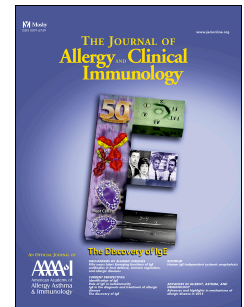


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Allergen-specific IgG antibodies signaling via FcγRIIb promote food tolerance

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Abstract

Background: Food-allergic subjects produce high-titer IgE antibodies that bind to mast cells via FcεRI and trigger immediate hypersensitivity reactions upon antigen encounter. Food-specific IgG antibodies arise in the setting of naturally resolving food allergy and

accompany the acquisition of food allergen unresponsiveness in oral immunotherapy (OIT).

Objective: In this study, we sought to delineate the effects of IgG and its inhibitory Fc receptor, FcγRIIb, on both *de novo* allergen sensitization in naïve animals and on established immune responses in the setting of pre-existing food allergy.

Methods: Allergen-specific IgG was administered to mice undergoing sensitization and desensitization to the model food allergen, ovalbumin (OVA). Cellular and molecular mechanisms were interrogated using mast cell- and FcγRIIb-deficient mice. The requirement for FcγRII in IgG-mediated inhibition of human mast cells was investigated using a neutralizing antibody.

Results: Administration of specific IgG to food allergy-prone *IL4raF709* mice during initial food exposure prevented the development of IgE antibodies, T helper (Th) 2 responses, and anaphylactic responses upon challenge. When given as an adjunct to oral desensitization in mice with established IgE-mediated hypersensitivity, IgG facilitated tolerance restoration, favoring the expansion of Foxp3⁺ regulatory T cells (Treg) along with suppression of existing Th2 and IgE responses. IgG and FcγRIIb suppresses the adaptive allergic responses via effects on mast cell function.

Conclusion: These findings suggest that allergen-specific IgG antibodies can act to induce and sustain immunological tolerance to foods.

Key Messages:

-Allergen-specific IgG prevents anaphylactic food allergy, including Th2 immune polarization.

-Allergen-specific IgG can be used to restore a non-responsive, Treg-dominated phenotype in previously allergic mice.

-The inhibitory Fcγ receptor, FcγRIIb, is required for these effects.

Capsule Summary:

IgG:FcγRIIb ligation restrains allergic pathology and provides a handle for tolerance restoration in food allergy.

Key Words: mast cell, IgG, Treg, food allergy, FcγRIIb, OIT, tolerance

Abbreviations: oral immunotherapy, OIT; subcutaneous immunotherapy, SCIT; regulatory T cell, Treg; ovalbumin, OVA; peanut, PN; mouse mast cell protease, MMCP; T cell receptor, TCR; T helper, Th; immunoglobulin, Ig; mesenteric lymph node, MLN; immunoreceptor tyrosine-based inhibitory motif, ITIM.

Introduction

Food allergy is a highly prevalent but intractable immunological disorder with no established cures and limited treatment options. Oral immunotherapy (OIT), currently under development in clinical trials, offers a promising approach for induction of tolerance to food allergens. OIT is performed by oral administration of increasing amounts of the allergenic food over several months. Allergic symptoms are common during the course of therapy and can be troublesome enough to cause safety concerns or discourage completion. Moreover, long-term success rates, defined as the maintenance of the food unresponsive state for 6 months or more after discontinuation of therapy, are in the range of only 15-30%¹⁻³, an outcome that suggests that true immunological tolerance is not fully induced. Adjunctive therapy with omalizumab (anti-IgE) during OIT has been implemented in some trials to reduce symptoms⁴ and facilitate more rapid escalation of allergen dosing⁵⁻⁷.

A consistent immunological feature of OIT is the marked increase (several logs) in serum levels of allergen-specific IgG antibodies. Whereas IgE binding to the high affinity receptor FcεRI triggers degranulation and cytokine production by mast cells and basophils, IgG bound to the inhibitory receptor FcγRIIb counters these effects, suppressing allergic reactions and anaphylaxis⁸⁻¹⁰ as well as the secretion of immunomodulatory cytokines that may exacerbate further reactions by amplifying pro-allergic T helper (Th)2 responses¹¹. In B cells, simultaneous engagement of FcγRIIb and the B cell receptor reduces immunoglobulin (Ig) production, exerting feedback inhibition on allergen-specific IgE synthesis¹².

Both induction of the allergic state in food-sensitive patients and maintenance of tolerance in normal subjects are orchestrated by allergen-specific T cells. Th2 cells promote IgE production and mast cell expansion in food allergy; regulatory T cells (Treg) counteract these responses and are essential to the non-responsive, tolerant phenotype

in healthy individuals¹³⁻¹⁷. Food allergy is associated with a reduction in Treg and switch to a Th2-like phenotype, changes that are pathogenic in mice^{18, 19}. The allergen-mediated crosslinking of IgE on mast cells is responsible for this disruption in Treg formation, and can occur directly when FcεRI-driven mast cell cytokines such as IL-4 and IL-6 divert naïve T cell fate decisions from Treg to Th2¹⁸⁻²⁰. In food-allergic mice undergoing OIT, interference with IgE receptor signaling in mast cells is permissive for the generation of Treg and leads to restoration of tolerance. While Treg induction has been associated with the natural outgrowth of food allergy, the data on Treg induction following OIT has been inconsistent and it is apparent that OIT fails to effectively restore Treg function in many patients^{3, 15, 21, 22}. Ultimately it is likely that Treg will be essential for long-term oral tolerance¹³, and designing OIT strategies that specifically boost allergen-specific Treg generation will be critical to the success of this therapy.

In this study, we characterize the effects of food allergen-specific IgG antibodies on emerging and established food allergic responses. We demonstrate that IgG and FcγRIIb modulate the severity of anaphylactic shock that occurs in sensitized animals upon ingestion of allergen. We show that in allergy-prone mice, administration of allergen-specific IgG prior to sensitization limits the production of IgE and regulates T cell responses, favoring the development of Treg over T helper (Th) 2 polarization. Furthermore, in mice with established allergy, combining IgG administration with OIT facilitates the expansion of Treg populations and tolerance, which is dependent on the presence of FcγRIIb specifically on mast cells.

Methods

Mice. All mice used in this study were bred and maintained under specific pathogen-free conditions in individually ventilated cages at Boston Children's Hospital. All work was performed under protocols reviewed and approved by the local Institutional Animal Care and Use Committee and Animal Research Children's Hospital. *Il4ra*^{F709} mice on BALB/c and C57BL/6J backgrounds were the kind gift of Dr. Talal Chatila and have been previously described, as have *Il4ra*^{F709} *Kit*^{W-sh} 18, 19, 23, 24. *FcγRIIb*^{-/-} (B6;129S-*Fcgr2b*^{tm1Ttk}/J) mice were purchased from The Jackson Laboratory (Bar Harbor, ME), and subsequently crossed with *Il4ra*^{F709} mice on the C57BL/6J background. IL-4Rα^{-/-} (BALB/c-*Il4ra*^{tm1Sz}/J) mice were obtained from The Jackson Laboratory (Bar Harbor, ME).

