

Immunoglobulin E Signal Inhibition during Allergen Ingestion Leads to Reversal of Established Food Allergy and Induction of Regulatory T Cells

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SUMMARY

Immunoglobulin E (IgE) antibodies are known for triggering immediate hypersensitivity reactions such as food anaphylaxis. In this study, we tested whether they might additionally function to amplify nascent antibody and T helper 2 (Th2) cell-mediated responses to ingested proteins and whether blocking IgE would modify sensitization. By using mice harboring a disinhibited form of the IL-4 receptor, we developed an adjuvant-free model of peanut allergy. Mast cells and IgE were required for induction of antibody and Th2-cell-mediated responses to peanut ingestion and they impaired regulatory T (Treg) cell induction. Mast-cell-targeted genetic deletion of the FcεRI signaling kinase Syk or Syk blockade also prevented peanut sensitization. In mice with established allergy, Syk blockade facilitated desensitization and induction of Treg cells, which suppressed allergy when transferred to naive recipients. Our study suggests a key role for IgE in driving Th2 cell and IgE responses while suppressing Treg cells in food allergy.

INTRODUCTION

Food allergy has emerged as a major public health issue worldwide (Burks et al., 2012). Sensitized individuals, who have high titers of food-specific IgE antibodies, experience a range of immediate hypersensitivity responses including acute-onset itching, hives, vomiting, and diarrhea all triggered when food proteins recognized by FcεRI-bound IgE induce mast cell activation. The most dramatic food hypersensitivity reaction is systemic anaphylaxis, in which vasoactive mast cell mediators induce plasma extravasation, shock, cardiopulmonary collapse, and death (Finkelman, 2007; Simons, 2010). The standard of care, namely recommendation to strictly avoid foods to which they

are allergic, paradoxically deprives patients of the chance to naturally develop oral tolerance, as would probably occur if they were able to continue to ingest them without experiencing harmful effects.

Following the very successful example of subcutaneous immunotherapy in which subjects with aeroallergen sensitivity are desensitized by injection of protein extracts ("allergy shots"), investigators have evaluated oral desensitization strategies for food allergy (Nowak-Węgrzyn and Sampson, 2011; Vickery and Burks, 2010). Although achieving substantial success, such approaches are associated with unpredictable IgE antibody-mediated allergic reactions, limiting their application in practice. Several groups have now performed oral desensitization under cover of the monoclonal anti-IgE antibody omalizumab, which effectively blocks food anaphylaxis, with the expectation that inhibiting IgE-mediated reactions would improve the patient experience (Nadeau et al., 2011; Schneider et al., 2013).

A growing body of evidence indicates that IgE antibodies and mast cells might serve not only as the effectors of immediate hypersensitivity in subjects with established sensitivity but also as amplifiers during initial antigen exposure in naive subjects, potentially providing early signals for nascent Th2 cell and antibody responses. IgE induces mast cell production of both Th2-cell-inducing and DC-activating cytokines (Asai et al., 2001; Kalesnikoff et al., 2001; Kawakami and Kita, 2005). We have reported that IgE and mast cells enhance immune sensitization in contact sensitivity (Bryce et al., 2004). By using an adjuvant-free asthma model, Nakae et al. (2007a, 2007b) demonstrated that the induction of airway inflammation and bronchial hyperreactivity are strongly amplified by mast cells in the airway mucosa. Additional evidence that effector cells of allergic responses might independently function as inducers of immune sensitization comes from studies implicating basophils (which, like mast cells, are FcεRI⁺) as early drivers of Th2 cell expansion (Mukai et al., 2005; Sokol et al., 2009). We similarly hypothesized that IgE antibodies and mast cells might serve not only as the effectors of food anaphylaxis but also as important early inducers of Th2 cell responses and suppressors of Treg cell responses to food allergens and that they might

provide an accessible therapeutic handle by which to dampen such responses.

Evaluation of this hypothesis required an animal model of food allergy in which immune sensitization could be accomplished directly by enteral sensitization and in the absence of the immunostimulatory effects of prior systemic parenteral immunization or ingestion of immunological adjuvants commonly used in food allergy models. For this purpose we took advantage of inherently atopic mice in which a mutation (F709) of the IL-4 receptor α chain (IL-4 α) ITIM results in amplified signaling upon interaction with IL-4 or IL-13, but not constitutive activation. These mice exhibit enhanced Th2 cell responses and IgE production and susceptibility to anaphylaxis after ingestion of the model antigen ovalbumin (OVA) in the absence of adjuvant (Mathias et al., 2011). We have now adapted this model to the clinically relevant food allergen peanut and have applied multiple parallel approaches that together provide strong evidence that IgE antibodies and mast cells enhance Th2 cell responses to ingested allergens. The mechanism for this Th2 cell induction is the suppression of Foxp3⁺ Treg cell responses, in terms of both Treg cell numbers and function. We show that established allergic sensitivity can be reversed when oral immunotherapy is paired with interventions that block IgE-mediated mast cell activation and demonstrate that pharmacologic inhibition of Syk activity can be used to inhibit mast cell function and achieve the same benefit as IgE blockade. Our results establish that IgE antibodies, signaling via FcεRI on mast cells, not only serve to induce anaphylactic reactions in individuals with food allergy but also exert critical regulatory effects on adaptive immunity to ingested proteins in emerging food allergy. Mast cells activated by IgE support Th2 cell responses and IgE production while suppressing Treg cell induction. Moreover, food ingestion under cover of IgE signaling blockade can lead to reversal of Th2 cell responses and induction of Treg cells in mice with established food allergy. These results suggest that pharmacologic IgE blockade might be of benefit to patients with food allergy.

RESULTS

IgE Antibodies Amplify Th2 Cell Responses and Suppress Treg Cell Induction to Ingested Peanut

Although IgE antibodies are best recognized for their unique ability to trigger immediate hypersensitivity reactions in previously sensitized subjects, there is evidence that they might serve an independent and important function in amplifying and skewing developing adaptive immune responses after initial antigen exposure (Gould and Sutton, 2008). For this study, we examined the possibility that IgE antibodies, signaling via FcεRI on mast cells, would support Th2 cell responses to ingested food allergens while suppressing the induction of Treg cells. In order to test this hypothesis, we developed a murine model of allergy to peanut, a very common food allergen and one associated with particularly severe anaphylactic reactions. We have previously reported the construction of mice (IL4 α ^{tm3.1Tch}) that harbor a targeted insertion of a mutant form of IL-4 α in which the immunoreceptor tyrosine-based inhibition motif (ITIM) motif is disrupted (F709 mutation). These mice have normal IL-4 α expression and do not have baseline signaling but exhibit enhanced signaling on ligand exposure and are highly susceptible to the in-

duction of allergy to ovalbumin (OVA) in an adjuvant-free ingestion model (Mathias et al., 2011; Tachdjian et al., 2010). They exhibit robust anaphylaxis after food allergen ingestion, a cardinal phenotype of food allergy that has previously been difficult to reproduce in animal models. We reasoned that these mice would provide an ideal tool for analysis of IgE and mast cell roles in food allergen sensitization to a physiologically relevant allergen, peanut.

Mice were sensitized by weekly gavage with 23 mg commercial peanut butter (5 mg protein) (Figure S1 available online). After four doses, peanut-fed mice carrying the IL-4 α F709 mutation, but not control animals, exhibited strong peanut-specific IgE and IgG1 responses as well as robust induction of peanut-specific Th2 cell responses (Figures 1A–1D and S2A). Consistent with the expected induction of oral tolerance, wild-type BALB/c mice developed a strong peanut-specific Foxp3⁺ Treg cell response (Figure 1E). Peanut-specific Treg cell induction was markedly impaired in mice with the F709 IL-4 receptor. In this study we measured Treg cell responses by ex vivo stimulation for 5 days with peanut antigen followed by flow cytometric evaluation of Foxp3⁺ dividing cells (Figure 1F).

Gavage challenge of peanut-sensitized mice with 450 mg peanut butter (100 mg protein) elicited anaphylaxis in the IL-4 α mutants, measured via implanted thermal transponders, as loss of core body temperature (Figure 1G; see Figure S1 for protocol schematic). Neither unsensitized IL-4 α mutant mice nor peanut-fed wild-type BALB/c mice had any temperature loss (Figure 1G and data not shown), and challenge of peanut-sensitized mutant mice with irrelevant protein had no effect on temperature (Figure S2B). Anaphylaxis was accompanied by elevated serum concentrations of mouse mast cell protease-1 (MMCP-1), a marker of mucosal mast cell activation (Figure 1H). These findings demonstrate that mice with a dysregulated F709 IL-4 receptor develop intense Th2 cell, IgE, and IgG responses to ingested peanut along with a defective induction of Treg cells and that this immunological profile renders them exquisitely sensitive to anaphylaxis after enteral peanut challenge. It is interesting to note that in addition to being susceptible to deliberate induction of sensitization with the food allergen peanut, these mice exhibit some spontaneous sensitization to food proteins within their standard chow diet (Figure S2C).

In order to evaluate the contributions of IgE antibodies to peanut sensitization and anaphylaxis, we crossed the IL-4 α mutant animals with IgE-deficient (*Igh*^{-/-}) BALB/c mice (Oettgen et al., 1994). As expected, IgE-deficient mice were fully resistant to peanut anaphylaxis (Figure 1G). Consistent with our hypothesis that IgE contributes to immunological sensitization in the gut during recurrent allergen ingestion, peanut-specific IgG1 and Th2 cell responses were dramatically reduced in peanut-sensitized mice lacking IgE (Figures 1B–1D). Treg cell induction, which had been markedly impaired in mice with the F709 form of IL-4 α , was fully restored in the absence of IgE (Figures 1E and 1F). Taken together, these findings provide strong evidence that IgE antibodies support the induction of Th2 cell and antibody responses to ingested allergens while suppressing the induction of Treg cells.

Our results with IgE-deficient mice suggest that the application of IgE blockade, which has been employed in clinical food desensitization studies to block undesired anaphylactic

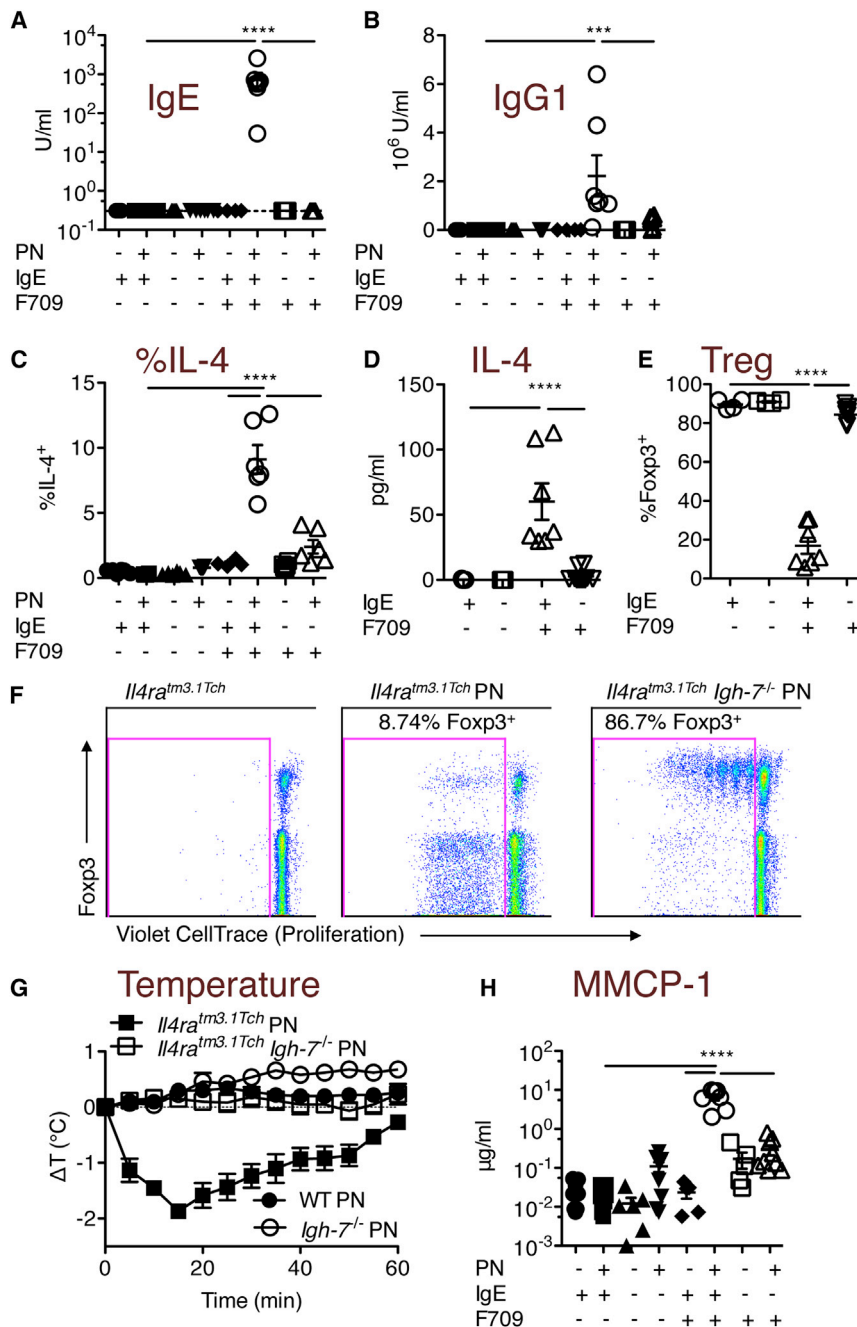


Figure 1. Effects of IgE and IL4 α Genotypes on Food Allergen Sensitization in the Absence of Adjuvant

The effects of IgE on sensitization were evaluated with completely IgE-deficient mice (*Igh*-7^{-/-}) both on the allergy-prone IL-4 α F709 background (*Il4ra*^{tm3.1Tch}) and on wild-type BALB/c.

(A) Peanut-specific IgE levels in sera of mice subjected to enteral gavage with peanut butter (PN). Mice (n = 5–10 per group) were gavaged with 23 mg PN once a week for 4 weeks. The horizontal line indicates the limit of detection in this ELISA. Presence of the activating IL-4 receptor, F709, and IgE are indicated by plus (+) or minus (–).

(B) Serum titers of peanut-specific IgG1 after sensitization.

(C) Flow cytometric analysis of intracellular IL-4 staining in CD4⁺ T cells from mesenteric lymph node (MLN) cells after PN sensitization.

(D) IL-4 levels as determined by ELISA in splenocyte cultures from PN-treated mice restimulated in vitro with PN extract for 5 days.

(E) Analysis of PN-specific Treg cells expanded from MLN cells of PN-treated mice.

(F) Representative flow cytometry plots demonstrate dye dilution (proliferation) among CD3e⁺ CD4⁺ MLN cells labeled with Violet CellTrace and cultured in vitro with PN extract for 5 days. The summary graph shows the Foxp3 phenotype of the divided cells, gating on CD4⁺ viable single cells.

(G) Temperature curves after enteral challenge with high-dose PN. p < 0.0001 by repeated measures (RM) two-way ANOVA, *Il4ra*^{tm3.1Tch} versus *Il4ra*^{tm3.1Tch} *Igh*-7^{-/-}.

(H) Serum MMCP-1 levels as measured by ELISA postchallenge. Data are represented as points for individual mice, with mean $\pm$ SEM in overlay. Similar data were obtained in at least three separate experiments.

prior to sensitization reduced Th2 cell responses and increased peanut-specific Treg cell frequencies (Figures 2B, 2D, and S2D).

Anti-IgE therapy in the peanut allergy model allowed us to also examine the effects of IgE blockade during allergen desensitization in animals with established peanut sensitivity. For desensitization, anti-IgE treatment was started a

reactions, might also modulate immune responses to ingested antigens. Omalizumab, the anti-IgE used in human trials, is “nonanaphylactogenic.” It does not bind to FcεRI-bound IgE and therefore does not activate mast cells. In contrast, available anti-mouse IgE reagents all exhibit some degree of mast cell activation. In order to use these antibodies in the peanut model, we had to mitigate this effect. Taking advantage of a rush desensitization protocol developed by Khodoun et al. (2013), we treated mice with anti-IgE in a 4-day build-up prior to the first exposure to peanut. Neutralization of IgE mimicked genetic deletion of IgE, rendering the IL-4 α mutant mice resistant to anaphylactic reactions (Figure 2A). Anti-IgE treatment

week after the final sensitizing peanut dose was administered, after which large doses (225 mg) of peanut were administered daily for 3 weeks. When finally challenged with peanut, mice that had received anti-IgE did not anaphylax, as expected given the requirement for IgE in anaphylaxis (Figure 2A). In contrast, mice subjected to the same peanut desensitization therapy without anti-IgE exhibited similar anaphylactic reactions to those seen in mice that received no therapy whatsoever. Th2 cell responses were reduced in mice treated with anti-IgE plus peanut, and peanut-specific Treg cell frequencies markedly increased (Figures 2C, 2D, and S2E). Control desensitization therapy with peanut alone did not elicit significant changes in Th2 cell or

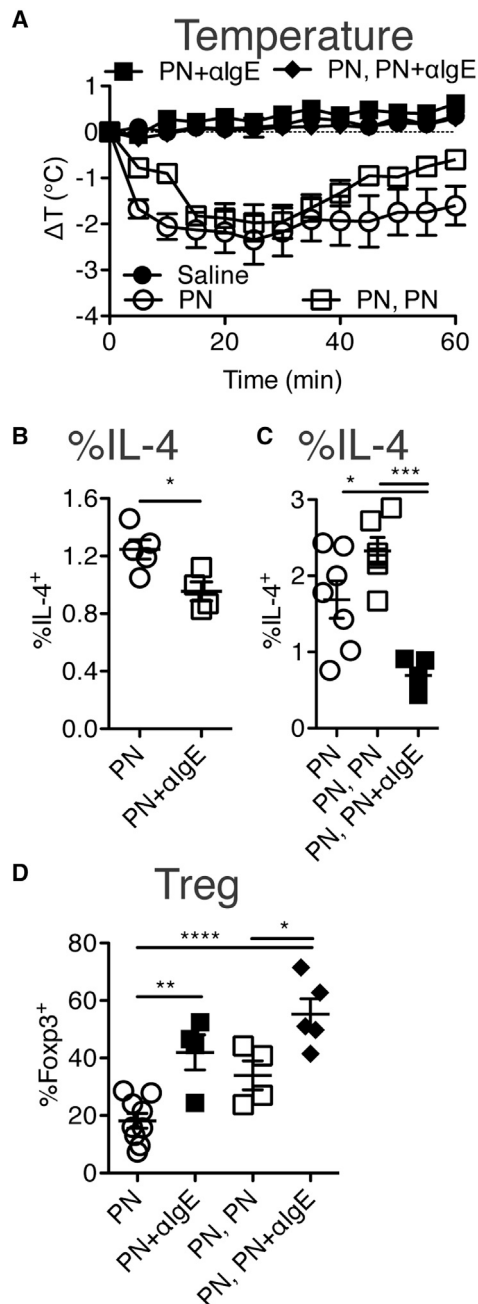


Figure 2. Effects of Anti-IgE on Food Allergen Sensitization

(A) Temperature curves after acute enteral challenge with PN in IL-4 α F709 mutant (*Il4ra*^{tm3.1Tch}) mice (n = 5) treated with PN and/or anti-IgE. Mice were sensitized by enteral gavage with PN (23 mg) once a week for 4 weeks, followed by desensitization with PN (225 mg) once a day for 3 weeks. Anti-IgE (100 μ g total) was injected i.p. in an incremental rush protocol prior to sensitization or desensitization. Abbreviations are as follows: PN+ α IgE: α IgE prior to PN sensitization; PN, PN+ α IgE: PN sensitization, α IgE prior to PN desensitization; PN, PN: PN sensitization, PN desensitization. PN versus PN+ α IgE: p = 0.0011; PN versus PN, PN+ α IgE: p = 0.0022; PN, PN versus PN, PN+ α IgE: p = 0.0002 by RM two-way ANOVA.

(B) Flow cytometric analysis of IL-4 by intracellular staining of CD4⁺ T cells in MLN (n = 4–5).

