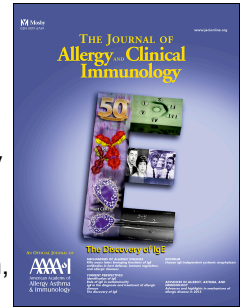


Accepted Manuscript

Anti-FcγRIIB monoclonal antibody suppresses murine IgG-dependent anaphylaxis by Fc domain targeting of FcγRIII

Corey D. Clay, M.D./Ph.D., Richard T. Strait, M.D., Ashley Mahler, Marat V. Khodoun, Ph.D., Fred D. Finkelman, M.D.



PII: S0091-6749(17)30934-X

DOI: [10.1016/j.jaci.2017.05.027](https://doi.org/10.1016/j.jaci.2017.05.027)

Reference: YMAI 12861

To appear in: *Journal of Allergy and Clinical Immunology*

Received Date: 14 July 2016

Revised Date: 21 April 2017

Accepted Date: 26 May 2017

Please cite this article as: Clay CD, Strait RT, Mahler A, Khodoun MV, Finkelman FD, Anti-FcγRIIB monoclonal antibody suppresses murine IgG-dependent anaphylaxis by Fc domain targeting of FcγRIII, *Journal of Allergy and Clinical Immunology* (2017), doi: 10.1016/j.jaci.2017.05.027.

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

**Anti-FcγRIIB monoclonal antibody suppresses murine IgG-dependent anaphylaxis
by Fc domain targeting of FcγRIII**

Corey D. Clay, M.D./Ph.D.¹, Richard T. Strait, M.D.^{2,4}, Ashley Mahler⁴, Marat V.
Khodoun, Ph.D.^{1,2}, Fred D. Finkelman, M.D.^{1,3,5†}

¹Division of Immunology, Allergy and Rheumatology, Department of Medicine,
University of Cincinnati Medical Center, Cincinnati, OH 45267; Departments of
²Research and ³Medicine, Cincinnati Veterans Affairs Medical Center,
Cincinnati, OH 45220; and Divisions of ⁴Emergency Medicine and ⁵Immunobiology,
Cincinnati Children's Hospital Medical Center, Cincinnati, OH 45229.

†Corresponding Author: Fred D. Finkelman, Division of Immunology, Allergy and
Rheumatology, University of Cincinnati College of Medicine, Cincinnati, OH 45267-
0563.

Phone: 513 636-6656; FAX: 513-636-6657; email: finkelfd@UCMAIL.UC.EDU

Key words: FcRs, anaphylaxis, mouse, signaling, IgG, IgE

Abbreviations: Ab, antibody; ACK, ammonium-chloride-potassium; Ag, antigen; EW,
chicken egg white; Ig, immunoglobulin; ITAM, immunoreceptor tyrosine-based
activation domain; ITIM, immunoreceptor tyrosine-based inhibitory domain; mAb,
monoclonal antibody; MFI, mean fluorescence intensity; OVA, ovalbumin; PBMC,

peripheral blood mononuclear cells; PI3K, phosphoinositide 3-kinase; R, receptor; SHIP,
SH-2 containing inositol polyphosphate-5-phosphatase; TNP, 2,4,6-trinitrophenyl; WT,
wild-type

Acknowledgements: Funding for this project was provided by NIH R01 AI113162, a
Merit Award to FDF from the United States 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.

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: Evaluate the effects of K9.361, a mouse IgG2a 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 IgG2b mAb that binds both Fc γ RIIB and the stimulatory receptor, Fc γ RIII); by actively immunizing IgE-deficient mice, then challenging with the immunizing Ag; and by passive immunization with IgG or IgE anti-TNP mAb, followed by injection of TNP-ovalbumin. Pretreatment with K9.361 was assessed for ability to influence anaphylaxis.

Results: Unexpectedly, K9.361 injection induced mild anaphylaxis, which was both Fc γ RIIB- and Fc γ RIII-dependent and was 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 failed to deplete Fc γ RIII or suppress IgG-mediated anaphylaxis; but prevented intact K9.361-induced anaphylaxis, without diminishing intact K9.36 suppression of IgG-mediated anaphylaxis.

Conclusion: Crosslinking Fc γ RIIB to stimulatory Fc γ Rs through the Fc domains of an anti-Fc γ RIIB mAb induces, then suppresses IgG-mediated anaphylaxis without

affecting IgE-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 the cellular level. Sequential treatment with the F(ab')₂ fragment of anti-FcγRIIB mAb followed by intact anti-FcγRIIB, safely prevents IgG-mediated anaphylaxis.

Key Messages:

- K9.361, an IgG2a anti-FcγRIIB mAb, inhibits IgG-mediated anaphylaxis in mice by 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 beta-blocker, induces mild anaphylaxis via 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 to be valuable therapeutic targets in wide-ranging allergic and inflammatory disorders.

74

75 ***Capsule Summary:***

76 Treatment with intact monoclonal anti-Fc γ RIIB and its F(ab')₂ fragment provides an
77 approach to safely inhibit IgG-mediated anaphylaxis in mice through an indirect
78 mechanism involving an Fc-dependent interaction with Fc γ RIII.

79

80 **Introduction:**

81 Anaphylaxis is an acute multi-system syndrome that results from rapid and
82 diffuse release of vasoactive mediators, cytokines and enzymes by myeloid cells minutes
83 to hours upon exposure to an inciting trigger. In mice, anaphylaxis can be induced
84 through both classical and alternative pathways (1-3). The classical pathway depends on
85 IgE-mediated activation of the high affinity Fcε receptor I (FcεRI) on mast cells and
86 basophils, while the alternative pathway depends on IgG-mediated activation of multiple
87 myeloid cell types via Fcγ receptors (FcγR), particularly FcγRIII. The classical pathway
88 is well-established in humans, while the importance of the alternative pathway in humans
89 is supported by considerable, but not conclusive evidence (4).

90 FcγRs regulate innate and adaptive immune responses in a coordinated manner
91 that involves positive signals that activate cells and cause pro-inflammatory responses as
92 well as negative signals that suppress cellular activation and inflammation. Dysregulation
93 of FcγR-mediated signaling has been implicated in wide-ranging disease processes (5-9).
94 Murine stimulatory FcγRs include FcγRI (expressed by dendritic cells and mononuclear
95 cell subpopulations in peripheral blood), FcγRIII (expressed in varying degrees by all
96 peripheral blood myeloid cells), and FcγRIV (expressed by neutrophils and peripheral
97 blood monocytes and dendritic cells) (6, 10). Activating FcγRs contain the
98 immunoreceptor tyrosine-based activation domain (ITAM) on the Fc receptor γ chain
99 (FcRγ). FcγRIIB, on the other hand, is inhibitory because it lacks FcRγ, but contains a
100 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 via FcγRs would have potential wide ranging therapeutic implications. We previously characterized a mouse model of IgG-mediated anaphylaxis triggered by 2.4G2, a rat IgG2b monoclonal antibody (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 a mAb that only binds to FcγRIIB, such as the mouse IgG2a allo-mAb K9.361 (26). Theoretically, this mAb might 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 crosslinked, 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 crosslinking of the stimulatory receptors to FcγRIIB. A third possibility is that K9.361 might bind to stimulatory FcγRs through its Fc domains and affect the expression and/or responsiveness 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; it 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 crosslinking FcγRIIB, but lacks the Fc domains required to interact with other FcγRs. Our results indicate that K9.361 indirectly induces mild anaphylaxis by an Fc-dependent interaction with FcγRIII, 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.

131

132 ***Materials and Methods:***

133 *Mice:* Male and female BALB/c and C57BL/6 mice were purchased from Taconic
134 (Hudson, NY) and were used at 7-15 weeks of age. Some experiments also used BALB/c
135 mice that were bred in-house. Fc γ RI- and Fc γ RIII-deficient mice on a C57BL/6
136 background were originally obtained from Jeffrey Ravetch, Rockefeller University (New
137 York, NY); these mice were bred to each other to generate mice deficient in both Fc γ RI
138 and Fc γ RIII. F2 offspring were typed by PCR to identify double-deficient offspring.
139 Fc γ RIIB-deficient mice and FcR γ - chain-deficient mice on a BALB/c background were
140 purchased from Jackson (Bar Harbor, ME). IgE-deficient mice on a BALB/c background
141 were a gift of Hans Oettgen, Boston Children's Hospital (Boston, MA). All transgenic
142 mice were bred in house. BALB/c females, 6-8 weeks old, were treated intra-tracheally 3
143 times per week for 3 weeks with chicken egg-white (EW) protein and assessed for airway
144 hypersensitivity using Buxco with gradient methacholine challenge. EW-sensitized mice
145 were subsequently challenged by oral gavage with 100 mg of protein and assessed for
146 temperature drop and diarrhea, then grouped according to the severity of sensitization.