IgG preparation. Murine OVA-specific IgG was prepared from pooled sera from mice immunized i.p. with alum (50μg protein precipitated to 1mg aluminum hydroxide gel (Sigma Aldrich, St. Louis, MO) on days 0 and 7, followed by 10μg protein on days 14 and 21). Control IgG was prepared from mock-immunized mice. Human IgG was prepared from pooled sera samples from patients who had undergone oral immunotherapy for peanut allergy or from non-allergic donors. IgG was isolated over Nab protein G spin columns (Thermo Scientific, Waltham, MA), concentrated and dialyzed with Macrosep Advance Centrifugal Devices carrying a 100 kDa cut-off (Pall Corporation, Westborough, MA) and filter-sterilized with 0.2μm syringe filters (Millex, EMD Millipore, Billerica, MA). Murine OVA-specific IgG concentrations were determined by ELISA, and mice were injected with 100μg IgG1. Peanut-specific IgG4 in human IgG preparations was quantified by ImmunoCAP on a Phadia 100 (ThermoScientific, Kalamazoo, MI).

Food allergen sensitization, desensitization and anaphylaxis. Mice were sensitized by oral gavage with 250μg OVA (grade V, Sigma Aldrich, St. Louis, MO) plus 20μg cholera toxin (CT, List Laboratories) in 200μl PBS once a week for 3-8 weeks as

indicated according to procedures we have developed^{18, 24}. Desensitization was performed in mice previously sensitized as above by daily administration of 5mg allergen protein i.g. for three weeks. Enteral challenge with 150mg OVA was performed 7-10 days after the discontinuation of sensitization or desensitization gavages in order to elicit anaphylaxis. Where specified, recombinant murine IL-4 (Shenandoah Biotechnology) was administered i.p. at 2µg per mouse once a week in conjunction with OVA dosing. IL-4 was administered as a purified protein (not a long-lived cytokine:antibody complex), only at times of OVA dosing and the last dose was given a week prior to challenge; this protocol was designed to mimic the episodic IL-4 release from mast cells undergoing allergen-driven IgE receptor activation without the potent potentiating effect of IL-4 on anaphylactic severity²⁵.

Intestinal leukocyte isolation. Lamina propria leukocytes were isolated from the jejunum as previously described²⁶.

ELISAs.

MMCP-1 in serum samples was measured according to the manufacturer's instructions (eBioscience). OVA-specific IgE and IgG1 were assessed as previously described, using clones TOε and TOSG1C6 (BioLegend, San Diego, CA) as standards²⁴. The sandwich ELISA for murine IL-4 was constructed using 11B11 to capture and biotinylated BVD6-24G2 to detect (BioLegend, San Diego, CA) with recombinant protein standard from Shenandoah Biotechnology (Warwick, PA). Murine IL-13 was detected using paired antibodies from Affymetrix/eBioscience (San Diego, CA): eBio13A and biotinylated eBio1316H. Human IL-4 was detected using the antibody pair 8D4-8 and biotinylated MP4-25D2 (BioLegend, San Diego, CA).

Detection of IL-4 production from murine mast cells was enabled by culture in the presence of azide-free anti-IL-4Rα (10µg/ml, clone mIL4R-M1, BD Biosciences, Franklin Lakes, NJ) to block cellular uptake of secreted IL-4. Similarly, IL-4 reuptake by human

mast cells was inhibited with anti-IL-4R α (clone 25463, R&D Systems/biotechne, Minneapolis, MN).

Quantitative PCR

Mast cells were stimulated for 1hr prior to RNA extraction using a Qiagen RNeasy Micro Kit (Qiagen, Hilden, Germany). cDNA was prepared using an iScript cDNA Synthesis Kit (BioRad, Hercules, CA), and transcripts were assessed in real-time using TaqMan probes (Applied Biosystems/ThermoFisher, Waltham, MA). *GAPDH* was used as a housekeeping gene.

Mast cell culture. Cells were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum (Atlanta Biologicals, Lawrenceville, GA), 1mM sodium pyruvate, 100U/ml penicillin, 100 μ g/ml streptomycin, 10 μ g/ml gentamicin, 1% Minimum Essential Medium non-essential amino acids, 55 μ M 2-mercaptoethanol, 10mM HEPES buffer, 2mM L-glutamine (all from ThermoFisher Scientific, Waltham, MA), henceforth referred to as complete RPMI. Murine bone marrow-derived mast cells (BMMC) were cultured from bone marrow progenitors in the presence of recombinant murine IL-3 and SCF (10-20ng/ml, Shenandoah Biotechnology, Warwick, PA), with repeated selection for non-adherent cells. Human mast cells were isolated from humanized mice using immunomagnetic selection for c-Kit-expressing cells, and subsequently maintained in recombinant human SCF (50ng/ml), IL-3 (20ng/ml) and IL-4 (20ng/ml)²⁷. Mast cell phenotypes were confirmed by flow cytometry (c-Kit⁺Fc ϵ R1 α ⁺).

Murine BMMC were sensitized for 72h with serum from OVA-allergic *Il4ra*^{F709} mice (1% by volume, 34ng/ml OVA-specific IgE final concentration), and washed prior to stimulation. In some cases, monoclonal IgG2a anti-OVA (clone TOSGAA1, BioLegend, San Diego, CA) was added 4hrs prior to stimulation. Cells (10⁵/well) were stimulated for 48h with 250ng/ml OVA.

Human mast cells were sensitized for 72h with IgG-depleted serum from peanut (PN)-allergic donors (1% by volume, 1.4kU/L PN-specific IgE final concentration), and washed prior to stimulation. In some cases, anti-CD32 (clone FL18.26, 10µg/ml, BD Biosciences, Franklin Lakes, NJ) and/or IgG (containing 0.9mg/L PN-specific IgG4 final concentration) was added 2h prior to stimulation. Cells (10⁵/well) were stimulated for 48h with 1µg/ml PN extract. Complete PN extract was prepared as previously described¹⁸.

Mast cell reconstitution. Mast cell-deficient *Il4raF709 Kit^{W-sh}* mice were injected i.p. with 5x10⁶ BMMC eight and twelve weeks prior to the initiation of sensitization¹⁸. For reconstitution studies, all mast cells used carried *Il4raF709* alleles. Reconstitution of the intestine was assessed by flow cytometry.

Flow Cytometry. Antibodies for flow cytometry were purchased from BioLegend (San Diego, CA) unless otherwise stated. Mast cells were identified by staining with APC anti-FcεR1α (MAR-1), PE anti-IgE (R35-72, BD Biosciences, Franklin Lakes, NJ), PE-Cy7 anti-c-Kit (2B8), AlexaFluor700 anti-CD45 (30-F11) and FITC-conjugated lineage antibodies against CD3ε (145-2C11), CD4 (RM4-5), CD8α (53-6.7), CD11c (N418), CD11b (M1/70), CD19 (6D5), B220 (RA3-6B2), NKp46 (29A1.4), Gr-1 (RB6-8C5), TCRβ (H57-597) and TCRγδ (GL3). Tregs were identified in cell suspensions by surface staining with PerCP-Cy5.5-conjugated anti-CD4 (RM4-5), PE-Cy7-conjugated anti-TCRβ (H57-597), and AlexaFluor700-conjugated anti-CD45 (30-F11), followed by intracellular staining using eBioscience's Foxp3 staining kit with APC-conjugated anti-Foxp3 (FJK-16s, eBioscience, San Diego, CA). Cytokine staining was performed after 4hrs stimulation with 500ng/ml ionomycin (Sigma Aldrich, St. Louis, MO), 500ng/ml 12,13-phorbol dibutyrate (bio-techne/Tocris Bioscience, Minneapolis, MN) and 1µg/ml brefeldin A (Sigma Aldrich, St. Louis, MO), using Cytofix and permeabilization buffers from BD Biosciences (Franklin Lakes, NJ) and staining overnight with anti-IL-4 PE-Dazzle594 (clone 11B11 at 670ng/ml). Non-viable cells were excluded using fixable viability dye

eFluor 780 (eBioscience, San Diego, CA). Use of FSC-W and FSC-H signals restricted analyses to single cells. Cells were acquired on an LSR Fortessa or a FACSCanto using DIVA software (BD Biosciences, Franklin Lakes, NJ), and analyzed in FlowJo 10.0.8 (Tree Star Software, Ashland, OR).

