanisms that minimize anaphylaxis severity,³¹ to determine whether this would make anaphylaxis easier to appreciate. Indeed, propranolol-pretreated mice had an approximately 4°C decrease in rectal temperature in response to intravenous anti-Fc γ RIIB mAb (Fig 1, A) but did not have hypothermia in response to intravenous saline or intravenous injection of a mouse IgG_{2a} alloantibody to H-2K^d (SF-1.1.1.10; Fig 1, B).

To support the interpretation that propranolol acts to suppress downstream compensatory mechanisms and not to enhance upstream mediator release, mice were treated with propranolol or saline and subsequently challenged with K9.361, the IgG_{2a} isotype control mAb CBPC-101, or EM95 (rat IgG_{2a} anti-IgE mAb used as a positive control for the capacity to induce histamine release). In fact, propranolol-treated mice had lower levels of plasma histamine on K9.361 challenge (see Fig E2 in this article's Online Repository at www.jacionline.org). Anti-Fc γ RIIB mAb induced anaphylaxis in propranolol-pretreated C57BL/6 mice, as well as BALB/c wild-type (WT) mice, but not in propranolol-pretreated BALB/c background mice that lack FcR γ (a required component of all stimulatory FcRs) or Fc γ RIIB or in C57BL/6 background Fc γ RIII-deficient mice or Fc γ RI/RIII double-deficient mice (Fig 1, C).

Taken together, these observations suggest that induction of anaphylaxis by anti-Fc γ RIIB mAb requires 2 interactions: with Fc γ RIIB through its antigen-binding site and with Fc γ RIII through its Fc domain. To confirm this, we compared the abilities of intact anti-Fc γ RIIB mAb and equimolar amounts of its F(ab')₂ fragment to induce hypothermia in propranolol-pretreated mice: only the intact form of this mAb had this effect, even when we varied the quantities of the intact mAb and its F(ab')₂ fragment over a 10-fold range (Fig 1, D). In contrast, the F(ab')₂ fragment of 2.4G2 induced hypothermia in propranolol-pretreated mice, although it was considerably less severe hypothermia than with intact 2.4G2, whereas the intact rat IgG_{2b} anti-CD11b mAb M1/70, which binds to most myeloid cells and also suppresses stimulatory Fc γ R expression,²⁵ induced less severe hypothermia in these mice than either intact 2.4G2 or its F(ab')₂ fragment (Fig 1, E, and see Fig E3 in this article's Online Repository at www.jacionline.org).

***In vivo* effects of anti-Fc γ RIIB mAb on Fc γ R expression**

Failure of the F(ab')₂ fragment of K9.361 anti-mouse Fc γ RIIB mAb to induce anaphylaxis did not result from a loss of binding activity inasmuch as it was more potent on a weight basis than intact K9.361 at blocking the binding of fluorochrome-labeled intact K9.361 to Fc γ RIIB⁺ spleen cells *in vitro* (Fig 2, A). Consistent with this, compared with injection with CBPC-101, injection of WTBALB/c mice with intact K9.361 or its F(ab')₂ fragment suppressed *ex vivo* Fc γ RIIB staining of peripheral blood monocytes, neutrophils, and basophils (the 3 cell types implicated in IgG-mediated anaphylaxis; Fig 2, B, and see Fig E4, A, in this article's Online Repository at www.jacionline.org).^{1,25} Note that mice were injected with 500 μ g of intact K9.361 but 1 mg of the F(ab')₂ fragment to partially compensate for the decreased *in vivo* half-life of F(ab')₂.³² Intact K9.361 was only slightly more effective than the F(ab')₂ fragment of this mAb at blocking *ex vivo* staining with intact fluorochrome-labeled K9.361, and similar suppression was observed 6 and 24 hours after injection of intact K9.361 or its F(ab')₂ fragment, although relatively little F(ab')₂ of K9.361 remained in serum 24 hours after injection (see Fig E5 in this article's Online Repository at www.jacionline.org). Intact K9.361 also differed from its F(ab')₂ fragment in that only the former decreased the total quantity of Fc γ RIIB on myeloid cells after *in vivo* injection (Fig 2, C, and see Fig E4, B). In contrast to intact anti-Fc γ RIIB mAb, the intact IgG_{2a} anti-H-2K^d allo-mAb SF-1.1.1.10 had no effect on expression of its ligand on myeloid cells.

In addition to decreasing Fc γ RIIB expression, intact K9.361 also induced loss of most Fc γ RIII on myeloid cells *in vivo*, an effect not observed in Fc γ RIIB-deficient mice, indicating that anti-Fc γ RIIB mAb binding to cells through its antigen-binding site is essential for its downmodulation of Fc γ RIII (Fig 2, D, and see Fig E4, C). As predicted, the F(ab')₂ fragment of anti-Fc γ RIIB mAb and intact CBPC-101 had no *in vivo* effect on the expression of Fc γ RIII (Fig 2, E, and see Fig E4, D). Intact mouse IgG_{2a} anti-H-2K^d mAb also had no effect on Fc γ RIII expression, even though its Fc domains should be able to interact with this receptor (Fig 2, E, and see Fig E4, D).

An additional experiment evaluated the ability of intact anti-Fc γ RIIB mAb to affect expression of Fc γ RI and Fc γ RIV, which are expressed on some mononuclear populations

and on mononuclear cells, as well as neutrophils, respectively.¹⁰ Although there appeared to be some decreases in expression of these stimulatory Fc γ Rs (Fig 2, F), they were variable, cell type dependent, and considerably less than those observed for Fc γ RIII. This might reflect the higher affinity of Fc γ RI and Fc γ RIV than Fc γ RIII for uncomplexed IgG antibody molecules,^{10,30} which would allow endogenous serum IgG to better inhibit an interaction between the Fc of Fc γ RIIB-bound K9.361 with Fc γ RI and Fc γ RIV than with Fc γ RIII.

Inhibition of anaphylaxis by anti-Fc γ RIIB mAb

The ability of the F(ab')₂ fragment of K9.361 to bind to Fc γ RIIB without interacting with stimulatory Fc γ Rs or inducing anaphylaxis, even in propranolol-pretreated mice, and the ability of intact K9.361 to cross-link Fc γ RIIB, modulate most Fc γ RIII from myeloid cells, and induce mild anaphylaxis suggested that they might differ in their abilities to inhibit subsequent Fc γ R-mediated anaphylaxis. To test this, we first treated WT mice with 1 mg of the F(ab')₂ fragment of K9.361 and then, 6 or 24 hours later, treated these mice with propranolol and injected them with intact K9.361. Pretreatment with the F(ab')₂ fragment of K9.361 strongly suppressed the anaphylactic response to intact K9.361 6 hours later and totally blocked anaphylaxis when the intact mAb was injected 24 hours after the F(ab')₂ fragment (Fig 3, A). In contrast, pretreatment of WT mice with intact K9.361 but not with its F(ab')₂ fragment suppressed completely the hypothermia response to intact 2.4G2 (anti-Fc γ RIIB/RIII mAb) in mice that were not treated with propranolol (Fig 3, B). As expected, K9.361 suppression of the anaphylactic response to 2.4G2 was Fc γ RIIB dependent (Fig 3, C), reflecting the Fc γ RIIB requirement for K9.361 binding to cells and was dose dependent (Fig 3, D). A single injection of intact K9.361 suppressed the anaphylactic response to 2.4G2, even when 2.4G2 was given in the presence of propranolol, for at least 3 days (Fig 3, E). K9.361 significantly suppressed 2.4G2-mediated anaphylaxis 6 hours after intravenous injection and protection increased 24 hours after injection (Fig 3, F).