147 *Antibodies, antigens, and pharmaceuticals:* Monoclonal Abs (mAbs) or the
148 hybridomas that produce them were obtained from the following sources: K9.361 (mouse
149 IgG2a anti-mouse Fc γ RIIB mAb, (a gift of Ulrich Hammerling (Sloan-Kettering, NY)),
150 2.4G2 (rat IgG2b anti-mouse Fc γ RII/RIII mAb (ATCC, Rockville, MD)), EM95 (rat
151 IgG2a anti-mouse IgE mAb (a gift of Zelig Eshhar (Rehovot, Israel)), CBPC-101 (mouse
152 IgG2a anti-myeloma protein, (John Abrams, DNAX, Palo Alto, CA)), SF1-1.1.10 (mouse
153 IgG2a anti-H-2K^d mAb, (ATCC)), M1/70 (rat IgG2b anti-mouse CD11b mAb, (ATCC)),

J1.2 (rat IgG2b anti-4-hydroxy-3-nitrophenylacetyl (gift from John Abrams, DNAX)), 1B7.11 (mouse IgG1 anti-TNP mAb (ATCC)), HY1.2 (mouse IgG2a anti-TNP mAb (Shozo Izui (University of Geneva, Geneva, Switzerland))), (mouse IgE anti-TNP (ATCC)). mAbs produced as ascites in Pristane-primed athymic nude mice were purified as described (28). A goat antiserum to mouse IgD was produced as described (28). Ovalbumin (OVA) was purchased from Sigma. OVA was labeled with 2,4,6-trinitrophenyl- ϵ -aminocaproyl-O-succinamide (TNP-OSu, (Biosearch)). Biotinylation was achieved by alkalizing stock mAb in NaHCO₃ at a pH of 8.0, then mixing mAb with EZ Link biotin (Thermo Scientific) 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, UK). Histamine ELISA kits were purchased from IBL-America (Minneapolis, MN).

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, then purified using the NAb Protein A Plus column (Thermo Scientific) and concentrated with centrifugal filter devices (Amicon). Aliquots were compared with undigested intact mAb under non-reducing conditions by SDS-PAGE with 7% polyacrylamide gels (NuPAGE Tris-Acetate, Life Technologies) and lacked detectable intact IgG (Supplemental Fig. 1) (25).

Detection of systemic anaphylaxis. Mice were challenged i.v. with the appropriate triggering Ab or Ag, after which serial body temperatures were determined every 5-15 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 tail vein incision was collected in microtainers that contained K₂EDTA (BD Bioscience). Red blood cells were lysed with ACK 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 described (29).

Immunofluorescence staining: 1x10⁶ peripheral blood cells or 2x10⁶ splenocytes in 0.1 ml HNA were stained with 0.25-2 µg each of fluorochrome labeled mAbs. Neutrophils were identified as Ly6G⁺ cells (Biolegend) with relatively high FSC and SSC. Mononuclear cells were identified as B220⁻, CD3⁻, CD11b⁺ cells with relative low FSC and SSC. Mononuclear cell subtypes were identified 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 FcγRIV mAb, a gift of Jeffrey Ravetch, Rockefeller University (New York, NY) (30)). bv421-labeled mouse IgG1 anti-mouse CD64 (anti-FcγRI) was purchased from Biolegend. FITC-labeled rat IgG2a anti-FcγRIII was purchased from R&D Systems. Fluorochrome labeled isotype antibodies; bv421-labeled mouse IgG1 (Biolegend), AF594-labeled CBPC101, FITC-labeled rat IgG2a (Biolegend), and AF647-labeled Hamster IgG1 (Biolegend) were used as negative controls. In some experiments, cells were incubated at 4°C with 10 µg of unlabeled K9.361 prior to staining, as an additional negative control. Cells were washed twice after staining, fixed with 2-4% paraformaldehyde, analyzed with a LSR II (BD Biosciences) and data were analyzed using FACSDiva software. Similar results were generated using cells obtained from male and female mice.

200 *Statistics:* One-tailed Mann-Whitney t-tests or 1-way ANOVA with Bonferroni's
201 multiple comparison post-testing were calculated to test for statistical significance.

202

Results:

Anti-Fc γ RIIB mAb induces Fc γ RIII-dependent anaphylaxis. Our previous studies demonstrated that 2.4G2 (a mAb that binds to both the stimulatory Fc γ R, Fc γ RIII, and the inhibitory Fc γ R, 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 IgG2a 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 drop rapidly developed in BALB/c mice injected with this mAb (Fig. 1A). Because the temperature drop was not always greater than that observed in mice injected with saline or isotype-control mAbs, we pre-treated mice with the β -adrenergic receptor antagonist propranolol, which prevents compensatory mechanisms that minimize anaphylaxis severity (31), to see if this would make anaphylaxis easier to appreciate. Indeed, propranolol-pretreated mice developed an $\sim 4^{\circ}\text{C}$ decrease in rectal temperature in response to i.v. anti-Fc γ RIIB mAb (Fig. 1A), but failed to develop hypothermia in response to i.v. saline or i.v. injection of a mouse IgG2a allo-Ab to H-2K^d (SF-1.1.1.10, Fig. 1B). 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 IgG2a isotype control mAb CBPC-101, or EM95 (rat IgG2a anti-IgE mAb used as a positive control for the capacity to induce histamine release). Propranolol treated mice, in fact, developed lower levels of plasma histamine upon K9.361 challenge (Supplemental Fig. 2). Anti-Fc γ RIIB mAb induced anaphylaxis in propranolol-pretreated C57BL/6 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. 1C). Taken together, these observations suggest that induction of anaphylaxis by anti-Fc γ RIIB mAb requires two interactions: with Fc γ RIIB via its Ag-binding site and with Fc γ RIII via 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. 1D). In contrast, the F(ab')₂ fragment of 2.4G2 induced hypothermia in propranolol-pretreated mice, albeit considerably less severe hypothermia than did intact 2.4G2, while intact rat IgG2b 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. 1E, Supplemental Fig. 3).

In vivo effects of anti-Fc γ RIIB mAb on Fc γ R expression. The 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. 2A). Consistent with this, compared to injection with CBPC-101, injection of WT BALB/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 three cell types implicated in IgG-mediated anaphylaxis (1, 25)) (Fig. 2B, Supplemental Fig. 4A). 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 hr 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 hr after injection (Supplemental Fig. 5). Intact K9.361 also differed from its F(ab')₂ fragment in that only the former decreased the total quantity of FcγRIIB on myeloid cells following in vivo injection (Fig. 2C, Supplemental Fig. 4B). In contrast to intact anti-FcγRIIB mAb, the intact IgG2a anti-H-2 K^d allo-mAb, SF-1.1.1.10, had no effect on the expression of its ligand on myeloid cells.

In addition to decreasing FcγRIIB expression, intact K9.361 also induced the loss of most FcγRIII on myeloid cells in vivo, an effect not observed in FcγRIIB-deficient mice, indicating that anti-FcγRIIB mAb binding to cells through its Ag binding site is essential for its downmodulation of FcγRIII (Fig. 2D, Supplemental Fig. 4C). As predicted, the F(ab')₂ fragment of anti-FcγRIIB mAb and intact CBPC-101 had no in vivo effect on the expression of FcγRIII (Fig. 2E, Supplemental Fig. 4D). Intact mouse IgG2a anti-H-2K^d mAb also had no effect on FcγRIII expression, even though its Fc domains should be able to interact with this receptor (Fig. 2E, Supplemental Fig. 4D).

An additional experiment evaluated the ability of intact anti-FcγRIIB mAb to affect expression of FcγRI and FcγRIV, 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 the expression of these stimulatory FcγRs (Fig. 2F), they were variable, cell type-dependent, and considerably less than those observed

for Fc γ RIII. This may reflect the higher affinity of Fc γ RI and Fc γ RIV than Fc γ RIII for uncomplexed IgG Ab 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 crosslink 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, then, 6 or 24 hours later, treated these mice with propranolol and injected them with intact K9.361. Pre-treatment with the F(ab')₂ fragment of K9.361 strongly suppressed the anaphylactic response to intact K9.361 6 hr later and totally blocked anaphylaxis when the intact mAb was injected 24 hr after the F(ab')₂ fragment (Fig. 3A). In contrast, pre-treatment of WT mice with intact K9.361, but not with its F(ab')₂ fragment, completely suppressed the hypothermia response to intact 2.4G2 (anti-Fc γ RIIB/RIII mAb) in mice that were not treated with propranolol (Fig. 3B). As expected, K9.361 suppression of the anaphylactic response to 2.4G2 was Fc γ RIIB-dependent (Fig. 3C), reflecting the Fc γ RIIB requirement for K9.361 binding to cells, and was dose-dependent (Fig. 3D). 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. 3E). K9.361 significantly suppressed 2.4G2-mediated anaphylaxis 6 hr post i.v. injection, and protection increased 24 hr post injection (Fig. 3F). These

observations suggested 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. 3G).