(C) IL-4 staining in CD4⁺ MLN cells in response to desensitization with PN $\pm$ anti-IgE (n = 5–7).

Treg cell responses to peanut, although there were trends for elevation in both.

Taken together these results indicate that IgE antibodies promote immune sensitization to ingested allergens. In the food-allergy-susceptible mice harboring a dysregulated form of IL-4 α , the presence of IgE during recurrent enteral peanut exposure promoted the induction of peanut-specific Th2 cell, IgE, and IgG1 responses while suppressing the expansion of antiallergic Treg cells. Moreover, IgE blockade in animals with established allergy served to reverse sensitivity with a reduction in Th2 cell responses and expansion of Treg cells.

Mast Cells Amplify Allergic Responses to Ingested Peanut and Suppress Peanut-Specific Treg Cell Induction

We hypothesized that mast cells were the likely effectors of IgE-mediated Th2 cell induction and Treg cell suppression observed in the peanut allergy model. In order to test this, we bred the F709 IL-4 α mutation onto the C57BL/6 genetic background and then intercrossed these mice with *Kit*^{W-sh} mice. These mast-cell-deficient mice exhibited a dramatic (3-log) reduction in peanut-specific IgE along with significantly impaired Th2 cell responses and markedly enhanced Foxp3⁺ Treg cell induction (Figures 3A–3C and S2F). Reconstitution of the mast-cell-deficient mice with WT bone-marrow-derived mast cells (BMMCs) restored Th2 cell responses while partially but significantly restoring IgE production (Figures 3A and 3B). Mast-cell-reconstituted mice exhibited impaired Treg cell generation (Figure 3C). Unlike their mast-cell-sufficient counterparts, mast-cell-deficient mice subjected to peanut sensitization were protected from anaphylaxis, but anaphylaxis was restored by mast cell reconstitution (Figure 3D). In contrast to WT BMMC-reconstituted mice, mice reconstituted with IL-4-deficient mast cells failed to develop peanut-specific IgE, Th2 cells, or anaphylactic responses, instead showing enhanced Treg cell induction (Figure 3). Mast cell reconstitution of the small intestine of mast-cell-deficient mice was confirmed by histologic examination (Figure S3).

Although *Kit*^{W-sh} mice exhibit complete mast cell deficiency and are amenable to reconstitution by BMMCs, making them an attractive model system in which to study mast cell biology, they have an array of immunological defects including dysregulation of granulocytes, introducing potential complications to the interpretation of our results. In order to further evaluate mast cell functions in the peanut allergy model, we took advantage of the Kit-independent Mcpt5^{cre} transgene system to genetically target mast cells. A two-pronged approach was applied that used Mcpt5 promoter-driven Cre expression either to mediate mast cell deletion via induction of iDTR followed by treatment with diphtheria toxin (*Il4ra*^{tm3.1Tch} Mcpt5^{cre} iDTR) or to drive mast-cell-specific excision of the *Sykb* gene (*Il4ra*^{tm3.1Tch} Mcpt5^{cre} *Sykb*^{fl/fl}). We reasoned that removing Syk, the proximal kinase in FcεRI signaling, would paralyze IgE-mediated mast cell activation, allowing us to isolate the role of IgE-mediated mast cell activation within this model (Figures S4 and S5).

(D) Flow cytometric analysis of Foxp3⁺ cells in the CD3 ϵ ⁺CD4⁺ MLN cells exhibiting proliferative responses to PN after in vitro restimulation with 100 μ g/ml PN for 5 days (n = 4–9).

Data are from one of two experiments and are represented as mean $\pm$ SEM.

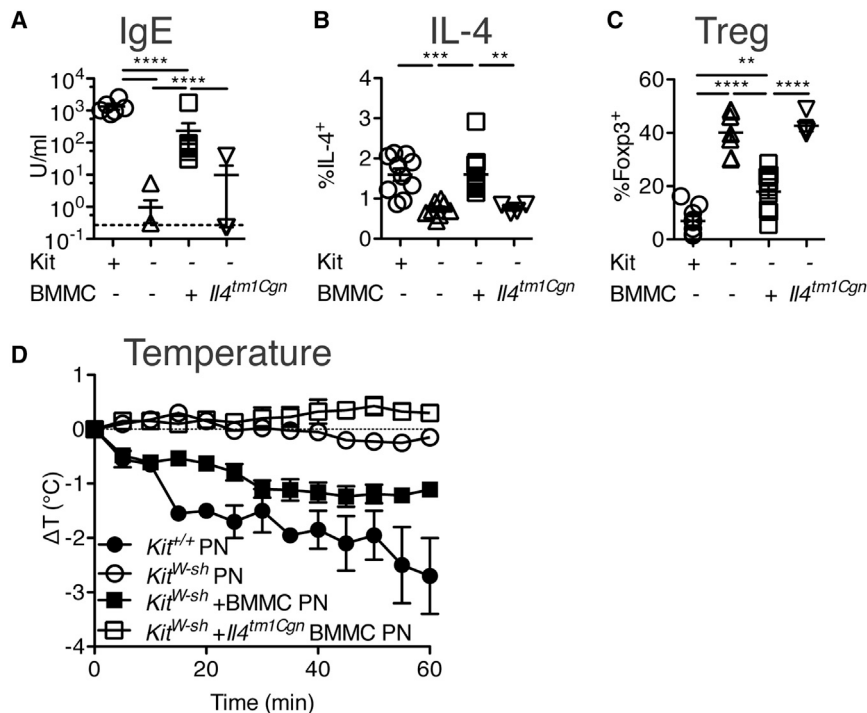


Figure 3. Mast Cells Are Required for Sensitization to Food Allergens

(A) PN-specific IgE levels in sera as determined by ELISA. IL-4 α F709 mutant (*IL4ra^{tm3.1Tch}*) and mast-cell-deficient *IL4ra^{tm3.1Tch}Kit^{W-sh}* mice ($n = 6-8$) were sensitized by enteral gavage with 23 mg PN once a week for 8 weeks. Mast cells were reconstituted in additional *IL4ra^{tm3.1Tch}Kit^{W-sh}* mice by i.p. injection of WT or IL-4-deficient (*IL4^{tm1Cgn}*) BMMCs (5×10^6) at 4 and 8 weeks prior to sensitization. Kit variants are abbreviated as + for WT and – for *Kit^{W-sh}*.

(B) IL-4⁺CD4⁺ T cells in the MLN.

(C) Foxp3⁺ Treg cell frequencies in CD3 ϵ ⁺CD4⁺ MLN cells exhibiting proliferative responses to PN.

(D) Temperature curves after acute enteral challenge with PN (450 mg) after sensitization of IL-4 α F709 mutant (*IL4ra^{tm3.1Tch}*).
 $p < 0.001$ by RM two-way ANOVA *Kit^{+/+}* versus *Kit^{W-sh}*, *Kit^{W-sh}* versus *Kit^{W-sh}* + BMMCs, and *Kit^{W-sh}* + BMMCs versus *Kit^{W-sh}* + *IL4^{tm1Cgn}* BMMCs. Data shown are from one of two experiments and are depicted as mean $\pm$ SEM.

Mice in which these genetic approaches were used to achieve mast cell ablation or mast cell paralysis behaved similarly, exhibiting both a restoration of oral tolerance and reduction in the peanut allergic phenotype. Specific IgE and Th2 cell responses were reduced in these mice relative to controls (Figures 4A and 4B). As we had observed with the *Kit^{W-sh}* model, removal of mast cells was sufficient to restore Treg cell generation to near wild-type levels even in the face of the proallergic IL-4 α F709 mutation (Figure 4C). Anaphylaxis was undetectable in peanut-sensitized *IL4ra^{tm3.1Tch} Mcpt5^{cre} iDTR* mice that received DT treatment prior to sensitization and was similarly absent in peanut-sensitized *IL4ra^{tm3.1Tch} Mcpt5^{cre} Syk^{fl/fl}* mice (Figure 4D). Strikingly, deletion of Syk solely from mast cells was sufficient to restore Treg cell induction, identifying IgE crosslinking of FcεRI on mast cells as the key signal tipping the balance between allergy and tolerance after food allergen exposure in an environment genetically permissive for the induction of food allergy.

In summary, these observations demonstrate that allergic sensitization (Th2 cell responses and IgE production) are reduced and Treg cell responses enhanced in two independent strains of mice harboring the activating IL-4 α mutation but lacking mast cells and in mice in which FcεRI signaling is impaired specifically in mast cells. Repletion of the mast cell compartment by wild-type but not IL-4-deficient BMMC transfer restores the food-allergy-prone phenotype of the IL-4 α mutant mice, confirming a direct role for mast cells and IL-4 produced by them in driving allergic sensitization and suppressing tolerance.

Syk Blockade Prevents Allergic Sensitization in Naive Mice and Facilitates Induction of Tolerance in Food-Allergic Animals

As the key kinase in FcεRI receptor signaling, Syk represents an attractive target for pharmacologic inhibition in the treatment of

allergic disease. We have recently characterized a new highly specific, potent, orally bioavailable Syk inhibitor, SYKi,

and applied this to probe the consequences of FcεRI blockade in the peanut model (Moy et al., 2013). SYKi displayed a dose-dependent inhibition of passive IgE-mediated anaphylaxis and completely abrogated the lethal anaphylactic reaction to acute food allergen challenge in sensitized IL-4 α mutant mice (Figure S6). We anticipated that inhibition of Syk would block sensitization to food allergens, much the way that sensitization was blocked in mice with targeted genetic deletion of Syk in mast cells. This proved to be the case. Peanut-specific IgE responses were very low or undetectable in mice treated with SYKi during sensitization, and T cell responses correspondingly shifted from Th2 cell biased to Treg cell dominated (Figures 5A–5C). Peanut-specific IgG1 responses remained largely intact in SYKi-treated mice, indicating that the absence of IgE did not result from a global suppression of B cell function. However, we cannot rule out effects on Syk-mediated B cell receptor signaling by this compound (Figure 5D). Treatment with SYKi during sensitization was sufficient to prevent the development of anaphylactic food allergy to peanut, limiting temperature loss and preventing MMCP-1 release (Figures 5E and 5F). To ensure complete clearance of SYKi, peanut challenge was performed after a 10-day “wash-out” period.

In order to test whether Syk inhibition, like anti-IgE treatment, might facilitate tolerance induction in peanut-allergic animals, mice were sensitized to peanut and then separated into groups that received (1) no further treatment, (2) high-dose peanut desensitization, or (3) high-dose daily peanut under cover of SYKi. Mice were evaluated 10 days after the discontinuation of therapy. Mice subjected to peanut sensitization and those with peanut-only desensitization therapy developed normal anaphylactic reactions; the SYKi plus peanut group tolerated the challenge and displayed no symptoms of anaphylaxis (Figures 6A and S7). MMCP-1 release was reduced by approximately 2

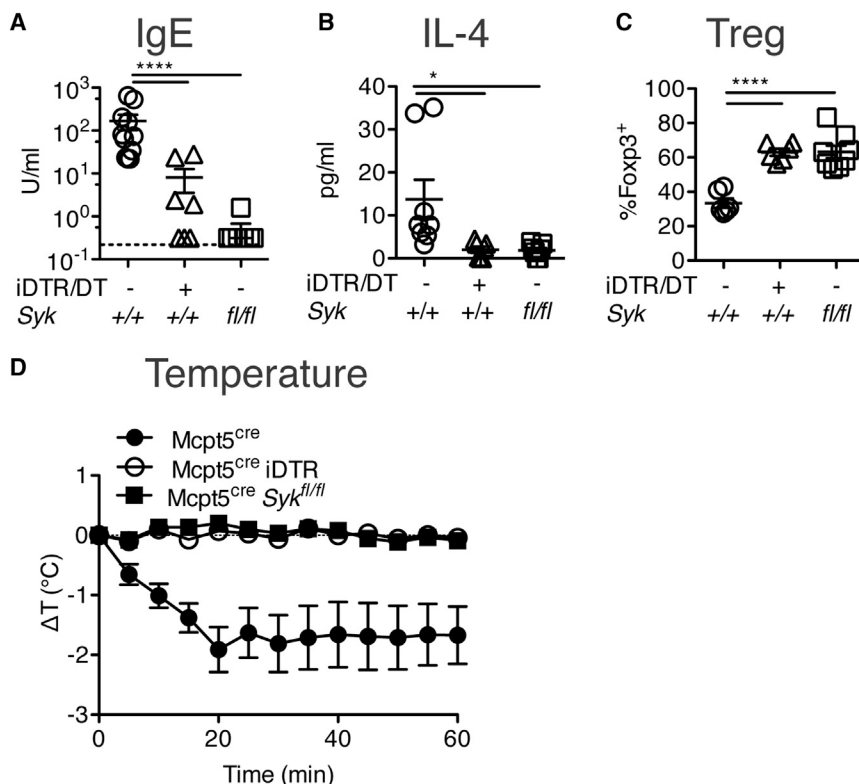


Figure 4. Deletion of Syk in Mast Cells Prevents Peanut Sensitization

(A) PN-specific IgE levels in sera. Mast-cell-directed induction of the diphtheria toxin receptor (DTR) or inactivation of Syk tyrosine kinase on the IL-4 α F709 (IL4 α ^{tm3.1Tch}) background were achieved by Mcpt5^{cre}-driven gene expression. Mice expressing iDTR on mast cells (IL4 α ^{tm3.1Tch} Mcpt5^{cre} iDTR) or with mast-cell-targeted Syk deletion (IL4 α ^{tm3.1Tch} Mcpt5^{cre} Syk^{fl/fl}) (n = 6–11) were sensitized once a week for 4 weeks with 23 mg PN i.g. Mast cells were depleted from Mcpt5^{cre} iDTR mice by i.p. injection of diphtheria toxin over 3 days (100 ng, 500 ng, 500 ng) 1 week prior to initiating PN sensitization (indicated as iDTR DT “+”).

(B) ELISA analysis of PN-specific IL-4 secretion in splenocyte cultures.

(C) Foxp3⁺ Treg cell frequencies among PN-responding CD3 ϵ ⁺CD4⁺ T cells from the MLN.

(D) Temperature curves from PN-treated IL4 α ^{tm3.1Tch} mice after enteral challenge with high-dose PN (450 mg).

p < 0.001 by RM two-way ANOVA Mcpt5^{cre} versus Mcpt5^{cre} iDTR and Mcpt5^{cre} versus Mcpt5^{cre} Syk^{fl/fl}. Data shown are a pool of three independent experiments, represented as mean $\pm$ SEM.

logs (Figure 6B). Peanut-specific Th2 cell responses were nearly absent in the tolerized mice, and specific Treg cell frequencies increased (Figures 6C and 6D). Peanut-specific IgE was reduced in mice receiving SYKi plus peanut compared with mice receiving peanut-only desensitization (Figure 6E). Our data demonstrate that the addition of SYKi to peanut desensitization markedly altered several critical aspects of the food allergen immune response in a manner consistent with tolerance induction (Figure S7).

Tolerance to foods is maintained in part by Treg cells and Treg cell deficiency is associated with severe food allergy (Bennett et al., 2001; Chatila et al., 2000; Wildin et al., 2001). Our analysis of Foxp3⁺ T cell frequencies in both the peanut and OVA food allergy models suggested that the mechanism underlying peanut desensitization in the setting of IgE:FcεRI signaling blockade was the induction of functional Treg cell, capable of suppressing effector T cell responses. This potential mechanism was directly evaluated by preparing Foxp3-eGFP⁺ Treg cells from mice that had been sensitized to peanut in the presence or absence of SYKi and transferring these cells into naive IL4 α ^{tm3.1Tch} recipients, which were in turn sensitized to peanut (Figure 7A). Treg cells from peanut+SYKi-treated donors almost completely ablated specific IgE responses in peanut-fed recipients (Figure 7B). T cell responses in the same mice exhibited significant shifts from Th2 cell to Treg cell biased (Figures 7C, 7D, S2G, and S2H). Only mice given Treg cells from peanut+SYKi-treated mice exhibited reductions in anaphylaxis and MMCP-1 release upon peanut challenge (Figures 7E and 7F). Recipients of Treg cells from mice exposed to peanut in the absence of SYKi had much more modest (and only signifi-

cant in the case of IgE) trends relative to mice that did not receive Treg cells.

Taken together, these results show that pharmacologic blockade of FcεRI signaling via a Syk inhibitor prevents food allergen sensitization and promotes tolerance induction in food-allergy-prone IL-4 α mutant mice. The Treg-cell-skewing effect of Syk inhibition can be exploited to restore tolerance in mice with established allergy. Our observations of food allergy suppression in recipients of Treg cells confirm the role of functional peanut-specific Foxp3⁺ Treg cells in oral tolerance. SYKi has no activity in T cells and in the context of our corroborating data using mice with lineage-specific Syk deletion in mast cells, mast cell deficiency models, and anti-IgE treatment, we attribute the block in functional Treg cell induction to FcεRI signaling blockade rather than any T-cell-intrinsic effect.

DISCUSSION

Mast cells reside at the interfaces between the body and the environment, stationing them as sentinels for the immune system. IgE antibodies arm these innate immune cells for specific antigen recognition. In the setting of food allergy, our findings suggest a triple function for these first-line defenders: (1) as effectors of anaphylactic reactions in allergen-exposed subjects, (2) as amplifiers of nascent Th2 cell and IgE responses during recurring sensitizing ingestions of food allergen, and (3) as suppressors of functional Foxp3⁺ Treg cell induction. Our report shows that mast cells and IgE:FcεRI signaling are required for the induction of adaptive Th2 cell immunity to ingested allergens and that they counter Treg cell expansion; in the absence of mast

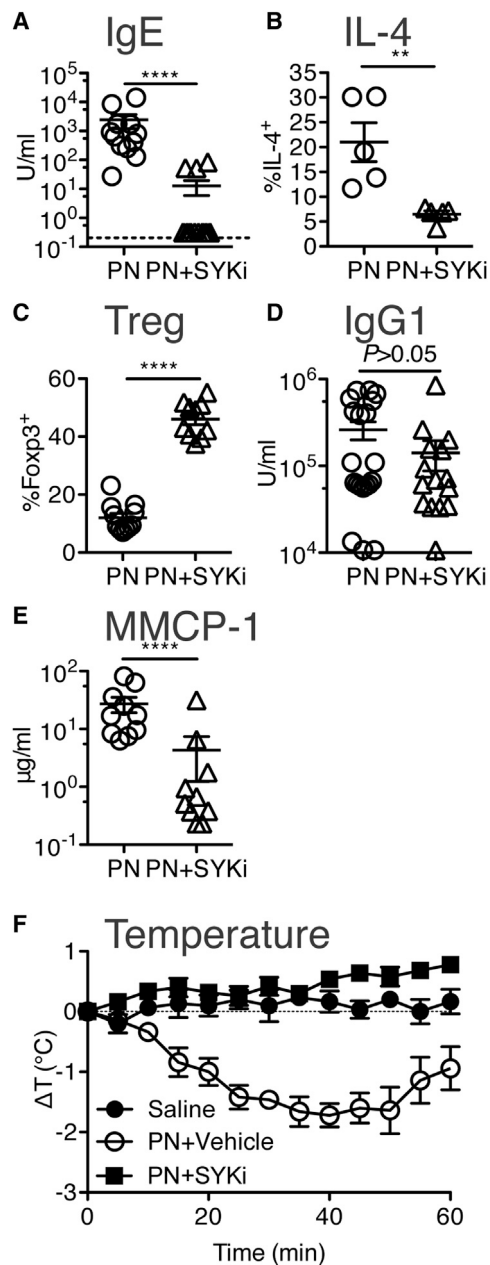


Figure 5. Targeted Pharmacologic Inhibition of Syk Blocks Food Allergen Sensitization

(A) PN-specific IgE levels in sera as measured by ELISA. IL-4 α F709 (*Il4ra*^{tm3.1Tch}) mutant mice (n = 12–15) were sensitized once a week by enteral gavage with PN (23 mg) for 4 weeks. Mice were also gavaged with the Syk inhibitor SYKi (30 mg/kg) or vehicle (10% Tween-80 [pH 8.0], 5 ml/kg) 15 min prior to PN, 10 hr post-PN, and 24 hr post-PN. Abbreviations: PN, PN plus vehicle during sensitization; PN+SYKi, PN plus SYKi during sensitization.