These observations suggest that suppression of the anaphylactic response to 2.4G2 might be accomplished without even the mild anaphylaxis that is induced by intact K9.361 by serially injecting mice with F(ab')₂ of K9.361, then intact K9.361, and finally 2.4G2. This was indeed the case. Even in mice that were treated with propranolol, initial treatment with F(ab')₂ of K9.361 did not diminish the ability of intact K9.361 to inhibit 2.4G2-induced hypothermia (Fig 3, G).

Intact anti-Fc γ RIIB mAb blocks active and passive IgG-mediated anaphylaxis

Suppression of 2.4G2-induced anaphylaxis by pretreatment with intact K9.361 but not its F(ab')₂ fragment suggested that treatment with intact anti-Fc γ RIIB mAb might be able to suppress antigen-induced IgG-mediated anaphylaxis. To test this, mice were pretreated with 500 μ g of intact K9.361 or CBPC-101 or 1 mg of the F(ab')₂ fragment of K9.361. Twenty-two hours later, mice were passively sensitized for IgG-mediated anaphylaxis by means of injection of 500 μ g of IgG₁ or IgG_{2a} anti-TNP mAb and then challenged intravenously 2 hours later with 100 μ g of TNP-OVA in the absence of propranolol. Pretreatment with intact K9.361 completely suppressed IgG₁- and IgG_{2a}-mediated anaphylaxis, whereas pretreatment with the F(ab')₂ fragment of K9.361 had no effect on the anaphylactic response (Fig 4, A).

BALB/c background IgE-deficient mice were immunized with goat anti-mouse IgD antiserum, which stimulates a very large IgG₁ anti-goat IgG antibody response, to determine whether intact K9.361 would also suppress IgG-mediated anaphylaxis in actively sensitized mice.^{33,34} Thirteen days later, mice were treated with 500 µg of intact K9.361 or CBPC-101 and then challenged intravenously 1 day after that with 100 µg of goat IgG. Severe anaphylaxis developed in the mice that had been treated with the control mAb, whereas no hypothermia was seen in the K9.361-treated mice (Fig 4, B).

Because FcγRIIB is present on mast cells and basophils and can inhibit IgE-mediated anaphylaxis, we determined whether K9.361 can suppress or exacerbate IgE-mediated anaphylaxis. BALB/c WT mice were treated with 500 µg of K9.361 or CBPC-101 and then passively sensitized 20 hours later by means of intravenous injection of 500 µg of IgG₁ anti-TNP or 10 µg of IgE anti-TNP and challenged intravenously 4 hours later with 100 µg of TNP-OVA. K9.361 protected against IgG₁-mediated anaphylaxis but not IgE-mediated anaphylaxis (Fig 4, C).

Because the binding of IgG-containing immune complexes to FcγRIIB can suppress IgE-mediated anaphylaxis, it was possible that K9.361 blocking of FcγRIIB might exacerbate IgE-mediated anaphylaxis in an active sensitization model in which mice produced both IgG and IgE antibodies to the immunogen. To evaluate this, we studied mice that had been sensitized intratracheally with EW and then challenged repeatedly with EW by means of oral gavage until they had both diarrhea and hypothermia in response to oral gavage EW. Although mice immunized by using this protocol generate both IgE and IgG anti-EW antibodies, the diarrhea and hypothermia responses to oral EW challenge are entirely IgE dependent. Intravenous treatment of these mice with EW allergy with 500 µg of K9.361, followed 24 hours later by oral gavage with 100 mg of EW, slightly ameliorated anaphylaxis severity (Fig 4, D).

DISCUSSION

Anaphylaxis is induced in mice and most likely in human subjects through aggregation of stimulatory FcγRs by IgG-containing immune complexes.⁴ We have now shown that K9.361, an IgG_{2a} mAb that binds to and cross-links the inhibitory receptor FcγRIIB, also induces mild anaphylaxis, which becomes severe when homeostatic compensatory mechanisms that depend on β-adrenergic receptors are blocked. Induction of anaphylaxis requires binding of K9.361 to cells through its antigen-binding site because this mAb does not induce anaphylaxis in FcγRIIB-deficient mice. It also requires binding to FcγRIII through this mAb's Fc domain because it does not induce anaphylaxis in FcγRIII- or FcγR-deficient mice. The induction of anaphylaxis by K9.361 is accompanied by and probably depends on FcγRIII cross-linking because this mAb both decreases the quantity of FcγRIIB expressed by myeloid cells and removes approximately 80% of FcγRIII from these cells, whereas its F(ab')₂ fragment, which does not induce anaphylaxis, has neither effect. This dependence on stimulatory FcγR cross-linking is also suggested by the inability of SF-1.1.1.10, an IgG_{2a} mAb to H-2K^d (an antigen that is strongly expressed by cells that express FcγRIIB), to induce anaphylaxis or decrease the expression of H-2K^d or FcγRIII on myeloid cells (Figs 1, B; 2, C; and 2, E).

Although previous studies have shown that mAbs that bind to one Fc γ R or other cell-surface targets can interact with other Fc γ Rs through their Fc domains and that this can affect cell function,^{25,35–39} ours provides the first evidence that antibody binding of an inhibitory receptor through the antibody's F(ab) domain can activate cells through an interaction between that antibody's Fc domain and a stimulatory receptor. These observations have important implications for the clinical use of IgG mAbs to cell membrane molecules that can also interact with stimulatory Fc γ Rs on those cells. Our observations also demonstrate that even though Fc γ RIIB inhibits cell signaling through activation of its ITIM, these inhibitory effects in our model are inadequate to totally suppress anaphylaxis if Fc γ RIII is also activated. Thus the stimulatory signaling of the Fc γ RIII-associated ITAM can dominate the inhibitory effects of the Fc γ RIIB-associated ITIM.

Our observations also establish the ability of the K9.361 anti-Fc γ RIIB mAb to inhibit anti-Fc γ RIIB/RIII mAb (2.4G2)–induced anaphylaxis, as well as IgG-mediated anaphylaxis induced by antigen challenge of actively or passively sensitized mice. More than 1 mechanism can be involved in this suppressive effect. First, the loss of approximately 80% of Fc γ RIII by myeloid cells in K9.361-treated mice might diminish the ability of Fc γ RIII cross-linking to induce anaphylaxis. However, this effect is probably not sufficient to suppress IgG-mediated anaphylaxis because treating mice with M1/70 rat IgG_{2b} anti-mouse CD11b mAb does not inhibit IgG-mediated anaphylaxis, even though it substantially decreases myeloid cell Fc γ RIII expression.²⁵ Additional possible mechanisms for suppression of IgG-mediated anaphylaxis are (1) negative signaling through Fc γ RIIB or (2) anergy induction by co–cross-linking of Fc γ RIIB with Fc γ RIII, thereby establishing a refractory state with potentially fewer available mediators for secretion. These possibilities are consistent with the considerable time required for maximal suppression of 2.4G2-induced anaphylaxis by K9.361 (greater after 24 hours than after 6 hours; Fig 3, F) and might in part explain K9.361-mediated protection in IgE-mediated anaphylaxis to food protein challenge (Fig 4, D).