Intact anti-FcγRIIB mAb blocks active and passive IgG-mediated anaphylaxis.

Suppression of 2.4G2-induced anaphylaxis by pretreatment with intact K9.361, but not by its F(ab')₂ fragment, suggested that treatment with intact anti-FcγRIIB mAb might be able to suppress antigen-induced IgG-mediated anaphylaxis. To test this, mice were pre-treated with 500 μg of intact K9.361 or CBPC-101 or with 1 mg of the F(ab')₂ fragment of K9.361. Twenty-two hr later, mice were passively sensitized for IgG-mediated anaphylaxis by injection of 500 μg of IgG1 or IgG2a anti-trinitrophenyl (TNP) mAb, then challenged i.v. 2 hr after that with 100 μg of TNP-ovalbumin (TNP-OVA), in the absence of propranolol. Pre-treatment with intact K9.361 completely suppressed IgG1- and IgG2a-mediated anaphylaxis, while pre-treatment with the F(ab')₂ fragment of K9.361 had no effect on the anaphylactic response (Fig. 4A). To see if intact K9.361 would also suppress IgG-mediated anaphylaxis in actively sensitized mice, 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 (33, 34). Thirteen days later, mice were treated with 500 μg of intact K9.361 or CBPC-101, then challenged i.v. one day after that with 100 μg of goat IgG. Severe anaphylaxis developed in the mice

that had been treated with the control mAb, while no hypothermia was seen in the K9.361-treated mice (Fig. 4B).

Because Fc γ RIIB 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 μ g of K9.361 or CBPC-101, then passively sensitized 20 hr later by i.v. injection of 500 μ g IgG1 anti-TNP or 10 μ g of IgE anti-TNP and challenged i.v. 4 hr after that with 100 μ g of TNP-OVA. K9.361 protected against IgG1-mediated anaphylaxis, but not IgE-mediated anaphylaxis (Fig. 4C). Because the binding of IgG-containing immune complexes to Fc γ RIIB can suppress IgE-mediated anaphylaxis, it was possible that K9.361 blocking of Fc γ RIIB 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 i.t. with egg white (EW), then challenged repeatedly with EW by oral gavage (o.g.), until they develop both diarrhea and hypothermia in response to o.g. EW. Although mice immunized by this protocol generate both IgE and IgG anti-EW antibodies, the diarrhea and hypothermia responses to oral EW challenge are entirely IgE-dependent. Treatment of these EW-allergic mice i.v. with 500 μ g of K9.361, followed 24 hours later by oral gavage with 100 mg of EW slightly ameliorated anaphylaxis severity (Fig. 4D).

Discussion:

Anaphylaxis is induced in mice, and most likely in humans, by the aggregation of stimulatory Fc γ Rs by IgG-containing immune complexes (4). We have now shown that K9.361, an IgG2a mAb that binds to and crosslinks the inhibitory receptor, Fc γ RIIB, 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 via its Ag-binding site, because this mAb does not induce anaphylaxis in Fc γ RIIB-deficient mice. It also requires binding to Fc γ RIII via this mAb's Fc domain, because it fails to induce anaphylaxis in Fc γ RIII- or FcR γ -deficient mice. The induction of anaphylaxis by K9.361 is accompanied by, and probably depends on Fc γ RIII crosslinking, because this mAb both decreases the quantity of Fc γ RIIB expressed by myeloid cells and removes ~80% of Fc γ RIII from these cells, while its F(ab')₂ fragment, which fails to induce anaphylaxis has neither effect. This dependence on stimulatory Fc γ R crosslinking is also suggested by the inability of SF-1.1.1.10, an IgG2a mAb to H-2K^d (an Ag that is strongly expressed by cells that express Fc γ RIIB) to induce anaphylaxis or decrease the expression of H-2K^d or Fc γ RIII on myeloid cells (Figs.1B, 2C and 2E). 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 Ab binding of an inhibitory receptor via the Ab's F(ab) domain can activate cells through an interaction between that Ab's Fc domain and a stimulatory receptor. These observations have important implications for the clinical use of IgG mAbs to cell membrane molecules

that may also interact with stimulatory Fc γ R_s 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 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 that is induced by Ag challenge of actively or passively sensitized mice. More than one mechanism may be involved in this suppressive effect. First, the loss of ~80% of Fc γ RIII by myeloid cells in K9.361-treated mice may diminish the ability of Fc γ RIII crosslinking to induce anaphylaxis. This effect, however, is probably not sufficient to suppress IgG-mediated anaphylaxis because treating mice with M1/70 rat IgG2b 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-crosslinking of Fc γ RIIB with Fc γ RIII and 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 hr than after 6 hr, Fig. 3F) and may in part explain K9.361-mediated protection in IgE-mediated anaphylaxis to food protein challenge (Fig. 4D).

The inhibitory effects of FcγRIIB generally depend upon co-aggregation of this inhibitory receptor with stimulatory receptors via a common surface ligand and the ensuing recruitment of SHIP-1 (SH-2 containing inositol polyphosphate-5-phosphatase), which terminates ITAM-induced PI3K-mediated signaling (40, 41). This may explain the failure of intact K9.361 to strongly suppress IgE-mediated anaphylaxis (Fig. 4C). Although this mAb binds the FcγRIIB that is expressed on mouse basophils and mast cells, its Fc domains will not interact with the FcεRI on these cells. Because mouse mast cells and basophils also express FcγRIII, 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 FcR-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 FcγRIII, to decrease FcγRIII expression, and/or its limited ability to activate SHIP-1 by crosslinking FcγRIIB. Although the F(ab')₂ fragment of an IgG Ab, like the intact Ab, is divalent and consequently has the potential to crosslink its ligand, F(ab')₂ fragments of IgG Abs are less potent than intact IgG Abs at crosslinking their ligands in vivo (39). The aggregation of IgG-bound molecules on one cell is enhanced by IgGFc interactions with FcγRs on adjacent cells and by Fc-dependent interactions with C1q (42). The greater crosslinking ability of intact K9.361 than its F(ab')₂ fragment is most likely required for the intact Ab's ability to decrease FcγRIIB and FcγRIII expression (Fig. 2B) through the endocytosis of Ab-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 hr), time-dependent suppression of the response to intact K9.361 (Fig. 3A), without suppressing the anaphylactic response to 2.4G2 (Fig. 3B) or antigen-induced, IgG-mediated anaphylaxis (Fig. 4A). 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 hr after F(ab')₂ injection as it is 18 hr later (Fig. 2B), when suppression of anaphylaxis induction by intact K9.361 is considerably greater (Fig. 3A). It is possibly that F(ab')₂ binding to FcγRIIB, in the absence of FcγRIIB crosslinking to a stimulatory receptor, has a cumulative suppressive effect on subsequent activation of stimulatory receptors, including FcγRIII. However, the failure of 24 hr of treatment with the F(ab')₂ fragment of K9.361 to inhibit FcγRIII-dependent anaphylaxis induction by 2.4G2 suggests that this putative inhibitory effect would have to be quite limited. An alternative possibility is that crosslinking of FcγRIIB by the F(ab')₂ fragment of K9.361 physically separates FcγRIIB from FcγRIII on the cell membrane, so that the Fc domain of intact K9.361 that has bound to FcγRIIB is less likely to bind FcγRIII.

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 Ag-induced IgG-mediated anaphylaxis suggests that sequential treatment with the F(ab')₂ fragment of anti-FcγRIIB mAb, followed by treatment with the intact mAb, provides a safe way to suppress IgG-mediated anaphylaxis (Fig. 3G) and, possibly, other FcγR-mediated immune disorders.

