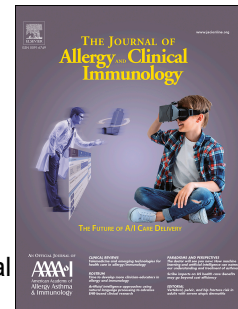


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Suppression of IgE-mediated anaphylaxis and food allergy with monovalent anti-FcεRIα monoclonal antibodies

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Key words: Mouse, humanized, mast cell, desensitization, Syk, crosslinking, IL-4, IgG1, IgG4

Abbreviations: Ag, antigen; γ_c , the cytokine receptor common γ chain, also known as IL-2 receptor γ chain; d, day; dv, divalent; hr, hour; hu, human, mAb, monoclonal antibody; min, minute; mv, monovalent; reNSGS mice; NSGS mice that have been reconstituted with T cell-depleted human cord blood cells; NSGS mice, NOD background SCID mice that lack γ_c and express transgenic human GM-CSF, IL-3, and stem cell factor (kit ligand); o.g., oral gavage; R, receptor; THSDT, Tukey’s honest significant difference test

Declaration of Interest Statement: SCM, MVK, CP, ABH, and FDF have rights in a patent for rapid desensitization with anti-Fc ϵ RI α mAbs that was awarded to the University of Cincinnati and could benefit from the licensing of this patent. FDF is a principal in a start-up company that is trying to commercialize rapid desensitization with anti-Fc ϵ RI α monoclonal antibodies. The other authors have no relevant conflicts of interest.

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Abstract

Background: Mast cell and basophil activation by antigen crosslinking of FcεRI-bound IgE is central to allergy pathogenesis. We previously demonstrated global suppression of this process by rapid desensitization with anti-FcεRIα mAbs.

Objective: Determine whether use of monovalent (mv) anti-FcεRIα mAbs increases desensitization safety without loss of efficacy.

Methods: mv anti-human (hu) FcεRIα mAbs were produced with mouse-derived immunoglobulin V regions and huIgG1 or huIgG4 C regions, and used to suppress murine IgE-mediated anaphylaxis and food allergy. mAbs were administered as a single dose or as serially increasing doses to mice that express hu instead of mouse FcεRIα, mice that additionally have an allergy-promoting IL-4Rα mutation, and hu cord blood-reconstituted immunodeficient, hu cytokine-secreting, mice that have large numbers of activated hu mast cells. Anaphylaxis susceptibility was sometimes increased by treatment with IL-4 or a β-adrenergic receptor antagonist.

Results: mv anti-huFcεRIα mAbs have considerably less ability than divalent mAbs to induce anaphylaxis and deplete mast cell and basophil IgE, but still strongly suppress IgE-mediated disease. The mv mAbs can be safely administered as a single large dose to mice with typical susceptibility to anaphylaxis, while a rapid desensitization approach safely suppresses disease in mice with increased susceptibility. Our huIgG4 variant of mv anti-huFcεRIα mAb is safer than our huIgG1 variant, apparently because reduced interactions with FcγRs decrease ability to indirectly crosslink FcεRI.

Conclusion: mv anti-FcεRIα mAbs more safely suppress IgE-mediated anaphylaxis and food allergy than divalent variants of the same mAbs. These mv mAbs may be useful for

68 suppression of huIgE-mediated disease.

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Clinical implication: Mouse model studies provide evidence that a monovalent monoclonal anti-FcεRIα antibody with low Fcγ receptor binding should safely and rapidly suppress IgE-mediated disease.

Capsule Summary: Our studies in humanized mice provide evidence that treatment with a monovalent monoclonal anti-FcεRIα antibody that interacts poorly with Fcγ receptors can safely, rapidly, and effectively suppress IgE-mediated anaphylaxis and food allergy.

Introduction:

The IgE-FcεRI-mast cell/basophil axis provides a mechanism for the rapid release of vasoactive mediators, cytokines, and enzymes that quickly induce an inflammatory response¹. Although this response protects against helminth and insect parasites²⁻⁵, it is also important in allergy pathogenesis, particularly those allergic disorders in which disease develops within minutes of allergen exposure^{6, 7}. The decreased importance of multicellular parasites and the increased prevalence of allergic disorders in developed countries has shifted the balance between the beneficial and adverse effects of this response and promoted the development of approaches to suppress the IgE-FcεRI-mast cell/basophil axis and its contributions to allergy.

One such approach is based on in vitro and in vivo evidence that IgE/FcεRI crosslinking does not invariably lead to mast cell/basophil activation and degranulation. Limited crosslinking, instead, has been associated with desensitization of these cells, characterized by the development of an anergic state in which even strong IgE/FcεRI crosslinking causes little or no activation and degranulation⁸⁻¹⁰. This process provides the basis for the therapeutic procedure known as rapid, or rush desensitization, in which patients who require a drug to which they have developed IgE-mediated allergy are treated with serially increasing doses of that drug, starting with a dose too small to induce disease and finishing with a full therapeutic dose¹¹⁻¹⁴.

During the past few years, we have used conventional, then humanized mouse models to study the feasibility of using a rapid desensitization approach that replaces a single allergen with a monoclonal antibody (mAb) to FcεRIα (the IgE-binding chain of the FcεRI heterotetramer¹⁵), with the goal of suppressing all IgE-mediated allergy rather

than only disease caused by the single allergen^{12, 16}. Our studies have demonstrated the feasibility and efficacy of this approach, which, in addition to suppressing all IgE-mediated anaphylaxis was safer and longer lasting in our models than allergen-mediated desensitization¹². However, we also found that an extended period of treatment with very slow increases in anti-FcεRIα mAb dose was required to desensitize mice that were particularly susceptible to anaphylaxis¹⁶.

This limitation has led us to modify our approach by using an engineered monovalent (mv) anti-FcεRIα mAb for desensitization that has only one Fab, which is associated with complete Fc domains. We initially expected that because a mv antibody would bind to, but not crosslink its target, it would be unable to either activate or desensitize basophils and mast cells, but could slow FcεRIα crosslinking by a subsequently injected divalent (dv) mAb that had the same V regions and bound to the same FcεRIα epitope. Indeed, this approach works well. However, as also shown herein, injection of the dv mAb is not necessary; although the mv mAb has much less ability than the dv mAb to activate mast cells and cause disease, it can still desensitize mast cells by limited, indirect FcεRI crosslinking that is predominantly mediated through interactions of its Fc with FcγRs.

Methods:

Mice. BALB/c mice were purchased from Taconic (Hudson, NY) or bred in the Cincinnati Children's Hospital Medical Center (CCHMC) animal facility. BALB/c background huFcεRIα mice, which lack mouse FcεRIα and express transgenic huFcεRIα; and huFcεRIα/F709 mice, which additionally express IL-4Rα with a mutation (F709) that allows only positive signaling by IL-4 and IL-13, were bred as previously described¹⁶⁻¹⁸. These mice, unlike otherwise identical mice that lack the IL-4Rα F709 mutation, develop egg allergy in response to epicutaneous (e.c), followed by oral gavage (o.g.) with egg¹⁶. Human IL-3/SCF/GM-CSF transgenic, immune-deficient NOD/LtSz-SCID IL-2RG^{-/-} (NSGS) or NOD/Rag1^{-/-}/IL-2RG^{-/-} (NRGS) mice were bred at a CCHMC core and engrafted with T cell-depleted human cord blood cells as previously described^{16, 19, 20}. 8-12 week old mice of both sexes were used. All mouse studies were approved by the Cincinnati Children's Hospital Research Foundation Institutional Animal Care and Use Committee (IACUC).

Reagents. Ovalbumin (OVA) and bovine serum albumin (BSA) were purchased from Sigma (St Louis, MO). TNP-OVA and TNP-BSA were prepared by haptenating OVA or BSA, respectively, with 2,4,6-trinitrophenyl-ε-aminocaproyl-O-succinamide (Biosearch Technologies, Novato, CA). Propranolol was purchased from West Ward Pharmaceuticals, Eatontown, NJ). Egg antigen (Ag) was prepared as previously described¹⁶. Briefly, egg white (EW) and egg yolk, removed under sterile conditions from fresh organic chicken eggs, were pooled separately. The EW was dialyzed against distilled water, analyzed for protein concentration using a BCA Protein Assay Kit (Pierce, Waltham, MA), then pressure concentrated with a stirred ultrafiltration cell

(Millipore, Temecula, CA) with a 30-kDa cut-off Diaflo membrane. The yolks were diluted 1:1 with 0.15M NaCl and centrifuged to remove egg yolk granules. The supernatant (egg yolk plasma (EYP)) was added to the EW while the EW was being concentrated to create a homogenous mixture with a final concentration of 333 mg of EW protein/ml. A long-acting formulation of IL-4 was produced by mixing the anti-IL-4 BVD4-1D11 mAb with recombinant murine IL-4 (Peprotech, Cranbury, NJ) at a 5:1 w/w ratio.

Antibodies. Generation of a hybridoma that secretes IE7 mouse IgG1 anti-huFcεRIα has been described¹⁶, as has generation of a hybridoma that secretes AER-37 (also called CRA-1) mouse IgG2b anti-human FcεRIα²¹. IE7 and AER-37 bind the same or closely positioned epitopes, as shown by mutual crossblocking¹⁶. IE7 and AER-37 bind huFcεRIα with similar affinities; however, AER-37 has considerably higher on- and off-rates than IE7¹⁶. A chimeric huIgG1 version of AER-37 was produced in our lab by subcloning synthetic coding DNA fragments (GeneArt, Thermo Fisher Scientific, Waltham, MA) into a pEE12.4 vector (Lonza, Basel, Switzerland). Transfected cells were seeded at low density into 96-well plates to obtain clones derived from single colonies. Clones were identified visually and then selected based on the amount of AER-37 detected in the culture medium by ELISA. CHO-K1 cells that stably expressed recombinant huIgG1 AER-37 were grown in glutamine-free medium in roller bottles. Cell culture medium was collected every 7 d and replaced with fresh medium. Conditioned medium was concentrated and dialyzed into pH 7.0 50 mM Tris / 0.15M NaCl, adsorbed to a column of GammaBind Plus Sepharose (GE Life Sciences, Marlborough, MA), and eluted with Gentle Ag/Ab Elution Buffer (Thermo Fisher

Scientific, Waltham, MA). The eluate was dialyzed into 0.15M NaCl, concentrated using an Amicon stirred cell, and its concentration of huIgG1 AER-37 was determined by ELISA. Mouse IgE anti-TNP mAb (clone IGELa2, from ATCC), human IgE anti-TNP (clone NS0 1F3.5C5), rat IgG2b anti-mouse Fc γ RIII/RIIb (clone 24G2), and control rat IgG2b (clone J1.2) mAbs were produced as ascites²² and in some cases were purified by DEAE ion exchange or protein G affinity chromatography (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer's instructions.

Generation of monovalent (mv) IE7 and AER-37 chimerized anti-huFc ϵ RI α mAb.

We generated a series of mv hIgG anti-huFc ϵ RI α recombinant constructs consisting of one full human κ light chain, one full human heavy chain and one truncated heavy chain resulting in formation of mv mAbs that had a single Fab linked through a hinge region to a complete Fc. Preferential assembling of the truncated and full heavy chains was determined by four Fc amino acid exchanges: E356K, D399K, K392D, and K409D (Supp. Fig. 1A)²³. Recombinant mv antibodies were generated by transient expression in CHO-S cells, using a Maxcyte STX flow electroporation system (Maxcyte, Inc., Gaithersburg, MD) according to the manufacturer's instructions. A three step purification strategy was used that consisted of: (1) human CH1-specific affinity chromatography; (2) size exclusion chromatography; and (3) human κ light chain-specific affinity chromatography. mv mAb structure and molecular mass were validated by protein gel electrophoresis and western blotting (Supp. Fig. 1B-D) and size exclusion chromatography (Supp. Fig. 1E). These mv Fab/Fc mAbs were used instead of mv mAb Fab fragments because the former have a much longer in vivo half-life than the latter as a result of efficient FcRn recycling²⁴.

Detection of systemic anaphylaxis. Anaphylaxis was detected as a change in mouse activity and the development of hypothermia. Mouse temperature was measured every 5 to 15 min with a rectal probe²⁵. Anaphylaxis-associated activity was scored on a 5 point scale, where a score of 0 indicates normal mobility; 1 = decreased spontaneous movement, 2 = spontaneous movement only after a pinch, 3 = slow or absent spontaneous movement after a pinch, 4 = inability to maintain upright posture, and 5 = death. Diarrhea was defined as profound liquid stool discharge, and was evaluated 30 min post-Ag challenge, unless otherwise indicated.

Measurement of serum levels of mouse mast cell protease 1 (MMCP1) and mouse mast cell protease 7 (mcpt7). Serum levels of proteases were measured in blood drawn 4 hrs (unless other specified) after challenge. MMCP1 was evaluated with a commercial ELISA kit (Thermo Fisher Scientific, Waltham, MA), according to the manufacturer's instructions. mcpt7 was evaluated by an ELISA developed in our lab. ELISA plate wells were coated overnight with 0.2 mg/ml of a goat anti-mouse mcpt7 polyclonal Ab (clone AF1937, R&D Systems, Minneapolis, MN). Serial dilutions of serum samples or recombinant standard (R&D Systems, Minneapolis, MN) were loaded into wells and bound mcpt7 was detected with 0.2 mg/ml of a biotin-labeled goat anti-mouse mcpt7 polyclonal Ab (BAF1937, R&D Systems, Minneapolis, MN), followed by streptavidin peroxidase conjugate (Thermo Fisher Scientific, Waltham, MA) and SuperSignal™ ELISA Femto Substrate (Pierce, Waltham, MA) according to the manufacturer's instructions. Although both assays show the greatest effects of desensitization when serum is obtained within 30 min of challenge, they still show marked effects when serum is obtained up to 4 hrs after challenge. Obtaining serum at the latter time point was

frequently necessary to simultaneously test for cytokine responses and because it is difficult to obtain serum when mice are recovering from shock.

Passive sensitization and induction of anaphylaxis. Unless otherwise indicated, mice were sensitized by i.v. injection of 10 µg of huIgE anti-TNP mAb and challenged i.v. the next d with 10 µg of TNP-OVA.

Active sensitization of huFcεRIα/F709 mice. Egg allergy was induced in huFcεRIα/F709 mice, as described¹⁶.

Immunization of mice with huIgG4 IE7 and detection of mouse antibodies to huIgG4 IE7. huFcεRIα/F709 Tg mice were immunized intraperitoneally (i.p.) with 10 µg of dv hIgG4 IE7 in 0.1 ml 0.15M NaCl, adsorbed to an equal volume of Imject Alum (Thermo Fisher Scientific, Waltham, MA) twice at a one month interval. 1 wk after the second immunization mice were bled and sera were tested by ELISA for level of mouse IgG anti-human IgG4 Ab, using wells coated with recombinant dv huIgG4 IE7. Bound Ab was detected with biotin-labeled anti-mouse IgGκ mAb (Clone RMK-12, Biolegend, San Diego, CA) followed by a streptavidin-peroxidase conjugate (Thermo Fisher Scientific, Waltham, MA) and SuperSignal™ ELISA Femto Substrate (Pierce, Waltham, MA) according to the manufacturer's instructions

Rapid desensitization. Unless otherwise indicated, rapid desensitization was accomplished by i.p. injection of doubling doses of an anti-FcεRIα mAb every 2 hrs, starting at 50 µg and ending with 400 µg.

Cell preparation. Peritoneal cells enriched in mast cells and blood, bone marrow, and splenic nucleated cells were prepared as described¹⁶.