The inhibitory effects of Fc γ RIIB generally depend on coaggregation of this inhibitory receptor with stimulatory receptors through a common surface ligand and the ensuing recruitment of SH2-containing inositol polyphosphate-5-phosphatase 1, which terminates ITAM-induced phosphoinositide 3-kinase–mediated signaling.^{40,41} This might explain the failure of intact K9.361 to strongly suppress IgE-mediated anaphylaxis (Fig 4, C). Although this mAb binds Fc γ RIIB, which is expressed on mouse basophils and mast cells, its Fc domains will not interact with the Fc γ RI on these cells. Because mouse mast cells and basophils also express Fc γ RIII, which mediates the participation of these cells in IgG-mediated anaphylaxis, the strong suppression of IgG-but not IgE-mediated anaphylaxis by K9.361 suggests that this suppression is stimulatory Fc receptor specific rather than a result of global desensitization of specific cell types.

The inability of the F(ab')₂ fragment of K9.361 to suppress IgG-mediated anaphylaxis could reflect its failure to bind to and activate Fc γ RIII to decrease Fc γ RIII expression and/or its limited ability to activate SH2-containing inositol polyphosphate-5-phosphatase 1 by cross-linking Fc γ RIIB. Although the F(ab')₂ fragment of an IgG antibody, like the intact antibody, is divalent and consequently has the potential to cross-link its ligand, F(ab')₂ fragments of

IgG antibodies are less potent than intact IgG antibodies at cross-linking their ligands *in vivo*.³⁹ The aggregation of IgG-bound molecules on one cell is enhanced by IgG-Fc interactions with Fc γ Rs on adjacent cells and by Fc-dependent interactions with C1q.⁴² The greater cross-linking ability of intact K9.361 than its F(ab')₂ fragment is most likely required for the intact antibody's ability to decrease Fc γ RIIB and Fc γ RIII expression (Fig 2, B) through the endocytosis of antibody receptor aggregates.

One result of our study that is somewhat difficult to explain is the ability of the F(ab')₂ fragment of K9.361 to cause relatively slow (>6 hours) time-dependent suppression of the response to intact K9.361 (Fig 3, A) without suppressing the anaphylactic response to 2.4G2 (Fig 3, B) or antigen-induced and IgG-mediated anaphylaxis (Fig 4, A).

The time dependence of the suppressive effect cannot be explained by the time required for the F(ab')₂ fragment to saturate its ligand because blocking of intact K9.361 binding to myeloid cells is at least as great 6 hours after F(ab')₂ injection as it is 18 hours later (Fig 2, B), when suppression of anaphylaxis induction by intact K9.361 is considerably greater (Fig 3, A). It is possible that F(ab')₂ binding to Fc γ RIIB in the absence of Fc γ RIIB cross-linking to a stimulatory receptor has a cumulative suppressive effect on subsequent activation of stimulatory receptors, including Fc γ RIII. However, the failure of 24 hours of treatment with the F(ab')₂ fragment of K9.361 to inhibit Fc γ RIII-dependent anaphylaxis induction by 2.4G2 suggests that this putative inhibitory effect would have to be quite limited. An alternative possibility is that cross-linking of Fc γ RIIB by the F(ab')₂ fragment of K9.361 physically separates Fc γ RIIB from Fc γ RIII on the cell membrane, so that the Fc domain of intact K9.361 that has bound to Fc γ RIIB is less likely to bind Fc γ RIII.

Regardless of the explanation for this phenomenon, the ability of the F(ab')₂ fragment of K9.361 to prevent any anaphylactic response to intact K9.361 and the ability of intact K9.361 to prevent antigen-induced and IgG-mediated anaphylaxis suggests that sequential treatment with the F(ab')₂ fragment of anti-Fc γ RIIB mAb, followed by treatment with the intact mAb, provides a safe way to suppress IgG-mediated anaphylaxis (Fig 3, G) and, possibly, other Fc γ R-mediated immune disorders.

Disclosure of potential conflict of interest:

Supported by National Institutes of Health (NIH) grant R01 AI113162, a Merit Award to F.D.F. from the US Department of Veterans Affairs, the NIH-supported Immunology/Allergy Fellowship Training Program (T32AI060515-11), and the Department of Internal Medicine, University of Cincinnati Medical Center.

C. D. Clay receives grant support from the National Institutes of Health (NIH). F. D. Finkelman serves as a consultant for Vedanta Bioscience, is an employee of the University of Cincinnati, receives grant support from the NIH and Food Allergy Research & Education (FARE), and has 3 patents pending with the University of Cincinnati. The rest of the authors declare that they have no relevant conflicts of interest.