426

427 **References:**

- 428 1. Strait RT, Morris SC, Yang M, Qu X-W, Finkelman FD. Pathways of
429 anaphylaxis in the mouse. *Journal of Allergy and Clinical Immunology*.
430 2002;109(4):658-68.
- 431 2. Strait RT, Morris SC, Finkelman FD. IgG-blocking antibodies inhibit IgE-
432 mediated anaphylaxis in vivo through both antigen interception and Fc γ RIIb cross-
433 linking. *J Clin Invest*. 2006;116(3):833-41.
- 434 3. Oettgen HC, Martin TR, Wynshaw-Boris A, Deng C, Drazen JM, Leder P.
435 Active anaphylaxis in IgE-deficient mice. *Nature*. 1994;370(6488):367-70.
- 436 4. Finkelman FD, Khodoun MV, Strait R. Human IgE-independent systemic
437 anaphylaxis. *J Allergy Clin Immunol*. 2016;137(6):1674-80.
- 438 5. Nimmerjahn F, Ravetch JV. Fc γ receptors as regulators of immune
439 responses. *Nat Rev Immunol*. 2008;8(1):34-47.
- 440 6. Bruhns P. Properties of mouse and human IgG receptors and their
441 contribution to disease models. *Blood*. 2012;119(24):5640-9.
- 442 7. Boruchov AM, Heller G, Veri MC, Bonvini E, Ravetch JV, Young JW.
443 Activating and inhibitory IgG Fc receptors on human DCs mediate opposing functions. *J*
444 *Clin Invest*. 2005;115(10):2914-23.
- 445 8. Boross P, Arandhara VL, Martin-Ramirez J, Santiago-Raber ML, Carlucci
446 F, Flierman R, et al. The inhibiting Fc receptor for IgG, Fc γ RIIB, is a modifier of
447 autoimmune susceptibility. *J Immunol*. 2011;187(3):1304-13.

9. Clynes R, Maizes JS, Guinamard R, Ono M, Takai T, Ravetch JV. Modulation of immune complex-induced inflammation in vivo by the coordinate expression of activation and inhibitory Fc receptors. *J Exp Med*. 1999;189(1):179-85.
10. Bruhns P, Jonsson F. Mouse and human FcR effector functions. *Immunol Rev*. 2015;268(1):25-51.
11. Gillis C, Gouel-Cheron A, Jonsson F, Bruhns P. Contribution of human Fc γ Rs to Disease with evidence from human polymorphisms and transgenic animal studies. *Front Immunol*. 2014;5:254.
12. Getahun A, Cambier JC. Of ITIMs, ITAMs, and ITAMis: revisiting immunoglobulin Fc receptor signaling. *Immunol Rev*. 2015;268(1):66-73.
13. Miyajima I, Dombrowicz D, Martin TR, Ravetch JV, Kinet JP, Galli SJ. Systemic anaphylaxis in the mouse can be mediated largely through IgG1 and Fc γ RIII. Assessment of the cardiopulmonary changes, mast cell degranulation, and death associated with active or IgE- or IgG1-dependent passive anaphylaxis. *J Clin Invest*. 1997;99(5):901-14.
14. Watanabe N, Akikusa B, Park SY, Ohno H, Fossati L, Vecchiotti G, et al. Mast cells induce autoantibody-mediated vasculitis syndrome through tumor necrosis factor production upon triggering Fc γ receptors. *Blood*. 1999;94(11):3855-63.
15. Radeke HH, Janssen-Graalfs I, Sowa EN, Chouchakova N, Skokowa J, Loscher F, et al. Opposite regulation of type II and III receptors for immunoglobulin G in mouse glomerular mesangial cells and in the induction of anti-glomerular basement membrane (GBM) nephritis. *J Biol Chem*. 2002;277(30):27535-44.

16. Ioan-Facsinay A, de Kimpe SJ, Hellwig SM, van Lent PL, Hofhuis FM, van Ojik HH, et al. FcγRI (CD64) contributes substantially to severity of arthritis, hypersensitivity responses, and protection from bacterial infection. *Immunity*. 2002;16(3):391-402.
17. Hashimoto T, Ishii N, Ohata C, Furumura M. Pathogenesis of epidermolysis bullosa acquisita, an autoimmune subepidermal bullous disease. *J Pathol*. 2012;228(1):1-7.
18. Gessner JE, Heiken H, Tamm A, Schmidt RE. The IgG Fc receptor family. *Ann Hematol*. 1998;76(6):231-48.
19. Fossati-Jimack L, Ioan-Facsinay A, Reininger L, Chicheportiche Y, Watanabe N, Saito T, et al. Markedly different pathogenicity of four immunoglobulin G isotype-switch variants of an antierythrocyte autoantibody is based on their capacity to interact in vivo with the low-affinity Fcγ receptor III. *J Exp Med*. 2000;191(8):1293-302.
20. Bergtold A, Gavhane A, D'Agati V, Madaio M, Clynes R. FcR-bearing myeloid cells are responsible for triggering murine lupus nephritis. *J Immunol*. 2006;177(10):7287-95.
21. Baudino L, Nimmerjahn F, Azeredo da Silveira S, Martinez-Soria E, Saito T, Carroll M, et al. Differential contribution of three activating IgG Fc receptors (FcγRI, FcγRIII, and FcγRIV) to IgG2a- and IgG2b-induced autoimmune hemolytic anemia in mice. *J Immunol*. 2008;180(3):1948-53.
22. Bandukwala HS, Clay BS, Tong J, Mody PD, Cannon JL, Shilling RA, et al. Signaling through FcγRIII is required for optimal T helper type (Th)2 responses and Th2-mediated airway inflammation. *J Exp Med*. 2007;204(8):1875-89.

23. Unkeless JC. Characterization of a monoclonal antibody directed against mouse macrophage and lymphocyte Fc receptors. *J Exp Med.* 1979;150(3):580-96.
24. Hirano M, Davis RS, Fine WD, Nakamura S, Shimizu K, Yagi H, et al. IgEb immune complexes activate macrophages through Fcγ₄ binding. *Nat Immunol.* 2007;8(7):762-71.
25. Khodoun MV, Kucuk ZY, Strait RT, Krishnamurthy D, Janek K, Clay CD, et al. Rapid desensitization of mice with anti-FcγRIIb/FcγRIII mAb safely prevents IgG-mediated anaphylaxis. *J Allergy Clin Immunol.* 2013;132(6):1375-87.
26. Kimura S, Tada N, Nakayama E, Liu Y, Hammerling U. A new mouse cell-surface antigen (Ly-m20) controlled by a gene linked to Mls locus and defined by monoclonal antibodies. *Immunogenetics.* 1981;14(1-2):3-14.
27. Muta T, Kurosaki T, Misulovin Z, Sanchez M, Nussenzweig MC, Ravetch JV. A 13-amino-acid motif in the cytoplasmic domain of FcγRIIB modulates B-cell receptor signalling. *Nature.* 1994;368(6466):70-3.
28. Finkelman FD, Kessler SW, Mushinski JF, Potter M. IgD-secreting murine plasmacytomas: identification and partial characterization of two IgD myeloma proteins. *J Immunol.* 1981;126(2):680-7.
29. Khodoun MV, Kucuk ZY, Strait RT, Krishnamurthy D, Janek K, Lewkowich I, et al. Rapid polyclonal desensitization with antibodies to IgE and FcεRIα. *J Allergy Clin Immunol.* 2013;131(6):1555-64.
30. Nimmerjahn F, Bruhns P, Horiuchi K, Ravetch JV. FcγRIV: a novel FcR with distinct IgG subclass specificity. *Immunity.* 2005;23(1):41-51.

31. Khodoun M, Strait R, Orekov T, Hogan S, Karasuyama H, Herbert DR, et al. Peanuts can contribute to anaphylactic shock by activating complement. *J Allergy Clin Immunol.* 2009;123(2):342-51.
32. Chames P, Van Regenmortel M, Weiss E, Baty D. Therapeutic antibodies: successes, limitations and hopes for the future. *Br J Pharmacol.* 2009;157(2):220-33.
33. Svetic A, Finkelman FD, Jian YC, Dieffenbach CW, Scott DE, McCarthy KF, et al. Cytokine gene expression after in vivo primary immunization with goat antibody to mouse IgD antibody. *J Immunol.* 1991;147(7):2391-7.
34. Champion BR, Buckham S, Page K, Obray H, Zanders ED. Secondary immunoglobulin responses of BALB/c mice previously stimulated with goat anti-mouse IgD. *Immunology.* 1991;72(3):336-43.
35. Tipton TR, Mockridge CI, French RR, Tutt AL, Cragg MS, Beers SA. Anti-mouse Fc γ RIV antibody 9E9 also blocks Fc γ RIII in vivo. *Blood.* 2015;126(24):2643-5.
36. Xu Y, Szalai AJ, Zhou T, Zinn KR, Chaudhuri TR, Li X, et al. Fc γ Rs modulate cytotoxicity of anti-Fas antibodies: implications for agonistic antibody-based therapeutics. *J Immunol.* 2003;171(2):562-8.
37. Lim SH, Vaughan AT, Ashton-Key M, Williams EL, Dixon SV, Chan HT, et al. Fc γ receptor IIb on target B cells promotes rituximab internalization and reduces clinical efficacy. *Blood.* 2011;118(9):2530-40.
38. Beers SA, Glennie MJ, White AL. Influence of immunoglobulin isotype on therapeutic antibody function. *Blood.* 2016;127(9):1097-101.