Immunofluorescence and flow cytometry. 1×10^6 cells in 0.1 ml of Hank's balanced salt solution with 10% newborn calf serum and 0.2% sodium azide (HNA) were incubated with 1 μ g of 2.4G2 or human IgG for 15 minutes on ice, then stained with 1 μ g each of fluorochrome-labeled mAbs specific for mouse IgE (clone RME-1, Biolegend), hu IgE (clone MHE-18, Biolegend), or the anti-huFc ϵ RI α mAbs AER-37 or 15.1, and/or mAbs to human c-kit (clone 104D2, Biolegend) or mouse c-kit (clone 2B8, Biolegend), and human IL-3 receptor (clone 6H6, Biolegend), or mouse IL-3 receptor (clone 5B11, Biolegend), mouse B220 (clone RA3-6B2, Biolegend) and/or human CD3 (clone OKT3, Biolegend), or mouse CD3 (clone 145-2C11) or mouse pan-NK cells marker CD49b (clone DX5, Biolegend); Syk (clone 4D10.2, Biolegend); phospho-Syk (clone moch1ct, ThermoFisher); phospho-SHIP (clone P1C1-A5, Biolegend) Akt (C67E7, from Cell Signaling Tech.), phospho-AKT (D9E, Cell Signaling Tech.), phospho-ERK (clone 4B11B69, Biolegend). Cells were washed twice, fixed with 2% paraformaldehyde, and analyzed with a Becton-Dickinson LSR II Flow Cytometer (BD Biosciences). Mast cells were identified as high side scatter, c-kit positive, IL-3 receptor positive, CD3 negative and B220 negative cells. Basophils were identified as forward/side scatter intermediate, IL-3 receptor positive, CD49b positive, CD3 negative cells¹⁶.

IVCCA for IL-4. IL-4 secretion was quantitated with the in vivo cytokine capture assay (IVCCA), as described²⁶.

Measurement of mast cell signaling. After surface staining to identify cell types, peritoneal wash cells were fixed with 4% formaldehyde solution, permeabilized with permeabilization/wash buffer (R&D System, Minneapolis, MN) according to the

manufacturer's instruction, stained with Abs specific for the intra-cellular targets, washed and analyzed by flow cytometry.

Detection of mAb aggregates and deaggregation procedure. For deaggregation, mAbs were loaded onto a HiLoad 16/600 Superdex 200 pg (GE Healthcare, Marlborough, MA) column equilibrated in PBS. Fractions containing the pure monomer were pooled so that the final concentration of the pooled monomer remained high enough to perform functional in vivo experiments without needing to concentrate the material. Evaluation of the mAb aggregation was performed by sedimentation velocity analytical ultracentrifugation (AUC) testing with a Beckman Coulter XL-I (Beckman, Brea, CA) using absorbance optics at 280 nm. Samples were loaded into 1.2 cm, two-sector epon-charcoal centerpieces with sapphire windows. Evaluations were performed at 48,000 rpm in an An-60 Ti rotor at 20°C. Data analysis was done in SEDFIT version 16.1 using the continuous c(s) distribution model²⁷. SEDNTERP was used to calculate the partial specific volume, buffer density, and buffer viscosity of each protein in PBS buffer²⁸.

Statistics. GraphPad Prism version 8.4.3 was used for statistical calculations. A 2-tailed Mann-Whitney U test, a Kruskal-Wallis test, a Tukey honest significant difference test (THSDT), and/or a 1-way ANOVA were used when appropriate to test for statistical significance. The tests used for each experiment are reported in figure legends. A p value of <.05 was considered significant.

Results:

mv anti-huFcεRIα mAb protects against dv anti-huFcεRIα mAb-induced shock.

Our initial experiment evaluated whether a mv anti-huFcεRIα mAb protects against anaphylaxis induction by a dv mAb that has the same V regions. Separate groups of huFcεRIα mice were treated with increasing doses of mv huIgG1 IE7 followed by 100 µg of dv huIgG1 IE7. We observed dose-dependent protection against hypothermia (Fig. 1A, left panel) and mast cell degranulation and basophil activation (detected, respectively, as decreased serum levels of mouse mast cell protease 1 (MMCP1) and interleukin-4, Fig. 1A, right panel). Sequential treatment with mv, followed by dv huIgG1 IE7 completely protected IgE anti-TNP mAb-sensitized mice from shock (hypothermia) induction by TNP-OVA (Fig. 1B). Surprisingly, single injections of mv huIgG1 IE7 and dv IE7 had similar abilities to remove IgE from mast cells at the doses used and times studied, even though IE7 does not compete with IgE for binding to FcεRI¹⁶ and although mv huIgG1 IE7 had much less ability than dv IE7 to activate mast cells and basophils (determined by MMCP1 and IL-4 levels, Fig. 1C). Protection against IgE-mediated anaphylaxis and mast cell activation could be maintained for at least 18 d in mice that had originally been treated with sequential mv huIgG1 IE7 and dv IE7 by repeated injections of the dv mAb (Fig. 1D).

mv huIgG1 IE7 safely protects against IgE-mediated anaphylaxis. The ability of mv huIgG1 IE7 to remove IgE from mast cells and to modestly activate mast cells and basophils raised the possibility that this mv mAb might protect mice against IgE-mediated anaphylaxis directly, without a need to subsequently administer dv IE7. To test this possibility, IgE anti-TNP mAb-sensitized huFcεRIα mice were injected with mv

huIgG1 IE7 or dv IE7, followed for the development of hypothermia, and 1 d later
 challenged with TNP-OVA. Although, as expected, dv, but not mv IE7 directly induced
 hypothermia, the mv and dv forms of this anti-huFcεRIα mAb both completely
 suppressed IgE-mediated anaphylaxis and strongly suppressed the mast cell (MMCP1)
 and basophil (IL-4) responses to Ag challenge (Fig. 2A). Further comparison of the in
 vivo effects of mv huIgG1 IE7 vs. dv IE7 showed that neither consistently depleted
 peritoneal or bone marrow mast cells, both depleted blood, but not bone marrow
 basophils, both removed most mast cell and basophil IgE (although the dv mAb did this
 more completely in this experiment), the dv mAb remained associated with FcεRI more
 than the mv mAb (when evaluated ex vivo by ability to block the binding of the anti-
 huFcεRIα mAb AER-37, which cross-blocks IE7¹⁶), and the dv mAb was more effective
 than the mv mAb at decreasing the expression of mast cell surface FcεRIα that is not
 associated with IgE (determined by the ex vivo binding of 15.1, an anti-huFcεRIα mAb
 that competes with IgE for binding and is not crossblocked by IE7^{16,29}) (Fig 2B). Kinetic
 experiments revealed that a single injection of mv hIgG1 IE7 protected IgE anti-TNP
 mAb-sensitized mice against anaphylactic shock (hypothermia) in 2 hrs or less, with
 protection lasting for at least 48 hrs (Fig. 2C, upper left panel). Protection coincided with
 mast cell loss of IgE (Fig. 2C, lower left panel), but persisted even when mast cells had
 regained considerable surface IgE and baseline levels of free FcεRIα (Fig. 2C, lower
 middle panel). mv mAb treatment had no significant effect on peritoneal mast cell
 number (Fig. 2C, upper middle panel). Thus; loss of mast cells or mast cell IgE cannot
 fully explain the suppressive effects of our mv mAb on anaphylaxis; an IgE-independent
 decrease in mast cell responsiveness (desensitization, or anergy) is also important. This is

particularly apparent when mast cell surface IgE (Fig. 2C, lower left panel) is compared with the MMCP1 response 48 hours after mv mAb injection (Fig. 2C, lower right panel). Surprisingly, although anaphylaxis was strongly suppressed 2 hrs after mv mAb administration, the MMCP1 response to Ag challenge was not significantly suppressed at that time (compare Fig. 2C upper left and lower right panels). This is probably explained by the development of hypothermia 5-10 minutes after Ag challenge, while we measured MMCP1 levels 4 hours after challenge. Consistent with this explanation, we find that desensitization has a considerably greater suppressive effect on the release of MMCP1 by mucosal mast cells and the release of mcpt7 by connective tissue mast cells³⁰ 2 minutes after Ag challenge than 4 hours later (Supplemental Fig. 2). Thus, desensitization with a single dose of mv anti-huFcεRIα mAb suppresses IgE-mediated anaphylaxis by delaying mast cell degranulation as well as by decreasing it.

Mast cell SYK and SHIP phosphorylation are suppressed by mv huIgG1 IE7. In vitro mast cell desensitization has been explained, at least partially, by decreases in the total quantity and activation by phosphorylation of the tyrosine kinase Syk, which has an early and critical role in IgE-mediated mast cell activation and degranulation³¹. Consequently, we evaluated the effects of in vivo mv anti-huFcεRIα mAb treatment on total mast cell Syk as well as the activation (phosphorylation) of Syk and 2 other relevant signaling molecules, ERK and AKT, as well as on the inhibitory phosphatase SHIP³². mv mAb treatment significantly decreased the phospho-Syk and phospho-SHIP responses to Ag challenge, but had no significant effects on phospho-AKT or ERK and had no significant effect on total Syk levels (Fig. 3). These results are consistent with mv anti-huFcεRIα mAb at least partially desensitizing mast cells by inhibiting Syk activation.

Aggregation and FcγRs contribute to mast cell activation by mv anti-

FcεRIα mAbs. As far as is known, signaling through FcεRI requires aggregation or crosslinking of this receptor³³⁻³⁵, although it is difficult to prove that signaling is never induced by ligand binding without crosslinking³⁶. A crosslinking requirement explains why mv anti-huFcεRIα mAb is much less potent than the dv mAb at activating mast cells, but does not explain how some mast cell and basophil activation and degranulation is induced by the mv mAb. One simple possibility is spontaneous in vitro mv mAb aggregation, which should allow crosslinking by making the mv mAb divalent or oligovalent for huFcεRIα. Consistent with this, dimers and larger aggregates were detectable by analytical ultracentrifugation and analytical gel filtration and could be removed by preparative gel filtration (Supplemental Fig. 3). These aggregates contributed to the toxicity of mv huIgG1 IE7, as shown when highly susceptible, egg-allergic huFcεRIα/F709 mice were injected i.v. with 100 μg of deaggregated or non-deaggregated mv huIgG1 IE7 (Fig. 4A, left panel). Although injection of the non-deaggregated mv mAb caused an average temperature drop of ~8°C with the death of 7 of 8 injected mice, injection of the same quantity of the deaggregated mAb caused a reversible temperature drop of ~4°C. However, the deaggregated mv mAb retained the ability to desensitize mice, as shown by complete inhibition of hypothermia and diarrhea when the treated mice were challenged with egg the next day by oral gavage (o.g.) (Fig. 4A, center panel). The deaggregated mv mAb also caused considerable loss of mast cell surface IgE (Fig. 4A, right panel). Thus, mv anti-huFcεRIα mAb aggregation appears to explain some, but not all, of this mAb's toxicity when injected into highly sensitized mice, but does not appear to contribute to mast cell desensitization.

In addition to aggregation, mv mAbs have been shown to crosslink their ligands indirectly by interacting through their Fc domains with FcγRs³⁷. To determine the importance of this mechanism in mv mAb toxicity and desensitization, we sensitized huFcεRIα/F709 mice with IgE anti-TNP mAb, and made them particularly susceptible to anaphylaxis by treating them with a long-acting form of IL-4 (IL-4C) and propranolol^{38, 39} (Supplemental Fig. 4). To block mv mAb interactions with FcγRs, some mice were also pre-treated with 2.4G2, a rat IgG2b mAb that directly blocks mouse FcγRIIB and FcγRIII and indirectly inhibits FcγRI and FcγRIV by binding them through its Fc domains³⁹. 2.4G2 treatment blocked the toxicity of deaggregated mv, but not dv anti-huFcεRIα mAb and considerably inhibited the ability of the mv, but not the dv mAb to suppress IgE-mediated anaphylaxis (Fig. 4B). This suggests that indirect, FcγR-mediated FcεRI crosslinking by mv huIgG1 IE7 is important for both the toxic and therapeutic effects of this mAb.

As an additional way to evaluate the importance of mv anti-huFcεRIα mAb - Fc interactions with FcγRs for mAb toxic and therapeutic effects, we engineered mv IE7 mAb that had Ab C regions derived from huIgG1, as before, or from huIgG4, which binds less strongly than huIgG1 to both mouse and hu FcγRs^{40, 41}. Consistent with the results of our anti-FcγR blocking experiment, mv huIgG4 IE7 was much less toxic than mv huIgG1 IE7 when injected i.v into IL-4C and propranolol pre-treated huFcεRIα mice (Fig. 4C, left panel), but also had less ability to suppress IgE-mediated anaphylaxis (Fig. 4C, right panel). Intravenous injection of a higher dose of mv huIgG4 IE7 into propranolol- + IL-4C-pre-treated huFcεRIα mice could induce hypothermia, particularly if the mv mAb were not deaggregated prior to injection (Fig. 4D). A dose-response study

showed that no single dose of mv huIgG4 IE7 could be injected i.v. into highly susceptible IL-4C pre-treated huFcεRIα/F709 mice that had no toxicity, but completely protected against IgE-mediated anaphylaxis (Fig. 4E, left 2 panels). In the latter experiment, increasing the dose of mv mAb injected had no consistent effect on peritoneal mast cell number, but decreased mast cell surface IgE (Fig. 4E, right 2 panels). Pre-treatment of mice with 2.4G2 has little effect on the small hypothermic response induced by injection of 400 μg of mv huIgG4 IE7 (Figure 4F, left panel), but blocked the ability of this mv mAb to inhibit IgE-mediated anaphylaxis (Fig. 4F, right panel). Taken together, our results show that FcεRI crosslinking by mv anti-huFcεRIα mAb contributes to toxicity whether mediated through mv mAb aggregation or by interaction with FcγRs, whereas FcγR interactions, but not aggregation, contribute to mast cell desensitization.

Rapid desensitization and prolonged treatment with mv huIgG4 IE7 safely protect against IgE-mediated anaphylaxis. Although injection of mice with mv huIgG4 IE7 was much safer than injection with dv IE7, the mv mAb still induced toxicity when injected into highly susceptible mice in a single dose that could totally suppress IgE-mediated anaphylaxis in these mice. This led us to test whether using the mv mAb with our previously validated rapid desensitization procedure¹⁶ could fully desensitize mice without toxicity and whether rapid desensitization with a mv mAb had any advantage over the same procedure with a dv mAb. An initial experiment showed that rapid desensitization with just 4 doses of the mv mAb permitted injection of 400 μg of the same mv mAb without detectable toxicity, although injection of 400 μg of this mAb into mock desensitized mice caused an ~7°C temperature drop (Fig. 5A, upper 2 panels) and that safe desensitization with mv IE7 could be accomplished much more quickly than

safe desensitization with dv IE7 (compare Fig. 5A with the results in our previous paper¹⁶). Both the rapid desensitization and the single dose approaches prevented lethal IgE-mediated anaphylaxis (Fig. 5A, lower left and center panels), although rapid desensitization was more effective than a single mv mAb dose at removing mast cell IgE (Fig. 5A, lower right panel). Rapid desensitization with mv huIgG4 IE7 also safely prevented hypothermia and considerably decreased mast cell degranulation (MMCP1), IgE, and the incidence of diarrhea in our food allergy model (Fig. 5B). An additional experiment demonstrated that repeated injection of 400 μ g of mv huIgG4 IE7 every 2 d for 7 d after rapid desensitization safely increased the protective effect of rapid desensitization with the same mv mAb in very susceptible mice and increased removal of mast cell surface IgE (Fig. 5C).

mv huIgG4 IE7 desensitizes human mast cells in vivo. To determine whether mv huIgG4 IE7 could safely desensitize hu mast cells in vivo, reNSGS mice were sensitized with huIgE anti-TNP mAb by injecting them i.p., then i.v. with 100 μ g doses of this mAb or control mAb, and challenging them i.v. 1 day later with TNP-OVA (Fig. 6A). This short procedure was relatively ineffective at removing IgE from mast cells (Fig. 6A, left panel), and 1 of the 5 treated mice died following the mv mAb treatment while the remaining 4 developed considerable hypothermia (Fig. 6A, center panel). Two of the 4 surviving treated mice showed complete protection against Ag challenge, while one became hypothermic and one died 40 min after Ag challenge; in contrast, all of the control mAb-treated mice died 5-10 min after Ag challenge (Fig. 6A, right panel). To improve safety and efficacy, IgE-sensitized reNSGS mice were first rapidly desensitized with 25-400 μ g of mv huIgG4 IE7, followed 3 d later by a second 400 μ g dose of the

same mv mAb and challenged one day after that with TNP-OVA. These mice developed no toxicity and were completely protected against IgE-mediated anaphylaxis, while 3 of 5 control mAb-treated mice developed lethal anaphylaxis in response to TNP-OVA challenge and the other 2 developed severe hypothermia and loss of mobility (Fig. 6B).