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Appendix

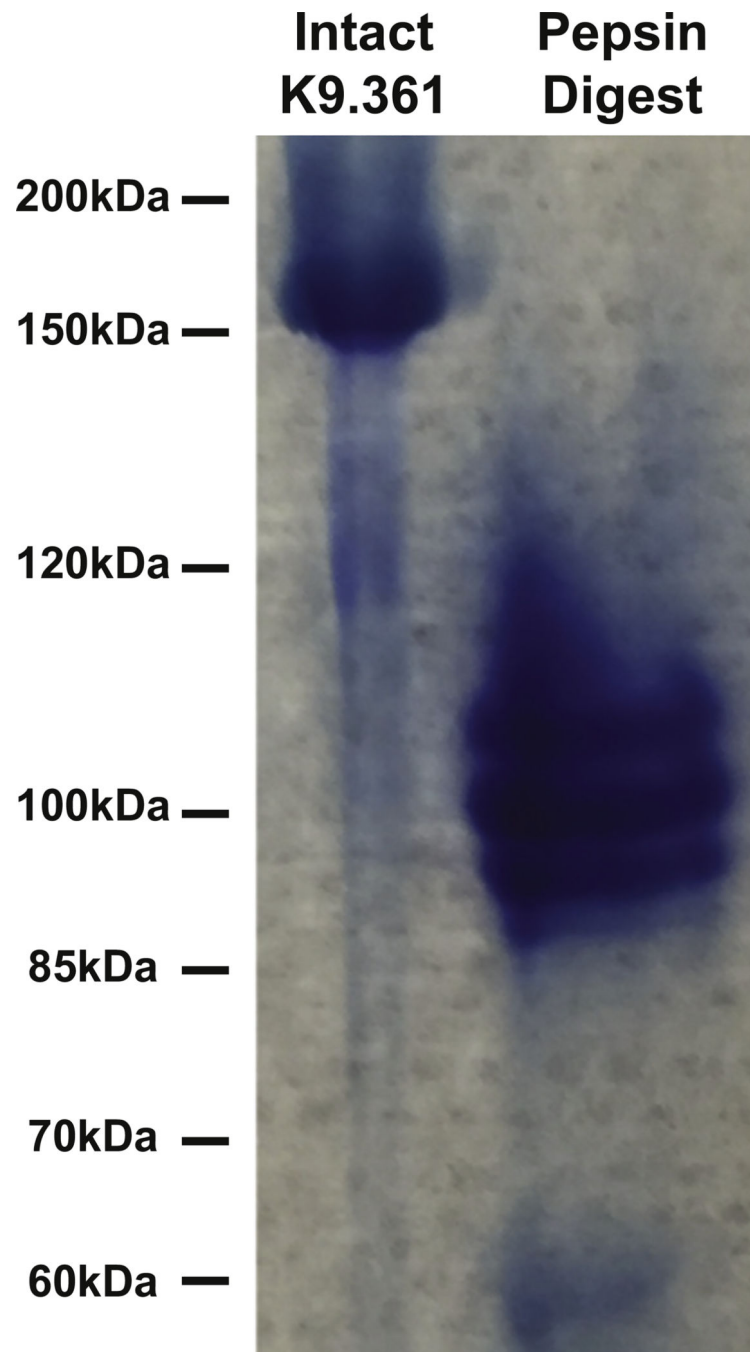


FIG E1. The $F(ab')_2$ fragment of K9.361 is devoid of intact mAb. Intact K9.361 mAb and product obtained after digestion with immobilized pepsin followed by Fc removal with protein A were analyzed by means of SDS-PAGE with 7% polyacrylamide gels under nonreducing conditions. Gels were stained with Coomassie blue. The band migrating between 150 and 200 kDa on the *left* is the typical size for intact mouse IgG_{2a} . $F(ab')_2$ fragments are typically

100 to 110 kDa, and F(ab) fragments are typically 50 kDa. The band on the *right* represents F(ab')₂.

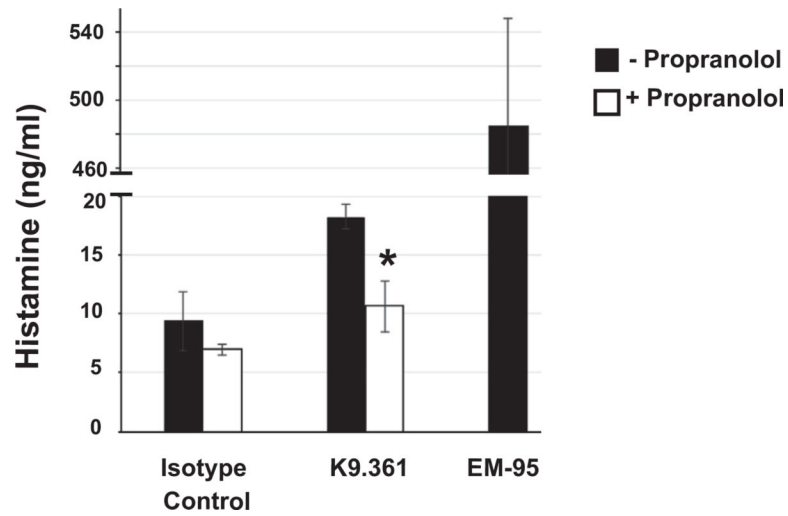


FIG E2.

Pretreatment with β -blocker does not enhance plasma histamine levels after challenge with anti-Fc γ RIIB mAb. BALB/c WT mice ($n = 4$) received saline or 35 μ g of propranolol administered intravenously before receiving 500 μ g of CBPC-101 (isotype control) or K9.361 intravenously or 50 μ g of EM95 (rat IgG_{2a} anti-mouse IgE mAb that generates robust histamine responses *in vivo*) intravenously. Plasma histamine concentrations 10 minutes after *in vivo* treatment \pm SEM are shown. * $P < .05$.

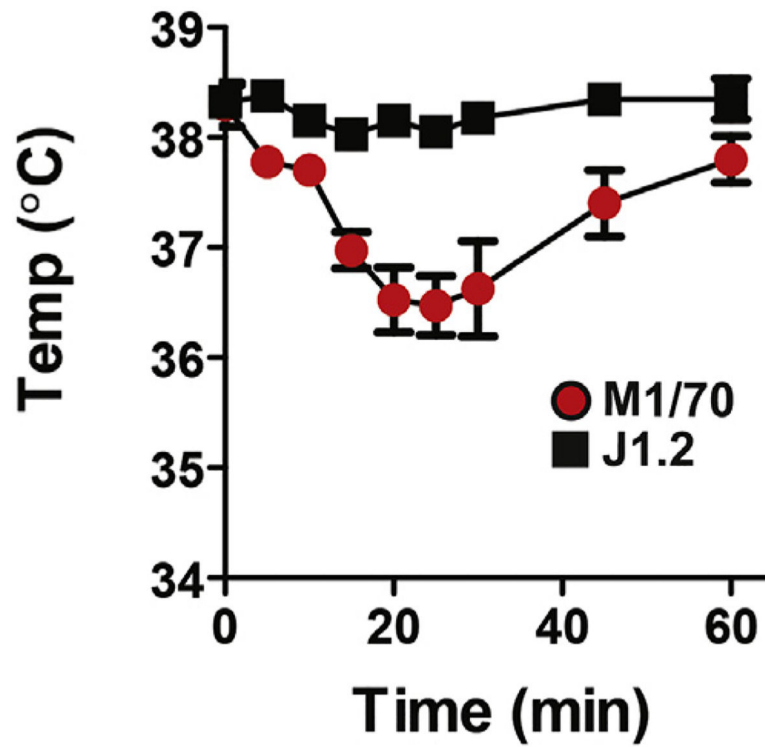


FIG E3.

Mild shock is induced by M1/70 and not J1.2. BALB/c WT mice ($n = 4$) received propranolol, followed by injection with 100 μg of M1/70 or J1.2 (rat IgG_{2b} isotype control mAb). Mean \pm SEM serial rectal temperatures are shown.