- 537 39. Goroff DK, Finkelman FD. Activation of B cells in vivo by a Fab/Fc
538 fragment of a monoclonal anti-IgD antibody requires an interaction between the antibody
539 fragment and a cellular IgG Fc receptor. *J Immunol.* 1988;140(9):2919-24.
- 540 40. Ono M, Bolland S, Tempst P, Ravetch JV. Role of the inositol
541 phosphatase SHIP in negative regulation of the immune system by the receptor Fc γ RIIB.
542 *Nature.* 1996;383(6597):263-6.
- 543 41. Bolland S, Ravetch JV. Inhibitory pathways triggered by ITIM-containing
544 receptors. *Adv Immunol.* 1999;72:149-77.
- 545 42. Vidarsson G, Dekkers G, Rispens T. IgG subclasses and allotypes: from
546 structure to effector functions. *Front Immunol.* 2014;5:520.
- 547

Figure Legends:

Figure 1. IgG2a anti-Fc γ RIIB mAb induces Fc γ RIIB- and Fc γ RIII-dependent anaphylaxis. A. BALB/c mice were injected i.v. with saline or propranolol and, 15 min later, with saline or 500 μ g of K9.361 mouse IgG2a allo-anti-Fc γ RIIB mAb. B. Propranolol-pretreated BALB/c mice were injected i.v. with 100 or 500 μ g of SF-1.1.1.10 IgG2a allo-anti-H-2K^d mAb or 500 μ g of K9.361. C. BALB/c background WT, Fc γ R γ -deficient, Fc γ RIIB-deficient, and C57BL/6 background WT, Fc γ RIII-deficient, and Fc γ RI/RIII-double-deficient mice were pre-treated with propranolol, then injected i.v. with 500 μ g of K9.361. D. BALB/c mice were pre-treated with propranolol, then injected i.v. with 375 μ g or 3.75 mg of intact K9.361 mAb or 250 μ g 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 failed to induce hypothermia (not shown). E. BALB/c mice were pre-treated with propranolol, then injected i.v. with 100 μ g of intact 2.4G2 rat IgG2b anti-mouse Fc γ RIIB/RIII mAb, 500 μ g of the F(ab')₂ fragment of 2.4G2, or 100 μ g of M1/70 rat IgG2b anti-mouse CD11b mAb. Skulls indicate deaths of individual mice. Means $\pm$ SEM (N=4) for serial rectal temperatures over 1-2 hr are shown. Similar results were seen in male and female mice.

Figure 2. K9.361 effects on myeloid cell Fc γ R expression. A. Isolated splenocytes were incubated 30 min at 4°C with increasing amounts of intact K9.361, K9.361-derived F(ab')₂, or without mAb (control) then stained with fluorochrome-labeled intact K9.361. Percent decrease in MFI (compared to controls) is shown. B. Mice received 500 μ g CBPC-101 (mouse IgG2a isotype control mAb), 500 μ g intact K9.361, or 1 mg K9.361-derived F(ab')₂ i.v. Six and 24 hr later, PBMCs were stained with fluorochrome-labeled

K9.361. Mean MFI $\pm$ SEM shown (* p < 0.005 compared to CBPC-101; # p < 0.05 compared to intact K9.361). C. Mice received 500 μ g intact biotin-labeled K9.361 or biotin-labeled SF-1.1.1.10 or 1 mg biotin-labeled K9.361-derived F(ab')₂ i.v. PBMCs at 6 and 24 hr were stained with labeled-streptavidin. Control PBMCs from untreated mice were incubated in vitro with 20 μ g intact biotin-labeled K9.361, SF-1.1.1.10, or K9.361-derived F(ab')₂ for 30 min at 4°C. Mean ratios shown (MFI relative to controls for the given mAb) $\pm$ SEM (* p < 0.01; 1-way ANOVA with post test). D. WT and Fc γ RIIB-deficient mice received 500 μ g intact K9.361 or CBPC-101 i.v. and PBMCs at 24 hr were stained for Fc γ RIII. Mean MFI $\pm$ SEM shown (* p < 0.005 compared to CBPC-101). E. Mice received 500 μ g CBPC-101, intact K9.361, or SF-1.1.1.10 or 1 mg K9.361-derived F(ab')₂ i.v. and PBMCs at 6 or 24 hr were stained for Fc γ RIII. Mean MFI $\pm$ SEM shown (* p < 0.01, 1-way ANOVA with post test). F. Mice received 500 μ g intact K9.361 or CBPC-101. Mononuclear cell subpopulations isolated at 24 hr were classified by Ly6c and CD11c expression and stained for Fc γ RI and Fc γ RIV. Mean MFI $\pm$ SEM shown (* p < 0.005). For D and F, percent decrease in MFI in parentheses. BALB/c background mice (at least 4/group) were used.

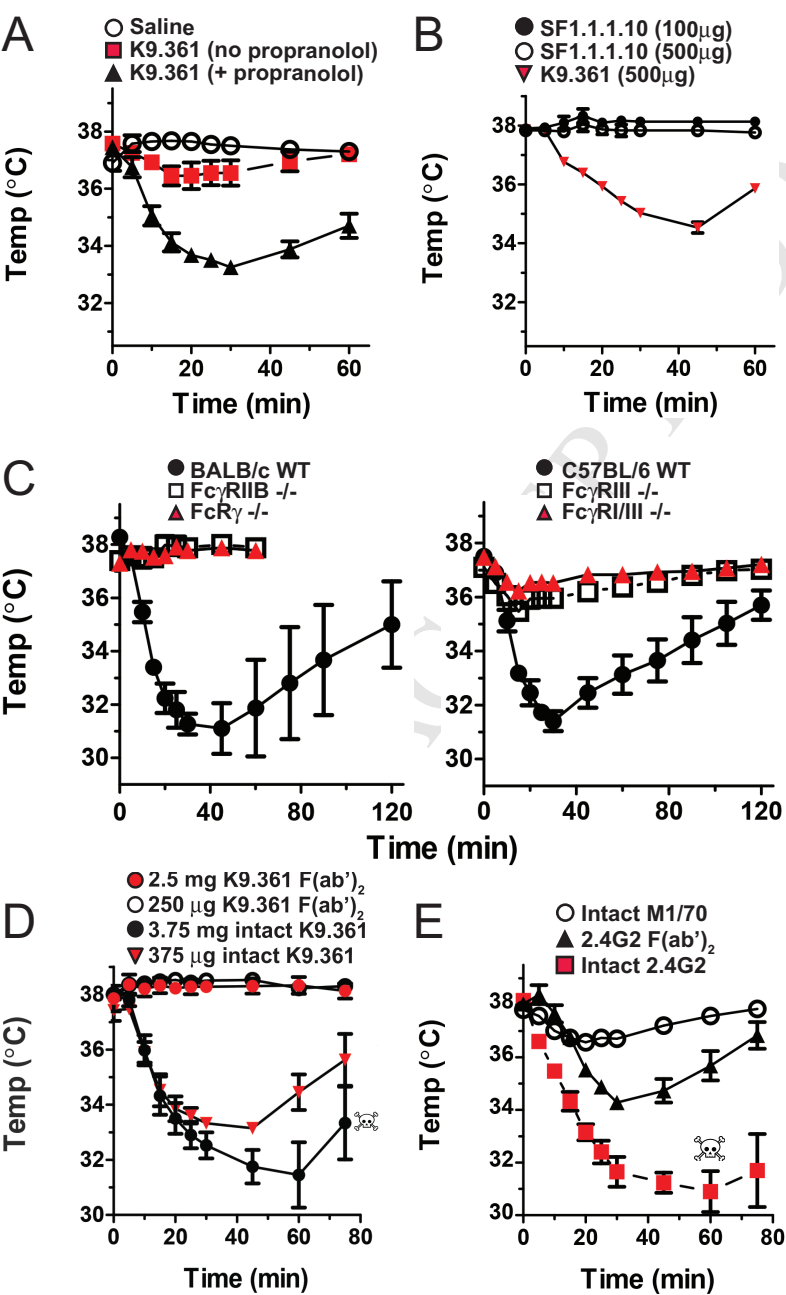
Figure 3. Suppression of IgG-mediated anaphylaxis by anti-Fc γ RIIB mAb. A.