(B) Flow cytometric analysis of IL-4 production by expanded CD4⁺ MLN cells exhibiting proliferative responses to PN in vitro (n = 5).

(C) Frequency of Foxp3⁺ cells among CD3e⁺CD4⁺ T cells proliferating to PN in vitro (n = 12–15).

(D) Serum levels of PN-specific IgG1 after sensitization, as determined by ELISA.

(E) Degranulatory release of MMCP-1 into serum after sensitization and challenge with PN (n = 10–11). There was a 10-day interval between the final SYKi treatment and the PN challenge.

cells or IgE signals, peanut-fed allergy-prone mice exhibited minimal Th2 cell and IgE responses but had robust Treg cell induction. This work was carried out with an innovative array of research tools including the novel IL-4 α mutant model of peanut allergy, in which enteral ingestion alone leads to anaphylactic food sensitivity, and a potent selective Syk inhibitor to modulate IgE:FcεRI signaling. The observation that inhibition of IgE signaling with anti-IgE antibody or Syk inhibition in the setting of pre-existing allergy leads to reversal of IgE and Th2 cell responses along with Treg cell expansion is completely new and quite striking. This result reveals significant plasticity in food allergy responses and points to Syk as an attractive therapeutic target in food allergy.

Food allergy has been notoriously difficult to model in mice. Even with the use of adjuvants and parenteral priming, which bypass normal immune-sensitization mechanisms, responses to food challenge are weak. Challenged animals do not exhibit anaphylaxis, a cardinal symptom of food allergy in patients. The IL-4 α mutant mice have provided a powerful genetic tool to address the pathogenesis of food allergy in a more physiologic setting. We believe the inherent predisposition to allergy, based on enhanced IL-4 α responses, in these mice reflects a cytokine-signaling imbalance that might be at work in atopic humans. We have previously reported that an IL-4 α polymorphism affecting signaling strength is linked to asthma (Hershey et al., 1997). In studies designed to identify loci linked with IgE production, the chromosome 5q31 cytokine cluster, which contains the gene encoding IL-4, is consistently identified (Marsh et al., 1994; Meyers et al., 1994; Xu et al., 1995), and one preliminary study reported a significant association between an *IL4Ra* polymorphism and food allergen-specific IgE levels (Brown et al., 2012). We believe that deletion of the ITIM in the IL-4 α of the mice we have studied and the resultant disinhibition of the receptor are paradigmatic of the situation in atopic humans in whom nascent allergic sensitization also appears to be affected by an altered IL-4 signaling axis.

We and others have previously demonstrated that IgE antibodies exert important effects on mast cells above and beyond the induction of degranulation and immediate hypersensitivity. Even in the absence of antigen, IgE regulates mast cell homeostasis in culture and in vivo and modulates mast cell cytokine production (Asai et al., 2001; Bryce et al., 2004; Kalesnikoff et al., 2001; Kawakami and Kitaura, 2005; Mathias et al., 2009). Similar effects on mast cell cytokine production are observed in systems involving antigen-driven IgE signaling as well (Galli et al., 2005; Nakae et al., 2007a). The effects of IgE-mediated mast cell signaling in modulating Th2 cell and Treg cell responses in the peanut allergy system in this report are corroborated by four independent lines of investigation: the study of IgE-deficient mice, anti-IgE treatment, *Mcpt5*^{cre} *Syk*^{fl/fl} mice, and SYKi treatment. Each of these experimental models demonstrated impairment of Th2 cell and IgE responses along with

(F) Temperature curves after acute challenge with PN (450 mg) in PN+vehicle-, PN+SYKi-, or saline-treated mice (n = 5).

p < 0.001 by RM two-way ANOVA PN versus PN+SYKi. Data in (B) and (F) are representative of two experiments each, whereas the data in (A), (C), (D), and (E) have been pooled from three independent experiments. Data are shown as points for individual mice with mean $\pm$ SEM.

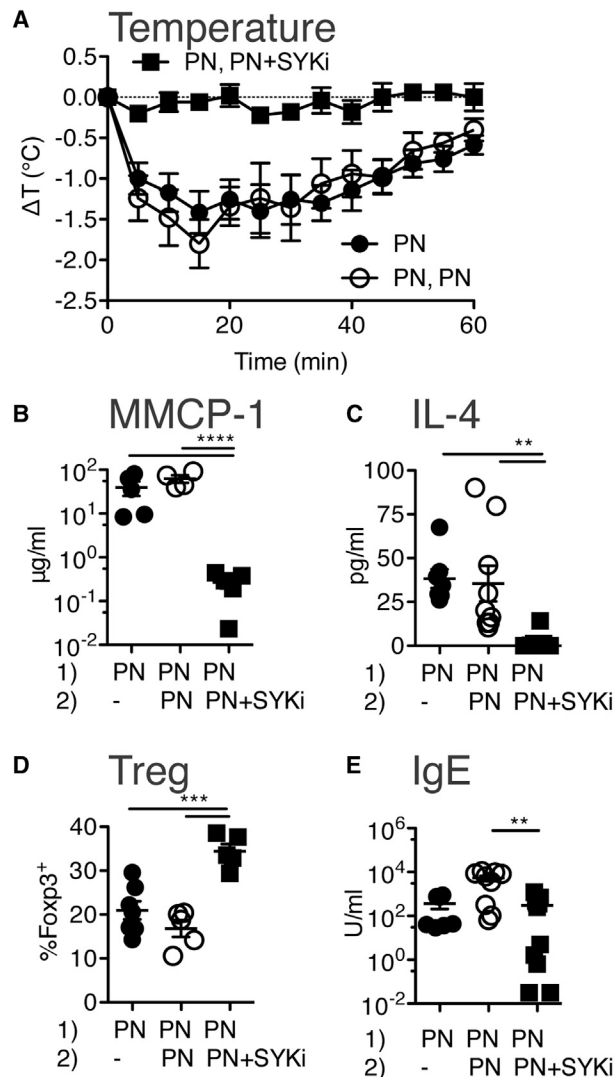


Figure 6. Pharmacological Inhibition of Syk Activity Enhances Food Allergen Desensitization

(A) Temperature curves after acute challenge with PN in PN-sensitized (PN), PN+SYKi-desensitized (PN, PN+SYKi), and mock-desensitized (PN, PN) mice ($n = 5$). PN-sensitized IL-4 α F709 (*Il4ra*^{tm3.1Tch}) mutant mice were subjected to 3 weeks of daily oral desensitization therapy with PN (225 mg) (PN), or PN paired with SYKi (30 mg/kg, p.o., b.i.d.) (PN, PN+SYKi). The final challenge was performed in the absence of SYKi, 10 days after the final SYKi dose. PN-sensitized mice that received no therapy after sensitization are shown for comparison (PN). $p = 0.0016$ by RM two-way ANOVA for PN versus PN, PN+SYKi or PN, PN versus PN, PN+SYKi.

(B) MMCP-1 levels in serum postchallenge ($n = 4-5$).

(C) IL-4 secretion by splenocyte cultures in response to PN stimulation ($n = 9-10$).

(D) PN-specific Treg cell frequencies in dividing MLN CD3 ϵ ⁺CD4⁺ cells ($n = 5-6$).

(E) Serum levels of peanut-specific IgE ($n = 9-10$).

Data shown are representative of two independent experiments (A, B, and D) or have been pooled from two experiments (C and E) and are represented as mean $\pm$ SEM.

enhanced Treg cell induction, providing a strong aggregate case for a Th2 cell adjuvant effect of IgE signaling in emerging food allergy responses. These results have potential implications for the design of strategies to achieve safe and effective oral desensitization in patients with peanut allergy. Although anti-IgE treatment during food desensitization has recently been applied in clinical trials, the immunomodulatory effects of such IgE blockade are not yet known.

The role of mast cells in murine disease models is challenging to assess given the limitations of each of the various mast cell deficiency models (Rodewald and Feyerabend, 2012). The fully consistent results we independently obtained with both Kit-dependent and -independent models of deficiency (*Kit*^{W-sh} and *Mcpt5*^{Cre} iDTR, respectively) provide strong converging support for a critical mast cell contribution to Th2 cell induction and Treg cell suppression. Whereas *Mcpt5*^{Cre} iDTR mice have previously been reported to have an intact mucosal mast cell compartment (based on baseline histologic analyses of the stomach), we have found that IL-4-driven and allergen-induced mucosal mast cell expansion in the jejunum is in fact suppressed in these animals, and it has been reported that MMCP-5 expression is not restricted to submucosal mast cells under Th2 cell conditions (Friend et al., 1996; Xing et al., 2011). DT-treated animals exhibited both impaired mast cell expansion and the same block in Th2 cell and IgE responses observed in IgE-deficient and *Kit*^{W-sh} mutants.

Although mast cells are not MHCII⁺ and may not function as antigen-presenting cells in this food allergy model system, we observed that they accumulate in the draining lymph nodes of sensitized animals, placing them proximal to T cell priming events. By immunofluorescent microscopy, both Treg cells and mast cells are present in the jejunal lamina propria and Peyer's patches (data not shown). Mast cells are known to produce significant amounts of the critical Th2-cell-inducing cytokine IL-4, which has the capacity both to subvert Treg cell generation, by destabilizing Foxp3 expression, and to simultaneously activate the Th2 and Th9 cell pathways via STAT6 signals (Dardalhon et al., 2008; Wang et al., 2010). We observe that when mast cells are cultured with naive T cells under Treg-cell-inducing conditions, FcεRI crosslinking inhibits TGF- β -driven expansion of Foxp3⁺ T cells. Furthermore, we find that *Il4* and *Il13* transcripts are very rapidly induced (within 1 hr) in the small intestine after allergen exposure and that these transcripts are Syk and IgE dependent, strongly implicating mast cells as cellular sources of IL-4 and IL-13 early after food ingestion. Our results from WT versus IL-4-deficient mast cell reconstitution of mice lacking mast cells demonstrate an essential role for mast-cell-derived IL-4 in promoting sensitization to ingested allergen.

Our findings that (1) peanut-specific Treg cell frequencies are higher in IgE-deficient, anti-IgE-treated, mast cell-deficient, mast cell Syk-deficient, and SYKi-treated mice and that (2) Treg cells from peanut-fed SYKi-treated mice functionally suppress the induction of peanut allergy in naive recipients identify a central mechanism whereby IgE antibodies and mast cells enhance emerging allergic sensitization: they suppress effective Treg cell responses. We and others have previously demonstrated that inherited Foxp3 deficiency leads to severe intractable food allergy and that patients with Foxp3 mutations lack

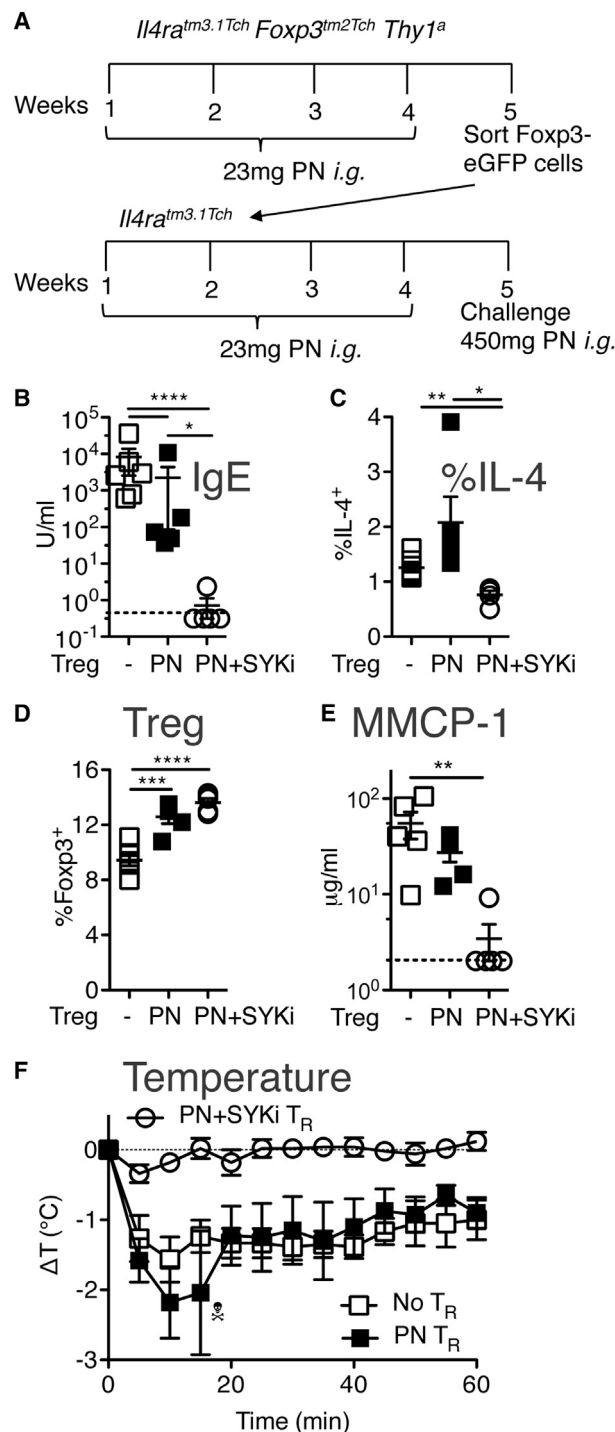


Figure 7. Treg Cells Induced while Mast Cells Are Paralyzed Effectively Control Food Allergen Sensitization

(A) Experimental schematic. IL-4 α F709 mutant Thy1.1-congenic Foxp3-eGFP (*Il4ra*^{tm3.1Tch} *Foxp3*^{tm2Tch} *Thy1*^a) donor mice ($n = 5$) were sensitized to peanut with or without SYKi for 4 weeks, at which point their CD4⁺Foxp3-eGFP⁺ Treg cells (T_R) were purified and transferred (4×10^5 /mouse) into naive IL-4 α F709 (*Il4ra*^{tm3.1Tch}) recipients (for PN and PN+SYKi T_R groups). Mice that did not receive Treg cells are included as positive controls for peanut sensitization. All recipient mice were sensitized with 23 mg PN once a week for 4 weeks and challenged with 450 mg PN by gavage ($n = 5$).

functional Treg cells (Bennett et al., 2001; Chatila et al., 2000; Wildin et al., 2001).

Syk inhibition offers an attractive pharmacologic approach for blocking IgE-induced FcεRI signaling. Previously available small-molecule Syk inhibitors have had significant off-target effects, however, especially on the closely related kinase ZAP70 in T cells. SYKi, used in this study, has >25-fold enhanced specificity for fully phosphorylated Syk versus ZAP70 (Moy et al., 2013). Although Syk is also the proximal signaling kinase for the B cell receptor (BCR), we believe that its major effects on allergic responses to peanut in our study resulted from its FcεRI inhibition. In support of this conclusion is our finding that the phenotype of peanut-fed mice in which Syk is specifically inactivated in mast cells, but not B cells, exactly mirrors that of the SYKi-treated animals. BCR activation is normal in the mice with mast-cell-targeted Syk gene deletion yet they exhibited the same impairment in Th2 cell and IgE induction and enhanced Treg cell responses to SYKi-treated mice, consistent with an FcεRI-driven effect.

In summary, our study provides clear evidence that IgE-mediated mast cell activation serves not only to elicit immediate hypersensitivity reactions but also to amplify evolving allergic sensitization to ingested proteins. We speculate that manipulations of the IgE axis might some day provide clinical benefit.

EXPERIMENTAL PROCEDURES

Mice

Detailed mouse information can be found in the Supplemental Experimental Procedures. All mice were bred and maintained under specific-pathogen-free conditions at Boston Children's Hospital. All animal experiments were conducted under procedures approved by the Boston Children's Hospital Institutional Animal Care and Use Committee.

Allergic Sensitization and Anaphylaxis

Mice were sensitized by enteral gavage with ball-tipped 18 g feeding needles. Peanut butter (Skippy, Hormel Foods) was gavage in 250 μ l 0.1 M sodium bicarbonate (pH 8.0). Mast cells were depleted by i.p. injection of diphtheria toxin (List Biological Laboratories) in saline over 3 days: 100 ng day 1, 500 ng day 2, and 500 ng day 3. SYKi was prepared as previously described (Moy et al., 2013) and solubilized in pH 8.0 aqueous isosmotic Tween-80 (10% w/v, Sigma). SYKi (30 mg/kg) and vehicle control were delivered by gavage in 5 ml/kg. Anti-IgE (clone R35-92, BD) was injected i.p. in tripling doses every hour starting at 50 ng, integrating to a total of 25 μ g the first day. Over the next 3 days, 25 μ g anti-IgE was injected once a day i.p., giving a total of 100 μ g anti-IgE per mouse. Core body temperature measurements taken to monitor anaphylaxis were recorded by subdermally implanted transponders (IPTT-300) and a DAS-6001 console (Bio Medic Data Systems) linked to a netbook. For sensitization protocols and further information, see Supplemental Experimental Procedures (Figure S1).

(B) PN-specific IgE production in recipient mice.

(C) Flow cytometric analysis of IL-4 in CD4⁺ T cells from the MLN.

(D) Foxp3⁺ Treg cell frequencies in the MLN of recipient mice as assessed by flow cytometric analysis of CD4⁺CD3 ϵ ⁺ cells taken directly ex vivo.

Data in (C) and (D) exclude Thy1.1⁺ donor cells from the analyses.

(E) Serum MMCP-1 release after acute enteral challenge of recipient mice with PN (450 mg).

(F) Core temperature loss after PN challenge in recipient mice ($p < 0.001$ by RM two-way ANOVA No T_R versus PN+SYKi T_R and PN+SYKi T_R versus PN T_R). The skull-and-crossbones symbol indicates a death from anaphylaxis in the PN T_R group.

Data are from a single experiment, and are shown as mean $\pm$ SEM.

Cell Culture

Bone-marrow-derived mast cells (BMMCs) were cultured as previously described (Burton et al., 2013). BMMCs were used for reconstitution experiments after 3–6 weeks of culture. Splenocytes were cultured in RPMI-1640 supplemented as previously described (Burton et al., 2013) and stimulated for antigen-specific recall with 100 µg/ml peanut extract. Endogenous allergen-specific T cells were identified by allergen-induced ex vivo proliferation, along the lines of assays developed for assessing human tetanus toxoid recall (Narendran et al., 2002). MLN cells (5×10^6) were labeled with Violet CellTrace (Life Technologies) and cultured with or without allergen (100 µg/ml peanut extract) for 5 days. Cells undergoing proliferation (dye dilution) were considered allergen specific, a conclusion supported by a lack of proliferation in the absence of allergen or in allergen-stimulated cells from unsensitized mice. We note that this approach does not provide the absolute number of Treg cells residing in the MLN but nevertheless gives a robust readout of the relative efficiency with which Treg cells are induced among experimental groups.

Mast Cell Reconstitution

Weanling *Il4ra^{tm3.1Tch} Kit^{W-sh}* mice were injected i.p. with 5×10^6 BMMCs and a second injection of BMMCs was performed 4 weeks later. Allergic sensitization was started 8 weeks after the initial transfer, at which point mast cell reconstitution of intestinal compartments has been shown to be evident (Grimbaldeston et al., 2005). Reconstitution was performed with matched cultures of WT C57BL/6J and IL-4-deficient *B6.129P2-Il4^{tm1Cgn}/J* (stock number 002253) mast cells prepared from age-matched female mice purchased from Jackson Laboratories.

Histology

Sections of the jejunum were taken approximately 10 cm from the pyloric sphincter and fixed overnight in 10% formalin (Sigma). Samples were stored in 70% ethanol prior to processing and toluidine blue staining by the Beth Israel Deaconess Medical Center Histology Core. Chloroacetate esterase staining, which stains mast cells with a characteristic red color, was performed as previously described (Burton et al., 2013; Friend et al., 1998). Samples were randomized and coded prior to histological processing, with scoring and staining being performed by an investigator unaware of the sample identities.