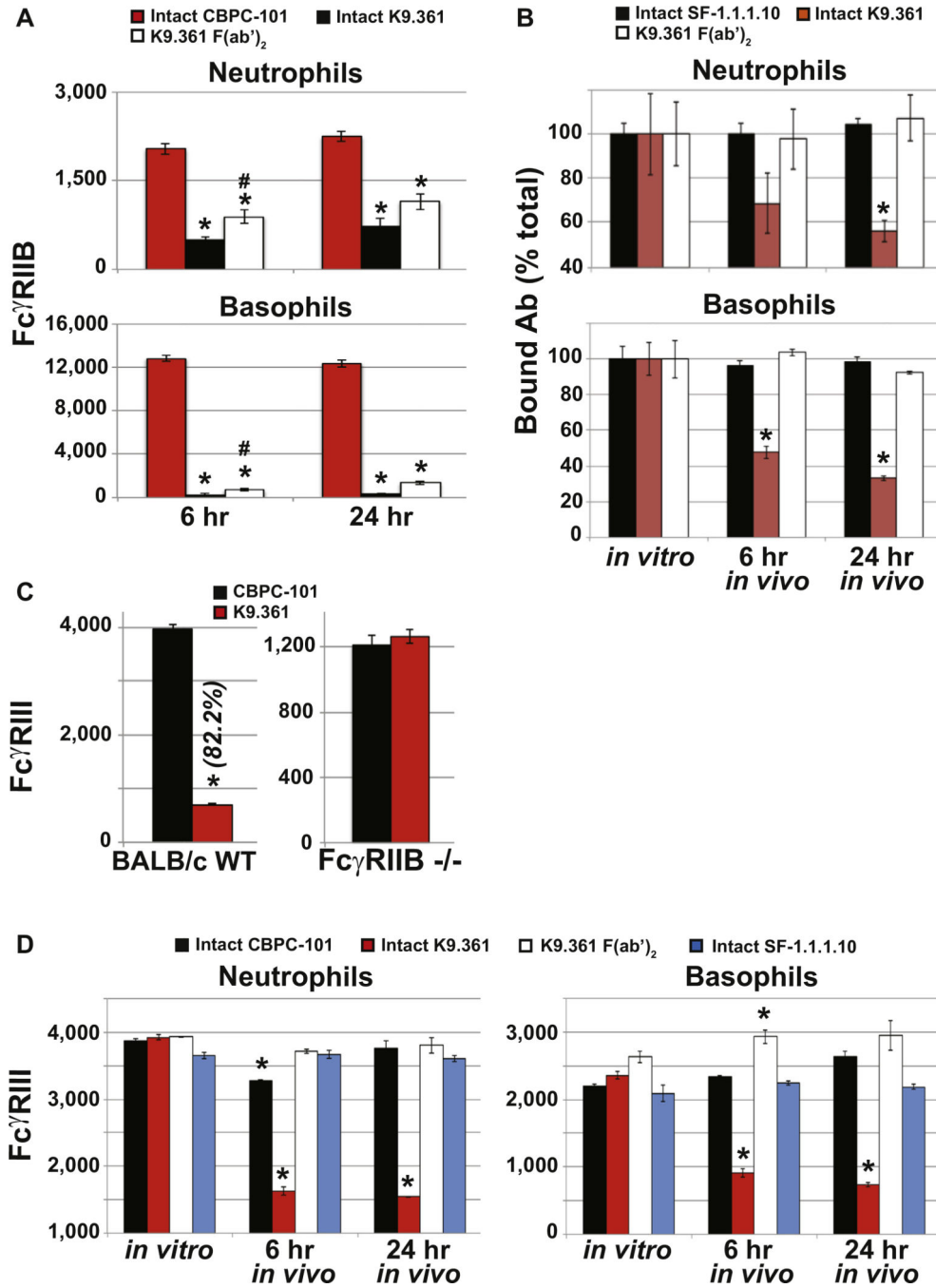


FIG E4. K9.361 effects on Fc γ RIIB and Fc γ RIII expression on neutrophils and basophils. **A**, BALB/c mice (4 per group) were injected intravenously with 500 μ g of CBPC-101 or intact K9.361 or 1 mg of K9.361 F(ab')₂. Six and 24 hours later, peripheral blood cells were stained to identify neutrophils and basophils and with fluorochrome-labeled K9.361. Mean fluorescence intensity (MFI) \pm SEM is shown. **P* < .005 compared with CBPC-101; #*P* < .05 compared with K9.361. **B**, BALB/c mice (4 per group) were injected intravenously with 500 μ g of intact biotin-labeled K9.361 or biotin-labeled SF-1.1.1.10 or 1 mg of the

biotin-labeled F(ab')₂ fragment of K9.361. Peripheral blood cells obtained 6 and 24 hours later were stained to identify neutrophils and basophils and with fluorochrome-labeled streptavidin. Additional cells from untreated mice were treated *in vitro* with 20 µg of intact biotin-labeled K9.361, SF-1.1.1.10, or the F(ab')₂ fragment of K9.361 for 30 minutes at 4°C and then stained with fluorochrome-labeled streptavidin. Data show mean ± SEM ratios (MFI relative to MFI on cells from untreated mice that were stained with the same mAbs *in vitro*). **P* < .001, 1-way ANOVA with posttest. **C**, BALB/c background WT and FcγRIIB-deficient mice (4 per group) were injected intravenously with 500 µg of intact K9.361 or CBPC-101. Neutrophils obtained 24 hours later were stained with fluorochrome-labeled anti-mouse FcγRIII. Mean ± SEM MFIs are shown. **P* < .005 compared with isotype control. Percentage decrease in mean MFI compared with isotype controls is shown in parentheses. **D**, BALB/c mice (4 per group) were injected intravenously with 500 µg of CBPC-101, intact K9.361, or SF-1.1.1.10 or with 1 mg of the F(ab')₂ fragment of K9.361. Neutrophils and basophils obtained 6 or 24 hours later were stained for FcγRIII, and mean ± SEM MFIs are shown. **P* < .001, 1-way ANOVA with posttest.

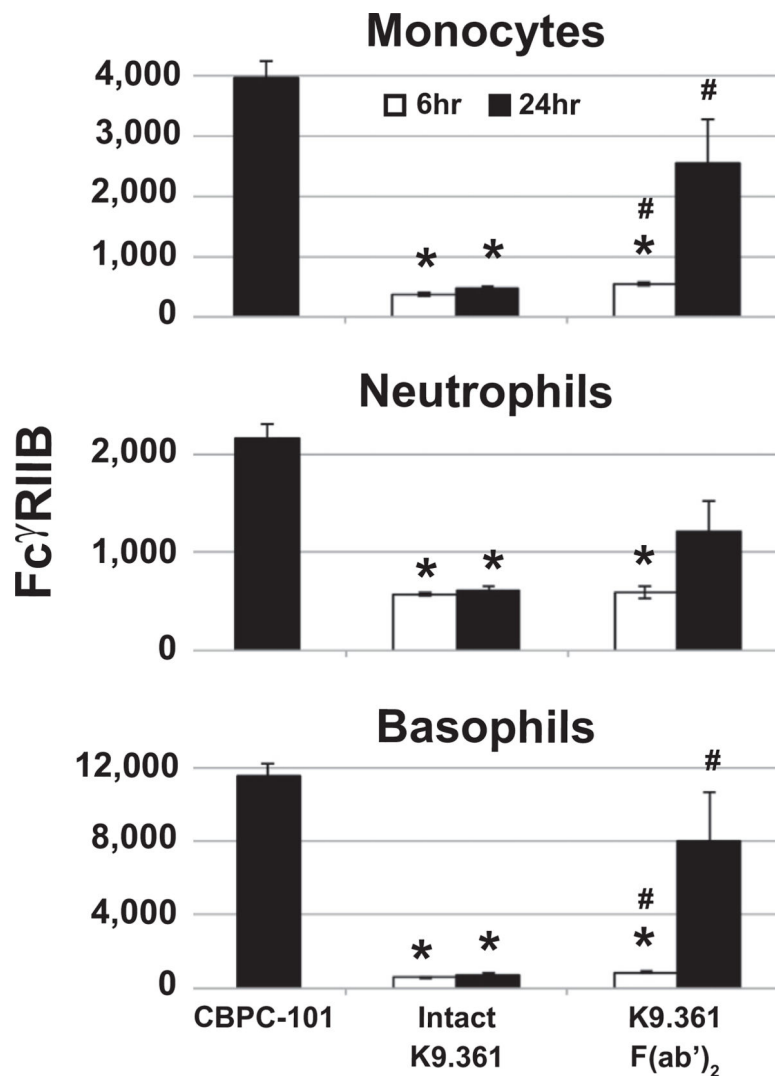


FIG E5.