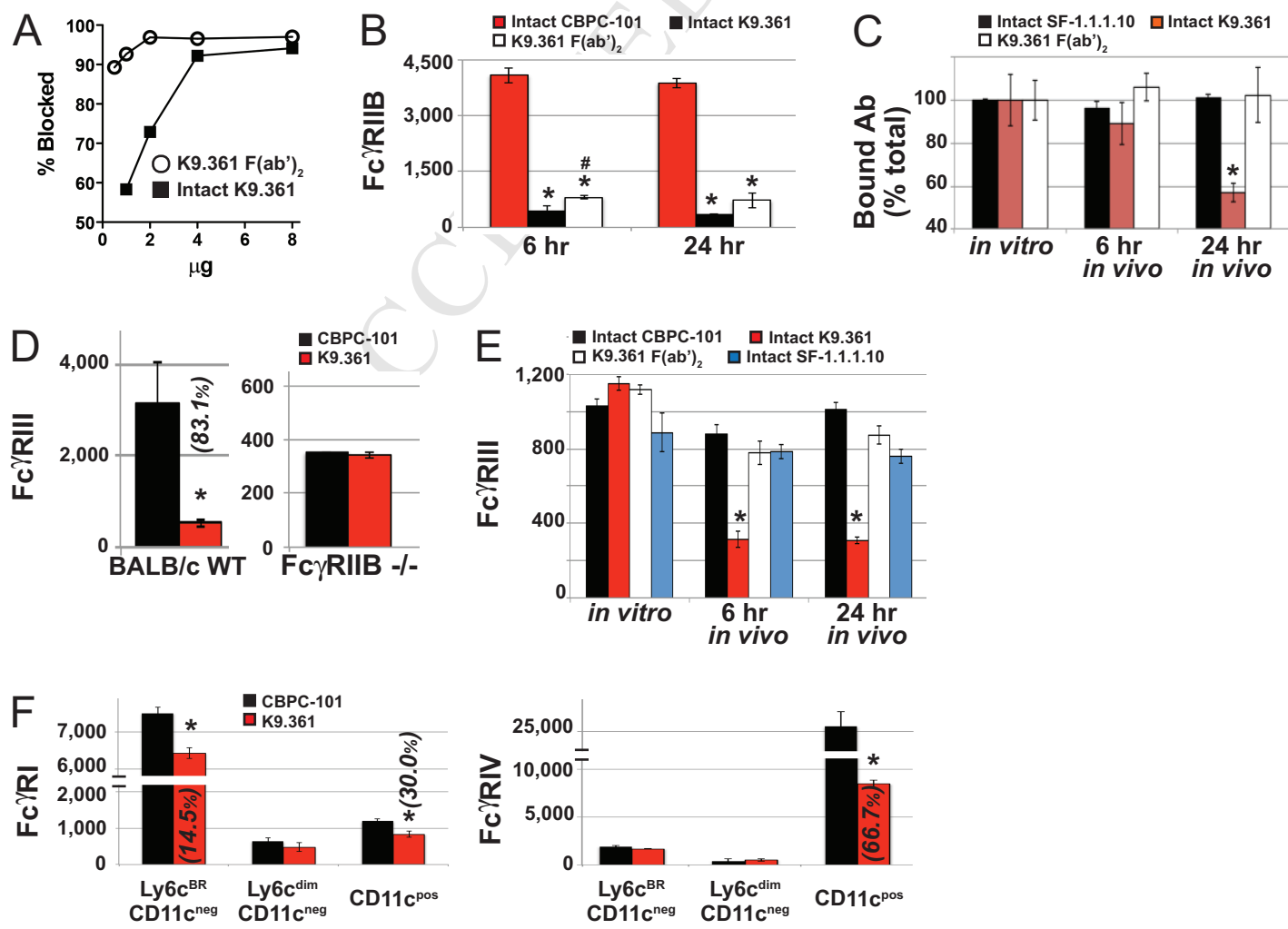
BALB/c mice (4/group) were injected i.v. with saline or with 1 mg of the F(ab')₂ fragment of K9.361. Mice were injected i.v. 6 or 24 hr later with propranolol, followed 15 min after that by i.v. injection of 500 μ g of intact K9.361. B. BALB/c mice (4/group) were injected i.v. with 500 μ g of intact CBPC-101 or K9.361 or with 5 mg of the F(ab')₂ fragment of K9.361. Mice were challenged i.v. 24 hr later with 500 μ g of 2.4G2. C. BALB/c background WT and Fc γ RIIB-deficient mice (4/group) were pre-treated by i.v.

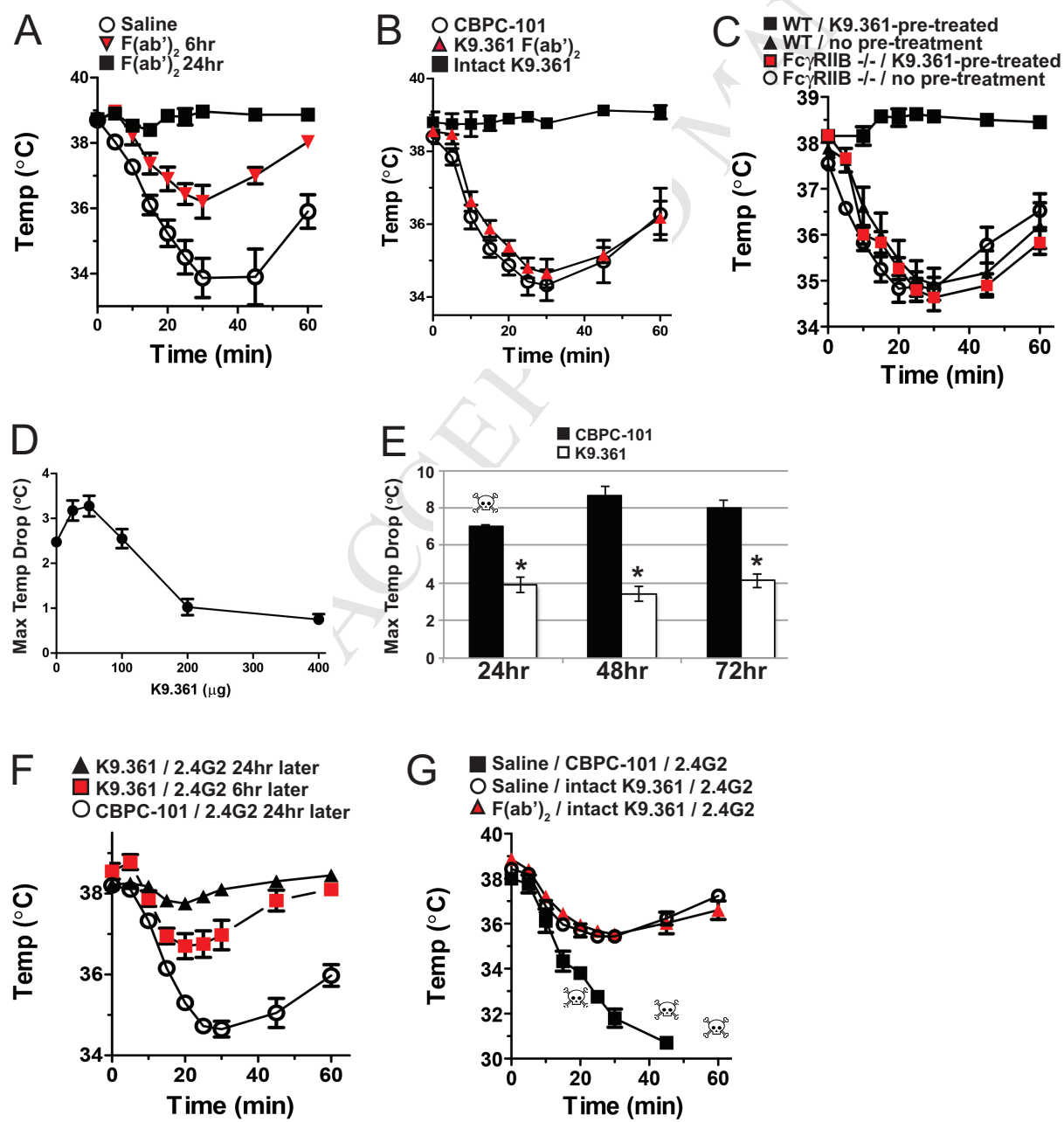
injection with 500 µg of intact CBPC-101 or K9.361 and challenged i.v. 24 hr later with
 500 µg of 2.4G2. A-C. Rectal temperatures were followed for the next hr following
 challenge and means ± SEM are shown. D. BALB/c mice (3/group) were injected i.v.
 with the doses of K9.361 shown and challenged 24 hr later with 500 µg of 2.4G2.
 Maximum temperature decreases ± SEM during the following hr are shown. E. BALB/c
 mice (4/group) were injected i.v. with 500 µg of intact CBPC-101 or K9.361 followed 24,
 48, or 72 hr later by i.v. injection of propranolol and 15 min after that by i.v. injection of
 100 µg of 2.4G2. Rectal temperatures were followed for the next 2 hr and mean
 maximum temp decreases ± SEM are shown. F. BALB/c mice (4/group) were injected i.v.
 with 500 µg of intact CBPC-101 or K9.361, followed 6 or 24hr later by i.v. injection of
 500 µg of 2.4G2. Rectal temperatures were followed for the next hr and means ± SEM
 are shown. G. BALB/c mice (3/group) were injected i.v. with saline or 1 mg of the
 F(ab')₂ fragment of K9.361. Twenty-four hr later, mice were injected i.v. with 500 µg of
 intact CBPC-101 or K9.361. After an additional 24 hr, mice were injected i.v. with
 propranolol, followed 15 min later by i.v. injection of 100 µg of 2.4G2. Rectal
 temperatures were followed for the next hr and means ± SEM are shown. Skulls indicate
 individual mouse deaths.