Statistics and Data Analysis

Data were graphed and analyzed with GraphPad Prism 5.0. Anaphylaxis temperature curves were analyzed by repeated-measures two-way ANOVA for overall distinction between treatment groups. Unpaired t tests were used to compute two-tailed p values for comparisons between two groups. For three or more groups, ANOVA was used, with individual p values coming from Bonferroni posttests. p values are abbreviated as *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. Because peanut-specific IgE and MMCP-1 levels varied by a few orders of magnitude, these data are presented on graphs with logarithmic y axes and were log-transformed prior to statistical analysis. Samples in which specific immunoglobulins or MMCP-1 were undetectable were assigned a nominal value corresponding to the limit of detection in the assay (0.3125 units/ml for peanut-specific IgE). Data are presented as mean ± SEM, with points representing individual mice where presented.

SUPPLEMENTAL INFORMATION

Supplemental Information include seven figures and Supplemental Experimental Procedures (including flow cytometry, cell sorting, and ELISA) and can be found with this article online at <http://dx.doi.org/10.1016/j.immuni.2014.05.017>.

AUTHOR CONTRIBUTIONS

O.T.B., H.C.O., M.N.-R., and T.A.C. designed experiments. O.T.B. and M.N.-R. performed experiments and developed experimental models. S.L.L. and J.S.Z. helped carry out experiments. K.J.K. and A.R.D. provided technical assistance and developed assays. H.H. and M.A.C. participated in the experimental design and data analysis for the SYKi experiments. A.R. provided *Mcp5^{Cre}* mice and participated in the analysis of the *Mcp5^{Cre}* data. O.T.B.

and H.C.O. wrote the manuscript. All authors participated in discussions of experimental results and edited the manuscript.

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Supplemental Information

Immunoglobulin E Signal Inhibition during Allergen Ingestion Leads to Reversal of Established Food Allergy and Induction of Regulatory T Cells

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Inventory of Supplemental Materials

Figure S1. Schematics of experimental food allergy procedures, related to Experimental Procedures and Figure 1.

Figure S2. Specificity of hypersensitivity reactions in *Il4raF709* mice, related to Figure 2.

Figure S3. Analysis of intestinal mast cell numbers in mast cell-deficient mice, related to Figure 3.

Figure S4. Characterization of Mcpt5-Cre mice, related to Figure 4.

Figure S5. IL-4-driven expansion of mast cells in Mcpt5-Cre/iDTR mice, related to Figure 4.

Figure S6. Effects of Syk inhibition on anaphylaxis, related to Figure 5.

Figure S7. Immunological effects of SYKi-facilitated food allergen desensitization, related to Figure 6.

Supplemental Experimental Procedures

Supplemental References

Supplemental Figure Legends

Supplemental Materials

Supplemental Figures

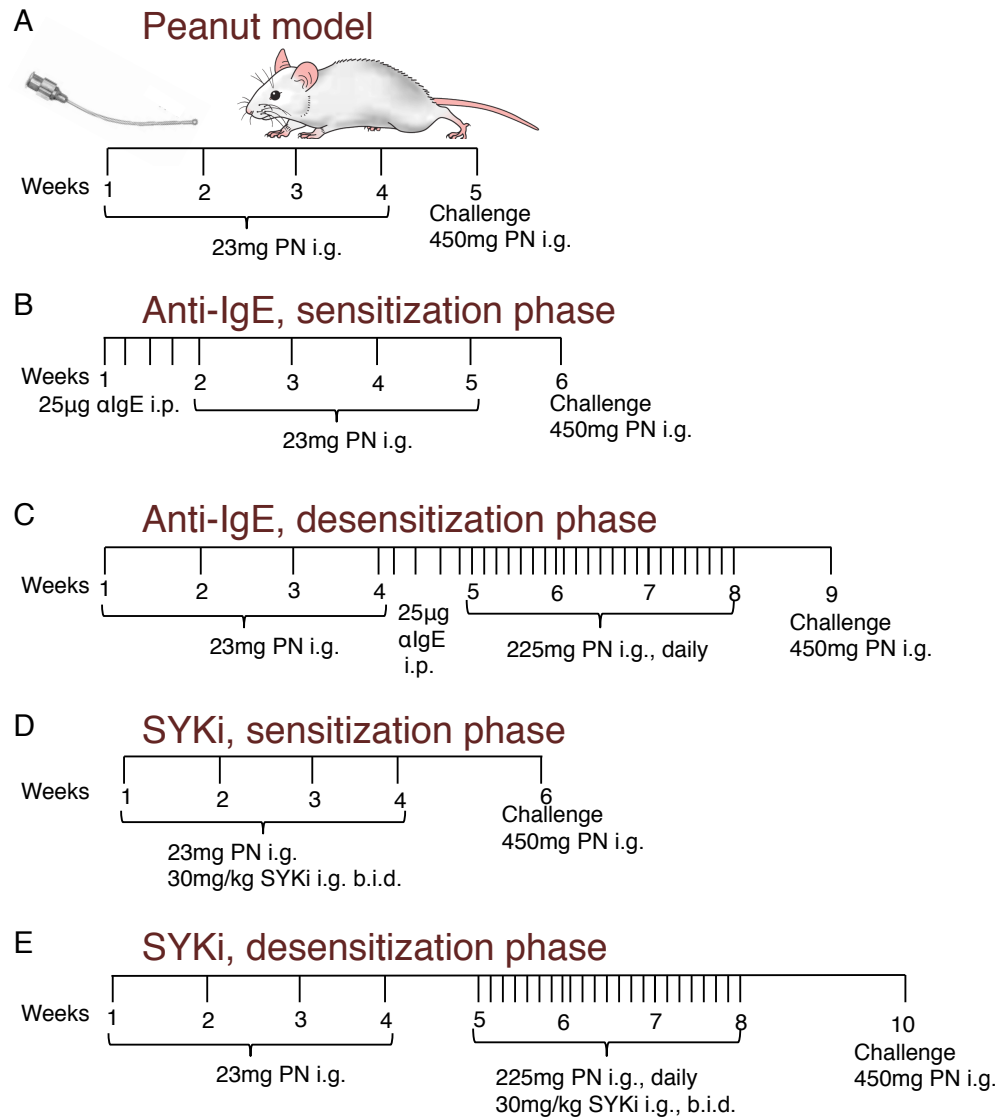


Figure S1, related to Experimental Procedures. Schematic of experimental food allergy procedures. **A)** Basic four-week peanut sensitization model in IL-4 α F709 mutant (*Il4ra*^{tm3.1Tch}) mice. Mice receive 23mg peanut butter (5mg protein) in 250 μ l 0.1M sodium bicarbonate pH 8.0 by oral gavage once a week over four weeks. Mice are challenged with an enteral bolus dose of 450mg peanut butter (~100mg protein) diluted with water 7-10 days after the final sensitization dose, and core body temperature readings are taken. **B)** Anti-IgE prevention therapy. To neutralize endogenous IgE, mice receive 100 μ g anti-IgE over the course of four days. On the first day, anti-IgE is injected *i.p.* in doubling or tripling doses every 90min starting at 50ng, and reaching a total of 25 μ g. On the second through fourth days, 25 μ g anti-IgE is injected *i.p.* in a single shot. Peanut sensitization is then accomplished as in A, starting three days after the completion of anti-IgE treatment. **C)** Anti-IgE assisted allergen desensitization model. Mice are sensitized to peanut as in A, and then treated with anti-IgE as in C. Desensitization therapy then begins, with mice receiving gavage doses of 225mg peanut butter (50mg protein) daily for three weeks. After 7-10 days of rest, mice are challenged with 450mg peanut butter (100mg protein) to assess anaphylaxis. **D)** SYKi-mediated allergy prevention model. Mice are sensitized to peanut as in A (or OVA as in B), but also receive two daily gavage doses of SYKi (30mg/kg) or vehicle (10% Tween-80 pH 8.0, 5ml/kg), the first 15min prior to allergen exposure, the second 10-12hrs later. SYKi is only administered once a week, in conjunction with allergen. Challenge occurs 10 days after the final sensitization dose, in the absence of SYKi. **E)** SYKi desensitization therapy.

Mice are sensitized to peanut. One week after the final sensitization dose, mice are subjected to daily dosing of 225mg peanut (50mg protein) and SYKi or vehicle (dosing as in E) for three weeks. Challenge occurs 10 days after the final desensitization dose, in the absence of SYKi.

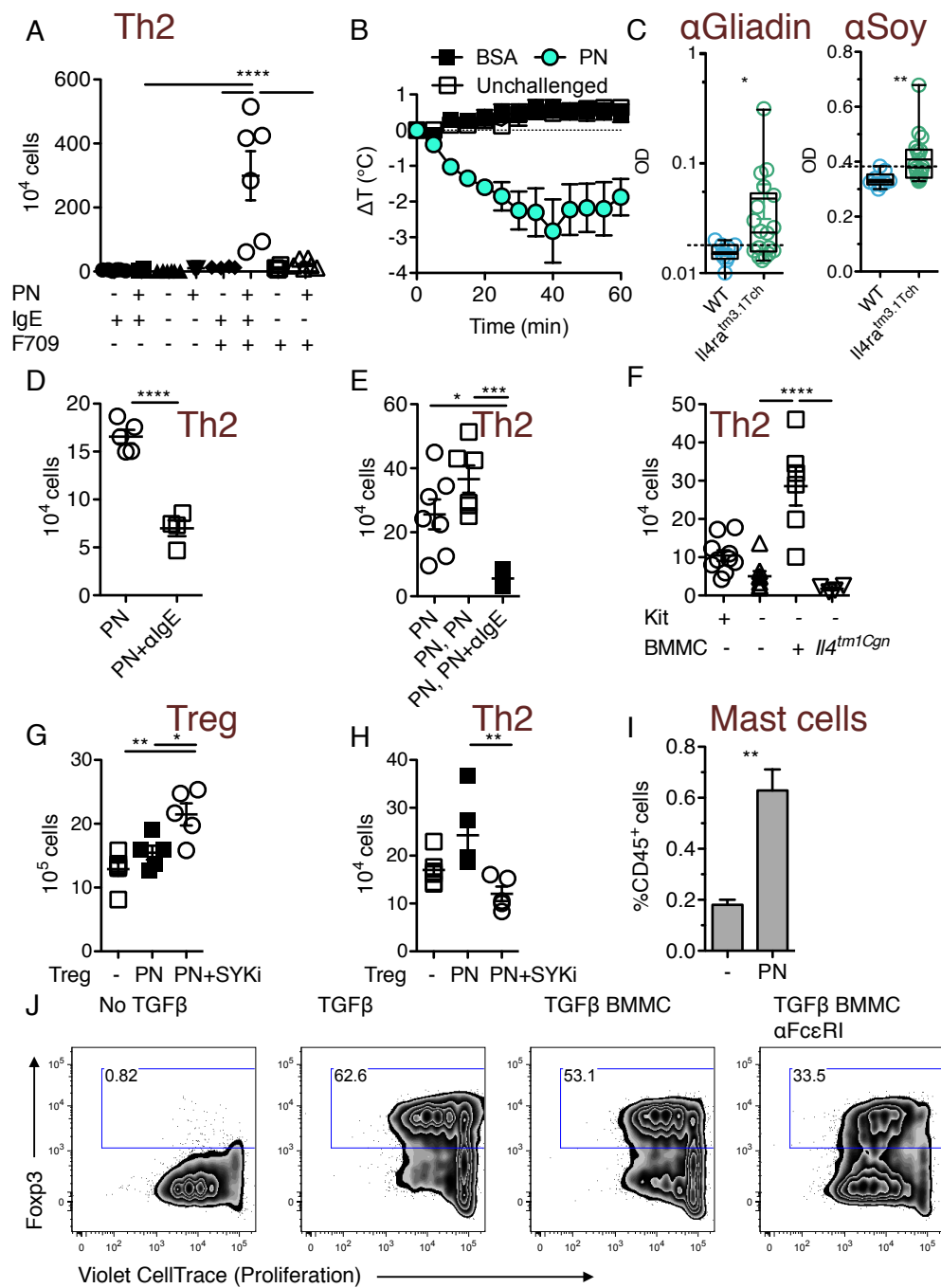


Figure S2, Related to Figures 1, 2, 3 and 7. Spontaneous sensitization to chow components and specificity of food hypersensitivity reactions in IL-4 α F709

mutant (Il4ra^{tm3.1Tch}) mice. A) Effect of IgE genotype on total numbers of Th2 cells in the MLN. Refer to Figure 1C. **B)** Challenge of peanut-sensitized IL-4 α F709 mutant *Il4ra^{tm3.1Tch}* mice ($n=5$) with an equivalent mass (100mg) of an irrelevant protein (BSA). $P=0.0041$ by repeated measures two-way ANOVA for BSA vs. PN challenge. **C)** Optical density (OD) values for IgE against gliadin (wheat) and soy in sera from 16-20 week old WT and *Il4ra^{tm3.1Tch}* mice. The dashed line indicates the background without serum. $*P<0.05$, $**P<0.01$ by unpaired t test on log-transformed values. **D)** Effect of α IgE treatment prior to sensitization on numbers of Th2 cells in the MLN. Refer to Figure 2B. **E)** Effect of α IgE prior to desensitization on Th2 cell numbers. Refer to Figure 2C. **F)** Effect of mast cells on Th2 cell numbers using *Il4ra^{tm3.1Tch} Kit^{W-sh}* mice. Refer to Figure 3B. **G)** Endogenous Thy1.2⁺ Treg cell numbers in the MLN of peanut-sensitized *Il4ra^{tm3.1Tch}* mice receiving Thy1.1⁺ Treg cells from SYKI-treated peanut-sensitized *Il4ra^{tm3.1Tch}* mice. Refer to Figure 7D. **H)** Th2 cell numbers in Treg cell recipients as in panel G. Refer to Figure 7C. **I)** Mast cell frequencies in the MLN following peanut sensitization. $**P<0.01$ by unpaired t test. **J)** Influence of mast cell activation on Treg cell induction *in vitro*. Violet CellTrace-labeled DO11.10 Rag-deficient Foxp3-reporter CD4⁺ T cells were cultured on plate-bound α CD3 and α CD28 for 5 days with TGF β . BMMC were added at a 1:1 ratio and activated by plate-bound α Fc ϵ RI. Foxp3 (eGFP) expression was measured by flow cytometry. Data in C are from a single cohort, while the others were replicated in at least one additional experiment. Where summarized, data are presented as mean $\pm$ SEM.

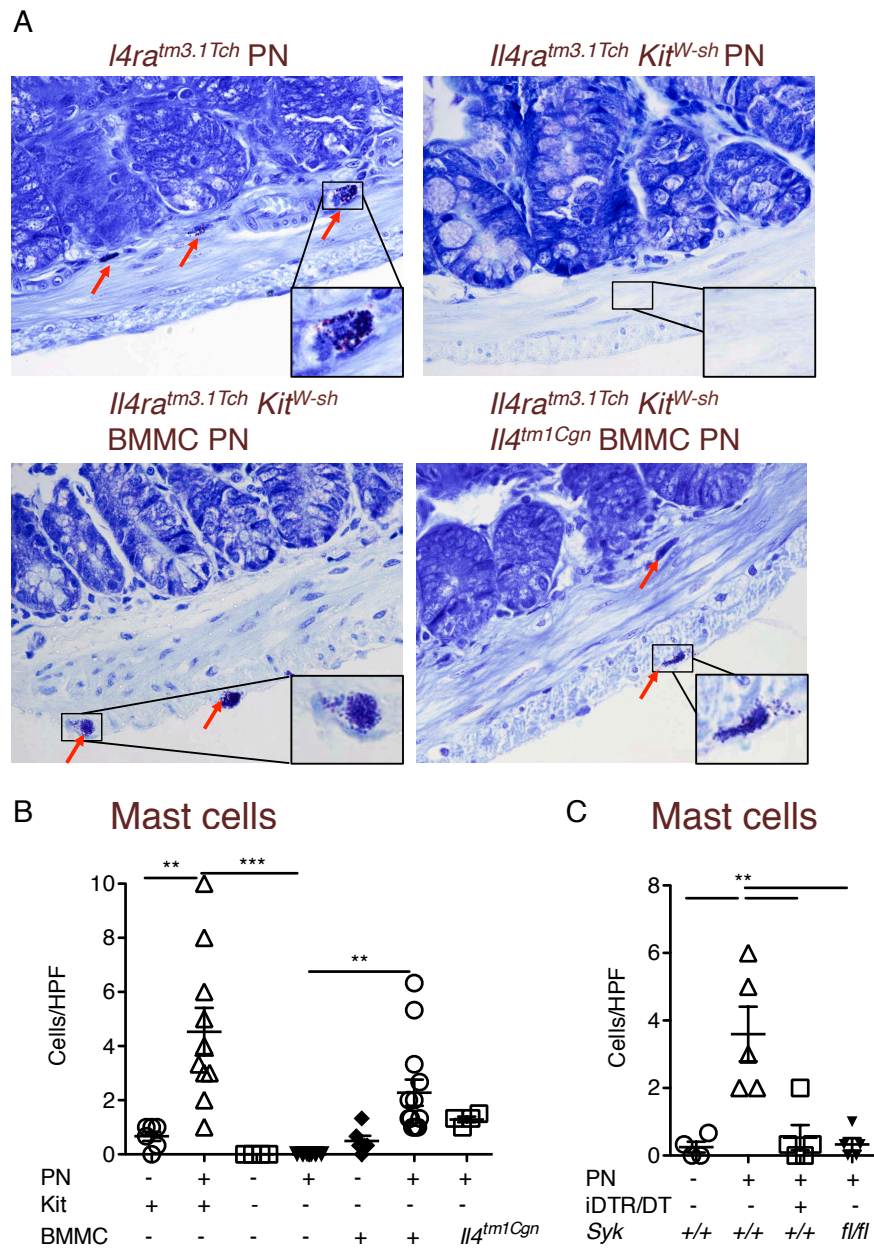


Figure S3, related to Figures 3 and 4. Analysis of intestinal mast cells in mast cell-deficient mice. A) Representative toluidine blue-stained sections of jejunum

with red arrows showing mast cells. Insets show selected mast cells at higher power. The inset for the unreconstituted IL-4 α F709 mutant *Il4ra*^{tm3.1Tch} *Kit*^{W-sh} shows a random section of jejunum without a mast cell. **B)** Mast cell counts from toluidine blue-stained sections of jejunum. Mast cell reconstitution and PN sensitization were performed as in Figure 3. **C)** Mast cell counts in chloroacetate esterase-stained sections of jejunum from mice with the Mcpt5^{cre} transgene. Diphtheria toxin (DT) was injected *i.p.* into *Il4ra*^{tm3.1Tch} Mcpt5^{cre} iDTR mice one week prior to sensitization, and the mice were sensitized to PN for four weeks as in Figure 4. Note that mast cells begin to recover from depletion after approximately three weeks (Dudeck et al., 2011; Scholten et al., 2008), and this analysis is performed five weeks post-depletion. Data in B are pooled from three experiments, while the data in C are from one of three experiments. Data are presented with mean $\pm$ SEM in B and C.