Statistical analysis. Data were plotted and analyzed in Prism 5.0f (GraphPad Software, Inc., La Jolla, CA). Anaphylaxis data were analyzed using repeated measures 2-way ANOVA; all other data were analyzed with standard ANOVA with Bonferroni post-tests between groups. ELISA values for IgE varied across multiple orders of magnitude and thus were subjected to log transformation prior to statistical analysis; for this purpose, null values were assigned a nominal value corresponding to the limit of detection in the assay. Two-tailed *P* values are summarized as follows: **P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001. Data are represented as mean ±SEM for anaphylaxis curves. Flow cytometry data are depicted as points showing individual mice overlaid with mean ±SEM. ELISA data are represented as box-and-whisker plots depicting median, quartiles and range for ex vivo data, or as bar charts with mean+SEM for in vitro data. *In vitro* mast cell analyses describe the results of three independent cultures.

Results

IgG antibodies prevent allergic sensitization to food

IgG antibodies, signaling via the inhibitory IgG receptor, FcγRIIb, provide a powerful brake on IgE mediated activation of mast cells and basophils, blocking both degranulation and mediator release in cultured cells and anaphylactic responses *in vivo*^{8, 11, 28}. IgE-mediated mast cell activation not only elicits anaphylactogenic mediator production but also the secretion of immunomodulatory cytokines critical to the allergic sensitization process^{18, 29}. We hypothesized that IgG-mediated inhibition of such cytokine responses would attenuate both developing and established allergic adaptive responses to food allergens. To test this hypothesis, we used a well-characterized mouse model of food allergy, the *Il4raF709* atopic mouse to investigate the impact of IgG antibodies on the development of food allergy^{18, 24}. *Il4raF709* mice have a strong atopic predisposition and defects in oral tolerance that result from disinhibited IL-4R signaling in mast cells and Treg²⁶. As a result, dietary exposure of naive animals to food proteins can trigger allergic sensitization, with expansion of intestinal mast cells and a failure to generate suppressive Foxp3⁺ Treg populations due to IgE-dependent IL-4 production from mast cells¹⁹.

We examined the effect of passive administration of IgG on sensitization to the model allergen ovalbumin (OVA) in *Il4raF709* mice. Administration of IgG purified from OVA-hyperimmunized mice to *Il4raF709* mice prior to enteral sensitization prevented the development of anaphylactic food allergy (Fig 1). IgG from non-immune mice did not have this effect. After four weeks of gavage sensitization with OVA in combination with the adjuvant cholera toxin, gavage challenge with a high dose of OVA (150mg) provokes a reproducible loss of core body temperature, which was entirely prevented by the OVA-specific IgG administration (Fig 1A). There was a corresponding reduction in mast cell degranulation, as assessed by measurement of challenge-dependent release of mouse

mast cell protease (MMCP)-1 and IL-4 into the serum (Fig 1C, D). This is consistent with prior observations that IgG prevents degranulation in cultured mast cells⁹, as well as our prior report that mast cells are an important source of IL-4 in food allergy^{18, 19, 30}. IL-4 is an important growth factor for mast cells^{26, 31-33}, and co-ligation of FcγRIIb with cytokine receptors has been reported to inhibit mast cell growth³⁴. Examination of intestinal mast cell burden by flow cytometry revealed a marked attenuation of allergen-induced mast cell expansion following IgG administration (Fig 1E). Consistent with their reduced systemic IL-4 levels, IgG-treated mice showed impaired induction of serum IgE against OVA (Fig 1B), and the dominant intestinal T cell phenotype shifted from IL-4-producing Th2 to Foxp3⁺ Treg (Fig 1F, G).

Allergen-specific IgG facilitates desensitization

While decreases in allergen-specific IgE levels are inconsistent, increases in allergen-specific IgG levels are uniformly observed in immunotherapy protocols, including subcutaneous immunotherapy (SCIT) for aeroallergens and OIT for foods. We reasoned that the high levels of IgG present upon completion of OIT could affect the allergic phenotype, and that providing similar levels of IgG by passive transfer at the inception of desensitization might block IgE-mediated mast cell activation thereby facilitating the acquisition of tolerance. We therefore administered specific IgG as an adjunct to oral desensitization in mice with established food allergy. Mice subjected to continued oral administration of allergen still developed anaphylaxis upon subsequent enteral challenge. Combining desensitization with specific IgG administration, however, greatly reduced anaphylaxis upon challenge (Fig 2A). MMCP-1 release into the serum, indicative of mast cell degranulation diminished by ~70% in mice treated with specific IgG (Fig 2B), and IL-4 release also decreased (Fig 2C). These effects were not observed with non-immune IgG, indicating that allergen-specificity is required. As we had observed in the experiments using specific IgG to prevent allergic sensitization,

adjunctive administration of specific IgG also reduced the intestinal mast cell burden, bringing it back down to approximately half the level seen in mice that did not receive specific IgG (Fig 2D). While control mice undergoing oral desensitization with OVA exhibited elevated IgE production, OVA-specific IgG administration prevented this increase (Fig 2E). CD4⁺ T cells in OVA-sensitized mice developed a Foxp3⁺ Treg-biased phenotype after specific IgG administration (Fig 2F), along with reduced Th2 frequencies (Fig 2G).

Tolerance restoration requires FcγRIIb

IgG can exert its inhibitory effects in several ways, including antigen interception and steric blockade of access to IgE as well as ligation of cellular inhibitory Fc receptors on immune effector cells. We therefore investigated the contribution of FcγRIIb signaling in IgG-mediated suppression of allergic sensitization. As previously observed in other systems, loss of FcγRIIb in *Il4raF709* mice greatly exacerbated the severity of anaphylactic shock in response to allergen challenge, amplifying a modest 1-2°C temperature loss on the C57BL/6 background into a substantial 7°C drop (Fig 3A). Serum IL-4 levels were decreased following IgG administration in wild-type but not FcγRIIb-deficient mice, and mast cell burden was substantially increased by the loss of FcγRIIb (Fig 3B, C). Production of IgE, IgG2b and IgA was enhanced in FcγRIIb-deficient mice (Fig 3D and Fig E1). Consistent with prior reports on the role of FcγRIIb in suppressing the induction of inflammatory T cell responses³⁵⁻³⁷, FcγRIIb^{-/-} *Il4raF709* mice exhibited elevated frequencies of Th2 cells in the small intestinal lamina propria, with a corresponding decrease in Foxp3⁺ Treg (Fig 3E, F and data not shown). These findings demonstrate the substantial effects that IgG: FcγRIIb ligation can exert in restraining allergic pathogenesis.