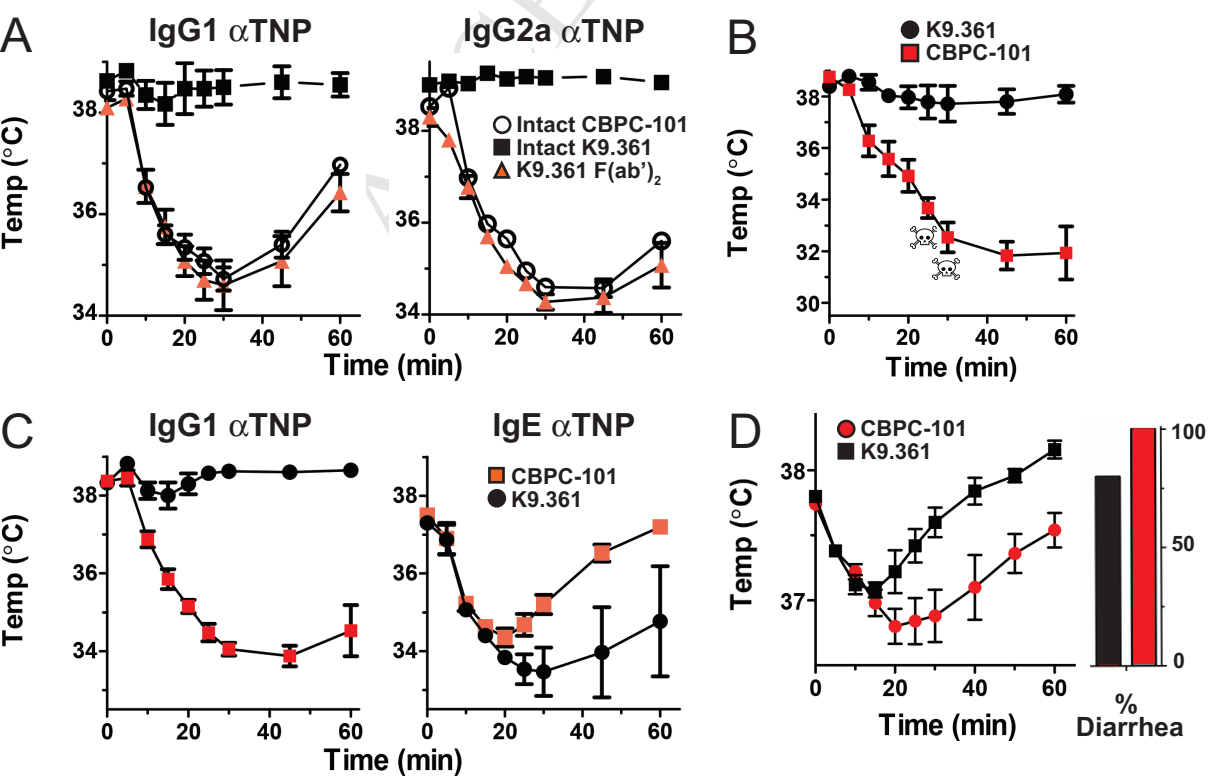
Figure 4. Treatment with anti-FcγRIIB mAb suppresses passive and active IgG-mediated anaphylaxis. A. BALB/c mice (4/group) were injected i.v. with 500 µg of intact CBPC-101 or K9.361 or with 1 mg of the F(ab')₂ fragment of K9.361. Mice were passively sensitized 22 hr later by i.p. injection of 500 µg of IgG1 or IgG2a anti-TNP mAb and challenged 2 hr after that by i.v. injection of 100 µg of TNP-OVA. Rectal temperatures were followed for the next hr. B. BALB/c background IgE-deficient mice were actively

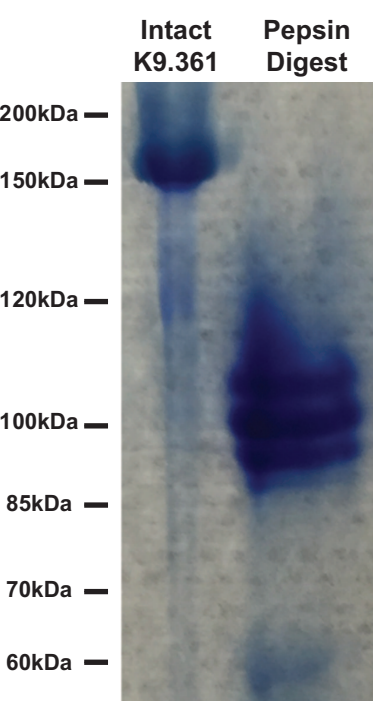
immunized by s.c. injection of 200 μ l of goat anti-mouse IgD antiserum. Mice were injected i.v. 13 d later with 500 μ g of CBPC-101 (N = 5) or K9.361 (N = 6) and challenged i.v. 1 d after that with 100 μ g of goat IgG. Rectal temperatures were followed for the next hr. C. BALB/c mice (4/group) were injected i.v. with 500 μ g of CBPC-101 or K9.361. Mice were passively sensitized 20 hr later by i.v. injection with 500 μ g of IgG1 anti-TNP mAb or with 10 μ g of IgE anti-TNP and challenged 4 hr after that by i.v. injection of 100 μ g of TNP-OVA. Rectal temperatures were followed for the next hr. Means $\pm$ SEM are shown. D. BALB/c WT mice (N=5) were sensitized to EW by i.t. inoculation, then injected i.v. with 500 μ g CBPC-101 or K9.361 24 hr prior to oral gavage with 100 μ g EW. Rectal temps $\pm$ SEM and the percent of mice that experienced diarrhea are shown. Skulls indicate deaths of individual mice.