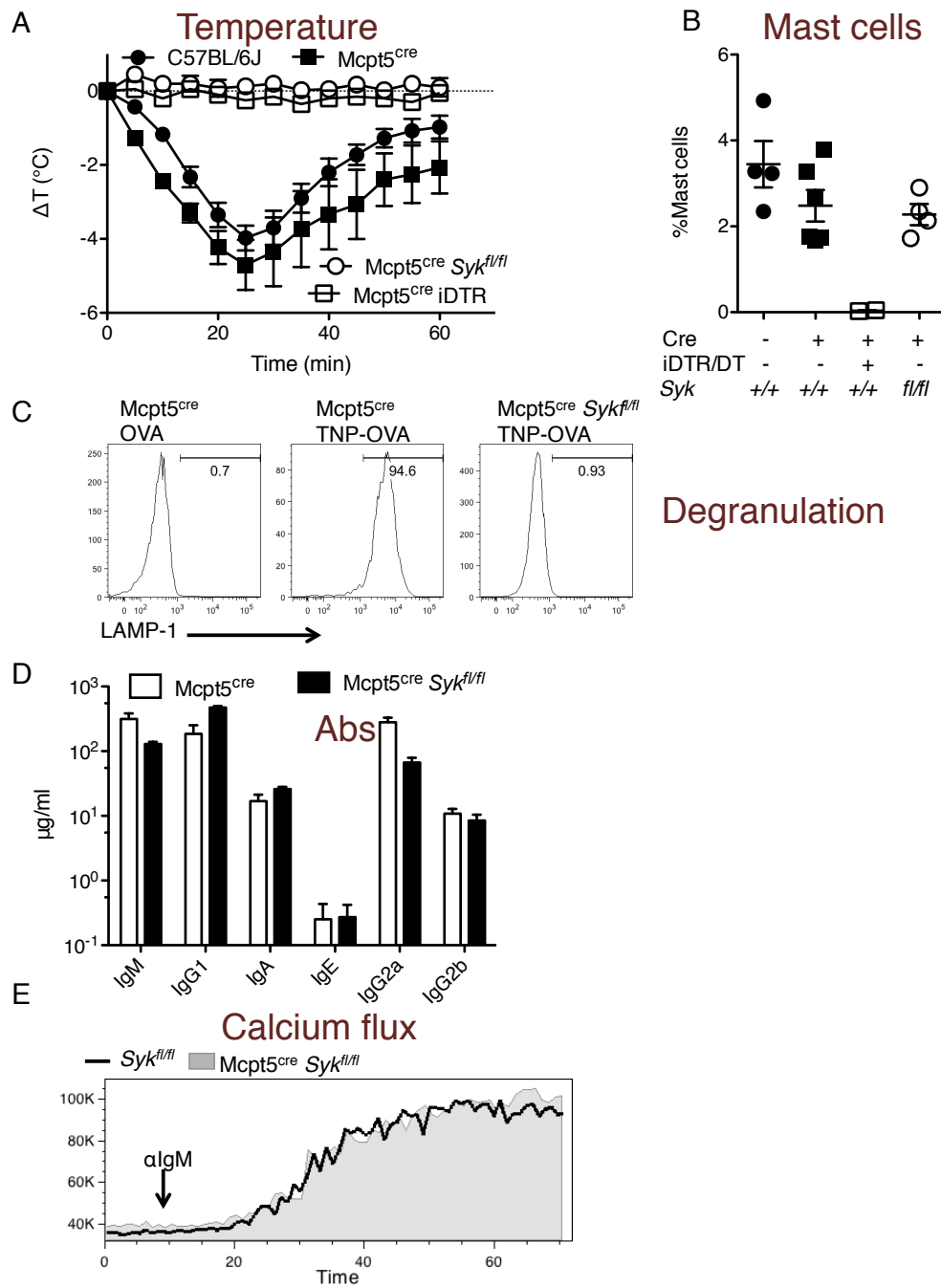


Figure S4, related to Figure 4. Characterization of *Mcpt5^{cre}* mice. A)

Temperature responses to passive anaphylactic challenge. Wild-type C57BL/6J,

Mcpt5^{cre}, Mcpt5^{cre} Syk^{fl/fl} and Mcpt5^{cre} iDTR mice ($n=4-6$) were injected *i.p.* with 2 μ g IgE anti-TNP, and challenged *i.p.* with 20 μ g TNP-OVA four days later. Mcpt5^{cre} iDTR mice were treated with DT in the interim to deplete mast cells as in Figure 4. Core body temperature was measured using subcutaneously implanted transponders. Data are presented as mean $\pm$ SEM. **B)** Frequencies of mast cells in peritoneal lavage fluid. Mast cells (c-Kit⁺Fc ϵ RI α ⁺) were detected by flow cytometry. **C)** Flow cytometric analysis of mast cell degranulation by LAMP-1 staining. Mast cells were expanded from peritoneal lavage cells by culture for two weeks in IL-3 (10ng/ml), SCF (20ng/ml) and IgE anti-TNP (100ng/ml). Mast cells were stimulated for ten minutes with OVA-TNP (100ng/ml) in the presence of anti-LAMP-1-PerCP-e710 to detect degranulation and co-stained with anti-c-Kit-PE. Unconjugated OVA (100ng/ml) was used as a negative control for manipulation. Non-viable cells were excluded using eFluor780 fixable viability dye. **D)** ELISA analysis of basal immunoglobulin levels in Mcpt5^{cre} and Mcp5^{cre} Syk^{fl/fl} mice ($n=4$). **E)** Kinetics of calcium flux in B cells from Mcpt5^{cre} and Mcpt5^{cre} Syk^{fl/fl} mice upon stimulation through the BCR (anti-IgM). Similar data were obtained in an additional set of experiments. Data in A and B are mean $\pm$ SEM, while in D the mean +SEM is presented.

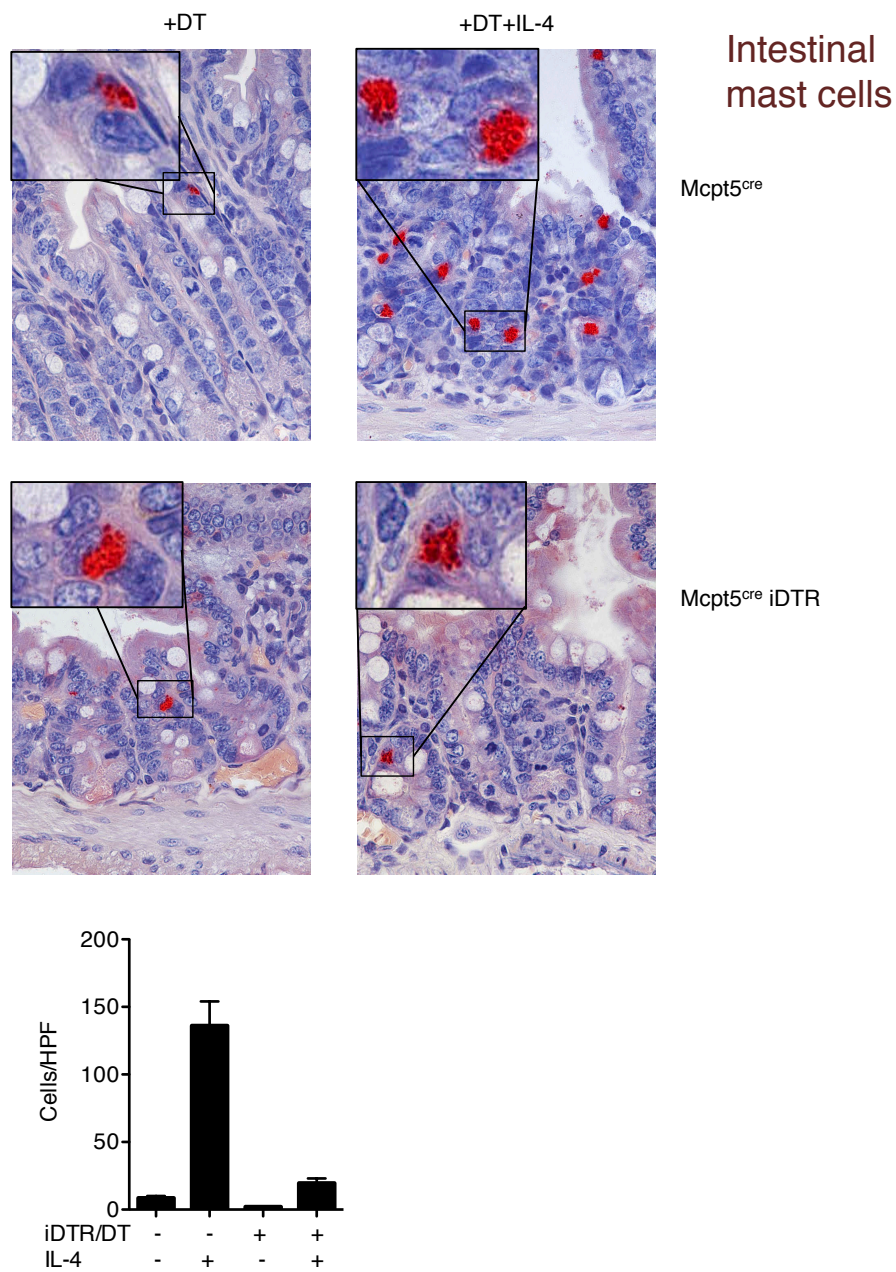


Figure S5, related to Figure 4. Representative chloroacetate esterase staining of jejunal sections showing mast cells in red and counts from three mice. Mice

were treated with DT (100ng day 1, 500ng day 2, 500ng day 3) to deplete mast cells, and IL-4:anti-IL-4 complexes (2 μ g IL-4 plus 10 μ g anti-IL-4, days 1, 4) to promote mast cell growth. Mice were sacrificed for analysis on day 7. Data derive from one of three experiments. The bar chart shows the mean +SEM.

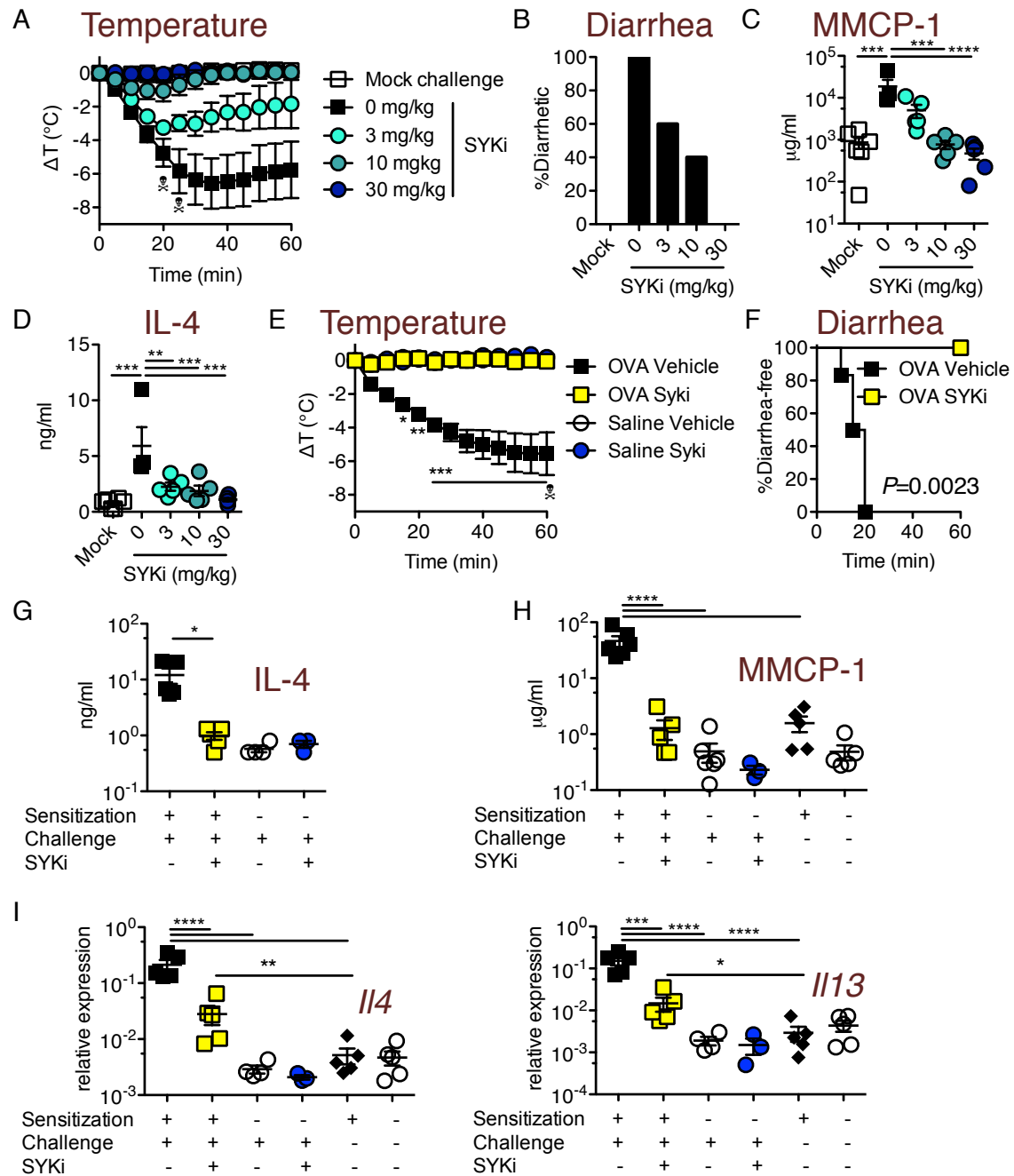


Figure S6, related to Figure 5. Effects of Syk inhibition on anaphylaxis. A)

Effects of escalating doses of SYKi on core temperature loss and lethality

following IgE-mediated anaphylaxis. IgE-deficient IL-4 α F709 mutant *Il4ra*^{tm3.1Tch} mice were sensitized with 20 μ g IgE anti-DNP (SPE-7) by intravenous injection into the retro-orbital sinus, and then challenged with 500 μ g DNP-BSA *i.p.* 16hrs later ($n=5-6$ per group). SYKi or vehicle was administered by oral gavage 15min prior to challenge. **B)** Effects of Syk inhibition on diarrhea onset ($n=5-6$). **C)** ELISA analysis of MMCP-1 release into serum ($n=4-6$). Serum was not available from mice that died during anaphylaxis, so the data for the vehicle group have effectively been stripped of the high responders. **D)** Serum IL-4 levels after IgE-mediated anaphylaxis as measured by *in vivo* cytokine capture assay. Only mice that survived the anaphylaxis were analyzed. Biotinylated anti-IL-4 (10 μ g) was injected intravenously 16hrs prior to challenge. Statistical analysis in C and D by ANOVA with Bonferroni post-tests. **E)** Effects of SYKi on acute food allergen-induced anaphylaxis. Temperature curves following enteral challenge with OVA. *Il4ra*^{tm3.1Tch} mice were sensitized for three weeks with 250 μ g OVA and 20 μ g cholera toxin or mock-sensitized with saline, and then subjected to oral challenge with 150mg OVA. SYKi (30mg/kg) or vehicle was administered 15min prior to challenge. **F)** Kinetics of diarrhea onset following acute food allergen challenge. **G)** Serum IL-4 levels as determined by IL-4 *in vivo* cytokine capture assay. **H)** MMCP-1 levels in serum of sensitized or sensitized and challenged mice. **I)** Induction of mRNA transcripts for IL-4 and IL-13 in the jejunum following acute food allergen challenge. Error bars show the mean $\pm$ SEM. Data are from a single experiment.

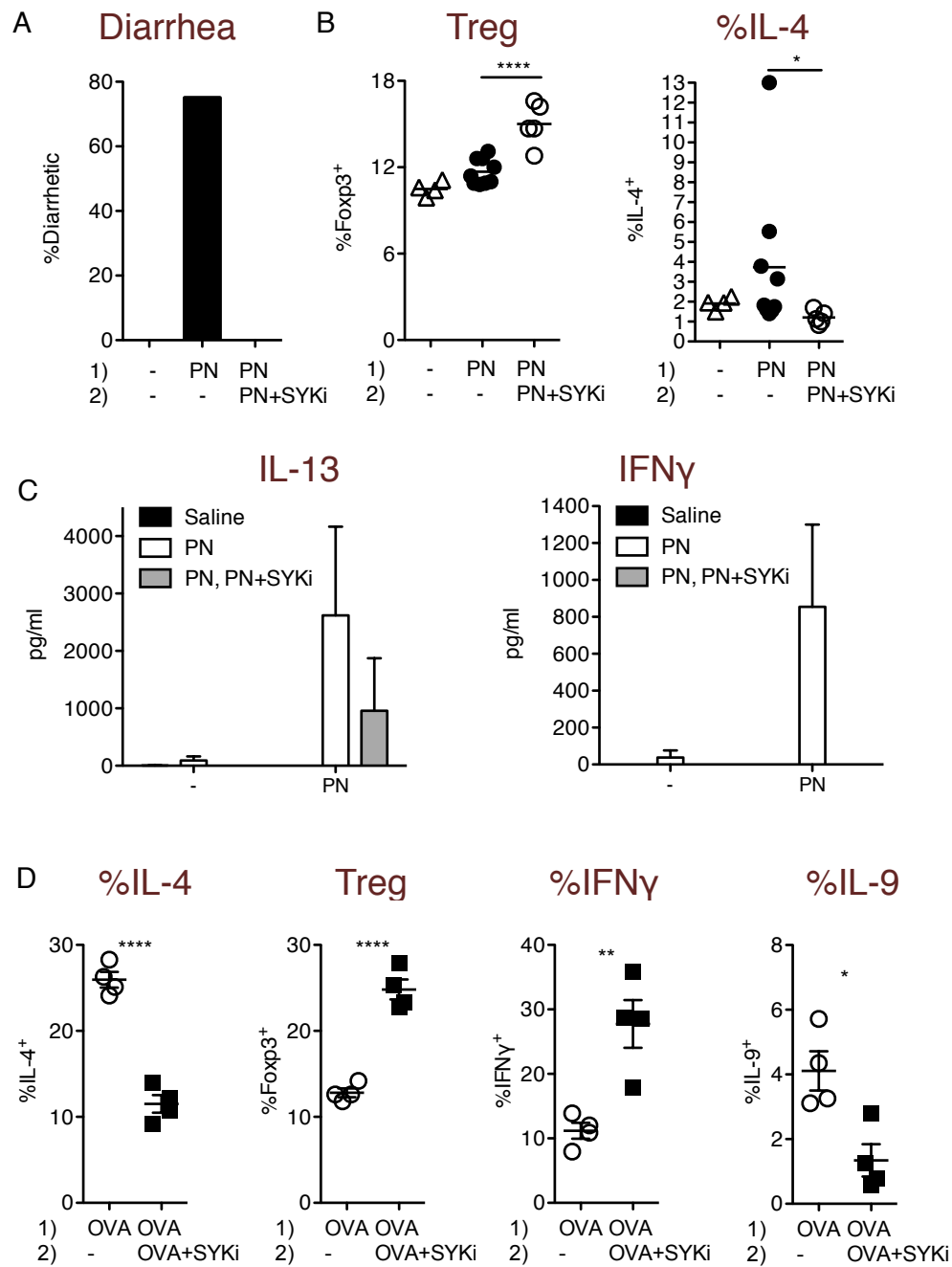


Figure S7, related to Figure 6. Immunological effects of SYKi-facilitated food allergen desensitization. A) Diarrhea incidence in PN-allergic and PN+SYKi-

desensitized mice. IL-4 α F709 mutant (*Il4ra*^{tm3.1Tch}) mice were treated as in Figure 6. **B)** Treg and Th2 cell frequencies in MLN cells following PN+SYKi desensitization therapy. Data were obtained by flow cytometric analysis of CD4⁺ T cell populations, assessing Foxp3 and IL-4 expression. **C)** ELISA analysis of IL-13 and IFN γ levels in splenocyte cell culture supernatants. Splenocytes (10⁶/ml) were cultured with PN (100 μ g/ml) for 5 days. **D)** Phenotypic analysis of CD4⁺ T cells exhibiting OVA-specific proliferative responses *in vitro*. MLN cells from *Il4ra*^{tm3.1Tch} mice ($n=4$) sensitized with OVA+CT and left untreated or desensitized with SYKi and high dose (150mg) OVA were labeled with Violet CellTrace and cultured for 5 days with 100 μ g/ml OVA. The cells were stimulated with ionomycin, phorbol 12,13-dibutyrate and brefeldin A, and then stained for intracellular cytokine and Foxp3 expression. Data shown derive from analyses of CD4⁺ viable single cells exhibiting dye dilution. * $P<0.05$, ** $P<0.01$, **** $P<0.0001$ by unpaired t test. Data are representative of three experiments, and are presented as mean +SEM in C or mean $\pm$ SEM in D.