Anti-huIgG4 Abs does not increase mv huIgG4 IE7 mAb toxicity. One concern about the therapeutic use of mv anti-huFcεRIα mAbs is that some patients might develop anti-therapeutic mAb Abs, which, theoretically, might aggregate the mv mAbs into polyvalent complexes that could induce mast cell degranulation and anaphylaxis. We initially tried to test this possibility by repeatedly injecting mice i.p. with mv huIgG4 IE7; however, although this mv mAb is foreign to mice, it failed to induce anti-mAb Ab development. Because of this, we immunized mice i.p. twice with the same mv mAb adjuvanted with alum; this led to an at least 500-fold increase in anti-mAb titer (Fig. 7A, upper left panel). These mice and mice inoculated i.p., as a control, with mouse IgG/alum, were then sensitized with IgE anti-TNP mAb, desensitized with mv huIgG4 IE7 or left undesensitized, and challenged with TNP-OVA. Desensitization failed to induce toxicity in mice that had pre-existing anti-mAb Abs (Fig 7A. upper right panel), although these mice were less protected against Ag-induced IgE-mediated anaphylaxis and developed higher MMCP1 levels than desensitized mice that lacked pre-existing anti-mAb Abs (Fig. 7A, lower panels). Because mv anti-huFcεRIα mAb-treated patients might develop anti-mAb Abs after desensitization and before receiving a large, single dose of the mv mAb to sustain mast cell suppression, a second experiment was performed in which mice that had first been induced to develop anti-mv mAb Abs were sensitized with IgE anti-TNP mAb, rapidly desensitized with mv huIgG4 IE7, injected with 400 µg

of the same mv mAb 3 d later, and challenged with TNP-OVA 1 d after that. No adverse effects were induced by either the rapid desensitization (as with the first experiment) or the subsequent injection of 400 μ g of the mv mAb and the treatment with this mAb still removed some surface IgE from mast cells and partially protected against IgE-mediated anaphylaxis (Fig. 7B). Thus, although anti-mv mAb Abs decrease the efficacy of mv anti-huFc ϵ RI α mAbs, presumably by decreasing their effective concentration, they do not increase the toxicity of mv anti-huFc ϵ RI α mAb therapy.

In vivo potency of mv anti-Fc ϵ RI α mAb is determined by its rate of binding to huFc ϵ RI α . The potency of a therapeutic mAb is influenced by the strength and speed of its interactions with its ligand⁴². To evaluate the effect of the binding kinetics of anti-huFc ϵ RI α mAbs on in vivo toxicity and therapeutic efficacy, we produced two mv anti-huFc ϵ RI α mAbs that had identical huIgG4 C regions but had V regions derived from either of 2 anti-huFc ϵ RI α mAbs: IE7 and AER-37. These mAbs bind to identical or closely positioned huFc ϵ RI α epitopes with similar affinity; however AER-37 has a considerably greater on-rate and off-rate than IE7¹⁶. In vivo, mv huIgG4 AER-37 was ~4 times as toxic as mv huIgG4 IE7 (Fig. 8A, upper panels and 8B, left panel), but was >4 times as potent at blocking IgE-mediated anaphylaxis (Fig. 8A, lower panels and 8B, right panel). Neither mv mAb decreased the percent of peritoneal mast cells (Fig. 8C, left panel, and mv huIgG4 AER-37 was 2-4-fold more potent than mv huIgG4 IE7 at removing mast cell surface IgE (Fig. 8C, right panel). The two mv mAbs had similar in vivo half-lives, even when mv huIgG4 AER-37 was injected into mice at one fourth the dose of mv huIgG4 IE7, to adjust for their differences in in vivo potency (Fig. 8D). Taken together, these results indicate that on-rate is more predictive than affinity or off-

483 rate of the in vivo potency of mv anti-huFc ϵ RI α mAbs and suggest that a therapeutic
484 mAb with AER-37 V regions would have the advantage over one with IE7 V regions of
485 being effective in lower quantity.
486

Discussion: The data presented here demonstrate that a mv anti-huFcεRIα mAb composed of one Fab linked to a complete Fc has advantages over an intact dv anti-huFcεRIα mAb that has the same variable (V) regions for suppression of IgE-mediated anaphylaxis and food allergy. Specifically: (1) mv anti-FcεRIα mAb is much less potent than dv anti-FcεRIα mAb at inducing anaphylaxis; (2) pretreatment with mv anti-FcεRIα mAb blocks the ability of a dv anti-FcεRIα mAb that has the same V regions to induce anaphylaxis; (3) mv anti-FcεRIα mAb protects mice against IgE-mediated anaphylaxis and food allergy; (4) mv anti-FcεRIα mAb's already limited ability to induce anaphylaxis can be decreased further by administration through a rapid desensitization approach, with safe desensitization requiring considerably less time than the same procedure with a dv mAb; (5) the ability of mv anti-FcεRIα mAb to suppress IgE-mediated anaphylaxis can be safely extended by repeated administration; and (6) preformed antibodies to mv anti-FcεRIα mAb decrease efficacy but do not pose a safety risk. These results suggest that mv anti-FcεRIα mAb would be a safer therapy for IgE-related disease than either dv anti-FcεRIα mAb or desensitization with the disease-causing allergen, without loss of either efficacy or ability to suppress disease.

In addition to these translational observations, our results support the hypothesis that prolonged, limited crosslinking of FcεRI leads to mast cell desensitization rather than to activation. In this regard, the effects of a mv anti-FcεRIα mAb somewhat resemble those of a very low affinity dv anti-IgE mAb, which also has been described to deactivate, rather than activate mast cells⁴³, presumably by usually dissociating from one FcεRI-linked IgE molecule before it can bind to a second and crosslink FcεRI. However,

targeting FcεRI rather than IgE has the theoretical advantage of not being influenced by the large range of IgE concentrations that are found in different allergic individuals⁴⁴.

In addition to their translational importance, our results increase understanding of the mechanisms involved in FcεRI-mediated mast cell activation and desensitization by a mv anti-FcεRIα mAb. Specifically, they demonstrate that: (1) treatment with mv anti-FcεRIα mAbs decreases both mast cell IgE expression and mast cell responsiveness to crosslinking of the remaining IgE; (2) the induction of decreased mast cell responsiveness by mv anti-FcεRIα mAb is associated with decreased levels of phospho-Syk, the activated form of a tyrosine kinase that is essential for mast cell activation^{31, 45}; (3) the limited ability of mv anti-FcεRIα mAb to induce anaphylaxis largely depends on aggregation and interactions with FcγRs; (4) mv anti-FcεRIα mAb treatment totally prevents the degranulation of some mast cells, but only delays the degranulation of others (as shown by serum MMCP1 and mcpt7 levels); and (5) mv anti-FcεRIα mAb potency correlates better with its on-rate than with its affinity or off-rate.

These translational and mechanistic observations both increase understanding and raise questions about how a mv anti-FcεRIα mAb suppresses IgE-mediated allergy and the optimal design of a therapeutic mv mAb. Most of our observations are consistent with the hypothesis that some FcεRI crosslinking is required for our mv mAb's therapeutic and residual untoward effects: (1) The removal of mAb aggregates prior to their administration decreases the ability of our mv mAbs to induce anaphylaxis, but has no observable effect on ability to suppress IgE-mediated anaphylaxis. This may be explained kinetically; FcεRIα-mediated induction of anaphylaxis occurs in a few minutes²⁵, while its induction of decreased mast cell responsiveness does not occur immediately and

increases over hours to days. Because IgG aggregates are rapidly removed from circulation⁴⁶⁻⁴⁸, they would be expected to have the ability to promote anaphylaxis to a much greater extent than they could promote unresponsiveness. This has a practical implication: administration through a route that would lead to relatively slow absorption (e.g.; subcutaneous administration) should minimize the adverse effects of aggregated mv mAbs. (2) Indirect crosslinking of FcεRI by mv mAbs is induced through mv mAb interactions with FcγRs. This is shown both by decreases in mv mAb adverse effects and efficacy when our mAbs are engineered with Fc domains that have decreased binding to FcγRs (i.e.; huIgG4 instead of huIgG1⁴¹) and by the effects of blocking FcγRs with the anti-FcγR mAb 2.4G2. Although these results are robust, they have limitations: huIgG4 retains some ability to interact with FcγRs⁴⁹ and we cannot exclude the possibility that 2.4G2 suppresses both anaphylaxis and induction of hyporesponsiveness through its interaction with the inhibitory receptor FcγRIIB^{39, 50}. Studies with mv anti-FcεRIα mAbs or mv fusion proteins that do not bind at all to FcγRs should be helpful, but even these may have undetectable, but sufficient levels of aggregated mAb to induce some level of FcεRIα crosslinking; it is also possible that mv anti-FcεRIα mAbs associate with each other after binding to mast cells. Thus, although crosslinking of FcεRIα is undoubtedly the main mechanism behind mv mAb activation and desensitization, it is difficult to rule out the possibility that binding without crosslinking has some effect. These same considerations are relevant to the issues of how mv anti-FcεRIα mAbs cause mast cell loss of IgE, even though the mAbs used do not directly interfere with IgE binding to FcεRI¹⁶, and how deaggregated IgE increases mast cell FcεRI expression^{36, 51, 52}.

Additional, related issues raised by our observations are the importance of Syk in mast cell desensitization by mv anti-FcεRIα mAbs, the variable extent of mast cell desensitization, and the importance of mAb affinity for FcεRIα in mast cell activation and desensitization. Syk is rapidly activated through tyrosine phosphorylation by mast cell or basophil FcεRI crosslinking and is essential for FcεRI-mediated induction of mast cell degranulation^{31, 45}. Unpublished in vivo work by our group (S. Morris et al., submitted for publication) shows that a Syk phosphorylation inhibitor blocks anti-FcεRIα mAb-induced mast cell desensitization as well as mast cell activation, although the opposite observation has been reported with in vitro studies⁸. Thus, either the small amount of FcεRIα crosslinking that is induced by our mv mAbs is sufficient to phosphorylate Syk, or this event can be induced to a sufficient extent by mv mAb binding without FcεRI crosslinking.

Our kinetic study of MMCP1 and mcpt7 responses to FcεRI crosslinking in IgE-sensitized, mv anti-FcεRIα mAb-treated mice demonstrated that although mv mAb treatment decreased levels of both the mucosal and connective tissue mast cell-associated proteases at all time points studied, percent suppression was greater shortly after Ag challenge than it was later. Thus, while mv mAb desensitization totally prevented degranulation in some mast cells, it only delayed it in others. This demonstrates that IgE-mediated desensitization is not an all or none processes, and may relate to different degrees of inhibition of activation of Syk or other signaling molecules in different mast cells. In this regard, mast cell desensitization resembles anergy induced in B lymphocytes by exposing them to their cognate Ag in the absence of a second stimulatory signal⁵³, a process that also involves Syk⁵⁴⁻⁵⁶. However, because the induction of anaphylaxis

requires rapid, relatively massive mast cell degranulation^{6, 25, 57} and anaphylaxis can be totally suppressed in our studies by mv anti-FcεRIα mAb treatment, delaying and spacing out mast cell degranulation appears to be sufficient to protect against anaphylaxis.

Our comparison of mv huIgG4 IE7 with mv huIgG4 AER-37 indicates that the latter mv mAb is ~4 times as potent as the former at both directly inducing anaphylaxis and at desensitizing mice, even though IE7 has a slightly higher affinity than AER-37¹⁶. This slight difference in affinity, as measured by surface plasmon resonance (SPR), reflects a considerably greater on-rate for AER-37 that is more than balanced by an even greater off-rate. Thus, to the extent to which SPR results reflect in vivo mv mAb binding and dissociation with a cell-associated ligand, the rate of binding of a mv mAb to FcεRI appears to be considerably more important than the duration of binding. This suggests that mv anti-FcεRIα mAb effects that contribute to anaphylaxis induction and desensitization occur very rapidly, before the rate of dissociation becomes consequential, at least within the range encompassed by our two mAbs. Taken together with our studies of the importance of FcγR binding, these observations suggest that an optimal therapeutic mv anti-FcεRIα mAb should have a relatively high on-rate, low or absent FcγR binding, and resist aggregation. Interestingly, ELISA measurements that correlate with mAb binding characteristics, unlike SPR measurements, are consistent with a 4-fold greater potency for AER-37 as compared to IE7¹⁶; thus, characterization of mAb binding by ELISA may be more useful than characterization of by SPR for predicting the in vivo potency of rapidly acting therapeutic mAbs.

An additional important consideration is the extent to which our humanized mouse model observations apply to humans. We have attempted to make our studies as

applicable as possible by including experiments with mice that have activated human mast cells and by going to considerable lengths to breed mice that are as susceptible to anaphylaxis as possible and are as difficult as possible to desensitize. Although it is impossible to be certain that humans will respond similarly to our mice, this strategy should increase the chance that our approach will be an effective human therapeutic and decrease the likelihood that it will be too risky for human use.

Additional studies and modifications of our mv mAb preparation and approach are necessary, however, before human trials would be appropriate. First, because mv mAb aggregates can increase toxicity, modifications in mv mAb handling, vehicle, and possibly amino acid sequence should be evaluated as ways to limit aggregate formation^{58,59}. Studies that determine the percent of complexed mv mAb that poses a hazard should be helpful for setting standards for preparations that would be used clinically. Because mv anti-FcεRIα mAb aggregation might promote FcεRI crosslinking by increasing avidity for FcγRs as well as by increasing valency for FcεRI, decreasing mv mAb interactions further by substituting an Ig heavy chain that has no ability to interact with huFcγRs, such as huIgG2σ⁶⁰, for huIgG4 might also decrease the risk of mv mAb aggregation. Beyond the issue of aggregation, studies of mv mAb safety and efficacy in a non-human primate whose FcεRIα is bound by our mAb could increase the likelihood of safe and effective use in humans.

Finally, consideration should be given to when and how a mv anti-FcεRIα mAb approach that has been proven safe and effective in mice and non-human primates could best be used to suppress IgE-mediated disease in humans. Although initial targets might be those disorders that can be clearly FcεRI-mediated, such as food allergy, drug allergy,

and chronic idiopathic urticaria, use in other disorders in which the IgE/FcεRI/mast cell/basophil axis has a partial role, such as severe atopic asthma and atopic eczema, might also be considered. In addition, although our studies have, so far, only evaluated our mv anti-FcεRIα mAbs as a sole therapy, combination with other approaches, including specific allergen desensitization and cytokine-directed therapies might also prove useful.

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Figure Legends:

Figure 1. mv anti-FcεRIα mAb protects mice against dv anti-FcεRIα mAb. A. Left panel:

huFcεRIα mice (3-4/group) were injected i.v. with 10 μg of biotin-anti-IL-4 mAb and the

doses of mv huIgG1 IE7 shown, followed 1 d later by i.v. injection of 100 μg of dv

huIgG1 IE7. Maximum temperature drops after each injection are shown. Fractions

indicate number of mice that showed reduced activity after injection ÷ number of mice

injected. Right panel: IL-4 and MMCP1 responses determined in serum 4 hrs after

injection of dv IE7. B. One d after treatment with dv IE7, naïve huFcεRIα mice and mice

that had originally been injected with 50 or 100 μg of mv huIgG1 IE7 (5/group) were

injected i.v. with 10 μg of mouse IgE anti-TNP mAb and challenged i.v. the next d with

10 μg of TNP-OVA. Rectal temperatures during the hr after challenge are shown. C.

huFcεRIα mice (3-4/group) were injected i.v. and i.p. with IgE anti-TNP mAb (5 μg each

route), followed 1 d later by saline, dv mouse IE7 or mv huIgG1 IE7 (50 μg i.v. + 50 μg

i.p.) and bled 3 hrs later for determination of serum IL-4 and MMCP1. Peritoneal lavage

was performed immediately afterwards and mast peritoneal cell IgE was determined. D.

huFcεRIα mice (4/group) were injected i.v. with 10 μg of mouse IgE anti-TNP mAb.