FcγRIIb-deficient mice exhibited enhanced anaphylactic severity despite high levels of endogenous IgG production during allergen sensitization (Fig E1). This

suggested the possibility that IgG:FcγRIIb interactions could be equally critical during allergen desensitization, in which allergen-specific IgG production typically increases by orders of magnitude^{2, 10, 38}. In order to test this hypothesis, *Il4raF709* and *Il4raF709* FcγRIIb^{-/-} mice were sensitized to OVA and then subjected to oral desensitization combined with administration of either OVA-specific or non-immune IgG. Following desensitization, *Il4raF709* mice carrying functional FcγRIIb continued to exhibit modest hypothermia upon allergen challenge (Fig 4A). Those mice treated with αOVA IgG, however, did not exhibit anaphylaxis following challenge. In contrast, mice lacking FcγRIIb expression were not protected by αOVA IgG. Serum IL-4 release was markedly enhanced in the absence of FcγRIIb (Fig 4B). Mast cell burden and IgE production was suppressed by IgG administration in wild-type but not FcγRIIb-deficient mice (Fig 4C, D). Treg frequencies were consistently elevated by αOVA IgG administration in *Il4raF709* mice (Fig 4E). In animals lacking FcγRIIb, αOVA IgG had no effect on Treg, and in fact Treg frequencies were consistently reduced, consistent with previous reports on FcγRIIb^{-/-} mice (Fig 4E)^{35, 36}. Th2 polarization was suppressed by IgG administration in an FcγRIIb-dependent manner (Fig 4F). Taken together, these findings support the hypothesis that FcγRIIb-mediates critical functions of IgG in attenuating allergic responses.

Mast cell-expressed FcγRIIb contributes to IgG-mediated desensitization

Having established a potent effect of FcγRIIb in both allergen-specific and general Treg induction by exposure to food in the gastrointestinal mucosa, we sought to determine the critical cellular effector in this response. Employing mast cell-deficient *Il4raF709 Kit^{W-sh}* mice, we tested the contributions of mast cells and specifically of mast cell FcγRIIb to IgG-mediated tolerance induction during allergen exposure. Mast cell-deficient mice failed to exhibit anaphylaxis after sensitization and challenge, but mice reconstituted with mast cells regained responsiveness, exhibiting modest losses of core

body temperature (Fig 5A), consistent with our prior observations using this model¹⁸. IgG administration abrogated anaphylaxis in mice engrafted with wild-type mast cells, but failed to do so in animals reconstituted with FcγRIIb^{-/-} mast cells. Among IgG-treated mice, serum IL-4 levels were higher in recipients of FcγRIIb-deficient cells, but we did not consistently observe differences in IgE production (Fig 5B, C). Engrafted intestinal mast cell numbers were similar in mice receiving WT vs. FcγRIIb^{-/-} mast cells (Fig 5D). IgG administration, however, reduced mast cell burden solely when FcγRIIb was present (Fig 5D and Fig E2). The absence of FcγRIIb on mast cells had no significant impact on Treg frequencies in the lamina propria except when allergen sensitization was combined with specific IgG administration (Fig 5E). Animals enterally exposed to OVA and given OVA-specific IgG exhibited increased intestinal Treg when populated with FcγRIIb-sufficient mast cells while failing to expand Treg following reconstitution with FcγRIIb-deficient mast cells. Th2 polarization followed the opposite pattern (Fig 5F). Data from these experiments show the importance of mast cell-expressed FcγRIIb in mitigating anaphylaxis and tempering Th2 bias.

FcγRIIb suppresses mast cell IL-4 secretion

Mast cells secrete copious quantities of cytokines upon crosslinking of FcεRI by IgE and allergen. Food allergy in *Il4ra*^{F709} mice requires rapid, production of IL-4 by mast cells, which enhances sensitization by promoting Th2 polarization, B cell production of IgE, and by disrupting Treg generation^{18, 19}. Since IgG and FcγRIIb regulated the systemic release of IL-4 upon challenge of sensitized mice, we tested the hypothesis that IgG blocks mast cell IL-4 production and that this effect is mediated by FcγRIIb. Co-ligation of FcγRIIb has been shown to inhibit FcεRI-dependent IL-4 release from murine and human basophils, and operates in a similar manner to suppress FcεRI-driven cytokine production in dendritic cells^{11, 39}. Detecting IL-4 in supernatants of cultured mast cells has often been challenging and has occasionally generated

controversy about their ability to produce the cytokine. This difficulty results at least in part from the rapid re-uptake of secreted IL-4 via IL-4 receptors precluding its detection in free, secreted form. We reasoned that this IL-4R-mediated re-uptake of the cytokine would be absent in IL-4R $\alpha^{-/-}$ BMMC cultures or blocked following addition of IL-4R α -neutralizing antibody, allowing for more efficient detection (Fig E3). Using the IL-4R α -neutralizing antibody to aid detection, we confirmed that allergen-specific IgG antibodies act in a dose-dependent manner to block IgE:Fc ϵ RI-induced production of IL-4 by murine mast cells (Fig 6A). This inhibitory effect required mast cell expression of Fc γ RIIb. A parallel inhibitory effect of IgG was observed with respect to IgE-stimulated murine mast cell production of IL-13 (Fig 6B).

Human mast cells have also been shown to produce IL-4⁴⁰ and have been reported to exhibit Fc γ RIIb-dependent suppression⁴¹. Both of these findings are somewhat controversial, and expression of IL-4 and Fc γ RIIb by human mast cells appears to depend considerably on the source of mast cells used. Due the difficulties in obtaining primary tissue-resident mast cells, many investigators opt for cell lines or cells cultured in isolation from progenitors. We chose instead to isolate mast cells from humanized mice²⁷, where they would have undergone maturation in the setting of tissue-specific cues⁴², albeit in a hybrid murine-human environment. Such mast cells express both activating and inhibitory Fc γ receptors and upregulate IL-4 upon Fc ϵ RI crosslinking. We therefore tested the hypothesis that ligation of Fc γ RIIb by IgG in human mast cells would also suppress IL-4 production. For this purpose, human mast cells were isolated from humanized mice and sensitized with sera from highly peanut (PN)-allergic donors containing IgE anti-PN. When human mast cells were stimulated with PN extract, mRNA transcripts for IL-4 increased approximately 70-fold (Fig 6C). IgG purified from the sera of food allergic patients who had successfully completed OIT suppressed IL4 expression by about 50%. Antibody blockade of CD32 (Fc γ RIIa and Fc γ RIIb)

restored *IL4* levels, consistent with an inhibitory effect of FcγRIIb. Similarly, OIT-derived IgG suppressed secretion of IL-4 protein in a largely CD32-dependent manner (Fig 6D). *IL-4 administration overrides aspects of IgG-enforced tolerance*

We hypothesized that the attenuated Th2 response observed in the presence of allergen-specific IgG resulted from $\alpha\gamma$ -mediated suppression of mast cell IL-4 production. This would predict that administration of exogenous IL-4 should counteract the suppressive effects of exogenous IgG on the adaptive response. To test this, *Il4ra*^{F709} mice were either 1) sensitized to OVA, 2) sensitized to OVA and given IgG αOVA, or 3) sensitized to OVA and given IgG along with IL-4. As observed in prior experiments, IgG administration suppressed the anaphylactic response to allergen. This effect was largely unaffected by IL-4 supplementation, consistent with the continuing suppression of degranulation and anaphylactic mediator release by IgG:FcγRIIb (Fig 7A). We did not observe statistically significant alterations in the IgG-mediated suppression of MMCP-1 release or IL-4 release following IL-4 administration (Fig 7C, D) consistent with the continued ability of IgG to suppress mast cell degranulation. IL-4 administration, however, restored intestinal mast cell burden to levels seen in normally sensitized mice (Fig 7E). Exogenous IL-4 administration also overrode the IgG-mediated shift from Th2 to Treg (Fig 7F, G). These findings suggest that while suppression of anaphylaxis results from a direct effect of IgG:FcγRIIb signals on mast cells, suppression of mast cell growth and Th2 conversion are secondary effects deriving from FcγRIIb-mediated suppression of IL-4 production.