Decreased half-life of the F(ab')₂ fragment of K9.361 versus intact K9.361. BALB/c mice (3 per group) were injected intravenously with 500 µg of CBPC-101, intact K9.361, or 1 mg of the F(ab')₂ fragment of K9.361. Six or 24 hours later, peripheral blood was obtained and centrifuged to produce serum. Pooled peripheral myelocytes from WT BALB/c mice treated with CBPC-101 were incubated for 30 minutes at 4°C with 20 µL of serum taken from antibody-injected mice; washed; stained with fluorochrome-labeled K9.361 and markers for monocytes, neutrophils, and basophils; and analyzed by means of flow cytometry. Mean ± SEM MFIs are shown. **P* < .05 compared with CBPC-101 controls; #*P* < .05 compared with intact K9.361.

Abbreviations used

EW	Chicken egg white
FcγR	Fcγ receptor
FcRγ	Fc receptor γ chain
FSC	Forward scatter
ITAM	Immunoreceptor tyrosine-based activation domain
ITIM	Immunoreceptor tyrosine-based inhibitory domain
MFI	Mean fluorescence intensity
OVA	Ovalbumin
SSC	Side scatter
TNP	2,4,6-Trinitrophenyl
WT	Wild-type

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Key messages

- K9.361, an IgG_{2a} anti-FcγRIIB mAb, inhibits IgG-mediated anaphylaxis in mice through a mechanism that involves cross-linking of FcγRIIB with stimulatory FcγRs. K9.361 has relatively little effect on IgE-mediated anaphylaxis.
- Although intact K9.361, in the presence of β-blocker, induces mild anaphylaxis through interaction primarily with FcγRIII, this can be inhibited by first treating mice with K9.361-derived F(ab')₂.
- This study contributes to the understanding of potential interactions between activating and inhibitory FcγRs, which are considered valuable therapeutic targets in patients with wide-ranging allergic and inflammatory disorders.

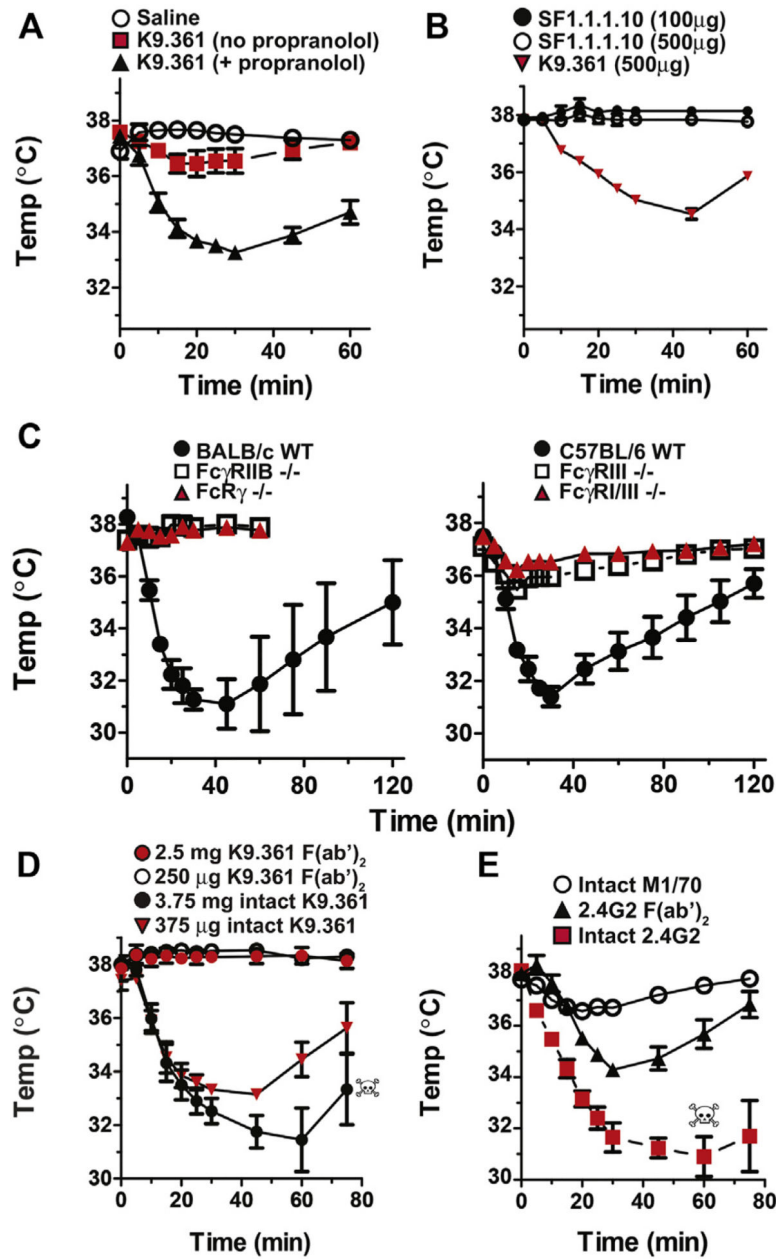


FIG 1.

IgG_{2a} anti-FcγRIIB mAb induces FcγRIIB- and FcγRIII-dependent anaphylaxis. **A**, BALB/c mice were injected intravenously with saline or propranolol and 15 minutes later with saline or 500 µg of K9.361 mouse IgG_{2a} allo-anti-FcγRIIB mAb. **B**, Propranolol-pretreated BALB/c mice were injected intravenously with 100 or 500 µg of SF1.1.1.10 IgG_{2a} allo-anti-H-2K^d mAb or 500 µg of K9.361. **C**, BALB/c background WT, FcRγ-deficient, and FcγRIIB-deficient and C57BL/6 background WT, FcγRIII-deficient, and FcγRI/III double-deficient mice were pretreated with propranolol and then injected intravenously with 500 µg of K9.361. **D**, BALB/c mice were pretreated with propranolol and then injected intravenously with 375 µg or 3.75 mg of intact K9.361 mAb or 250 µg of 2.5 mg of the F(ab')₂ fragment of K9.361. Injection of 5 mg of the F(ab')₂ fragment of mAb

K9.361 was also performed and did not induce hypothermia (not shown). **E**, BALB/c mice were pretreated with propranolol and then injected intravenously with 100 μg of intact 2.4G2 rat IgG_{2b} anti-mouse Fc γ RIIB/RIII mAb, 500 μg of the F(ab')₂ fragment of 2.4G2, or 100 μg of M1/70 rat IgG_{2b} anti-mouse CD11b mAb. *Skulls* indicate deaths of individual mice. Means \pm SEMs (n = 4) for serial rectal temperatures over 1 to 2 hours are shown. Similar results were seen in male and female mice.