One d later, mice were injected i.v. with saline or 25 or 50 μg of mv huIgG1 IE7. Starting

1 d after that and continuing 2x/wk for 18 d, mice that had received mv IE7 were injected

i.p. with dv IE7, while mice originally injected with saline continued to receive saline.

All mice were injected i.v. on d 18 with 10 μg of mouse IgE anti-TNP mAb and on d 19

with TNP-OVA (50 μg i.v.). Rectal temperatures of these mice and naïve mice on d 19

were followed for 1 hr after this last injection. MMCP1 levels on d 19 were determined

for sera obtained 4 hrs after TNP-OVA injection. Statistical analysis: For all figures, ns =

not significant; * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$; all tests 2-tailed unless otherwise noted. B: Mann-Whitney U test; C: 1-way ANOVA and THSDT; D: 1-way ANOVA and THSDT.

Figure 2. mv huIgG1 IE7 safely protects against IgE-mediated anaphylaxis. A. huFcεRIα

mice (4/group) were injected i.v. with 10 μg of mouse IgE anti-TNP mAb. One d later, mice were injected i.v. with 100 μg of dv mouse IE7, 100 μg of mv huIgG1 IE7, or saline and challenged i.v. the next d with saline or 10 μg of TNP-OVA. B. huFcεRIα mice (3/group) were injected i.v. with 10 μg of IgE anti-TNP mAb plus saline, 100 μg of dv mouse IE7, or 100 μg of mv huIgG1 IE7. Cells obtained 1 d later were stained for mast cell and basophil markers and mouse IgE, as well as with mAb AER-37 and mAb 15.1. Numbers show percent inhibition as compared to cells from saline-injected mice. C.

huFcεRIα mice (3-4/group) were injected on d 0 with mouse IgE anti-TNP mAb (5 μg i.v.; 5 μg i.p.). All mice received an additional 10 μg of mouse IgE anti-TNP mAb i.p. on d 2. Groups of mice were injected with mv huIgG1 IE7 (50 μg i.p., 50 μg i.v.) on d 1, d 2, or day 3. Two hrs after the d 3 injection of mv huIgG1 IE7, all mice were injected i.v. with 20 μg of TNP-OVA. Rectal temperatures were followed for 1 hr after the last injection. Mice were bled 4 hrs after the last injection and blood levels of MMCP1 and IE7 were determined. Peritoneal cells were obtained immediately after the mice were bled and analyzed by flow cytometry for percent of mast cells, mast cell surface IgE, and mast cell FcεRI that was not bound by IgE (free FcεRIα). Statistical analysis: All panels: 1-way ANOVA and THSDT.

Figure 3. Mast cell Syk phosphorylation is inhibited by mv huIgG1 IE7. huFcεRIα mice (6/group) were treated as diagrammed; the 3 groups were treated with hu IgG control

mAb and left unchallenged; with huIgG control mAb and challenged with TNP-OVA; or
 with non-deaggregated mv huIgG2 σ IE7 and challenged with TNP-OVA. Peritoneal cells
 obtained 5 min after i.p. TNP-OVA challenge were stained for mast cell-specific
 markers, permeablized, and stained for Syk, phospho-Syk, phospho-SHIP, phospho-
 AKT, and phospho-ERK and analyzed for fluorescence intensity by flow cytometry.
 Statistical analysis: Kruskal-Wallis test followed by 2-tailed Mann-Whitney test.

Figure 4. Aggregation and Fc γ Rs contribute to mast cell activation by mv anti-Fc ϵ RI α
mAbs. A. Egg allergy was induced in huFc ϵ RI α /F709 mice (5/group). Left panel: Mice
were injected i.v. with 100 μ g of control huIgG, non-deaggregated mv huIgG1 IE7, or
deaggregated mv huIgG1 IE7 and rectal temperatures were followed for 1 hr. Middle and
right panel: 10 hrs after control Ab or deaggregated mv huIgG1 IE7 injection, mice were
challenged o.g. with 100 mg of EW + EYP and followed for 1 hr for development of
diarrhea and hypothermia. Mice were bled 3.5 hrs after Ag challenge and serum MMCP1
levels were determined. Surface IgE expression was determined for peritoneal mast cells
obtained 4 hrs after Ag challenge. B. Six huFc ϵ RI α mice were sensitized with IgE anti-
TNP, IL-4C, and propranolol and treated with 2.4G2 to block Fc γ Rs. Two mice each
were injected i.v. with control IgG mAb, mv huIgG1 IE7, or dv huIgG1 IE7, followed 1 d
later by i.v. injection of TNP-OVA. Rectal temperatures during the hr following i.v.
injections are shown. C. huFc ϵ RI α mice (4/group) were sensitized by treatment with IgE
anti-TNP mAb, IL-4C, and propranolol, then injected i.v. with 100 μ g of control IgG, mv
huIgG1 IE7 or mv huIgG4 IE7, and, the next d, injected i.v. with 20 μ g of TNP-OVA.
Rectal temperatures were followed for 1 hr after each injection. D. huFc ϵ RI α mice
(4/group) were sensitized by treatment with IgE anti-TNP mAb, IL-4C, and propranolol,

then injected i.v. with 200 μ g of control IgG, non-deaggregated huIgG4 IE7, or deaggregated huIgG4 IE7 and followed for 1 hr for development of hypothermia. E. huFc ϵ RI α /F709 mice (4/group) that had been inoculated e.c. with EW + EYP, followed by repeated o.g. with the same Ags, but had never developed hypothermia or diarrhea in response to the o.g., were sensitized with IgE anti-TNP mAb plus IL-4C, then injected with the doses of deaggregated mv huIgG4 IE7 shown, followed 1 d later by i.v. challenge with TNP-OVA. Rectal temperatures were followed for 1 hr after each i.v. injection. Mast cell percentage and surface IgE staining were determined by flow cytometry for peritoneal cells obtained 1 hr after TNP-OVA injection. F. A protocol similar to Fig. 4B was followed, as shown on the time line, but with mv huIgG4 IE7 instead of mv huIgG1 IE7. 4 mice/group. Statistical analysis: A: 1-way ANOVA and THSDT (left panel), Mann-Whitney U test, or Fisher's exact test; B: 1-way ANOVA and THSDT; C: 1-way ANOVA and THSDT; D: 1-way ANOVA and THSDT or Fisher's exact test.

Figure 5. Rapid desensitization with mv huIgG4 IE7. A. huFc ϵ RI α /F709 mice (4/group) were sensitized with mouse IgE anti-TNP mAb and IL-4C. One day later, mice were serially injected i.p. every 2 hrs with 50, 100 and 200 μ g of control huIgG or mv huIgG4 IE7. Two hrs after the last i.p. injection, mice were injected i.v. with 400 μ g of control huIgG or mv huIgG4 IE7. One d later all mice were challenged i.v. with 20 μ g of TNP-OVA. Mice were followed after each injection for development of hypothermia and changes in mobility (anaphylaxis score). Peritoneal cells were obtained 1 hr after Ag challenge and analyzed for mast cell surface IgE by flow cytometry. B. Egg allergy was induced in huFc ϵ RI α /F709 mice (4-5/group). Mice were rapidly desensitized or mock

desensitized by serial i.p. injection every 2 hrs with 25, 50, 100, 200, and 400 μ g of control huIgG4 or mv huIgG4 IE7. Mice were challenged o.g. with 100 mg of EW + EYP and followed for 1 hr for development of diarrhea and hypothermia. Peritoneal cells and sera were obtained 2 hrs after the o.g. challenge and analyzed for mast cell surface IgE and MMCP1 levels, respectively. C. huFc ϵ RI α /F709 mice (5/group) were sensitized with mouse IgE anti-TNP mAb and IL-4C, injected with GK1.5 anti-CD4 mAb, then mock desensitized with control huIgG, rapidly desensitized for 1 d with mv huIgG4 IE7, or rapidly desensitized with mv huIgG4 IE7, followed by injection of 400 μ g of mv huIgG4 IE7 every other d for 7 d, as shown in the flow diagram. All mice were challenged i.v. with TNP-OVA one d after the last dose of control IgG or mv huIgG4 IE7 and followed for 1 hr for hypothermia, changes in mobility (anaphylaxis score) and death. Peritoneal cells obtained 1 hr after Ag challenge with analyzed for percent of mast cells and mast cell surface IgE. Statistical analysis: A: 1-way ANOVA and THSDT; B: Mann-Whitney U test; C: 1-way ANOVA and THSDT.

Figure 6. In vivo desensitization of human mast cells with mv huIgG4 IE7. A. reNSGS mice (4-5/group) were sensitized with huIgE anti-TNP mAb and the next d injected i.p. with 100 μ g of control huIgG or deaggregated mv huIgG4 IE7, followed 3 hrs later by i.v. injection of 100 μ g of the same mAb. Mice were challenged i.v. 1 d later with 20 μ g of TNP-OVA. Mice were followed for 1 hr for hypothermia and death after the i.v. injection. Spleens and peritoneal cells were obtained 1 hr after Ag challenge or immediately after death and analyzed for mast cell surface IgE. B. reNSGS mice (6/group) were sensitized with huIgE anti-TNP mAb on d 0 and 3, rapidly desensitized or mock desensitized i.p. with five doubling doses of deaggregated mv huIgG4 IE7 or

huIgG control every 2 hrs, starting with 25 µg of mAb; injected i.p. on d 3 with 400 µg of deaggregated mv huIgG4 IE7 or huIgG control, and challenged i.v. on d 4 with 20 µg of TNP-OVA. Mice were followed for 1 hr for hypothermia and death after the i.v. injection. Statistical analysis: 1-way ANOVA and THSDT or Fisher's exact test. B: Mann-Whitney U test.

Figure 7. The presence of anti-huIgG4 Abs does not increase the adverse effects of injecting mv huIgG4 IE7. A. huFcεRIα/F709 mice (3-4/group) were immunized twice i.p. with dv huIgG4 IE7 + alum to induce anti-huIgG4 Ab (upper left panel) or with mouse IgG + alum (control). Mice with high anti-huIgG4 Ab titers, control mice, and naïve mice were sensitized with IgE anti-TNP mAb. One d later all except the naïve mice were desensitized by serial i.p. injection every 2 hrs with 25, 50, 100, 200, and 400 µg of mv huIgG4 IE7. Mice were followed for 1 hr after each injection for development of hypothermia. All mice were challenged i.v. with TNP-OVA 1 d after desensitization and followed for 1 hr for development of hypothermia and changes in mobility. Serum was obtained 1 hr after Ag challenge and analyzed for MMCP1 level. B. huFcεRIα/F709 mice (4/group) were immunized twice s.c. with dv huIgG4 IE7 + alum or mouse IgG + alum. All immunized mice were then sensitized with mouse IgE anti-TNP mAb. One d later, one group each of huIgG4-immunized and mouse IgG-immunized mice was rapidly desensitized i.p. with mv huIgG4 IE7, while one group of mouse IgG-immunized mice received no further treatment. Four d later, mice that had been rapidly desensitized received an additional 400 µg of mv huIgG4 IE7 (half i.p.; half i.v.). All mice were challenged i.v. with TNP-OVA one d later. Mice were followed for the development of hypothermia and death after each mAb and Ag administration. Peritoneal cells obtained 1

hr after Ag challenge were analyzed for percent mast cells and mast cell surface IgE. Statistical analysis: A: Mann-Whitney U test (left panel) or 1-way ANOVA and THSDT; B. 1-way ANOVA and THSDT or Fisher's exact test.

Figure 8. mv anti-FcεRIα mAb on-rate determines in vivo potency. huFcεRIα/F709 mice (4/group) were treated with IL-4C and sensitized with 10 μg of IgE anti-TNP mAb. One d later, mice were injected i.v. with 200 μg of huIgG control mAb or the doses of mv huIgG4 IE7 or mv huIgG4 AER-37 shown and followed for development of hypothermia (A, upper panels) and changes in mobility (B, left panel). All mice were challenged i.v. with 20 μg of TNP-OVA and followed for death and hypothermia (A, lower panels) and changes in mobility (B, right panel). Peritoneal cells obtained 1 hr after challenge were evaluated for percent of mast cells and mast cell IgE fluorescence (C). D. huFcεRIα mice (4/group) were injected s.c. with 400 μg of mv huIgG4 IE7 or 100 μg of mv huIgG4 AER-37. Different groups of mice were bled 2, 24, 48, 72, or 120 hr later. mv mAb levels were determined by ELISA. Percent decreases in serum mAb levels from 24 to 120 hrs did not differ significantly for mv huIgG4 IE7 and mv huIgG4 AER-37 (2-tailed Mann-Whitney U test). Statistical analysis: A: Kruskal-Wallis test and Mann-Whitney U test or Fisher's exact test; B. Mann-Whitney U test with compensation for multiple comparisons.

Supplemental Figures:

Supplemental Figure 1. Design and validation of mv anti-FcεRIα mAbs. **A.** Proposed structure of mv mAb. Amino acid exchanges were introduced to induce preferred association of the full heavy chain with the truncated heavy chain. **B.** SDS-PAGE

analysis of purified mv huIgG1 IE7 under non-reducing and reducing conditions. C. Western blotting of reduced mv mAb with anti-huIgG and anti-huk chain Abs. D. SDS-PAGE analysis and Coomassie blue staining of purified mv huIgG4 IE7 and mv huIgG4 AER-37 mAbs under reducing and non-reducing conditions. E. Size exclusion chromatography of mv mAb derivatives after 3 step purification. The blue marker demarcates the size of wild-type huIgG1 (~150 kDa). Representative results from independent batches of mAbs are displayed.

Supplemental Figure 2. Desensitization slows kinetics of mast cell degranulation.

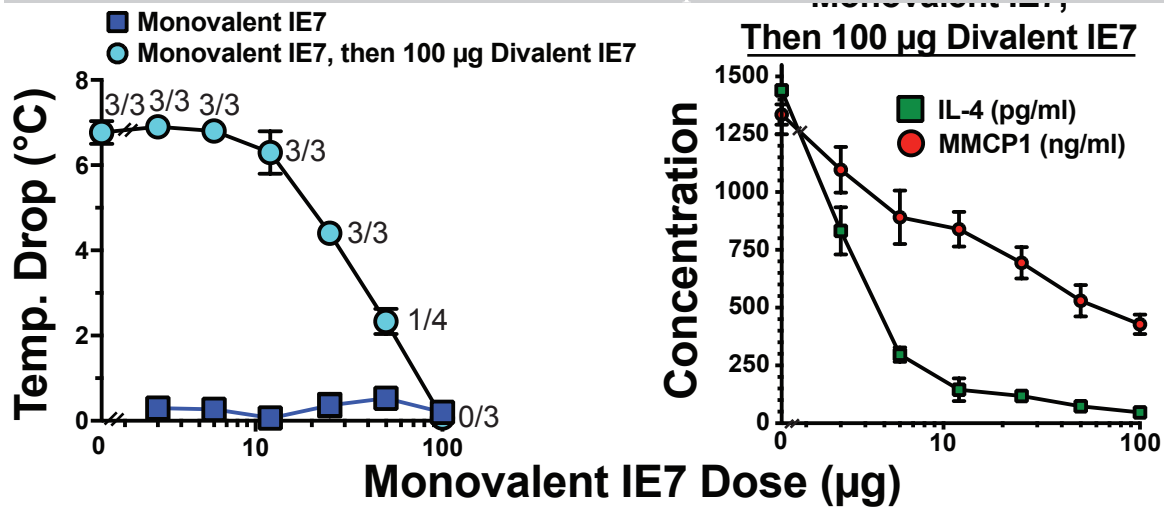
huFcεRIα/F709 mice (3-4/group) were sensitized with mouse IgE anti-TNP mAb and, one d later, injected i.v. with 50 µg of control human IgG or mv huIgG1 IE7. One d after that, mice were treated with 200 µg of triprolidine to limit the severity of anaphylaxis without affecting mast cell degranulation and challenged i.v. with 20 µg of TNP-OVA. Mice were bled at the time points indicated and serum MMCP1 and mcpt7 levels were determined by ELISA. Numbers below curves show the ratio of serum protease concentrations in undesensitized vs. desensitized mice at the indicated time points after Ag challenge. Statistical analysis: 1-tailed Mann-Whitney U test with compensation for multiple comparisons.