Discussion

IgE and mast cells are central players food allergy. They exert dual roles in pathogenesis, both priming the pump for Th2 responses and IgE production following allergen exposure, and also triggering acute reactions to allergen challenge^{18, 43}. The evidence gathered in this study indicates that high-titer allergen-specific IgG antibodies can inhibit both functions. Our results establish that the inhibitory Fc receptor FcγRIIb is the central mediator of this process, and that it potently suppresses allergen-induced anaphylaxis and mast cell-driven modulation of T cell phenotypes. We now show that supplemental administration of allergen-specific IgG not only prevents allergic sensitization but can also restore a desensitized state with tolerogenic T cell responses in previously allergic mice. We demonstrate the ability of IgG to suppress IL-4 secretion by both human and mouse FcεRI-expressing cells, and show that the diminished levels of IL-4 that exist after IgG administration play a role in the shifting Treg:Th2 balance. These findings have the potential to inform our understanding of the successes and failures of OIT, as they suggest that the development of higher specific IgG:IgE ratios would be more likely to produce lasting tolerance. Furthermore, we suggest that direct modulation of allergen-specific IgG levels in food allergic patients may facilitate the restoration of tolerance during OIT while simultaneously decreasing adverse reactions, such as has been already been demonstrated in venom immunotherapy⁴⁴.

IgG has a lengthy history associating it with clinical tolerance in allergic pathology^{45, 46}. Notable observations from studies of bee venom immunotherapy demonstrated a link between the production of IgG anti-venom antibodies and successful clinical outcomes^{47, 48}. A recent multi-cohort study found a strong association between the production of specific IgG1 and the development of benign rather than pathological Th2 responses to aeroallergens in atopic children⁴⁹. In the context of food allergy, murine models have established the importance of FcγRIIb and IgG in regulating

anaphylactic shock^{9, 10, 50}. Moreover, the regulatory activity of IgG has recently been demonstrated to underlie the desensitized (non-reactive) state observed in patients who have undergone immunotherapy for food allergies as well as naturally tolerant people who exhibit food-specific IgE but do not experience adverse reactions upon ingestion^{10, 51, 52}. Interestingly, IgG production to ingested foods, including common food allergens, is in fact the norm in tolerant, unsensitized individuals⁵³, which raises the possibility that such IgG may normally preclude the development of allergy⁵⁴. Since IgG, unlike IgE, readily crosses the placenta and is secreted in breast milk, food-specific IgG can be inherited by the immunologically naïve infant before ever ingesting the food.

Circulating IgG has access to a range of innate and adaptive effector and regulatory cells distributed throughout the body, including mucosal tissues. In this study, we have focused on the role of FcγRIIb on mast cells, but there are multiple Fcγ receptors and a variety of effector cells that express them. FcγRIIb-mediated suppression has been shown to favor tolerogenic phenotypes in dendritic cells and B cells^{35-37, 39}. The ability of FcγRIIb to influence a wide array of cellular functions derives from the relative simplicity in its mechanism of action. The intracellular portion of FcγRIIb contains an immunoreceptor tyrosine-based inhibitory motif (ITIM) that recruits inhibitory phosphatases—SHIP, Shp1 and Shp2—which between them have the ability to dephosphorylate and thus deactivate a substantial range of kinases. In the context of IgE receptor signaling, co-aggregation of FcεRI with FcγRIIb allows FcεRI-associated Lyn to phosphorylate FcγRIIb, thus recruiting SHIP to the nascent signaling complex⁵⁵. SHIP can then dampen calcium flux through direct degradation of inositol phosphate, as well as regulating the activity of the PI3K and MAPK pathways, thus inhibiting both degranulation and downstream cytokine synthesis^{8, 11, 28, 41, 56}. In this way, both immediate hypersensitivity and subsequent T cell polarization can be abrogated.

The inverse correlation between allergen-specific IgG responses and allergic symptoms in clinical cohorts^{49, 51, 52} along with the apparent immunomodulatory effects arising in the course of SCIT^{45, 57, 58} and OIT¹⁰ all support the clinical significance of the immunoregulatory IgG effects we report here. We have observed FcγRIIb-mediated inhibition of FcεRI activation in human mast cells derived from humanized mice in this study as have Kepley and colleagues using human mast cells cultured from umbilical cord blood⁴¹. Similarly, human mast cells from these two sources are capable of producing IL-4^{59, 60}. We caution, however, against generalizing these to be features of all human mast cells, since mast cells derived from human skin express neither IL-4 nor FcγRIIb^{61, 62}, and reports on IL-4 from primary human mast cells within various tissues differ in their conclusions^{40, 59, 61, 63-72}. Tissue-dependent heterogeneity of mast cells appears to restrict FcγRIIb expression to certain organs in humans^{62, 73}, and may correlate with mucosal versus serosal location.

Several investigators have tried to harness the negative signal induced by crosslinking of FcεRI and FcγRIIb to control hypersensitivity reactions. A variety of engineered chimeric proteins designed to crosslink allergen-specific IgE on FcεRI with FcγRIIb have been tested in mouse models of allergy and some in clinical trials⁷⁴⁻⁷⁶. Our findings predict that such constructs might additionally exert immunomodulatory effects. However such effects would be limited to a single allergen (defined in the construct), while the polyclonal and polyspecific IgG response arising in subjects with multiple food allergies would have a more encompassing suppressive effect.

Taken together, the presence of food-specific IgG antibodies in healthy individuals, their induction in patients successfully completing OIT and the immunomodulatory properties of these antibodies as demonstrated in this study suggest that the IgG response may constitute a common and important mechanism in the homeostatic pathways inducing and maintaining immunological tolerance to foods.

Investigating the role of IgG in these settings has the potential not only to more clearly define the regulatory mechanisms at play in physiologic host:food interactions but also to point to new approaches to testing for food allergy and predicting clinical sensitivity as well as innovative strategies for manipulating the allergic response.

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Figure Legends

Figure 1. Effect of IgG on food allergen sensitization. A) Anaphylaxis: Core body temperature decrease following enteral OVA challenge of sensitized BALB/c background *Il4raF709* mice. n=4 saline, n=5 for OVA and OVA+IgG. B) Serum OVA-specific IgE. n.d., not detected. C) Levels of the mast cell granule protease, MMCP-1, in challenged mice. D) Serum IL-4 after challenge. E) Intestinal mast cell numbers. F) Small intestinal Th2 and G) Treg frequencies. Data shown are from one of three independent experiments.