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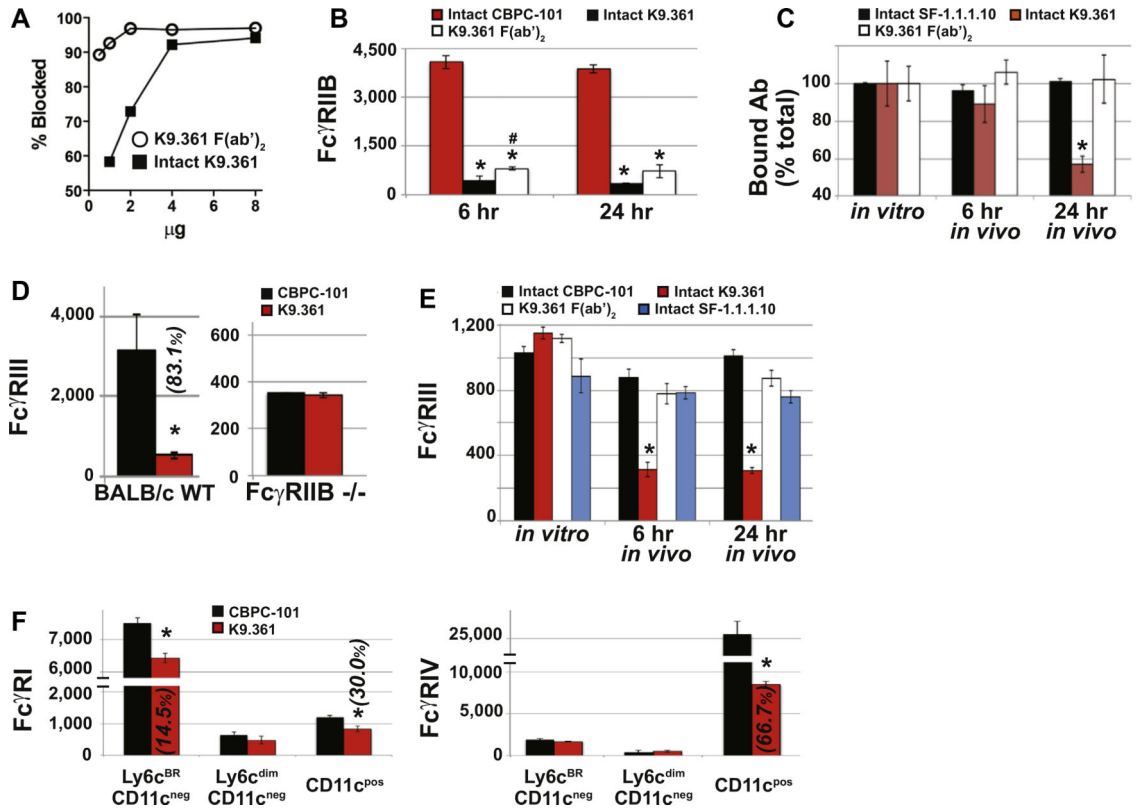


FIG 2. K9.361 effects on myeloid cell Fc γ R expression. **A**, Isolated splenocytes were incubated for 30 minutes at 4°C with increasing amounts of intact K9.361 or K9.361-derived F(ab')₂ or without mAb (control) and then stained with fluorochrome-labeled intact K9.361. Percentage decrease in mean fluorescence intensity (compared with control values) is shown. **B**, Mice received 500 μ g of CBPC-101 (mouse IgG_{2a} isotype control mAb), 500 μ g of intact K9.361, or 1 mg of K9.361-derived F(ab')₂ intravenously. Six and 24 hours later, PBMCs were stained with fluorochrome-labeled K9.361. Mean \pm SEM MFIs are shown. **P* < .005 compared with CBPC-101; #*P* < .05 compared with intact K9.361. **C**, Mice received 500 μ g of intact biotin-labeled K9.361 or biotin-labeled SF-1.1.1.10 or 1 mg of biotin-labeled K9.361-derived F(ab')₂ intravenously. PBMCs at 6 and 24 hours were stained with labeled streptavidin. Control PBMCs from untreated mice were incubated *in vitro* with 20 μ g of intact biotin-labeled K9.361, SF-1.1.1.10, or K9.361-derived F(ab')₂ for 30 minutes at 4°C. Mean \pm SEM ratios (MFI relative to controls for the given mAb) are shown. **P* < .01, 1-way ANOVA with posttest. **D**, WT and Fc γ RIIB-deficient mice received 500 μ g of intact K9.361 or CBPC-101 intravenously, and PBMCs at 24 hours were stained for Fc γ RIII. Mean \pm SEM MFIs are shown. **P* < .005 compared with CBPC-101. **E**, Mice received 500 μ g of CBPC-101, intact K9.361, or SF-1.1.1.10 or 1 mg of K9.361-derived F(ab')₂ intravenously, and PBMCs at 6 or 24 hours were stained for Fc γ RIII. Mean \pm SEM MFIs are shown. (**P* < .01, 1-way ANOVA with posttest). **F**, Mice received 500 μ g of intact K9.361 or CBPC-101. Mononuclear cell subpopulations isolated at 24 hours were classified based on Ly6c and CD11c expression and stained for Fc γ RI and Fc γ RIV. Mean \pm SEM

MFIs are shown. (* $P < .005$). For Fig 2, *D* and *F*, percentage decrease in MFI is shown in parentheses. BALB/c background mice (at least 4 per group) were used.