Supplemental Figure 3. Detection and removal of mv huIgG aggregates. Upper panel: mv mAb solutions were evaluated for aggregates by analytical ultracentrifugation (AUC). The 5 S peak represents the monomeric huIgG; the peaks at 7 S and above are dimeric or aggregated species. Lower panel: mv mAb solutions were depleted of aggregates by preparative gel filtration, as shown by repeated AUC analysis. Note the apparently complete loss of material with an S value of 7 or greater. A small amount of low

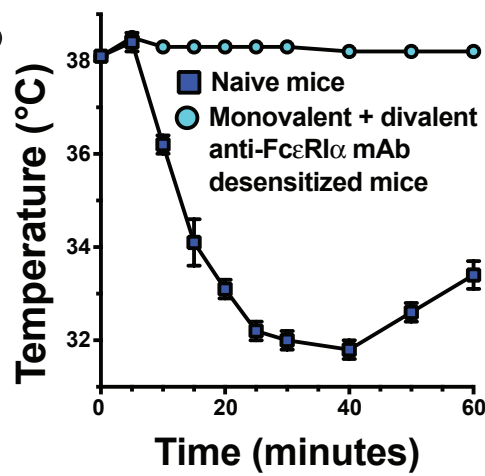
molecular weight material, S value 1 - 2, may represent mv mAb breakdown products.

Supplemental Figure 4. Exacerbation of anaphylaxis by IL-4 and propranolol. huFcεRIα mice (1 or 3/group) and huFcεRIα/F709 mice (3/group) were sensitized with IgE anti-TNP mAb, pretreated with IL-4C and propranolol, and challenged i.v. with control IgG mAb, deaggregated mv huIgG1 IE7, or dv huIgG1 IE7. Mice were followed for 1 hour for death and hypothermia. Statistical analysis (left panel only): 1-tailed Mann-Whitney U test and 1-tailed Fisher's exact test.

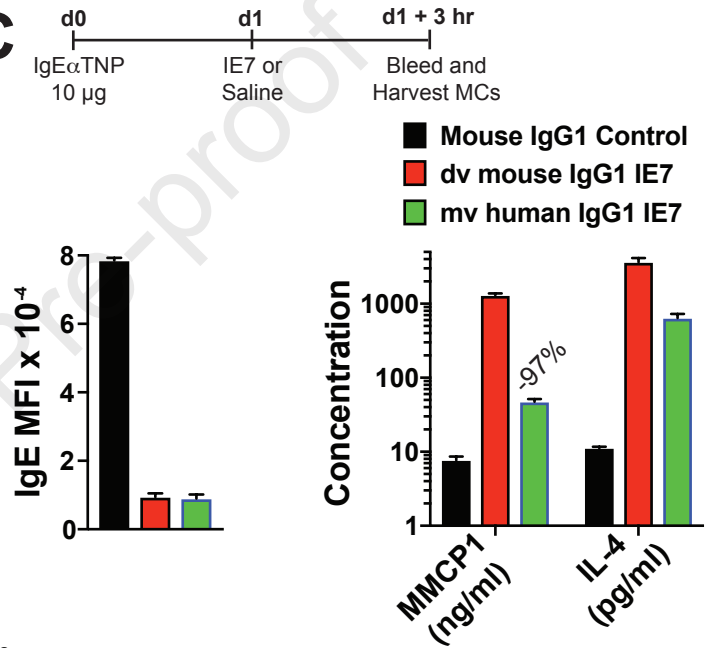
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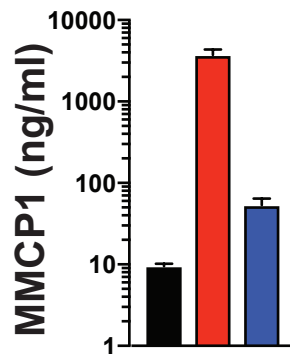
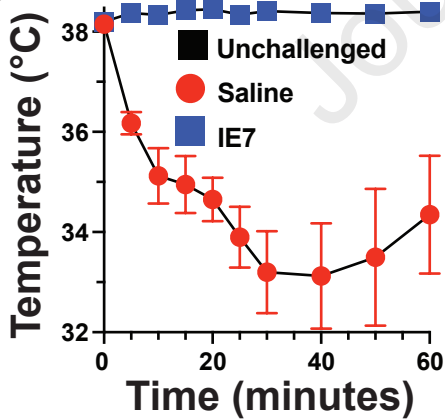
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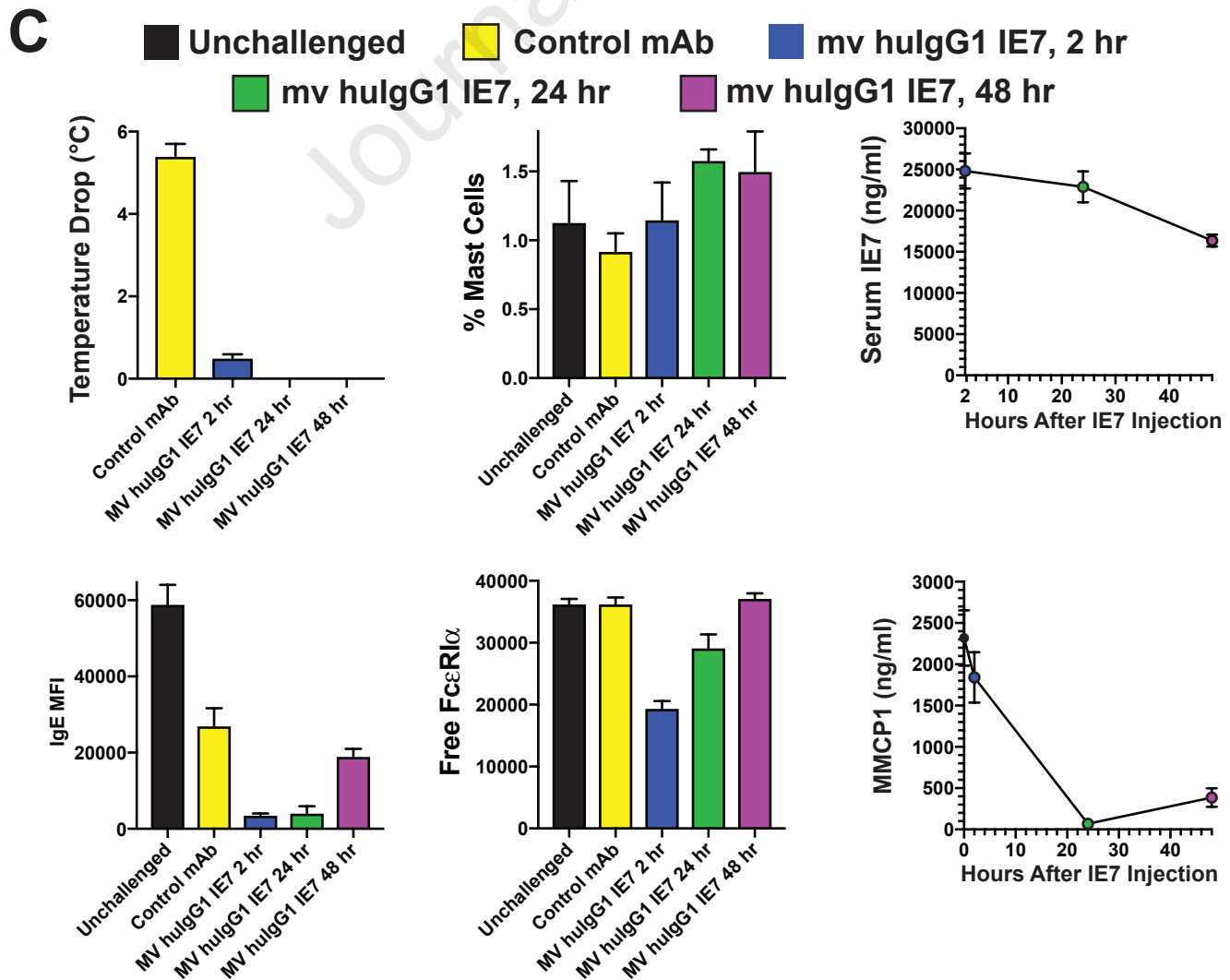
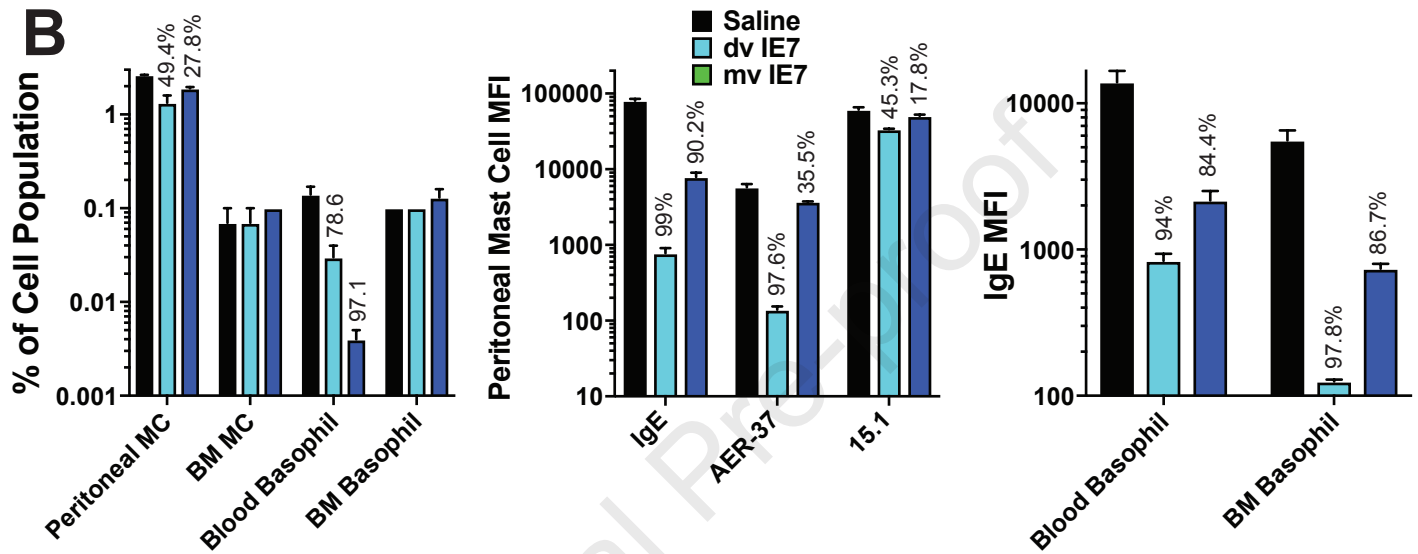
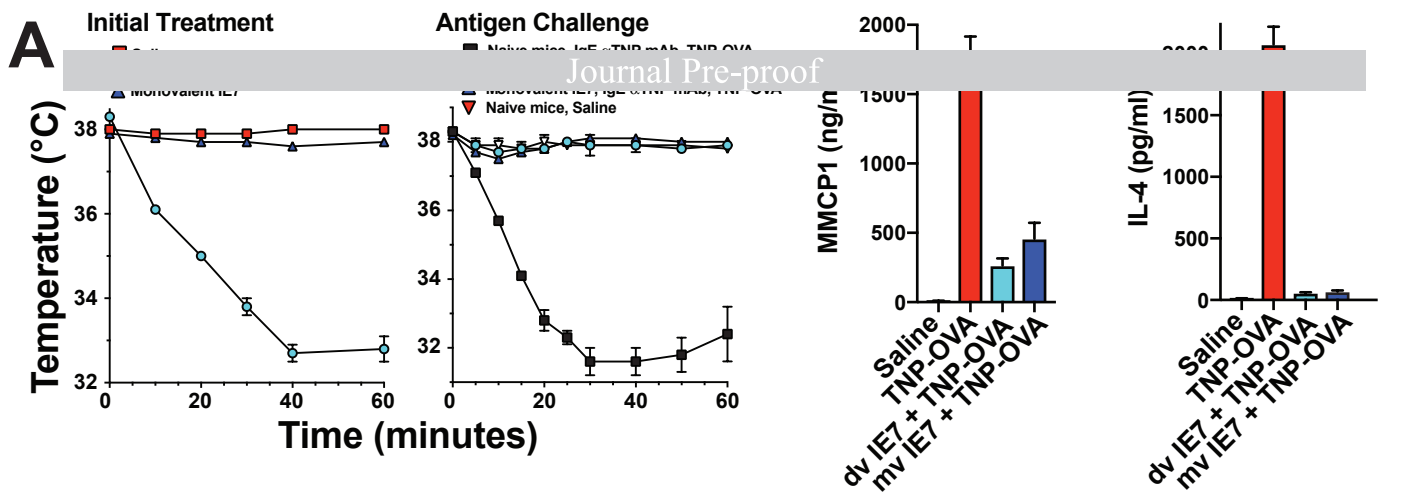