Supplemental Experimental Procedures

Mice

The generation of IL-4 α F709 mutant (C.129X1-*Il4ra*^{tm3.1Tch}), IgE-deficient (*Igh-7*^{-/-}) and *Il4ra*^{tm3.1Tch} *Igh-7*^{-/-} mice on the BALB/c background has been previously described (Burton et al., 2013). *Kit*^{W^{-sh}} mice (B6.Cg-*Kit*^{W^{-sh}}/HNihrJaeBsmGlliJ, stock number 005051) were purchased from The Jackson Laboratory and crossed with B6.*Il4ra*^{tm3.1Tch} mice (N8 C57BL/6J background). B6.Tg(Mcpt5^{cre})

mice (Scholten et al., 2008) were interbred with B6.*Il4ra*^{tm3.1Tch} mice, and then crossed with iDTR mice (C57BL/g-Gt(*ROSA*)26Sor^{tm1(HBEGF)Awai}/J, stock number 007900, The Jackson Laboratory) to create *Il4ra*^{tm3.1Tch} Mcpt5^{cre} iDTR mice, and separately crossed with *Sykb*^{fl/fl} mice (B6.129P2-*Sykb*^{tm1.2Tara}/J, stock number 017309, The Jackson Laboratory) to generate *Il4ra*^{tm3.1Tch} Mcpt5^{cre} *Sykb*^{fl/fl}. For the purposes of analyzing the specificity of the Mcpt5^{cre} transgene for targeting mast cells, we generated the following Mcpt5^{cre}-transgenic mice with normal IL-4R alleles: Mcpt5^{cre} (B6.Tg(Mcpt5^{cre})), Mcpt5^{cre} iDTR (B6.Tg(Mcpt5^{cre}) Gt(*ROSA*)26Sor^{tm1(HBEGF)Awai}), Mcpt5^{cre} *Sykb*^{fl/fl} (B6.Tg(Mcpt5^{cre}) *Sykb*^{tm1.2Tara}). *Il4ra*^{tm3.1Tch}, *Syk*, *Kit* and *Igh-7* alleles were maintained in homozygous states; iDTR and Mcpt5^{cre} alleles were checked for presence or absence using PCR. Thy1.1-congenic Foxp3-eGFP-reporter IL-4rα F709 mutant mice (C.129X1-*Il4ra*^{tm3.1} *Foxp3*^{tm2Tch} *Thy1*^a) and Thy1.1 DO11.10 TCR-transgenic Foxp3-eGFP-reporter Rag-deficient mice (C.Cg-Tg(DO11.10) *Thy1*^a *Foxp3*^{tm2Tch} *Rag1*^{tm1Mom}) on the BALB/c background have been previously described (Noval Rivas et al., 2013). Wild-type BALB/cAnNTac breeders were initially purchased from Taconic Farms, and C57BL/6J breeders were originally obtained from The Jackson Laboratory. Both were then bred in house for experimental use.

Reagents

Grade V ovalbumin was purchased from Sigma (St. Louis, MO), and cholera toxin was obtained from List Biological Laboratories (Campbell, CA). BSA was obtained from Sigma (St. Louis, MO). IL-3, SCF and IL-4 were purchased from Shenandoah Biotechnology, Inc. (Warwick, PA). Anti-IL-4 (clone 11B11) was

enriched from hybridoma supernatants using a 100kDa molecular weight cut-off centrifugal filter (Pall Corporation, Northborough, MA) as previously described (Burton et al., 2013).

Cell culture

Peritoneal mast cells were enriched from peritoneal lavage by culturing them in complete RPMI supplemented with 10ng/ml IL-3, 20ng/ml SCF and 100ng/ml IgE anti-TNP (clone C38-2, BD Biosciences, Franklin Lakes, NJ) for two weeks and removing adherent cells (macrophages). BMMC were cultured as previously described (Burton et al., 2013). Bone marrow-derived dendritic cells were cultured as previously described (Zaccone et al., 2009). For BMMC and T cell co-culture experiments, 10^5 CD4⁺ T cells were cultured for 5 days with an equal number of BMMC. T cells were purified using CD4 microbeads and an AutoMACS (Miltenyi Biotec, Cambridge, MA). Cells were stimulated with 2ng/ml recombinant human TGFβ1 (Shenandoah Biotechnology, Warwick, PA), 1μg/ml αCD3 (145-2C11, Biolegend, San Diego, CA), 5μg/ml αCD28 (37.51, Biolegend, San Diego, CA) and 5μg/ml αFcεRI (MAR-1, eBioscience, San Diego, CA). Antibodies were coated overnight on 48-well plates. Cell suspensions were counted using a hemocytometer, and Th2 and Treg numbers were calculated using frequencies of IL-4⁺ or Foxp3⁺ cells among CD4⁺CD3ε⁺ T cells as determined by flow cytometry.

Flow cytometry and cell sorting

Treg cells were stained with Foxp3-APC (FJK-16s, Affymetrix eBioscience, San Diego, CA), CD4-PerCP-Cy5.5 (RM4-5, Biolegend, San Diego, CA) and CD3ε-FITC (145-2C11, BD Bioscience, Franklin Lakes, NJ) using the Foxp3 staining kit (Affymetrix eBioscience, San Diego, CA). For the Thy1.1 Foxp3-eGFP congenic experiment, cells were stained with Thy1.1-eFluor450 (HIS51, Affymetrix eBioscience, San Diego, CA), IL-17-Brilliant Violet 605 (TC11-18H10.1, Biolegend, San Diego, CA), Foxp3-FITC or eGFP (FJK-16s, Affymetrix eBioscience, San Diego, CA, or endogenous Foxp3-eGFP), IL-4-PE-CF594 (11B11, BD Biosciences, Franklin Lakes, NJ), IL-13-PerCP-eFluor710 (eBio13a, Affymetrix eBioscience, San Diego, CA), CD4-PE-Cy7 (RM4-5, Biolegend, San Diego, CA), IFNγ-APC (XMG1.2, BD Biosciences, Franklin Lakes, NJ) and IL-10-AlexaFluor700 (JES5-16E3, Affymetrix eBioscience, San Diego, CA). Robust coordinate analysis of Foxp3 and Th2 cytokines was achieved using the BD CytoFix CytoPerm kit (BD Biosciences, Franklin Lakes, NJ) and staining overnight with reduced concentrations of antibodies after a 4hr stimulation with 500ng/ml ionomycin (Sigma-Aldrich, St. Louis, MO), 500ng/ml phorbol 12,13-dibutyrate (Tocris Bioscience, Bristol, UK), and 1μg/ml brefeldin A (Sigma-Aldrich, St. Louis, MO). Dead cells were routinely excluded from analyses on the basis of staining with fixable viability dye eFluor780 (Affymetrix eBioscience, San Diego, CA), low affinity Fc receptor interactions were blocked with anti-FcγRII/III (clone 93, Biolegend, San Diego, CA) and analyses were restricted to single cells using FSC-H/W signals. Data were acquired using DIVA software on an LSRIIFortessa (BD Biosciences, Franklin Lakes, NJ) with 405nm, 488nm, 561nm

and 640nm lasers, and analyzed using FlowJo version 8.8.7 (Tree Star Inc., Ashland, OR). T cells from *Il4ra^{tm3.1Tch}* Foxp3-eGFP Thy1.1 mice were enriched under sterile conditions from pooled splenic and MLN cells using CD4 microbeads on an AutoMACS (Miltenyi Biotec, Cambridge, MA). Foxp3⁺ Treg cells were then sorted on the basis of CD4-PE-Cy7⁺Foxp3-eGFP⁺ on a FACSAria (BD Biosciences, Franklin Lakes, NJ). Mast cells in the mesenteric lymph node were identified by staining with c-Kit-PE-Cy7 and IgE-PE, and by excluding B220-APC⁺ and non-viable eFluor780⁺ cells.

LAMP-1 degranulation assay

Mast cell degranulation was assessed by fusion of LAMP-1 (CD107a)⁺ granules the plasma membrane. IgE-sensitized mast cells were stimulated for ten minutes in RPMI containing IL-3 (10ng/ml), SCF (10ng/ml), TNP-OVA (100ng/ml, Biosearch Technologies, Petaluma, CA) and anti-LAMP-1-PerCP-eFluor710 (2µg/ml, clone eBio1D4B, Affymetrix eBioscience, San Diego, CA). Mast cells were then promptly washed twice in ice-cold FACS buffer (PBS, 0.5% BSA, 0.01% sodium azide) prior to staining with anti-c-Kit-PE-Cy7 (ACK2, Affymetrix eBioscience, San Diego, CA) and subsequent flow cytometric analysis.

Calcium flux

Splenic B cells were interrogated for calcium flux following B cell receptor stimulation as follows: single cell suspensions of splenocytes were subjected to ammonium chloride-induced erythrocyte lysis, and then loaded with Fluor-4 AM (3µM) and Fura Red AM (6µM, both Life Technologies, Carlsbad, CA) for 30min

at 37°C in HBSS containing divalent cations. B cells were rested for 20min at room temperature, and then stained with anti-B220-PE-Cy7 (RA3-6B2, Biolegend, San Diego, CA). After acquiring basal fluorescence signals for 20s, B cells were stimulated with anti-IgM (10µg/ml goat polyclonal, Southern Biotech, Birmingham, AL) to elicit calcium flux. Ionomycin (500ng/ml, Sigma-Aldrich, St. Louis, MO) was used as a control to elicit maximal calcium flux. Signals were acquired in linear set-up on an LSRFortessa, and analyzed as a kinetic of the algebraic parameter Fluo-4/Fura Red.

Anaphylaxis

Passive systemic anaphylaxis was induced by sensitizing 16-24-week old *Il4ra^{tm3.1Tch} Igh-7^{-/-}* mice overnight by i.p. injection with 20µg IgE anti-DNP (clone SPE-7, Sigma-Aldrich, St. Louis, MO), and challenging the mice the following day by i.p. injection with 500µg DNP-BSA (Biosearch Technologies, Petaluma, CA). Mice were randomly selected for treatment with SYKi or vehicle gavage, given 15min prior to challenge. A mock challenge was performed using unconjugated BSA (Sigma-Aldrich, St. Louis, MO). In some experiments, IgE anti-TNP (2µg, clone C38-2) and TNP-OVA (20µg) were used to induce anaphylaxis.

To address the immunological specificity of food allergen-induced anaphylaxis in *Il4ra^{tm3.1Tch}* mice (Figure S7B), enterally peanut-sensitized mice were sequentially monitored for temperature fluctuations over the course of an hour, followed by challenge with 100mg of BSA i.g., and then challenged with peanut.

ELISA

IL-4 was detected in cell culture supernatants by sandwich ELISA using purified 11B11 and biotinylated BVD4-24G2 (both Biolegend) followed by streptavidin-HRP (R&D) and trimethylbenzidine substrate (BD). MMCP-1 in serum was analyzed using MCPT-1 (MMCP-1) ELISA Ready-Set-Go! (Affymetrix eBioscience). Peanut-specific IgE was captured onto ELISA coated overnight with 3µg/ml anti-IgE (R35-92, BD) and detected using 10µg/ml biotinylated peanut extract. Peanut-specific IgG1 ELISAs were performed similarly using goat anti-mouse IgG1 for capture purposes (Southern Biotech). Peanut extract was prepared as previously described and biotinylated using an EZ-Link Sulfo-NHS-Biotinylation kit (Thermo Fisher) (Varshney et al., 2011). Anti-peanut serum from BALB/c mice immunized and primed with peanut extract adsorbed to alum was used as a source of peanut-specific immunoglobulins to develop a standard curve based on arbitrarily assigned units/ml.

Sandwich ELISA analysis of total immunoglobulin isotype levels was performed using highly adsorbed goat polyclonal anti-mouse isotype antibody pairs from Southern Biotech (Birmingham, AL). Purified anti-isotype antibodies were coated overnight in PBS at 2µg/ml, and biotinylated secondary antibodies were used for detection at 500ng/ml. IL-13 and IFNγ ELISAs were performed according to the manufacturer's instructions (Affymetrix eBioscience, San Diego, CA). To detect circulating IL-4 levels, mice were injected either i.p. or i.v. with 10µg biotinylated anti-IL-4 (clone BVD6-24G2, Biolegend, San Diego, CA). The biotinylated anti-IL-4:IL-4 complexes that subsequently formed were captured from serum onto plates coated with anti-IL-4 (clone 11B11, Biolegend, San Diego, CA) and

detected using streptavidin-HRP (R&D Systems, Minneapolis, MN). OVA-specific IgE was captured onto plates coated with OVA and detected using biotinylated anti-IgE (R35-72, BD Biosciences, Franklin Lakes, NJ) using IgE anti-OVA clone TO ϵ as a standard. IgE against chow components was assessed by coating plates with gliadin (MyBioSource, San Diego, CA) or soy extract at 100 μ g/ml, incubating with sera at a 1:5 dilution, and developing with the same biotinylated anti-IgE as for the OVA-IgE ELISA. Soy extract is an aqueous extract of soy flour (Bob's Red Mill, Milwaukie, OR).

Quantitative PCR

Real-time quantitative RT-PCR was performed as previously described (Mathias et al., 2011) using TaqMan probes from Life Technologies.

Supplemental References.

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Immunoglobulin E Signal Inhibition during Allergen Ingestion Leads to Reversal of Established Food Allergy and Induction of Regulatory T Cells

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SUMMARY

Immunoglobulin E (IgE) antibodies are known for triggering immediate hypersensitivity reactions such as food anaphylaxis. In this study, we tested whether they might additionally function to amplify nascent antibody and T helper 2 (Th2) cell-mediated responses to ingested proteins and whether blocking IgE would modify sensitization. By using mice harboring a disinhibited form of the IL-4 receptor, we developed an adjuvant-free model of peanut allergy. Mast cells and IgE were required for induction of antibody and Th2-cell-mediated responses to peanut ingestion and they impaired regulatory T (Treg) cell induction. Mast-cell-targeted genetic deletion of the FcεRI signaling kinase Syk or Syk blockade also prevented peanut sensitization. In mice with established allergy, Syk blockade facilitated desensitization and induction of Treg cells, which suppressed allergy when transferred to naive recipients. Our study suggests a key role for IgE in driving Th2 cell and IgE responses while suppressing Treg cells in food allergy.

INTRODUCTION

Food allergy has emerged as a major public health issue worldwide (Burks et al., 2012). Sensitized individuals, who have high titers of food-specific IgE antibodies, experience a range of immediate hypersensitivity responses including acute-onset itching, hives, vomiting, and diarrhea all triggered when food proteins recognized by FcεRI-bound IgE induce mast cell activation. The most dramatic food hypersensitivity reaction is systemic anaphylaxis, in which vasoactive mast cell mediators induce plasma extravasation, shock, cardiopulmonary collapse, and death (Finkelman, 2007; Simons, 2010). The standard of care, namely recommendation to strictly avoid foods to which they

are allergic, paradoxically deprives patients of the chance to naturally develop oral tolerance, as would probably occur if they were able to continue to ingest them without experiencing harmful effects.

Following the very successful example of subcutaneous immunotherapy in which subjects with aeroallergen sensitivity are desensitized by injection of protein extracts ("allergy shots"), investigators have evaluated oral desensitization strategies for food allergy (Nowak-Węgrzyn and Sampson, 2011; Vickery and Burks, 2010). Although achieving substantial success, such approaches are associated with unpredictable IgE antibody-mediated allergic reactions, limiting their application in practice. Several groups have now performed oral desensitization under cover of the monoclonal anti-IgE antibody omalizumab, which effectively blocks food anaphylaxis, with the expectation that inhibiting IgE-mediated reactions would improve the patient experience (Nadeau et al., 2011; Schneider et al., 2013).

A growing body of evidence indicates that IgE antibodies and mast cells might serve not only as the effectors of immediate hypersensitivity in subjects with established sensitivity but also as amplifiers during initial antigen exposure in naive subjects, potentially providing early signals for nascent Th2 cell and antibody responses. IgE induces mast cell production of both Th2-cell-inducing and DC-activating cytokines (Asai et al., 2001; Kalesnikoff et al., 2001; Kawakami and Kita, 2005). We have reported that IgE and mast cells enhance immune sensitization in contact sensitivity (Bryce et al., 2004). By using an adjuvant-free asthma model, Nakae et al. (2007a, 2007b) demonstrated that the induction of airway inflammation and bronchial hyperreactivity are strongly amplified by mast cells in the airway mucosa. Additional evidence that effector cells of allergic responses might independently function as inducers of immune sensitization comes from studies implicating basophils (which, like mast cells, are FcεRI⁺) as early drivers of Th2 cell expansion (Mukai et al., 2005; Sokol et al., 2009). We similarly hypothesized that IgE antibodies and mast cells might serve not only as the effectors of food anaphylaxis but also as important early inducers of Th2 cell responses and suppressors of Treg cell responses to food allergens and that they might

provide an accessible therapeutic handle by which to dampen such responses.

Evaluation of this hypothesis required an animal model of food allergy in which immune sensitization could be accomplished directly by enteral sensitization and in the absence of the immunostimulatory effects of prior systemic parenteral immunization or ingestion of immunological adjuvants commonly used in food allergy models. For this purpose we took advantage of inherently atopic mice in which a mutation (F709) of the IL-4 receptor α chain (IL-4 α) ITIM results in amplified signaling upon interaction with IL-4 or IL-13, but not constitutive activation. These mice exhibit enhanced Th2 cell responses and IgE production and susceptibility to anaphylaxis after ingestion of the model antigen ovalbumin (OVA) in the absence of adjuvant (Mathias et al., 2011). We have now adapted this model to the clinically relevant food allergen peanut and have applied multiple parallel approaches that together provide strong evidence that IgE antibodies and mast cells enhance Th2 cell responses to ingested allergens. The mechanism for this Th2 cell induction is the suppression of Foxp3⁺ Treg cell responses, in terms of both Treg cell numbers and function. We show that established allergic sensitivity can be reversed when oral immunotherapy is paired with interventions that block IgE-mediated mast cell activation and demonstrate that pharmacologic inhibition of Syk activity can be used to inhibit mast cell function and achieve the same benefit as IgE blockade. Our results establish that IgE antibodies, signaling via FcεRI on mast cells, not only serve to induce anaphylactic reactions in individuals with food allergy but also exert critical regulatory effects on adaptive immunity to ingested proteins in emerging food allergy. Mast cells activated by IgE support Th2 cell responses and IgE production while suppressing Treg cell induction. Moreover, food ingestion under cover of IgE signaling blockade can lead to reversal of Th2 cell responses and induction of Treg cells in mice with established food allergy. These results suggest that pharmacologic IgE blockade might be of benefit to patients with food allergy.

RESULTS

IgE Antibodies Amplify Th2 Cell Responses and Suppress Treg Cell Induction to Ingested Peanut

Although IgE antibodies are best recognized for their unique ability to trigger immediate hypersensitivity reactions in previously sensitized subjects, there is evidence that they might serve an independent and important function in amplifying and skewing developing adaptive immune responses after initial antigen exposure (Gould and Sutton, 2008). For this study, we examined the possibility that IgE antibodies, signaling via FcεRI on mast cells, would support Th2 cell responses to ingested food allergens while suppressing the induction of Treg cells. In order to test this hypothesis, we developed a murine model of allergy to peanut, a very common food allergen and one associated with particularly severe anaphylactic reactions. We have previously reported the construction of mice (IL4 α ^{tm3.1Tch}) that harbor a targeted insertion of a mutant form of IL-4 α in which the immunoreceptor tyrosine-based inhibition motif (ITIM) motif is disrupted (F709 mutation). These mice have normal IL-4 α expression and do not have baseline signaling but exhibit enhanced signaling on ligand exposure and are highly susceptible to the in-

duction of allergy to ovalbumin (OVA) in an adjuvant-free ingestion model (Mathias et al., 2011; Tachdjian et al., 2010). They exhibit robust anaphylaxis after food allergen ingestion, a cardinal phenotype of food allergy that has previously been difficult to reproduce in animal models. We reasoned that these mice would provide an ideal tool for analysis of IgE and mast cell roles in food allergen sensitization to a physiologically relevant allergen, peanut.

Mice were sensitized by weekly gavage with 23 mg commercial peanut butter (5 mg protein) (Figure S1 available online). After four doses, peanut-fed mice carrying the IL-4 α F709 mutation, but not control animals, exhibited strong peanut-specific IgE and IgG1 responses as well as robust induction of peanut-specific Th2 cell responses (Figures 1A–1D and S2A). Consistent with the expected induction of oral tolerance, wild-type BALB/c mice developed a strong peanut-specific Foxp3⁺ Treg cell response (Figure 1E). Peanut-specific Treg cell induction was markedly impaired in mice with the F709 IL-4 receptor. In this study we measured Treg cell responses by ex vivo stimulation for 5 days with peanut antigen followed by flow cytometric evaluation of Foxp3⁺ dividing cells (Figure 1F).