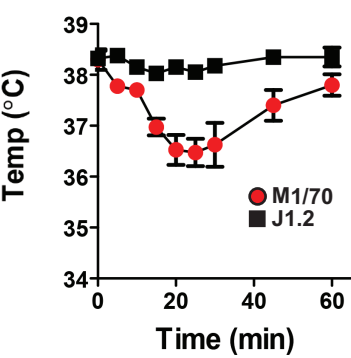


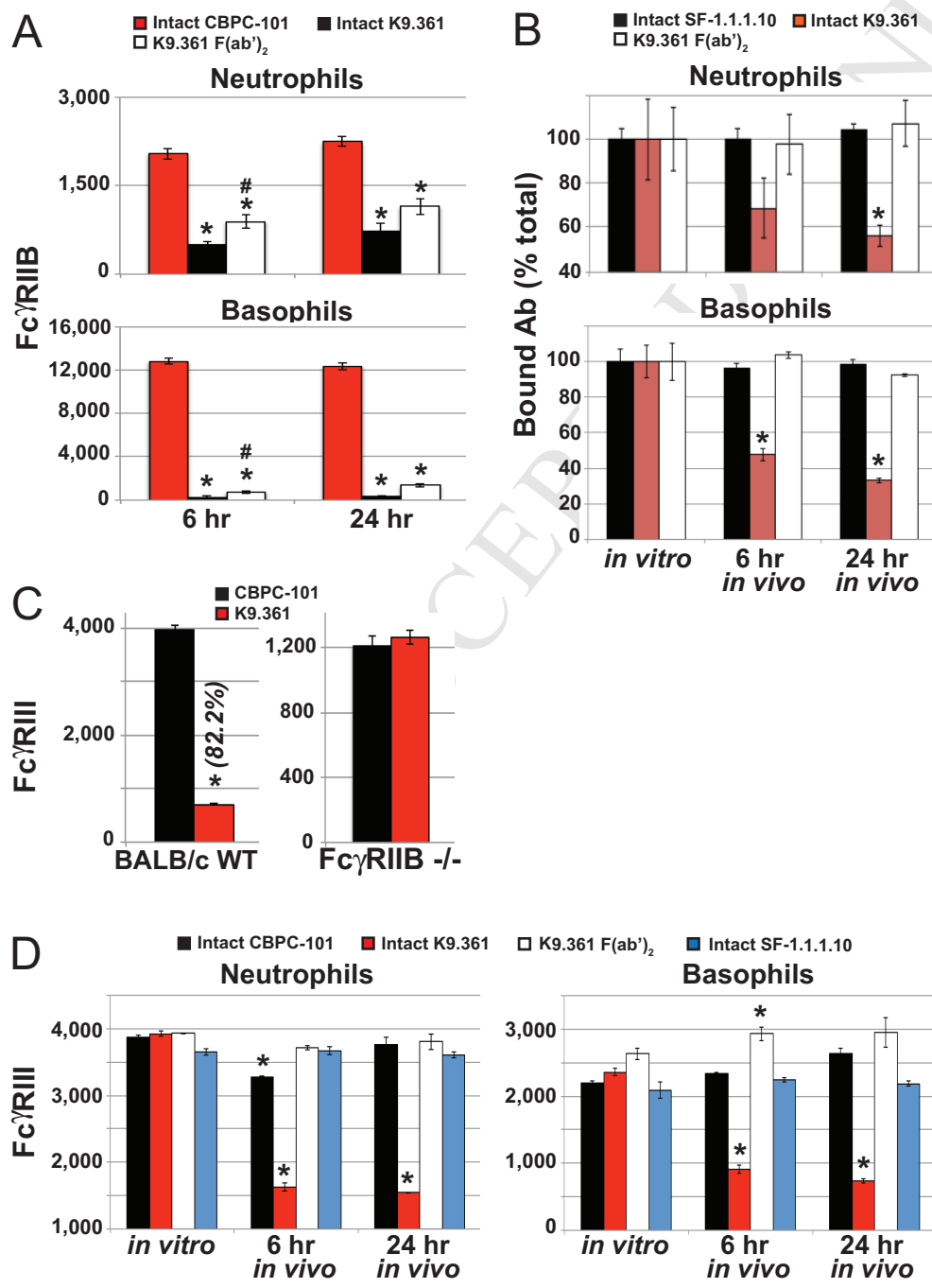


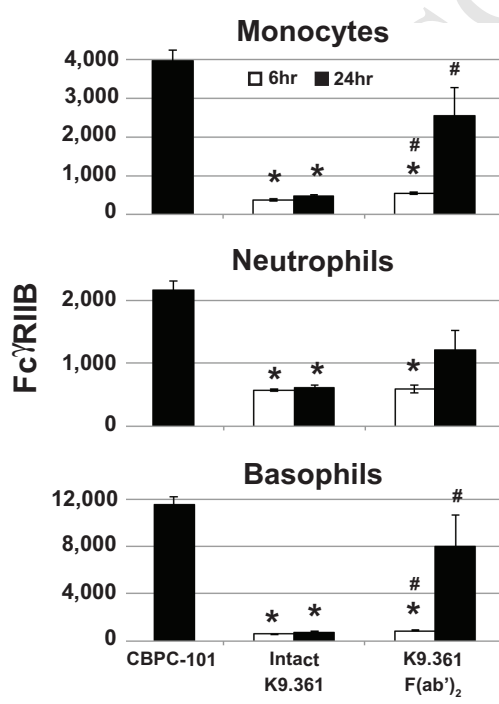


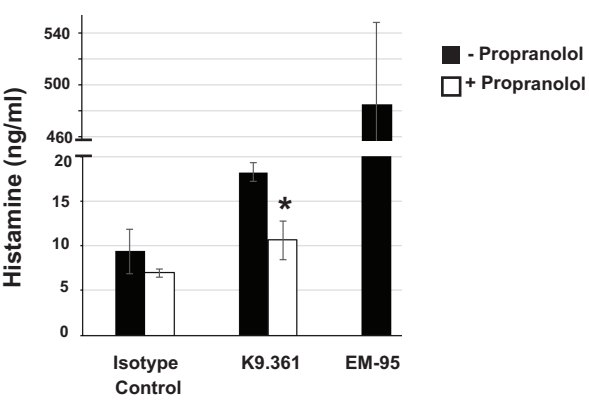












1 *Supplemental Figure 1. F(ab')₂ fragment of K9.361 is devoid of intact mAb.* Intact
 2 K9.361 mAb and product obtained after digestion with immobilized pepsin followed by
 3 Fc removal with protein A were analyzed by SDS 7% PAGE under non-reducing
 4 conditions. Gels were stained with Coomassie blue. The band migrating between 150-200
 5 kDa on the left is the typical size for intact mouse IgG2a. F(ab')₂ fragments are typically
 6 100-110kDa and F(ab) fragments are typically 50kDa. The band on the right represents
 7 F(ab')₂.

8 *Supplemental Figure 2. Pretreatment with beta blocker does not enhance plasma*
 9 *histamine levels after challenge with anti-FcγRIIB mAb.* BALB/c WT mice (N=4)
 10 received saline or 35 μg propranolol i.v. prior to receiving 500 μg CBPC-101 (isotype
 11 control) or K9.361 i.v. or 50 μg EM95 (rat IgG2a anti-mouse IgE mAb that generates
 12 robust histamine responses in vivo) i.v. Plasma histamine concentrations 10 min post in
 13 vivo treatment ± SEM shown (**p* < 0.05).

14 *Supplemental Figure 3. Mild shock is induced by M1/70 and not J1.2.* BALB/c
 15 WT mice (N=4) received propranolol followed by injection with 100 μg M1/70 or J1.2
 16 (rat IgG2b isotype control mAb). Mean serial rectal temps ± SEM shown.

17 *Supplemental Figure 4. K9.361 effects on FcγRIIB and FcγRIII expression on*
 18 *neutrophils and basophils.* A. BALB/c mice (4/group) were injected i.v. with 500 μg of
 19 CBPC-101 or intact K9.361, or 1 mg of K9.361 F(ab')₂. Six and 24 hr later, peripheral
 20 blood cells were stained to identify neutrophils and basophils and with fluorochrome-
 21 labeled K9.361. Mean MFI ± SEM are shown (**p* < 0.005 compared to CBPC-101, #*p*
 22 < 0.05 compared with K9.361). B. BALB/c mice (4/group) were injected i.v. with 500 μg
 23 of intact biotin-labeled K9.361 or biotin-labeled SF-1.1.1.10 or 1 mg of biotin-labeled

F(ab')₂ fragment of K9.361. Peripheral blood cells obtained 6 and 24 hr 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, then stained with fluorochrome-labeled streptavidin. Data show mean ratios (MFI relative to MFI on cells from untreated mice that were stained with the same mAbs in vitro) ± SEM (**p* < 0.001, 1-way ANOVA with post test). C. BALB/c background WT and FcγRIIB-deficient mice (4/group) were injected i.v. with 500 µg of intact K9.361 or CBPC-101. Neutrophils obtained 24 hr later were stained with fluorochrome-labeled anti-mouse FcγRIII. Mean MFI ± SEM are shown (**p* < 0.005 compared to isotype control).

Percent decrease in mean MFI compared with isotype controls are given in parentheses.

D. BALB/c mice (4/group) were injected i.v. 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 hr later were stained for FcγRIII and mean MFI ± SEM are shown (**p* < 0.001, 1-way ANOVA with post test).

Supplemental Figure 5. Decreased half-life of F(ab')₂ fragment of K9.361 vs. intact K9.361. BALB/c mice (3/group) were injected i.v. with 500 µg of CBPC-101, intact K9.361 or 1 mg 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 min at 4°C with 20 µl serum taken from Ab-injected mice, washed, stained with fluorochrome-labeled K9.361 and markers for monocytes, neutrophils and basophils, and analyzed by flow cytometry.

46 Mean MFI $\pm$ SEM are shown (* $p < 0.05$ compared to CBPC-101 controls, # $p < 0.05$
47 compared to intact K9.361).