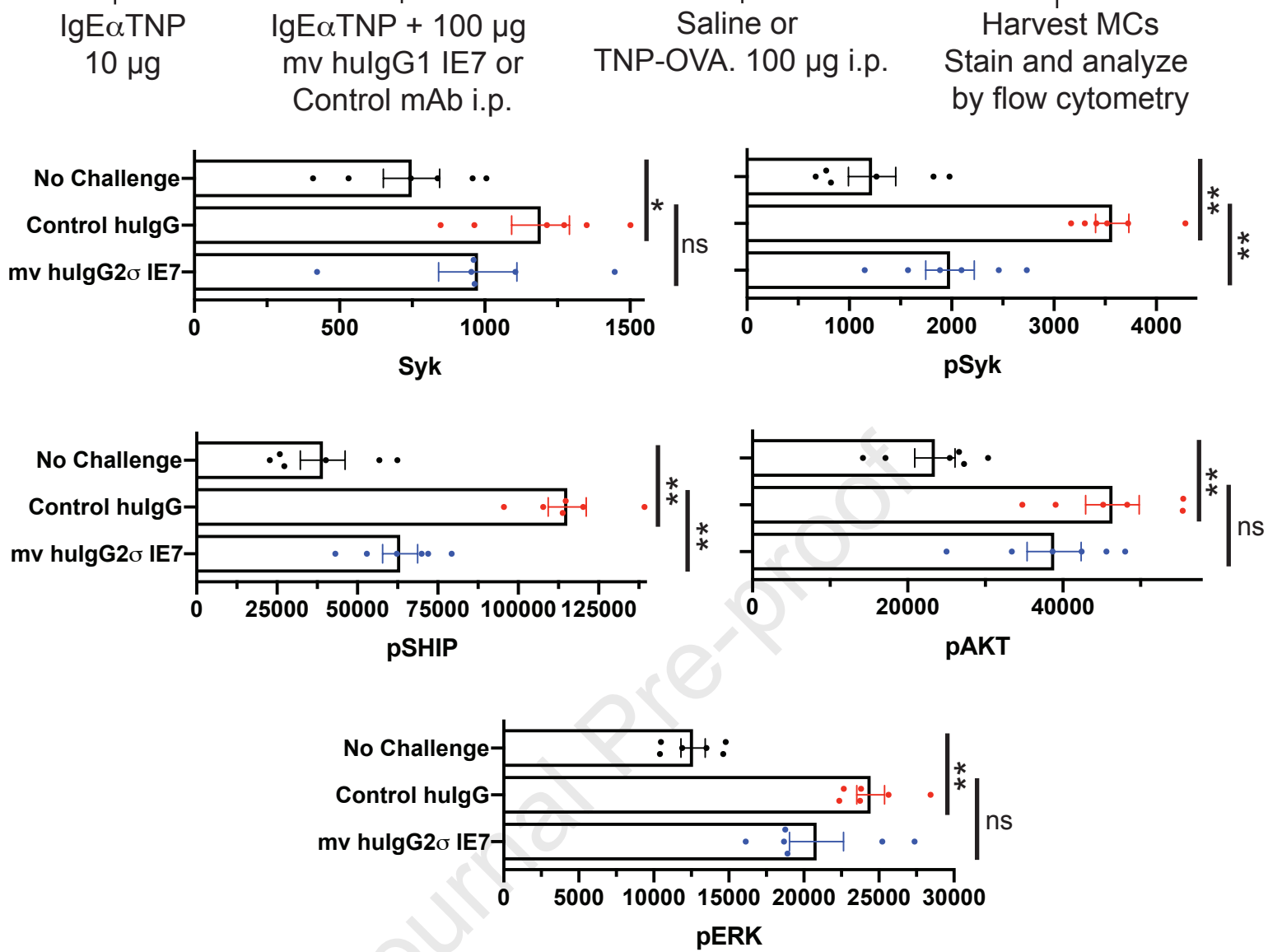
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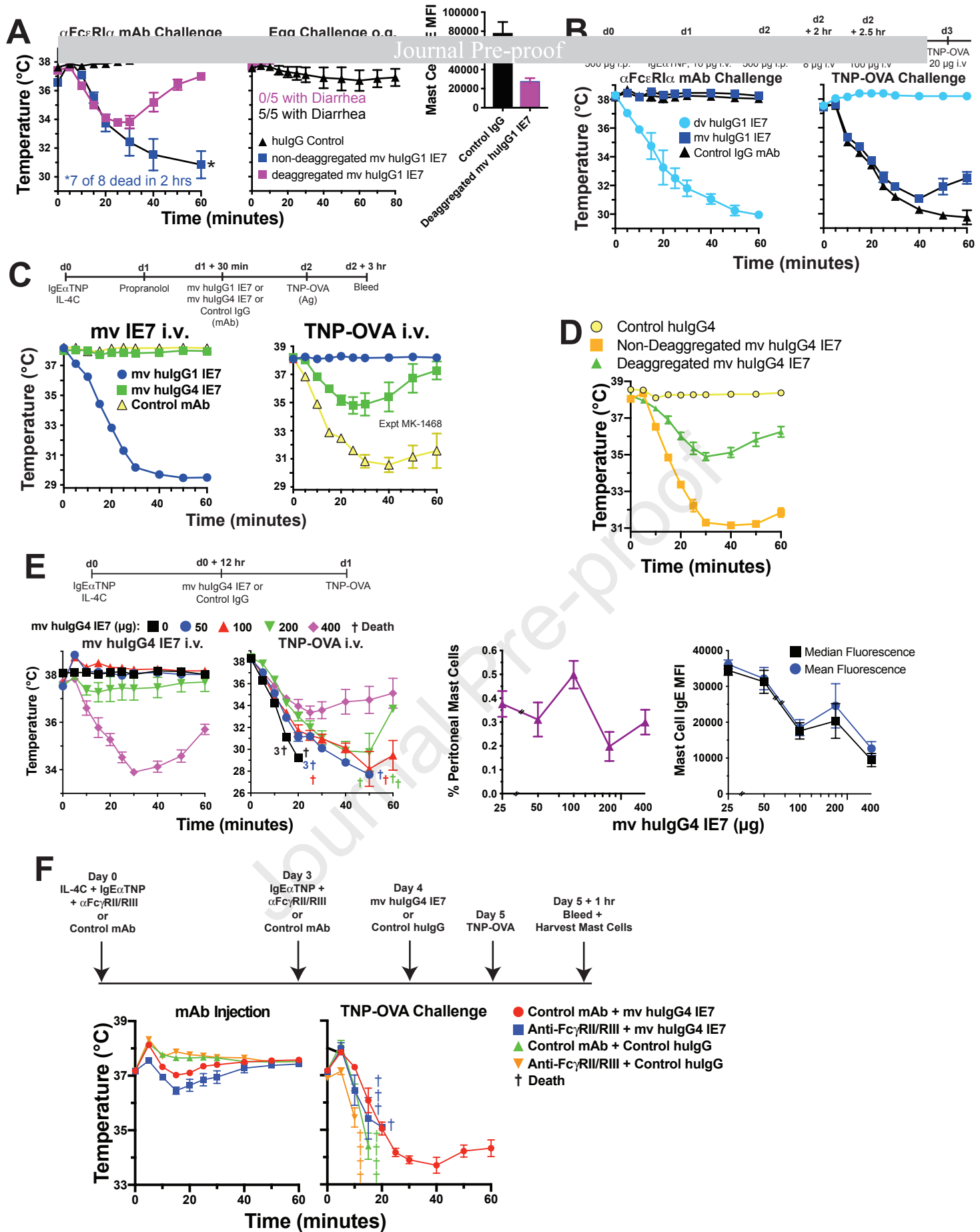


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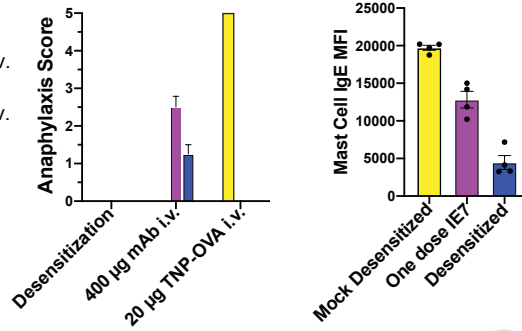
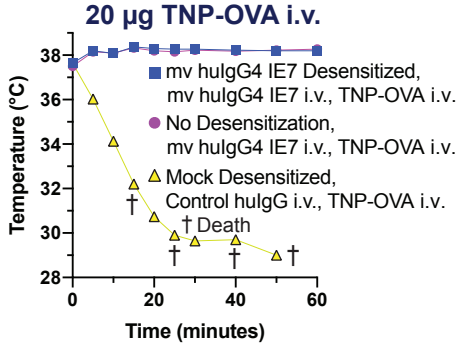
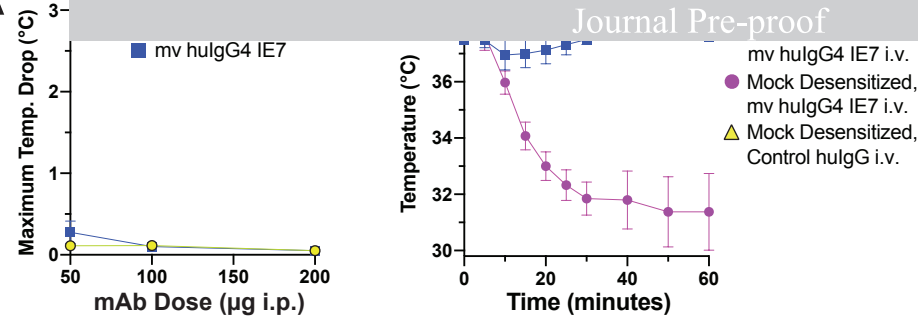




A

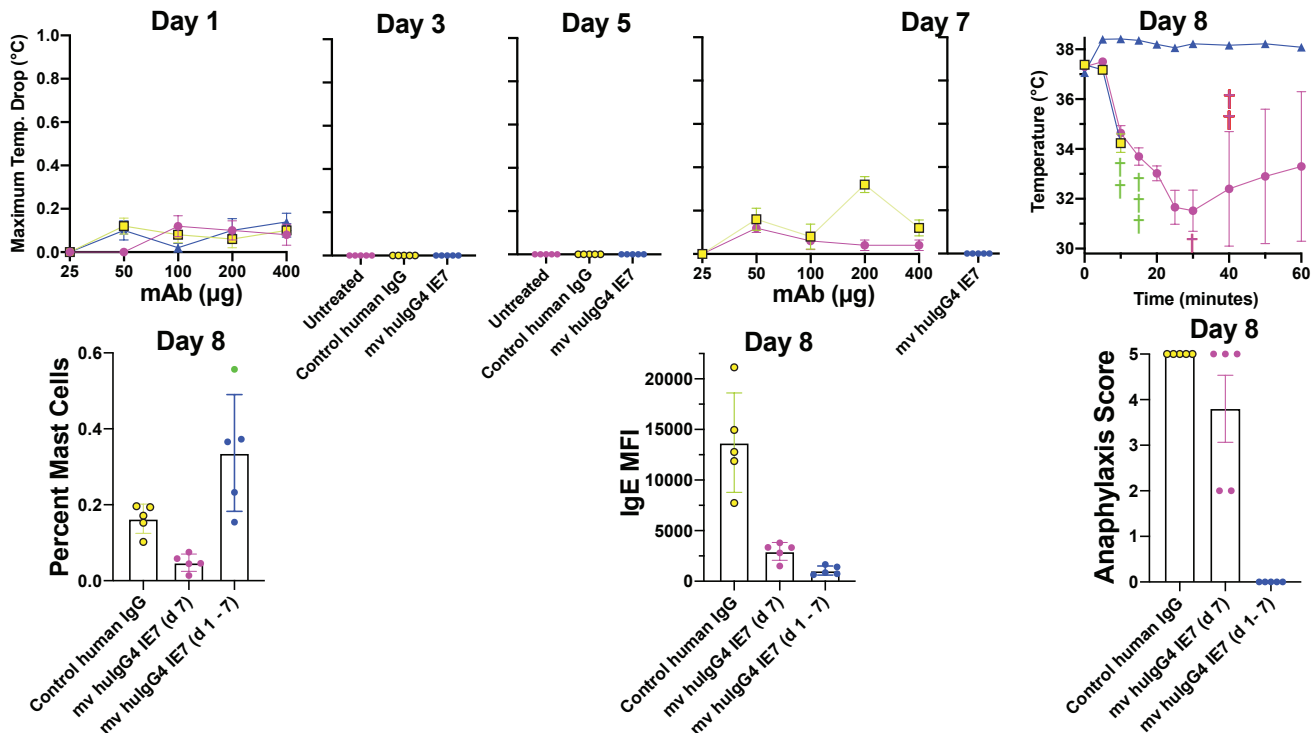
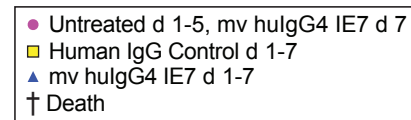
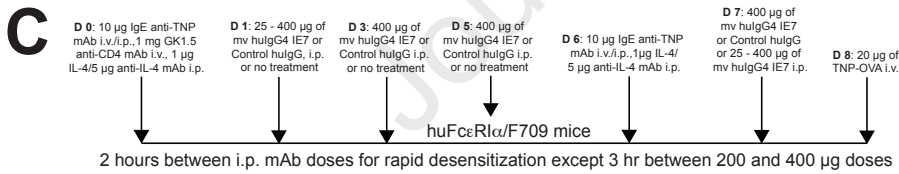
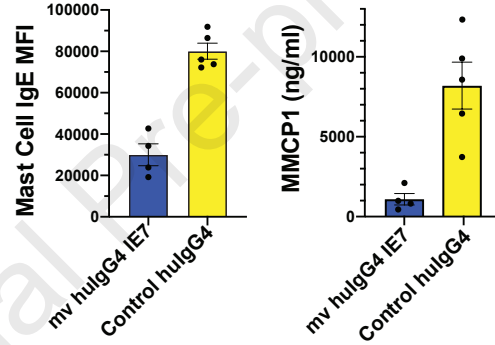
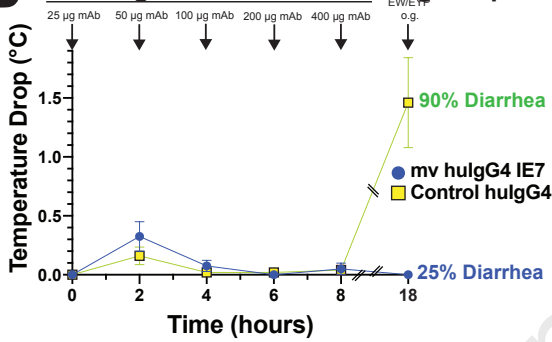
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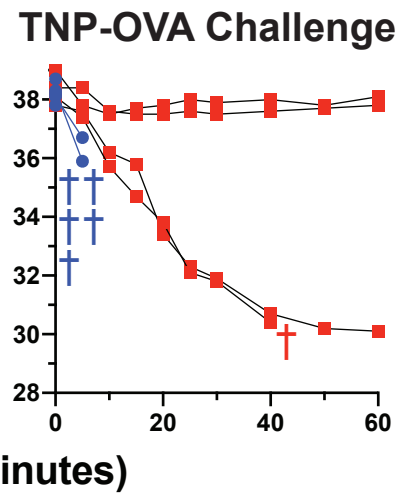
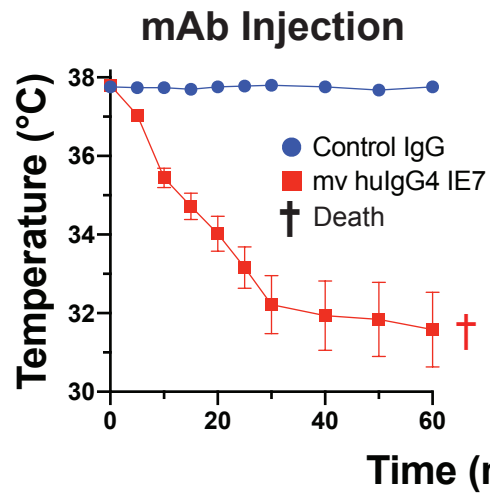
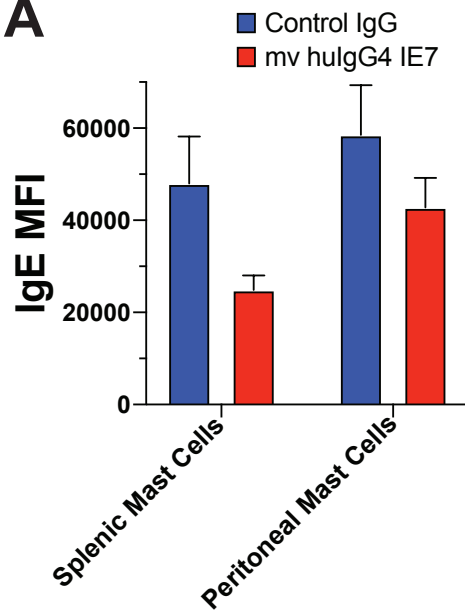
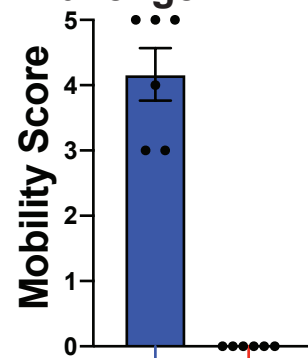
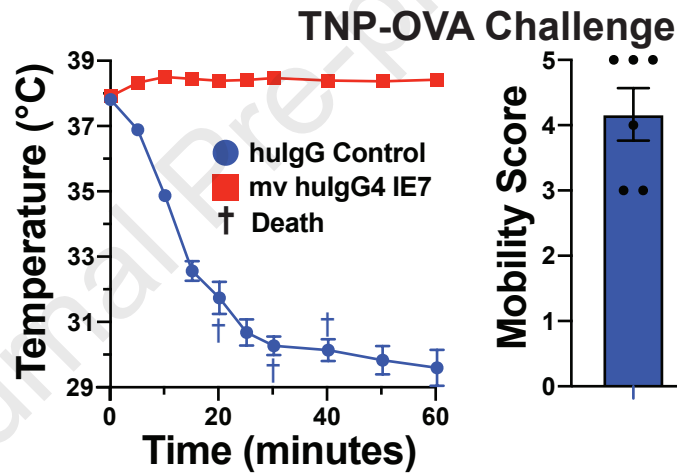
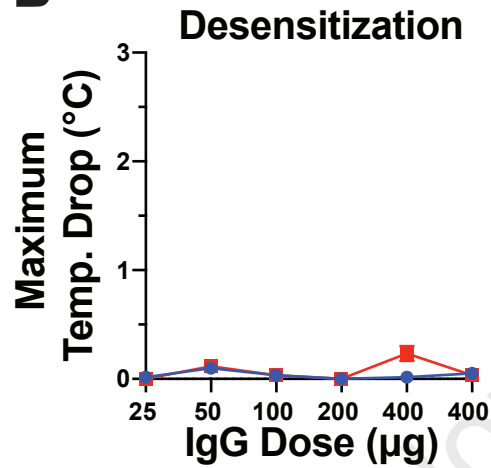
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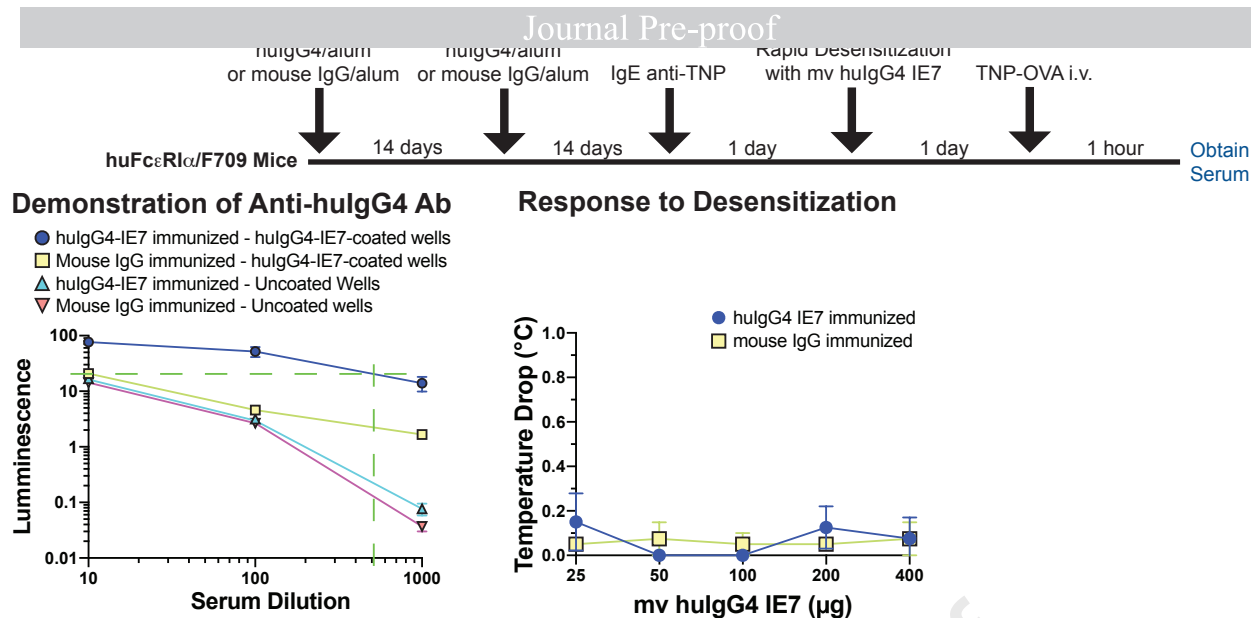
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mv hulG4 IE7 or Control hulG4 i.p.