Gavage challenge of peanut-sensitized mice with 450 mg peanut butter (100 mg protein) elicited anaphylaxis in the IL-4 α mutants, measured via implanted thermal transponders, as loss of core body temperature (Figure 1G; see Figure S1 for protocol schematic). Neither unsensitized IL-4 α mutant mice nor peanut-fed wild-type BALB/c mice had any temperature loss (Figure 1G and data not shown), and challenge of peanut-sensitized mutant mice with irrelevant protein had no effect on temperature (Figure S2B). Anaphylaxis was accompanied by elevated serum concentrations of mouse mast cell protease-1 (MMCP-1), a marker of mucosal mast cell activation (Figure 1H). These findings demonstrate that mice with a dysregulated F709 IL-4 receptor develop intense Th2 cell, IgE, and IgG responses to ingested peanut along with a defective induction of Treg cells and that this immunological profile renders them exquisitely sensitive to anaphylaxis after enteral peanut challenge. It is interesting to note that in addition to being susceptible to deliberate induction of sensitization with the food allergen peanut, these mice exhibit some spontaneous sensitization to food proteins within their standard chow diet (Figure S2C).

In order to evaluate the contributions of IgE antibodies to peanut sensitization and anaphylaxis, we crossed the IL-4 α mutant animals with IgE-deficient (*Igh*^{-/-}) BALB/c mice (Oettgen et al., 1994). As expected, IgE-deficient mice were fully resistant to peanut anaphylaxis (Figure 1G). Consistent with our hypothesis that IgE contributes to immunological sensitization in the gut during recurrent allergen ingestion, peanut-specific IgG1 and Th2 cell responses were dramatically reduced in peanut-sensitized mice lacking IgE (Figures 1B–1D). Treg cell induction, which had been markedly impaired in mice with the F709 form of IL-4 α , was fully restored in the absence of IgE (Figures 1E and 1F). Taken together, these findings provide strong evidence that IgE antibodies support the induction of Th2 cell and antibody responses to ingested allergens while suppressing the induction of Treg cells.

Our results with IgE-deficient mice suggest that the application of IgE blockade, which has been employed in clinical food desensitization studies to block undesired anaphylactic

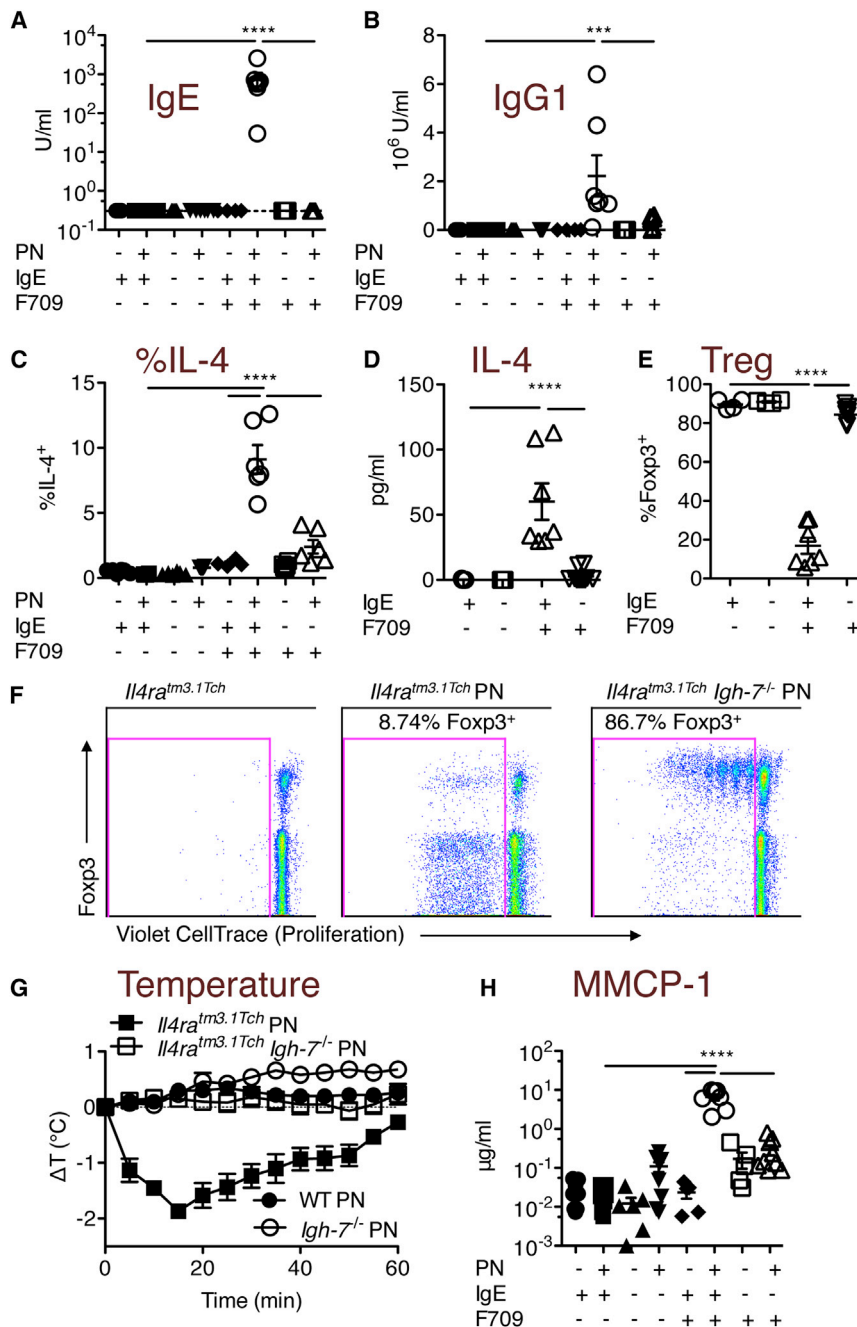


Figure 1. Effects of IgE and IL4 α Genotypes on Food Allergen Sensitization in the Absence of Adjuvant

The effects of IgE on sensitization were evaluated with completely IgE-deficient mice (*Igh*-7^{-/-}) both on the allergy-prone IL-4 α F709 background (*Il4ra*^{tm3.1Tch}) and on wild-type BALB/c.

(A) Peanut-specific IgE levels in sera of mice subjected to enteral gavage with peanut butter (PN). Mice (n = 5–10 per group) were gavaged with 23 mg PN once a week for 4 weeks. The horizontal line indicates the limit of detection in this ELISA. Presence of the activating IL-4 receptor, F709, and IgE are indicated by plus (+) or minus (–).

(B) Serum titers of peanut-specific IgG1 after sensitization.

(C) Flow cytometric analysis of intracellular IL-4 staining in CD4⁺ T cells from mesenteric lymph node (MLN) cells after PN sensitization.

(D) IL-4 levels as determined by ELISA in splenocyte cultures from PN-treated mice restimulated in vitro with PN extract for 5 days.

(E) Analysis of PN-specific Treg cells expanded from MLN cells of PN-treated mice.

(F) Representative flow cytometry plots demonstrate dye dilution (proliferation) among CD3e⁺ CD4⁺ MLN cells labeled with Violet CellTrace and cultured in vitro with PN extract for 5 days. The summary graph shows the Foxp3 phenotype of the divided cells, gating on CD4⁺ viable single cells.

(G) Temperature curves after enteral challenge with high-dose PN. p < 0.0001 by repeated measures (RM) two-way ANOVA, *Il4ra*^{tm3.1Tch} versus *Il4ra*^{tm3.1Tch} *Igh*-7^{-/-}.

(H) Serum MMCP-1 levels as measured by ELISA postchallenge. Data are represented as points for individual mice, with mean $\pm$ SEM in overlay. Similar data were obtained in at least three separate experiments.

prior to sensitization reduced Th2 cell responses and increased peanut-specific Treg cell frequencies (Figures 2B, 2D, and S2D).

Anti-IgE therapy in the peanut allergy model allowed us to also examine the effects of IgE blockade during allergen desensitization in animals with established peanut sensitivity. For desensitization, anti-IgE treatment was started a

reactions, might also modulate immune responses to ingested antigens. Omalizumab, the anti-IgE used in human trials, is “nonanaphylactogenic.” It does not bind to FcεRI-bound IgE and therefore does not activate mast cells. In contrast, available anti-mouse IgE reagents all exhibit some degree of mast cell activation. In order to use these antibodies in the peanut model, we had to mitigate this effect. Taking advantage of a rush desensitization protocol developed by Khodoun et al. (2013), we treated mice with anti-IgE in a 4-day build-up prior to the first exposure to peanut. Neutralization of IgE mimicked genetic deletion of IgE, rendering the IL-4 α mutant mice resistant to anaphylactic reactions (Figure 2A). Anti-IgE treatment

week after the final sensitizing peanut dose was administered, after which large doses (225 mg) of peanut were administered daily for 3 weeks. When finally challenged with peanut, mice that had received anti-IgE did not anaphylax, as expected given the requirement for IgE in anaphylaxis (Figure 2A). In contrast, mice subjected to the same peanut desensitization therapy without anti-IgE exhibited similar anaphylactic reactions to those seen in mice that received no therapy whatsoever. Th2 cell responses were reduced in mice treated with anti-IgE plus peanut, and peanut-specific Treg cell frequencies markedly increased (Figures 2C, 2D, and S2E). Control desensitization therapy with peanut alone did not elicit significant changes in Th2 cell or

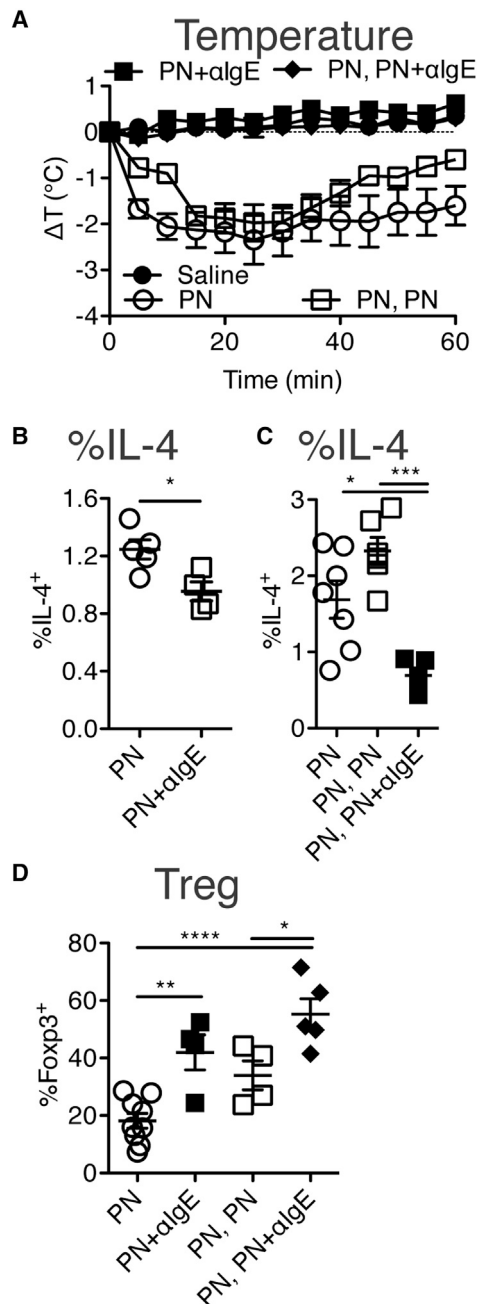


Figure 2. Effects of Anti-IgE on Food Allergen Sensitization

(A) Temperature curves after acute enteral challenge with PN in IL-4 α F709 mutant (*Il4ra*^{tm3.1Tch}) mice (n = 5) treated with PN and/or anti-IgE. Mice were sensitized by enteral gavage with PN (23 mg) once a week for 4 weeks, followed by desensitization with PN (225 mg) once a day for 3 weeks. Anti-IgE (100 μ g total) was injected i.p. in an incremental rush protocol prior to sensitization or desensitization. Abbreviations are as follows: PN+ α IgE: α IgE prior to PN sensitization; PN, PN+ α IgE: PN sensitization, α IgE prior to PN desensitization; PN, PN: PN sensitization, PN desensitization. PN versus PN+ α IgE: p = 0.0011; PN versus PN, PN+ α IgE: p = 0.0022; PN, PN versus PN, PN+ α IgE: p = 0.0002 by RM two-way ANOVA.

(B) Flow cytometric analysis of IL-4 by intracellular staining of CD4⁺ T cells in MLN (n = 4–5).

(C) IL-4 staining in CD4⁺ MLN cells in response to desensitization with PN $\pm$ anti-IgE (n = 5–7).

Treg cell responses to peanut, although there were trends for elevation in both.

Taken together these results indicate that IgE antibodies promote immune sensitization to ingested allergens. In the food-allergy-susceptible mice harboring a dysregulated form of IL-4 α , the presence of IgE during recurrent enteral peanut exposure promoted the induction of peanut-specific Th2 cell, IgE, and IgG1 responses while suppressing the expansion of antiallergic Treg cells. Moreover, IgE blockade in animals with established allergy served to reverse sensitivity with a reduction in Th2 cell responses and expansion of Treg cells.

Mast Cells Amplify Allergic Responses to Ingested Peanut and Suppress Peanut-Specific Treg Cell Induction

We hypothesized that mast cells were the likely effectors of IgE-mediated Th2 cell induction and Treg cell suppression observed in the peanut allergy model. In order to test this, we bred the F709 IL-4 α mutation onto the C57BL/6 genetic background and then intercrossed these mice with *Kit*^{W-sh} mice. These mast-cell-deficient mice exhibited a dramatic (3-log) reduction in peanut-specific IgE along with significantly impaired Th2 cell responses and markedly enhanced Foxp3⁺ Treg cell induction (Figures 3A–3C and S2F). Reconstitution of the mast-cell-deficient mice with WT bone-marrow-derived mast cells (BMMCs) restored Th2 cell responses while partially but significantly restoring IgE production (Figures 3A and 3B). Mast-cell-reconstituted mice exhibited impaired Treg cell generation (Figure 3C). Unlike their mast-cell-sufficient counterparts, mast-cell-deficient mice subjected to peanut sensitization were protected from anaphylaxis, but anaphylaxis was restored by mast cell reconstitution (Figure 3D). In contrast to WT BMMC-reconstituted mice, mice reconstituted with IL-4-deficient mast cells failed to develop peanut-specific IgE, Th2 cells, or anaphylactic responses, instead showing enhanced Treg cell induction (Figure 3). Mast cell reconstitution of the small intestine of mast-cell-deficient mice was confirmed by histologic examination (Figure S3).

Although *Kit*^{W-sh} mice exhibit complete mast cell deficiency and are amenable to reconstitution by BMMCs, making them an attractive model system in which to study mast cell biology, they have an array of immunological defects including dysregulation of granulocytes, introducing potential complications to the interpretation of our results. In order to further evaluate mast cell functions in the peanut allergy model, we took advantage of the Kit-independent Mcpt5^{cre} transgene system to genetically target mast cells. A two-pronged approach was applied that used Mcpt5 promoter-driven Cre expression either to mediate mast cell deletion via induction of iDTR followed by treatment with diphtheria toxin (*Il4ra*^{tm3.1Tch} Mcpt5^{cre} iDTR) or to drive mast-cell-specific excision of the *Sykb* gene (*Il4ra*^{tm3.1Tch} Mcpt5^{cre} *Sykb*^{fl/fl}). We reasoned that removing Syk, the proximal kinase in FcεRI signaling, would paralyze IgE-mediated mast cell activation, allowing us to isolate the role of IgE-mediated mast cell activation within this model (Figures S4 and S5).

(D) Flow cytometric analysis of Foxp3⁺ cells in the CD3 ϵ ⁺CD4⁺ MLN cells exhibiting proliferative responses to PN after in vitro restimulation with 100 μ g/ml PN for 5 days (n = 4–9).

Data are from one of two experiments and are represented as mean $\pm$ SEM.

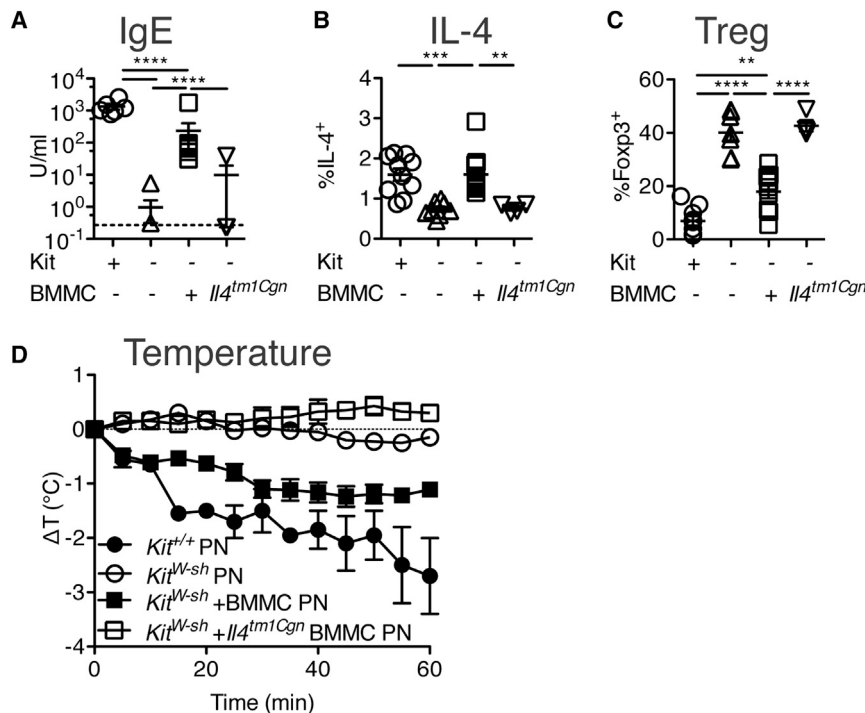


Figure 3. Mast Cells Are Required for Sensitization to Food Allergens

(A) PN-specific IgE levels in sera as determined by ELISA. IL-4 α F709 mutant (*IL4ra^{tm3.1Tch}*) and mast-cell-deficient *IL4ra^{tm3.1Tch}Kit^{W-sh}* mice ($n = 6-8$) were sensitized by enteral gavage with 23 mg PN once a week for 8 weeks. Mast cells were reconstituted in additional *IL4ra^{tm3.1Tch}Kit^{W-sh}* mice by i.p. injection of WT or IL-4-deficient (*IL4^{tm1Cgn}*) BMMCs (5×10^6) at 4 and 8 weeks prior to sensitization. Kit variants are abbreviated as + for WT and – for *Kit^{W-sh}*.

(B) IL-4⁺CD4⁺ T cells in the MLN.

(C) Foxp3⁺ Treg cell frequencies in CD3 ϵ ⁺CD4⁺ MLN cells exhibiting proliferative responses to PN.

(D) Temperature curves after acute enteral challenge with PN (450 mg) after sensitization of IL-4 α F709 mutant (*IL4ra^{tm3.1Tch}*).

$p < 0.001$ by RM two-way ANOVA *Kit^{+/+}* versus *Kit^{W-sh}*, *Kit^{W-sh}* versus *Kit^{W-sh}* + BMMCs, and *Kit^{W-sh}* + BMMCs versus *Kit^{W-sh}* + *IL4^{tm1Cgn}* BMMCs. Data shown are from one of two experiments and are depicted as mean $\pm$ SEM.