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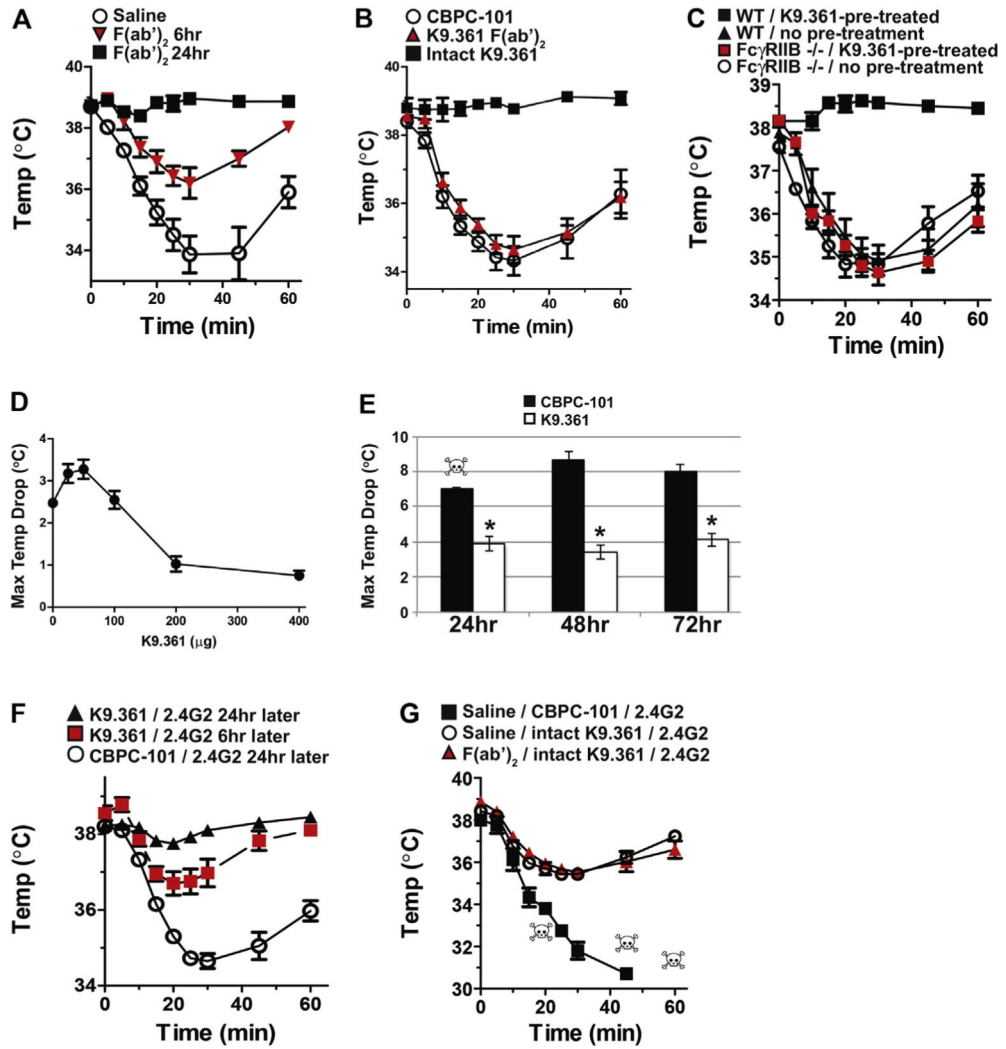
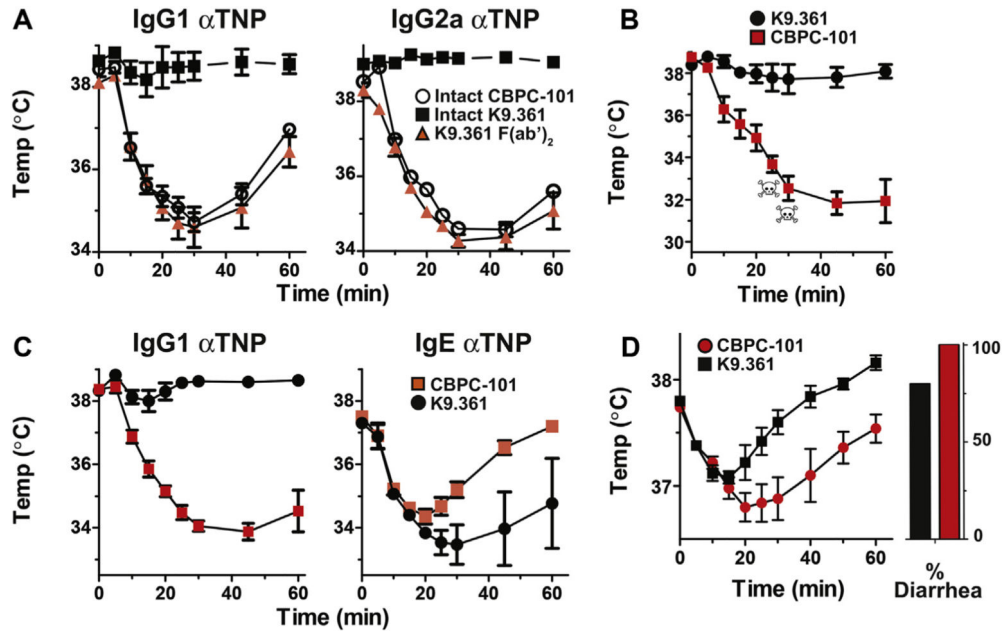


FIG 3. Suppression of IgG-mediated anaphylaxis by anti-Fc γ RIIB mAb. **A**, BALB/c mice (4 per group) were injected intravenously with saline or with 1 mg of the F(ab')₂ fragment of K9.361. Mice were injected intravenously 6 or 24 hours later with propranolol, followed 15 minutes later by intravenous injection of 500 μ g of intact K9.361. **B**, BALB/c mice (4 per group) were injected intravenously with 500 μ g of intact CBPC-101 or K9.361 or with 5 mg of the F(ab')₂ fragment of K9.361. Mice were challenged intravenously 24 hours later with 500 μ g of 2.4G2. **C**, BALB/c background WT and Fc γ RIIB-deficient mice (4 per group) were pretreated by means of intravenous injection with 500 μ g of intact CBPC-101 or K9.361 and challenged intravenously 24 hours later with 500 μ g of 2.4G2. In Fig 3, A–C, Rectal temperatures were followed for the next hour after challenge, and means \pm SEMs are shown. **D**, BALB/c mice (3 per group) were injected intravenously with the doses of K9.361 shown and challenged 24 hours later with 500 μ g of 2.4G2. Maximum temperature decreases \pm SEM during the following hour are shown. **E**, BALB/c mice (4 per group) were injected intravenously with 500 μ g of intact CBPC-101 or K9.361 followed 24, 48, or 72 hours later by intravenous injection of propranolol and 15 minutes later by intravenous

injection of 100 μg of 2.4G2. Rectal temperatures were followed for the next 2 hours, and mean \pm SEM maximum temperature decreases are shown. **F**, BALB/c mice (4 per group) were injected intravenously with 500 μg of intact CBPC-101 or K9.361, followed 6 or 24 hours later by intravenous injection of 500 μg of 2.4G2. Rectal temperatures were followed for the next hour, and means \pm SEM are shown. **G**, BALB/c mice (3 per group) were injected intravenously with saline or 1 mg of the F(ab')₂ fragment of K9.361. Twenty-four hours later, mice were injected intravenously with 500 μg of intact CBPC-101 or K9.361. After an additional 24 hours, mice were injected intravenously with propranolol, followed 15 minutes later by intravenous injection of 100 μg of 2.4G2. Rectal temperatures were followed for the next hour, and means \pm SEMs are shown. *Skulls* indicate individual mouse deaths.

**FIG 4.**

Treatment with anti-Fc γ RIIB mAb suppresses passive and active IgG-mediated anaphylaxis. **A**, BALB/c mice (4 per group) were injected intravenously with 500 μ g of intact CBPC-101 or K9.361 or with 1 mg of the F(ab')₂ fragment of K9.361. Mice were passively sensitized 22 hours later by means of intraperitoneal injection of 500 μ g of IgG₁ or IgG_{2a} anti-TNP mAb and challenged 2 hours after that by means of intravenous injection of 100 μ g of TNP-OVA. Rectal temperatures were followed for the next hour. **B**, BALB/c background IgE-deficient mice were actively immunized by means of subcutaneous injection of 200 μ L of goat anti-mouse IgD antiserum. Mice were injected intravenously 13 days later with 500 μ g of CBPC-101 (n = 5) or K9.361 (n = 6) and challenged intravenously 1 day later with 100 μ g of goat IgG. Rectal temperatures were followed for the next hour. **C**, BALB/c mice (4 per group) were injected intravenously with 500 μ g of CBPC-101 or K9.361. Mice were passively sensitized 20 hours later by means of intravenous injection with 500 μ g of IgG₁ anti-TNP mAb or with 10 μ g of IgE anti-TNP and challenged 4 hours later by means of intravenous injection of 100 μ g of TNP-OVA. Rectal temperatures were followed for the next hour. Means \pm SEM are shown. **D**, BALB/c WT mice (n = 5) were sensitized to EW by means of intratracheal inoculation and then injected intravenously with 500 μ g of CBPC-101 or K9.361 24 hours before oral gavage with 100 μ g EW. Rectal temperatures \pm SEM and the percentage of mice that experienced diarrhea are shown. *Skulls* indicate deaths of individual mice.