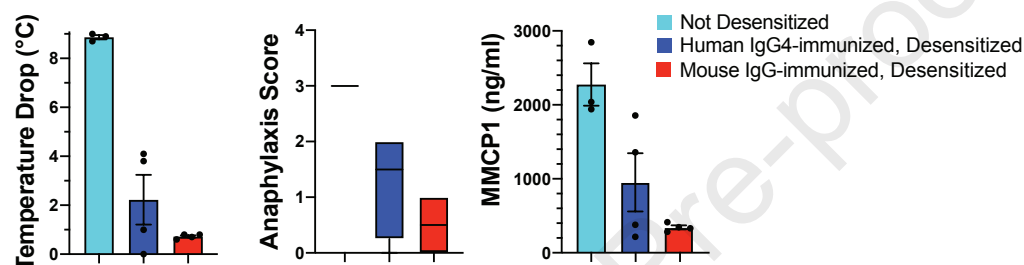


A**B**

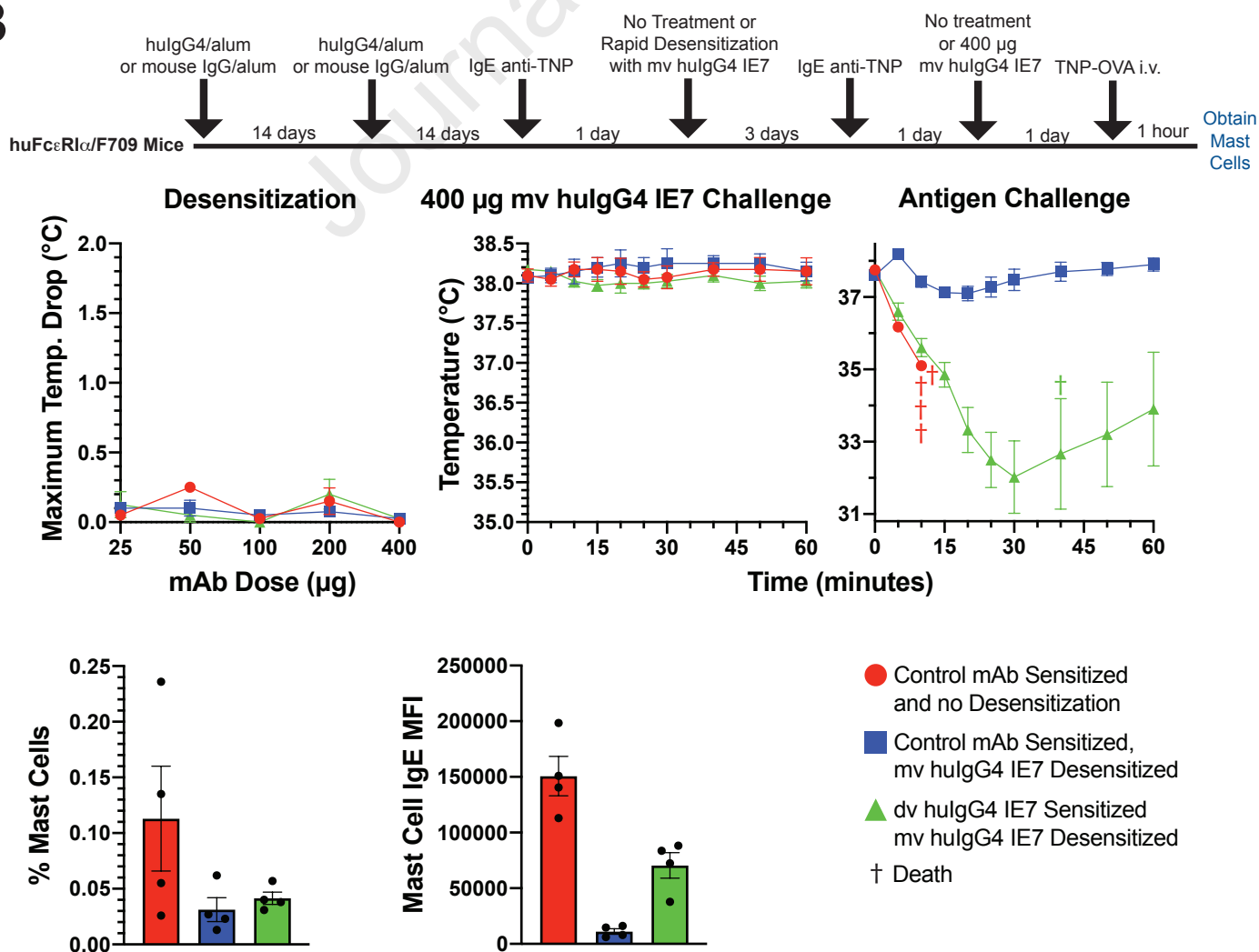
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Response to Ag Challenge



B



A

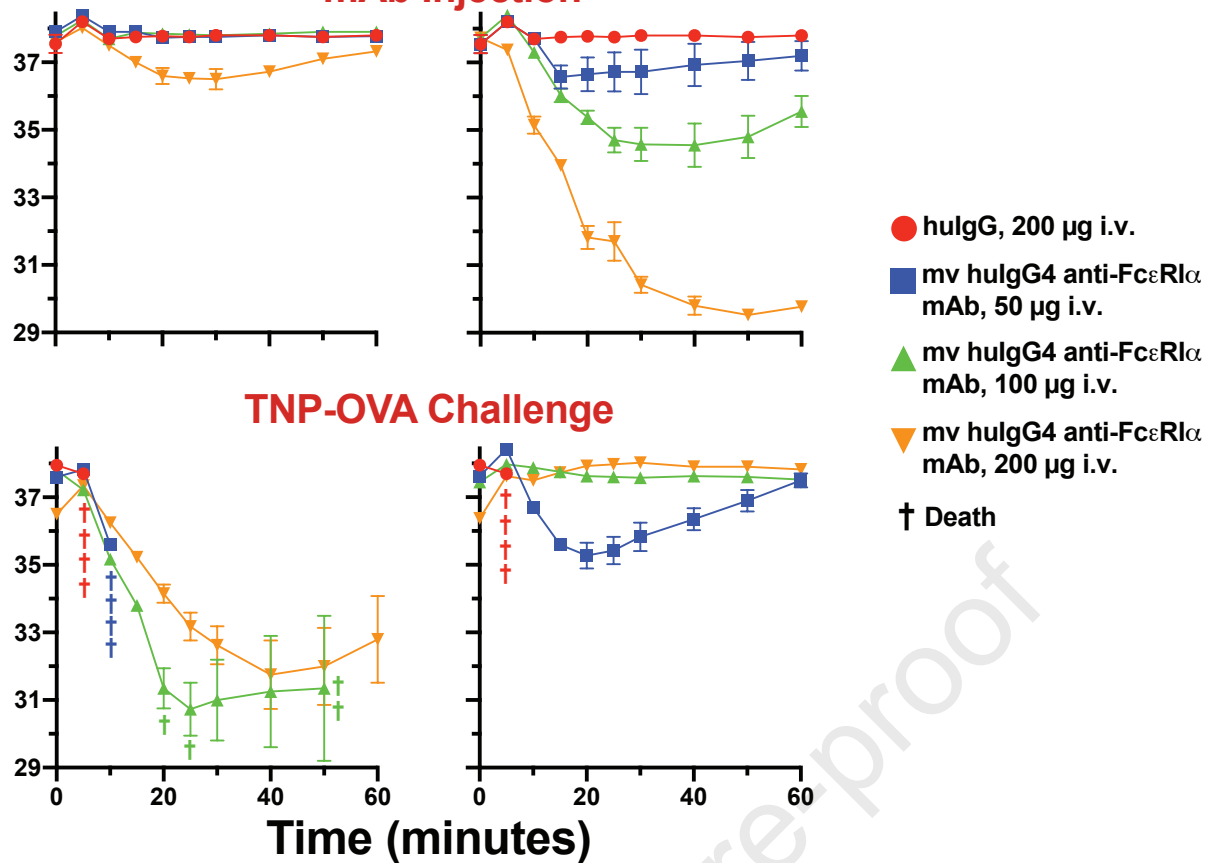
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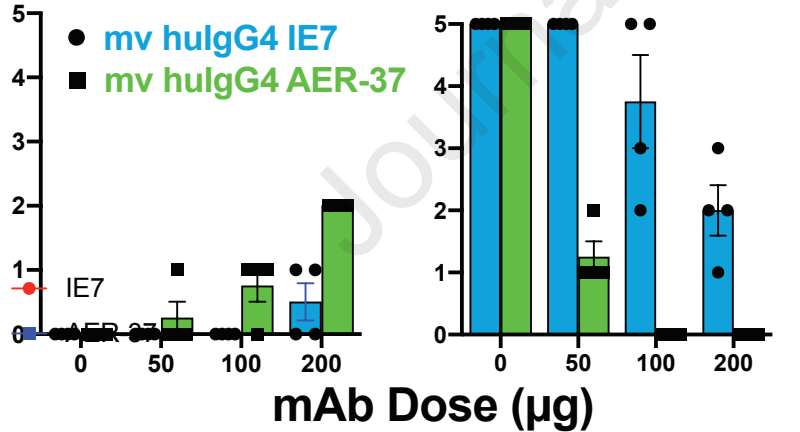
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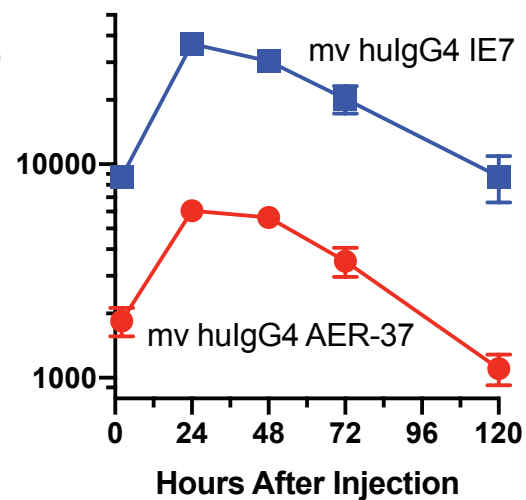
**B**

mAb Injection

TNP-OVA Challenge

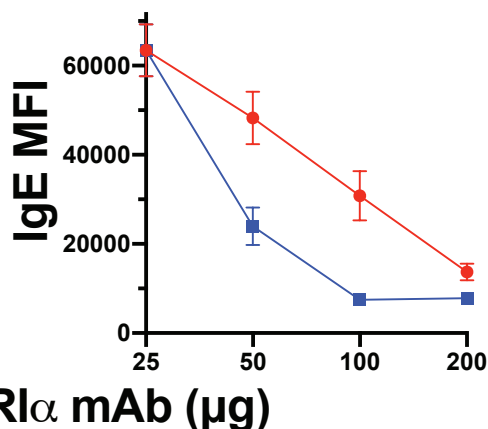
**D**

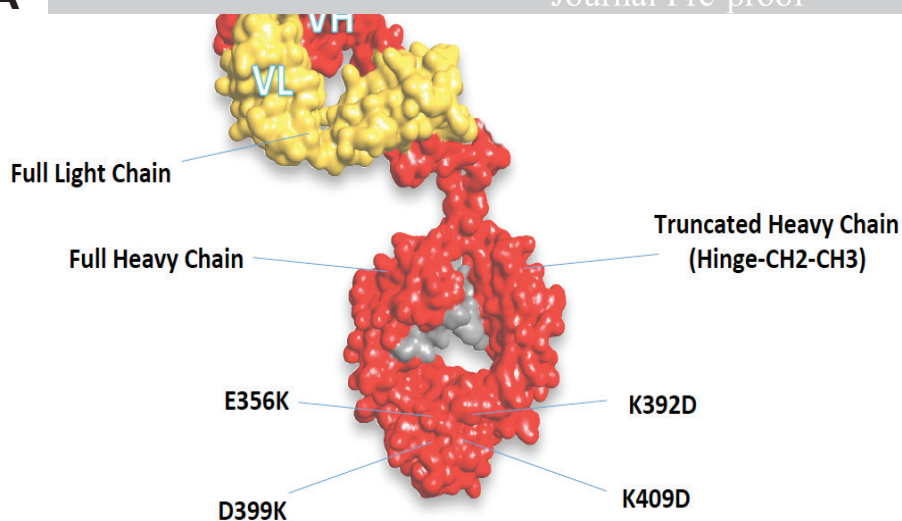
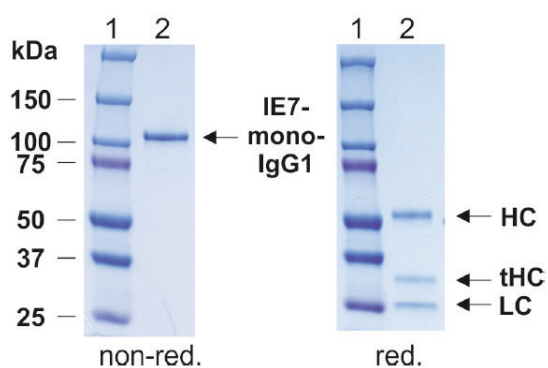
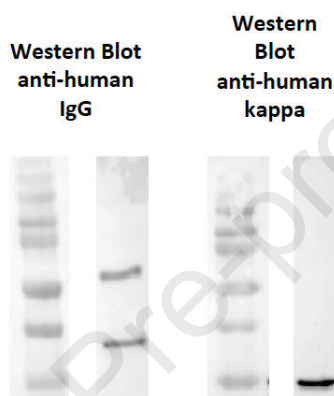
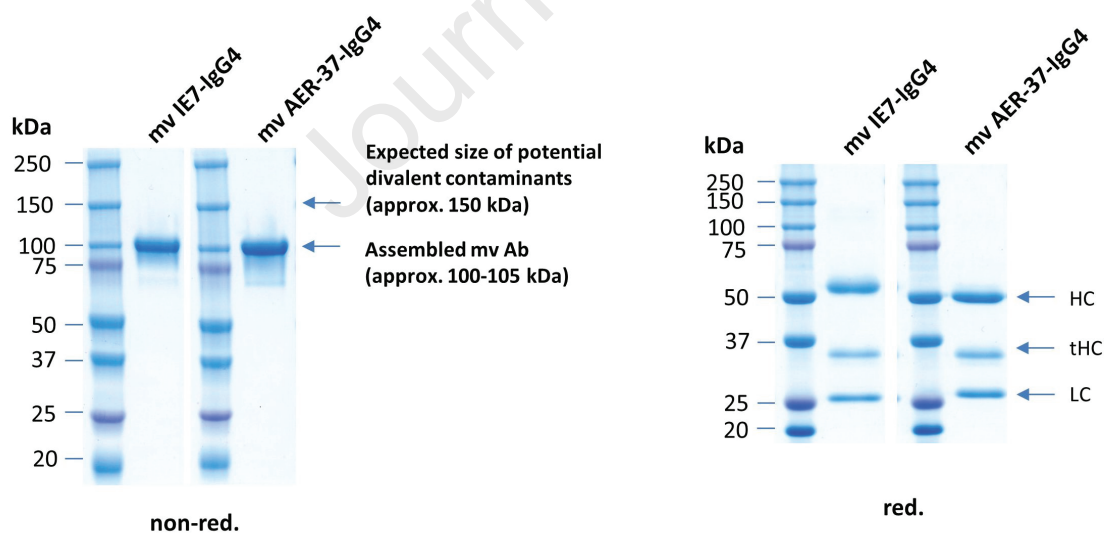
Serum Concentration (ng/ml)