Figure 2. Impact of exogenous IgG on allergen desensitization. A) Anaphylaxis in OVA-sensitized and desensitized BALB/c background *Il4raF709* mice after challenge. n=4 Sensitized only, n=5 OVA + control IgG, and n=4 OVA + α OVA IgG. B) MMCP-1 release. C) IL-4 release. D) Intestinal mast cell numbers. E) OVA-specific IgE levels. F) Intestinal Treg frequencies. G) Intestinal Th2 frequencies. Data shown are representative of three independent experiments.

Figure 3. Role of Fc γ RIIb in allergic sensitization. A) Anaphylaxis in OVA-sensitized *Il4raF709* and *Il4raF709* Fc γ RIIb^{-/-} mice on the C56BL/6 background. B) IL-4 release. C) Intestinal mast cell numbers. D) Specific IgE levels. E) Intestinal Treg frequencies. F)

Intestinal Th2 frequencies. n=3 (A, C, E), and n=4 WT, n=5 FcγRIIb^{-/-} (B, D, F). Data shown are representative of three independent experiments.

Figure 4. Role of IgG and FcγRIIb and in tolerance restoration during desensitization. A) Anaphylaxis following enteral challenge in OVA-sensitized and desensitized *Il4ra*F709 and *Il4ra*F709 FcγRIIb^{-/-} mice on the C57BL/6 background. ns, not significantly different. B) Serum levels IL-4. C) Intestinal mast cells. D) Specific IgE levels. E) Intestinal Treg frequencies. F) Intestinal Th2 frequencies. n=4-5 WT Control IgG, n=3-4 WT αOVA IgG, n=4-5 FcγRIIb^{-/-} Control IgG and n=4-5 FcγRIIb^{-/-} αOVA IgG. Data shown are from one of two independent experiments.

Figure 5. Impact of mast cell FcγRIIb ligation on the development of Treg responses during sensitization. A) Anaphylaxis in *Il4ra*F709 *Kit*^{W-sh} mice on the C57BL/6 background reconstituted with WT or FcγRIIb^{-/-} mast cells. n=3 F709 *Kit*^{W-sh} No MC, n=5 WT MC, n=4 WT MC αOVA IgG, n=4 FcγRIIb^{-/-} MC, n=5 FcγRIIb^{-/-} MC αOVA IgG. B) Serum IL-4 after challenge. C) Serum OVA-specific IgE. n=6 F709 *Kit*^{W-sh} No MC, n=8 WT MC, n=8 WT MC αOVA IgG, n=8 FcγRIIb^{-/-} MC, n=9 FcγRIIb^{-/-} MC αOVA IgG. D) Intestinal mast cells. E) Proportional representation of Treg in the small intestine. F) Intestinal Th2 frequencies. Data shown are from one of three independent experiments.

Figure 6. Requirement for FcγRIIb in IgG-mediated suppression of IgE:FcεRI-triggered IL-4 expression. A) Supernatant levels of IL-4 in WT and FcγRIIb-deficient murine mast cells (C57BL/6 background, n=3) sensitized with polyclonal IgE αOVA and stimulated with OVA in the presence of monoclonal IgG2a αOVA. B) IL-13 secretion. C) mRNA levels of *IL4* in human mast cells (n=3) sensitized with IgE αPN, and stimulated with PN in the presence of IgG αPN and/or αFcγRII (CD32) blocking antibody. D) IL-4 secretion by human mast cells. Data are representative of three independent experiments.

534 **Figure 7.** IL-4 administration partially reverses IgG-mediated inhibition of allergic
535 responses. A) Anaphylaxis in *Il4raF709* mice (n=5) on the BALB/c background
536 sensitized to OVA in combination with IgG α OVA and/or IL-4. B) Specific IgE levels. ns,
537 not significantly different. C) MMCP-1 release in serum. D) Serum IL-4 levels. E)
538 Intestinal mast cell numbers. F) Intestinal Th2 frequencies. G) Intestinal Treg
539 frequencies. Data are representative of three independent experiments.