**C**

● IE7
 ■ AER-37

% Mast Cells

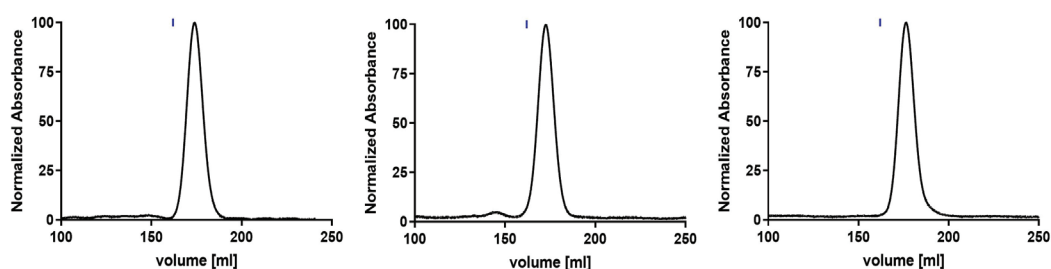


A**B****C****D****E**

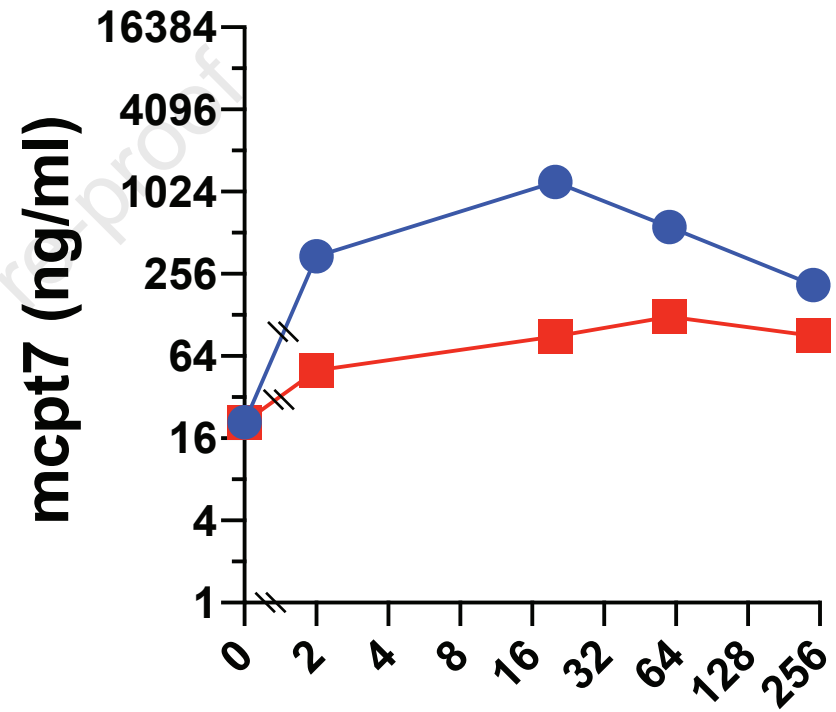
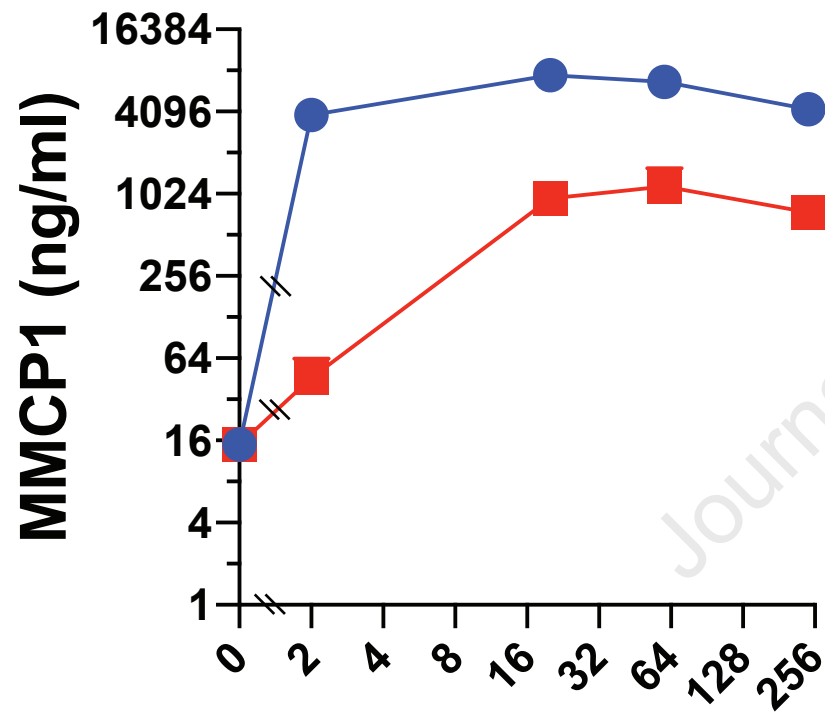
mvIE7-IgG1

mvIE7-IgG4

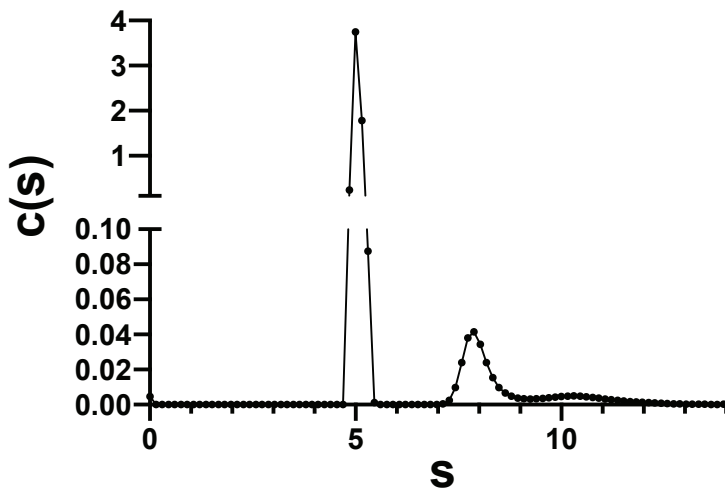
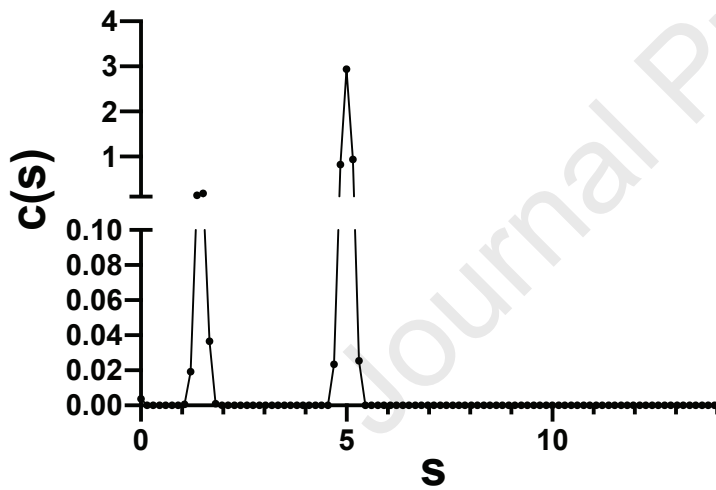
mvAER-37-IgG4

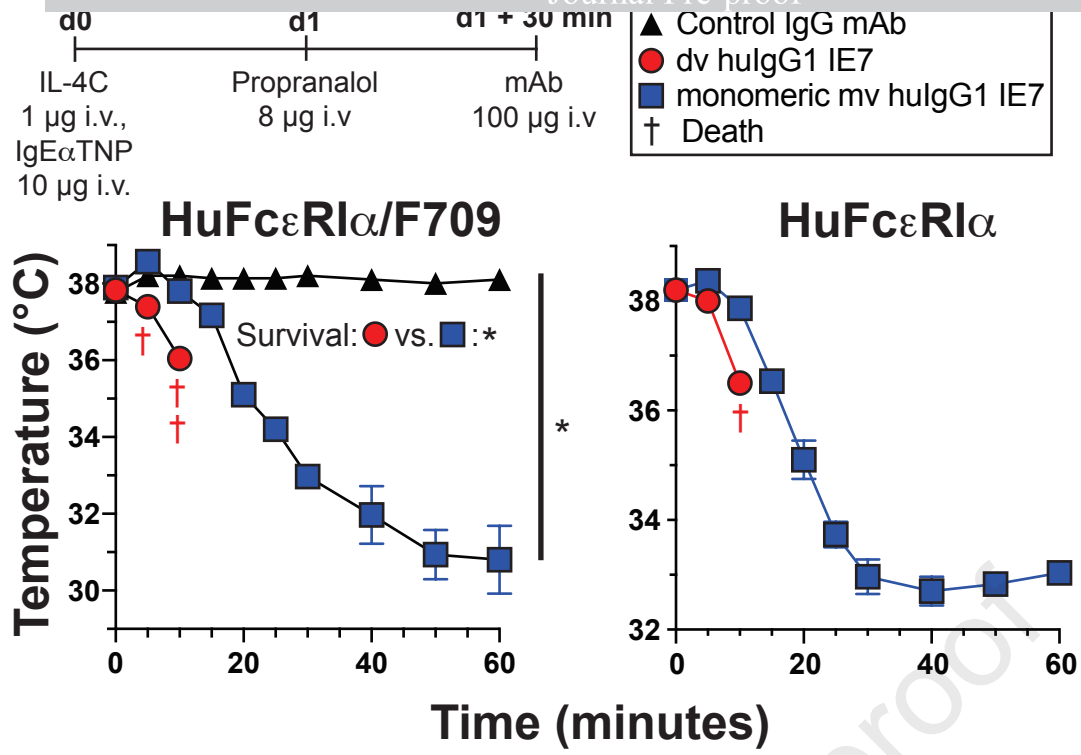


● Naive
■ mv hulgG1 IE7-Desensitized



Time (minutes)

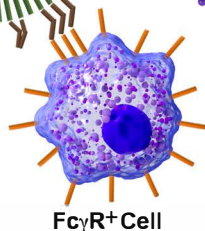
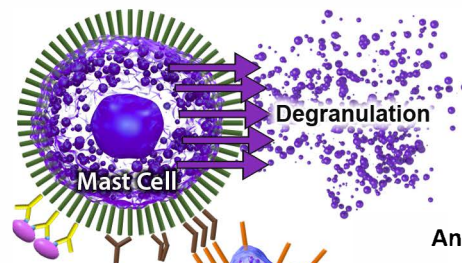
Before Gel Filtration**After Gel Filtration**





Inhibition of IgE-Mediated Anaphylaxis by Mast Cell Desensitization with Monovalent Anti-Fc ϵ R1 α Monoclonal Antibodies

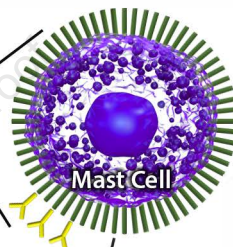
Different Stimuli Degranulate Mast Cells by Crosslinking Fc ϵ R1 Through Different Mechanisms



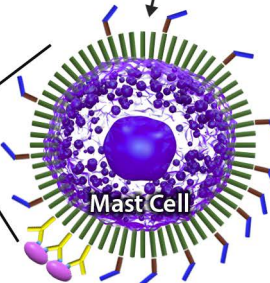
Desensitization



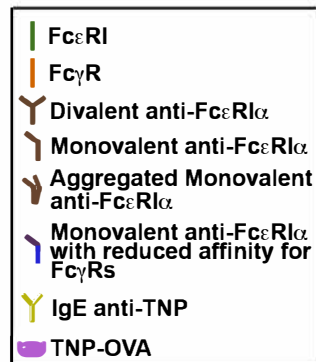
Antigen Challenge



Time



Decreased Syk Phosphorylation and No Degranulation



Supplemental Figures:

Supplemental Figure 1. Design and validation of mv anti-FcεRIα mAbs. **A.** Proposed structure of mv mAb. Amino acid exchanges were introduced to induce preferred association of the full heavy chain with the truncated heavy chain. **B.** SDS-PAGE analysis of purified mv huIgG1 IE7 under non-reducing and reducing conditions. **C.** Western blotting of reduced mv mAb with anti-huIgG and anti-huk chain Abs. **D.** SDS-PAGE analysis and Coomassie blue staining of purified mv huIgG4 IE7 and mv huIgG4 AER-37 mAbs under reducing and non-reducing conditions. **E.** Size exclusion chromatography of mv mAb derivatives after 3 step purification. The blue marker demarcates the size of wild-type huIgG1 (~150 kDa). Representative results from independent batches of mAbs are displayed.

Supplemental Figure 2. Desensitization slows kinetics of mast cell degranulation. huFcεRIα/F709 mice (3-4/group) were sensitized with mouse IgE anti-TNP mAb and, one d later, injected i.v. with 50 µg of control human IgG or mv huIgG1 IE7. One d after that, mice were treated with 200 µg of triprolidine to limit the severity of anaphylaxis without affecting mast cell degranulation and challenged i.v. with 20 µg of TNP-OVA. Mice were bled at the time points indicated and serum MMCP1 and mcpt7 levels were determined by ELISA. Numbers below curves show the ratio of serum protease concentrations in undesensitized vs. desensitized mice at the indicated time points after Ag challenge. Statistical analysis: 1-tailed Mann-Whitney U test with compensation for multiple comparisons.

Supplemental Figure 3. Detection and removal of mv huIgG aggregates. Upper panel: mv mAb solutions were evaluated for aggregates by analytical ultracentrifugation (AUC).

The 5 S peak represents the monomeric huIgG; the peaks at 7 S and above are dimeric or aggregated species. Lower panel: mv mAb solutions were depleted of aggregates by preparative gel filtration, as shown by repeated AUC analysis. Note the apparently complete loss of material with an S value of 7 or greater. A small amount of low molecular weight material, S value 1 - 2, may represent mv mAb breakdown products.

Supplemental Figure 4. Exacerbation of anaphylaxis by IL-4 and propranolol. huFcεRIα mice (1 or 3/group) and huFcεRIα/F709 mice (3/group) were sensitized with IgE anti-TNP mAb, pretreated with IL-4C and propranolol, and challenged i.v. with control IgG mAb, deaggregated mv huIgG1 IE7, or dv huIgG1 IE7. Mice were followed for 1 hour for death and hypothermia. Statistical analysis (left panel only): 1-tailed Mann-Whitney U test and 1-tailed Fisher's exact test.