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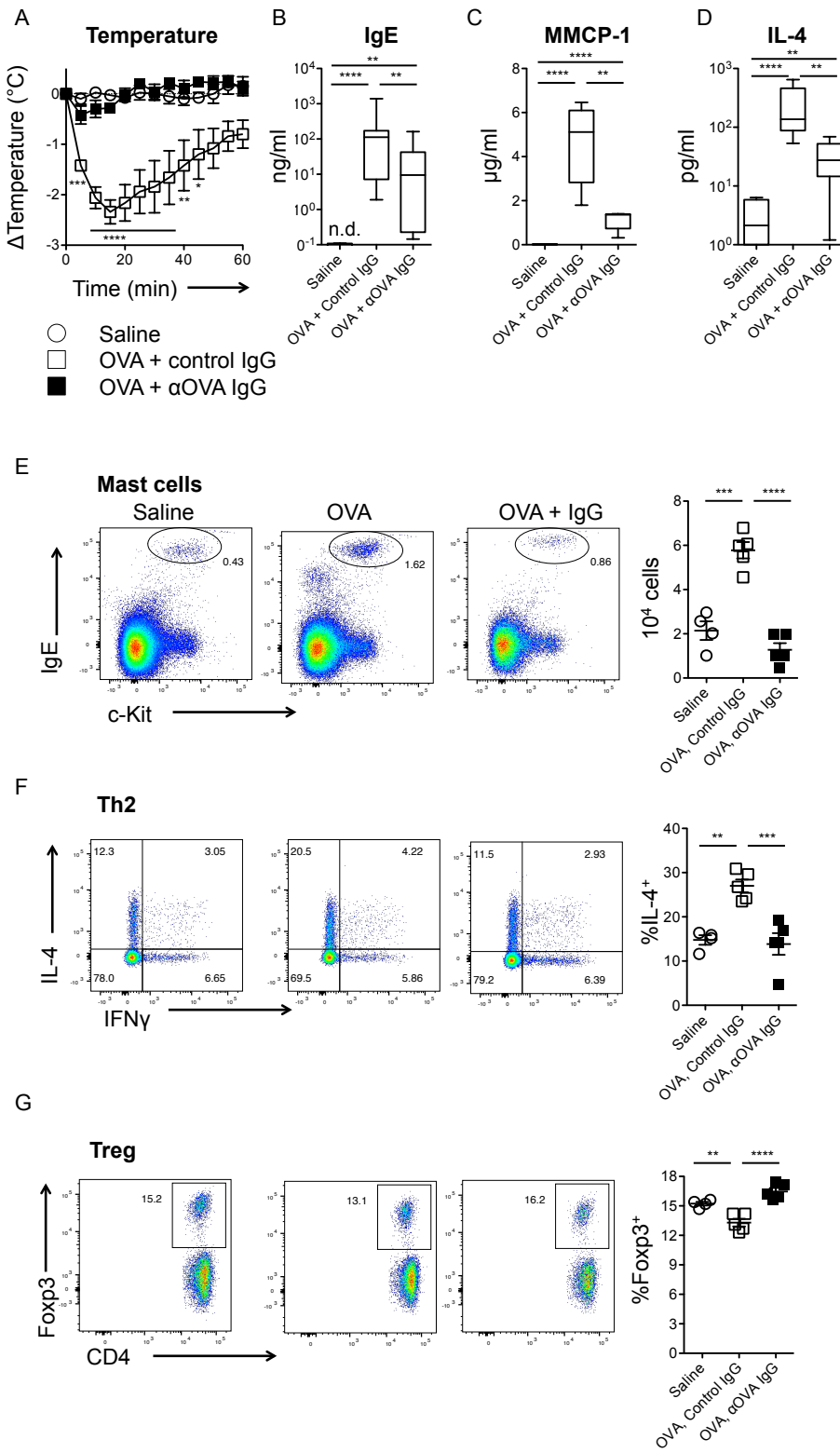
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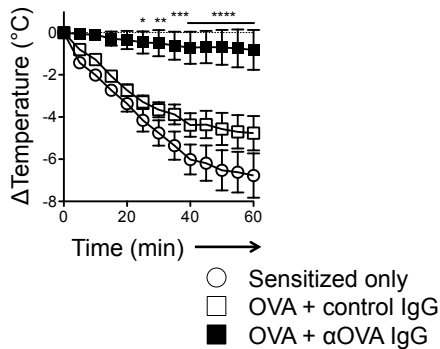
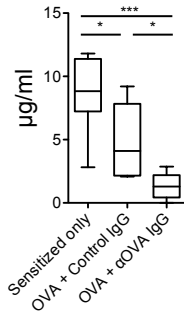
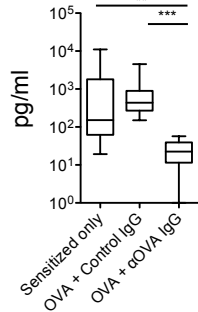
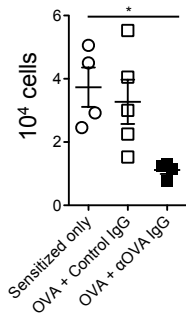
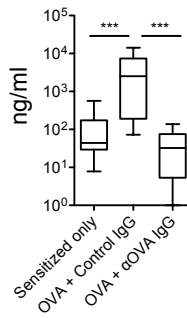
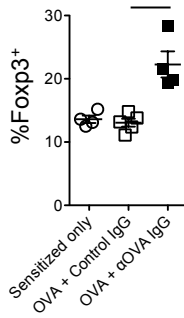
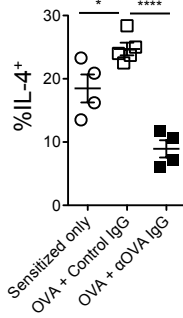
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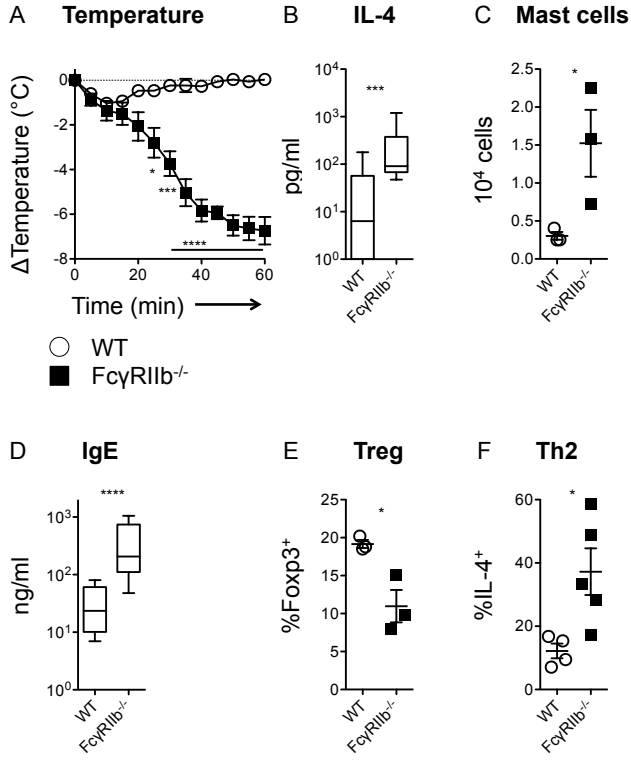
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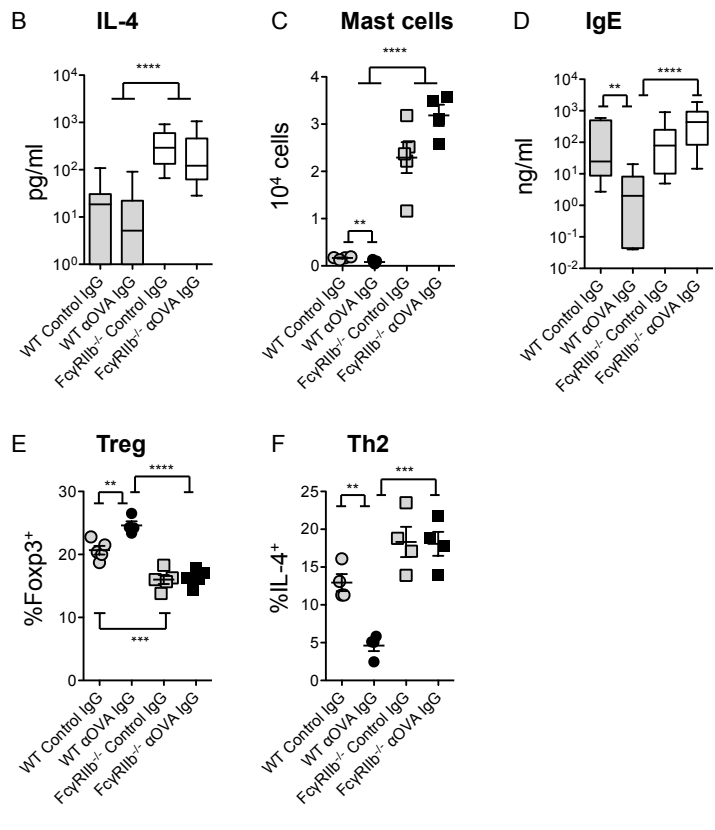
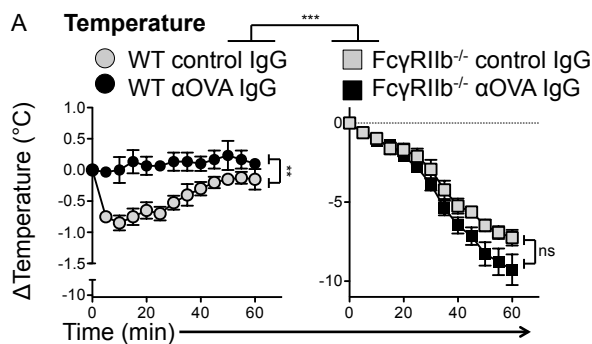
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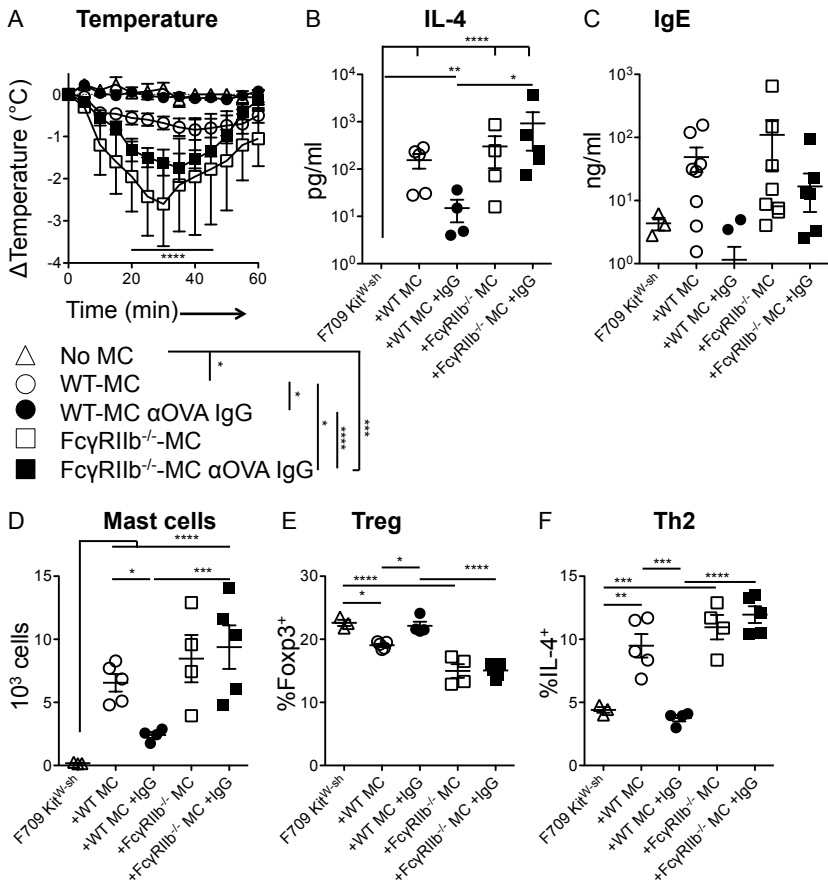
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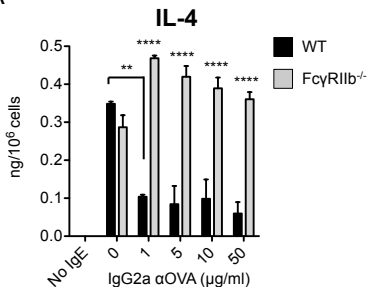
A Temperature**B MMCP-1****C IL-4****D Mast cells****E IgE****F Treg****G Th2**



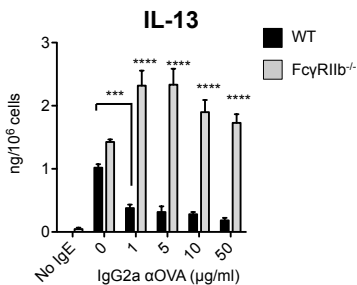




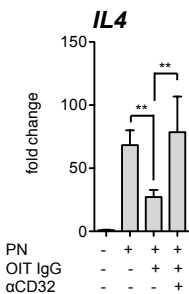
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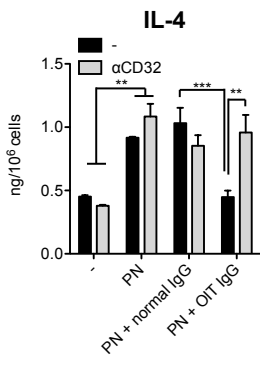
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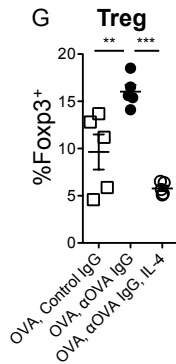
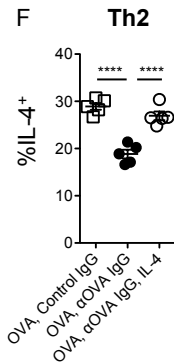
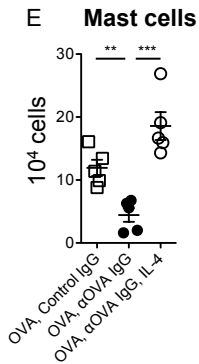
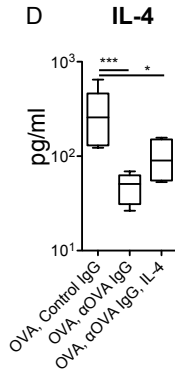
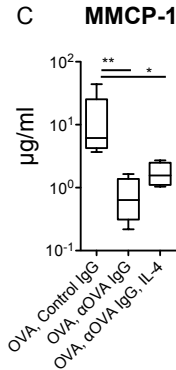
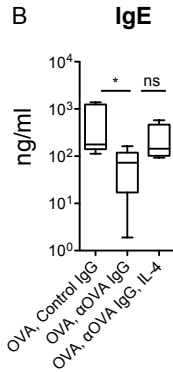
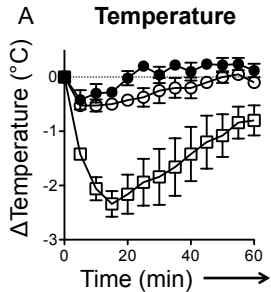


C



D



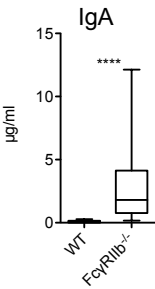
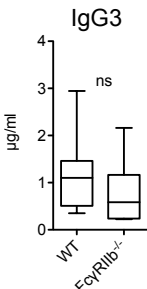
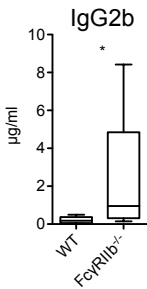
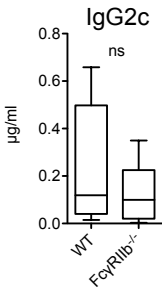
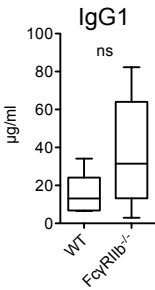


1 **Online Repository**

2 **Figure E1.** Anti-OVA immunoglobulin levels in sera of OVA-sensitized *Il4raF709* mice
3 with or without FcγRIIb, as determined by ELISA. * $P < 0.05$, **** $P < 0.0001$ by unpaired *t*
4 test on log-transformed values. Data are representative of three independent
5 experiments.

6 **Figure E2.** Intestinal mast cell reconstitution in *Il4raF709 Kit^{W-sh}* mice. Flow cytometric
7 analysis of jejunal lamina propria leukocytes showing c-Kit⁺IgE⁺ mast cells in mice
8 engrafted with WT or FcγRIIb^{-/-} mast cells carrying the *Il4raF709* mutation. All mice were
9 subjected to OVA sensitization, and some were additionally administered IgG anti-OVA.
10 Representative flow plots are shown, gating on viable CD45⁺ leukocytes.

11 **Figure E3.** Detection of secreted IL-4 is enhanced in the absence of IL-4Rα-mediated
12 uptake. BALB/c or IL-4Rα^{-/-} murine bone marrow-derived mast cells (2×10^5) were
13 cultured for 48hr, and IL-4 levels in the supernatant were determined by ELISA. For
14 IgE/Ag stimulation, cells were sensitized with polyclonal IgE αOVA from allergic mouse
15 serum, then stimulated with 1μg/ml OVA. PI: phorbol 12,13-dibutyrate and ionomycin
16 (500ng/ml each). * $P < 0.05$, *** $P < 0.001$, **** $P < 0.0001$ by Bonferroni post-test on ANOVA.



No MC

WT MC

FcγRIIb^{-/-} MC

IgE

5.33E-3

0.091

0.088

0.030

0.060

IgG αOVA

c-Kit



IL-4