Mice in which these genetic approaches were used to achieve mast cell ablation or mast cell paralysis behaved similarly, exhibiting both a restoration of oral tolerance and reduction in the peanut allergic phenotype. Specific IgE and Th2 cell responses were reduced in these mice relative to controls (Figures 4A and 4B). As we had observed with the *Kit^{W-sh}* model, removal of mast cells was sufficient to restore Treg cell generation to near wild-type levels even in the face of the proallergic IL-4 α F709 mutation (Figure 4C). Anaphylaxis was undetectable in peanut-sensitized *IL4ra^{tm3.1Tch} Mcpt5^{cre} iDTR* mice that received DT treatment prior to sensitization and was similarly absent in peanut-sensitized *IL4ra^{tm3.1Tch} Mcpt5^{cre} Syk^{fl/fl}* mice (Figure 4D). Strikingly, deletion of Syk solely from mast cells was sufficient to restore Treg cell induction, identifying IgE crosslinking of FcεRI on mast cells as the key signal tipping the balance between allergy and tolerance after food allergen exposure in an environment genetically permissive for the induction of food allergy.

In summary, these observations demonstrate that allergic sensitization (Th2 cell responses and IgE production) are reduced and Treg cell responses enhanced in two independent strains of mice harboring the activating IL-4 α mutation but lacking mast cells and in mice in which FcεRI signaling is impaired specifically in mast cells. Repletion of the mast cell compartment by wild-type but not IL-4-deficient BMMC transfer restores the food-allergy-prone phenotype of the IL-4 α mutant mice, confirming a direct role for mast cells and IL-4 produced by them in driving allergic sensitization and suppressing tolerance.

Syk Blockade Prevents Allergic Sensitization in Naive Mice and Facilitates Induction of Tolerance in Food-Allergic Animals

As the key kinase in FcεRI receptor signaling, Syk represents an attractive target for pharmacologic inhibition in the treatment of

allergic disease. We have recently characterized a new highly specific, potent, orally bioavailable Syk inhibitor, SYKi, and applied this to probe the consequences of FcεRI blockade in the peanut model (Moy et al., 2013). SYKi displayed a dose-dependent inhibition of passive IgE-mediated anaphylaxis and completely abrogated the lethal anaphylactic reaction to acute food allergen challenge in sensitized IL-4 α mutant mice (Figure S6). We anticipated that inhibition of Syk would block sensitization to food allergens, much the way that sensitization was blocked in mice with targeted genetic deletion of Syk in mast cells. This proved to be the case. Peanut-specific IgE responses were very low or undetectable in mice treated with SYKi during sensitization, and T cell responses correspondingly shifted from Th2 cell biased to Treg cell dominated (Figures 5A–5C). Peanut-specific IgG1 responses remained largely intact in SYKi-treated mice, indicating that the absence of IgE did not result from a global suppression of B cell function. However, we cannot rule out effects on Syk-mediated B cell receptor signaling by this compound (Figure 5D). Treatment with SYKi during sensitization was sufficient to prevent the development of anaphylactic food allergy to peanut, limiting temperature loss and preventing MMCP-1 release (Figures 5E and 5F). To ensure complete clearance of SYKi, peanut challenge was performed after a 10-day “wash-out” period.

In order to test whether Syk inhibition, like anti-IgE treatment, might facilitate tolerance induction in peanut-allergic animals, mice were sensitized to peanut and then separated into groups that received (1) no further treatment, (2) high-dose peanut desensitization, or (3) high-dose daily peanut under cover of SYKi. Mice were evaluated 10 days after the discontinuation of therapy. Mice subjected to peanut sensitization and those with peanut-only desensitization therapy developed normal anaphylactic reactions; the SYKi plus peanut group tolerated the challenge and displayed no symptoms of anaphylaxis (Figures 6A and S7). MMCP-1 release was reduced by approximately 2

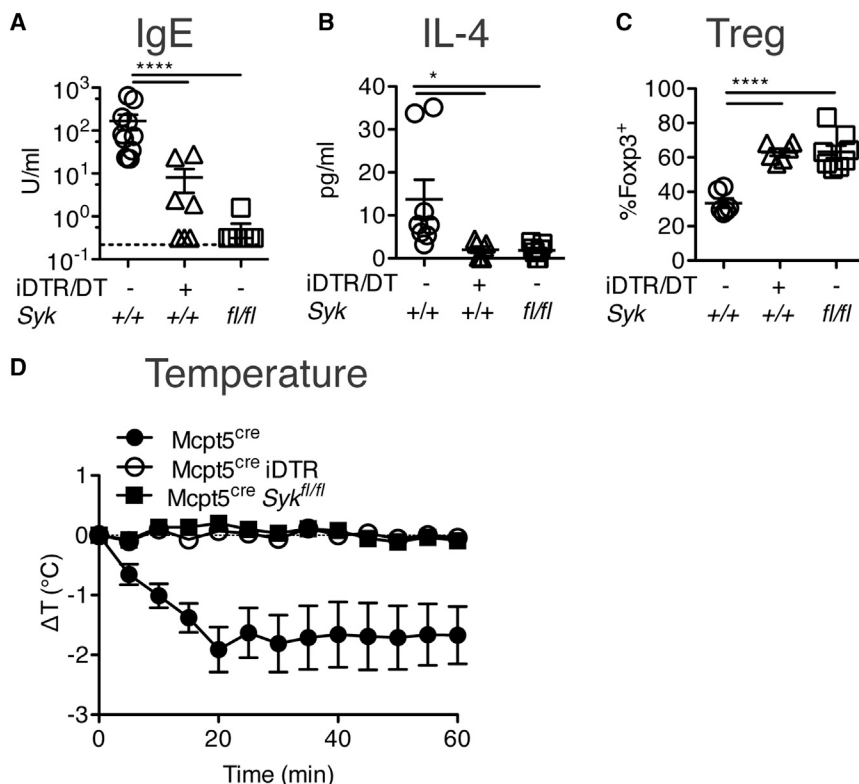


Figure 4. Deletion of Syk in Mast Cells Prevents Peanut Sensitization

(A) PN-specific IgE levels in sera. Mast-cell-directed induction of the diphtheria toxin receptor (DTR) or inactivation of Syk tyrosine kinase on the IL-4 α F709 (IL4 α ^{tm3.1Tch}) background were achieved by Mcpt5^{cre}-driven gene expression. Mice expressing iDTR on mast cells (IL4 α ^{tm3.1Tch} Mcpt5^{cre} iDTR) or with mast-cell-targeted Syk deletion (IL4 α ^{tm3.1Tch} Mcpt5^{cre} Syk^{fl/fl}) (n = 6–11) were sensitized once a week for 4 weeks with 23 mg PN i.g. Mast cells were depleted from Mcpt5^{cre} iDTR mice by i.p. injection of diphtheria toxin over 3 days (100 ng, 500 ng, 500 ng) 1 week prior to initiating PN sensitization (indicated as iDTR DT “+”).

(B) ELISA analysis of PN-specific IL-4 secretion in splenocyte cultures.

(C) Foxp3⁺ Treg cell frequencies among PN-responding CD3 ϵ ⁺CD4⁺ T cells from the MLN.

(D) Temperature curves from PN-treated IL4 α ^{tm3.1Tch} mice after enteral challenge with high-dose PN (450 mg).

p < 0.001 by RM two-way ANOVA Mcpt5^{cre} versus Mcpt5^{cre} iDTR and Mcpt5^{cre} versus Mcpt5^{cre} Syk^{fl/fl}. Data shown are a pool of three independent experiments, represented as mean $\pm$ SEM.

logs (Figure 6B). Peanut-specific Th2 cell responses were nearly absent in the tolerized mice, and specific Treg cell frequencies increased (Figures 6C and 6D). Peanut-specific IgE was reduced in mice receiving SYKi plus peanut compared with mice receiving peanut-only desensitization (Figure 6E). Our data demonstrate that the addition of SYKi to peanut desensitization markedly altered several critical aspects of the food allergen immune response in a manner consistent with tolerance induction (Figure S7).

Tolerance to foods is maintained in part by Treg cells and Treg cell deficiency is associated with severe food allergy (Bennett et al., 2001; Chatila et al., 2000; Wildin et al., 2001). Our analysis of Foxp3⁺ T cell frequencies in both the peanut and OVA food allergy models suggested that the mechanism underlying peanut desensitization in the setting of IgE:FcεRI signaling blockade was the induction of functional Treg cell, capable of suppressing effector T cell responses. This potential mechanism was directly evaluated by preparing Foxp3-eGFP⁺ Treg cells from mice that had been sensitized to peanut in the presence or absence of SYKi and transferring these cells into naive IL4 α ^{tm3.1Tch} recipients, which were in turn sensitized to peanut (Figure 7A). Treg cells from peanut+SYKi-treated donors almost completely ablated specific IgE responses in peanut-fed recipients (Figure 7B). T cell responses in the same mice exhibited significant shifts from Th2 cell to Treg cell biased (Figures 7C, 7D, S2G, and S2H). Only mice given Treg cells from peanut+SYKi-treated mice exhibited reductions in anaphylaxis and MMCP-1 release upon peanut challenge (Figures 7E and 7F). Recipients of Treg cells from mice exposed to peanut in the absence of SYKi had much more modest (and only signifi-

cant in the case of IgE) trends relative to mice that did not receive Treg cells.

Taken together, these results show that pharmacologic blockade of FcεRI signaling via a Syk inhibitor prevents food allergen sensitization and promotes tolerance induction in food-allergy-prone IL-4 α mutant mice. The Treg-cell-skewing effect of Syk inhibition can be exploited to restore tolerance in mice with established allergy. Our observations of food allergy suppression in recipients of Treg cells confirm the role of functional peanut-specific Foxp3⁺ Treg cells in oral tolerance. SYKi has no activity in T cells and in the context of our corroborating data using mice with lineage-specific Syk deletion in mast cells, mast cell deficiency models, and anti-IgE treatment, we attribute the block in functional Treg cell induction to FcεRI signaling blockade rather than any T-cell-intrinsic effect.

DISCUSSION

Mast cells reside at the interfaces between the body and the environment, stationing them as sentinels for the immune system. IgE antibodies arm these innate immune cells for specific antigen recognition. In the setting of food allergy, our findings suggest a triple function for these first-line defenders: (1) as effectors of anaphylactic reactions in allergen-exposed subjects, (2) as amplifiers of nascent Th2 cell and IgE responses during recurring sensitizing ingestions of food allergen, and (3) as suppressors of functional Foxp3⁺ Treg cell induction. Our report shows that mast cells and IgE:FcεRI signaling are required for the induction of adaptive Th2 cell immunity to ingested allergens and that they counter Treg cell expansion; in the absence of mast

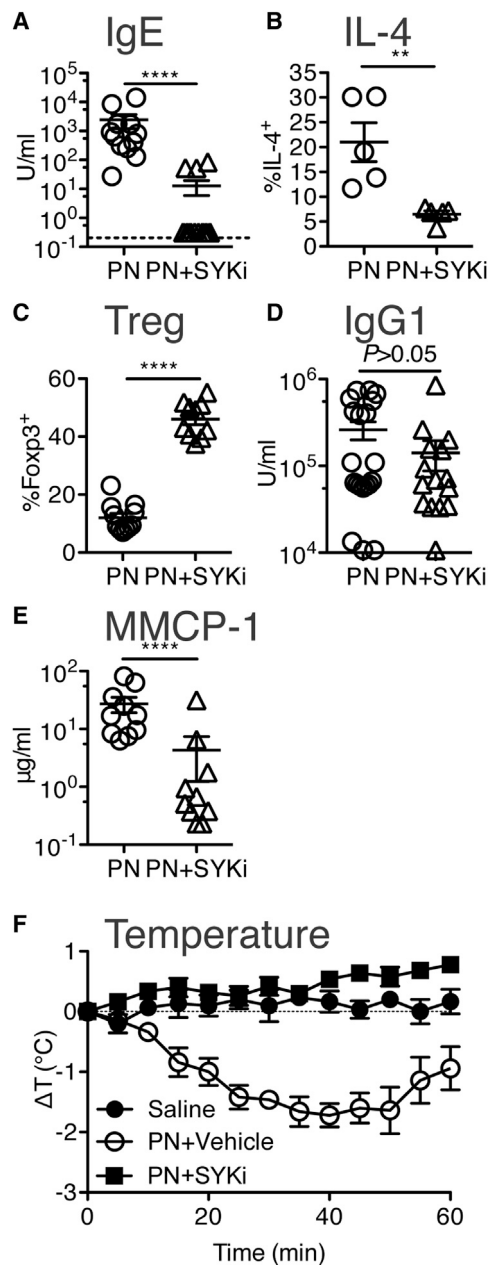


Figure 5. Targeted Pharmacologic Inhibition of Syk Blocks Food Allergen Sensitization

(A) PN-specific IgE levels in sera as measured by ELISA. IL-4 α F709 (IL4 α ^{tm3.1Tch}) mutant mice (n = 12–15) were sensitized once a week by enteral gavage with PN (23 mg) for 4 weeks. Mice were also gavaged with the Syk inhibitor SYKi (30 mg/kg) or vehicle (10% Tween-80 [pH 8.0], 5 ml/kg) 15 min prior to PN, 10 hr post-PN, and 24 hr post-PN. Abbreviations: PN, PN plus vehicle during sensitization; PN+SYKi, PN plus SYKi during sensitization.

(B) Flow cytometric analysis of IL-4 production by expanded CD4⁺ MLN cells exhibiting proliferative responses to PN in vitro (n = 5).

(C) Frequency of Foxp3⁺ cells among CD3e⁺CD4⁺ T cells proliferating to PN in vitro (n = 12–15).

(D) Serum levels of PN-specific IgG1 after sensitization, as determined by ELISA.

(E) Degranulatory release of MMCP-1 into serum after sensitization and challenge with PN (n = 10–11). There was a 10-day interval between the final SYKi treatment and the PN challenge.

cells or IgE signals, peanut-fed allergy-prone mice exhibited minimal Th2 cell and IgE responses but had robust Treg cell induction. This work was carried out with an innovative array of research tools including the novel IL-4 α mutant model of peanut allergy, in which enteral ingestion alone leads to anaphylactic food sensitivity, and a potent selective Syk inhibitor to modulate IgE:FcεRI signaling. The observation that inhibition of IgE signaling with anti-IgE antibody or Syk inhibition in the setting of pre-existing allergy leads to reversal of IgE and Th2 cell responses along with Treg cell expansion is completely new and quite striking. This result reveals significant plasticity in food allergy responses and points to Syk as an attractive therapeutic target in food allergy.

Food allergy has been notoriously difficult to model in mice. Even with the use of adjuvants and parenteral priming, which bypass normal immune-sensitization mechanisms, responses to food challenge are weak. Challenged animals do not exhibit anaphylaxis, a cardinal symptom of food allergy in patients. The IL-4 α mutant mice have provided a powerful genetic tool to address the pathogenesis of food allergy in a more physiologic setting. We believe the inherent predisposition to allergy, based on enhanced IL-4 α responses, in these mice reflects a cytokine-signaling imbalance that might be at work in atopic humans. We have previously reported that an IL-4 α polymorphism affecting signaling strength is linked to asthma (Hershey et al., 1997). In studies designed to identify loci linked with IgE production, the chromosome 5q31 cytokine cluster, which contains the gene encoding IL-4, is consistently identified (Marsh et al., 1994; Meyers et al., 1994; Xu et al., 1995), and one preliminary study reported a significant association between an IL4R α polymorphism and food allergen-specific IgE levels (Brown et al., 2012). We believe that deletion of the ITIM in the IL-4R α of the mice we have studied and the resultant disinhibition of the receptor are paradigmatic of the situation in atopic humans in whom nascent allergic sensitization also appears to be affected by an altered IL-4 signaling axis.

We and others have previously demonstrated that IgE antibodies exert important effects on mast cells above and beyond the induction of degranulation and immediate hypersensitivity. Even in the absence of antigen, IgE regulates mast cell homeostasis in culture and in vivo and modulates mast cell cytokine production (Asai et al., 2001; Bryce et al., 2004; Kalesnikoff et al., 2001; Kawakami and Kitaura, 2005; Mathias et al., 2009). Similar effects on mast cell cytokine production are observed in systems involving antigen-driven IgE signaling as well (Galli et al., 2005; Nakae et al., 2007a). The effects of IgE-mediated mast cell signaling in modulating Th2 cell and Treg cell responses in the peanut allergy system in this report are corroborated by four independent lines of investigation: the study of IgE-deficient mice, anti-IgE treatment, Mcpt5^{cre} Syk^{fl/fl} mice, and SYKi treatment. Each of these experimental models demonstrated impairment of Th2 cell and IgE responses along with

(F) Temperature curves after acute challenge with PN (450 mg) in PN+vehicle-, PN+SYKi-, or saline-treated mice (n = 5).

p < 0.001 by RM two-way ANOVA PN versus PN+SYKi. Data in (B) and (F) are representative of two experiments each, whereas the data in (A), (C), (D), and (E) have been pooled from three independent experiments. Data are shown as points for individual mice with mean $\pm$ SEM.

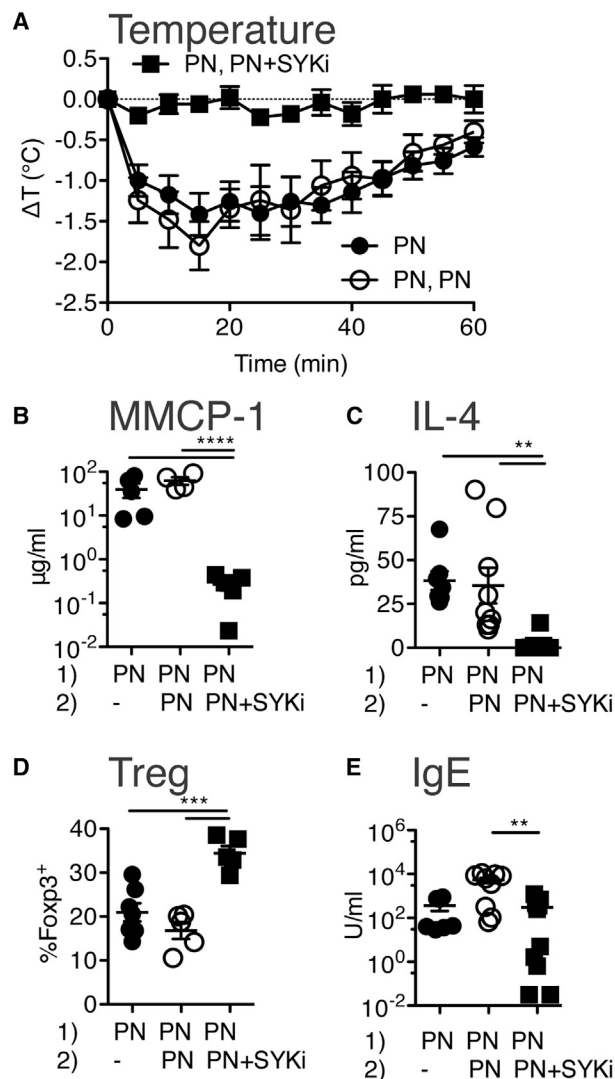


Figure 6. Pharmacological Inhibition of Syk Activity Enhances Food Allergen Desensitization

(A) Temperature curves after acute challenge with PN in PN-sensitized (PN), PN+SYKi-desensitized (PN, PN+SYKi), and mock-desensitized (PN, PN) mice ($n = 5$). PN-sensitized IL-4 α F709 (*Il4ra*^{tm3.1Tch}) mutant mice were subjected to 3 weeks of daily oral desensitization therapy with PN (225 mg) (PN), or PN paired with SYKi (30 mg/kg, p.o., b.i.d.) (PN, PN+SYKi). The final challenge was performed in the absence of SYKi, 10 days after the final SYKi dose. PN-sensitized mice that received no therapy after sensitization are shown for comparison (PN). $p = 0.0016$ by RM two-way ANOVA for PN versus PN, PN+SYKi or PN, PN versus PN, PN+SYKi.

(B) MMCP-1 levels in serum postchallenge ($n = 4-5$).

(C) IL-4 secretion by splenocyte cultures in response to PN stimulation ($n = 9-10$).

(D) PN-specific Treg cell frequencies in dividing MLN CD3 ϵ ⁺CD4⁺ cells ($n = 5-6$).

(E) Serum levels of peanut-specific IgE ($n = 9-10$).

Data shown are representative of two independent experiments (A, B, and D) or have been pooled from two experiments (C and E) and are represented as mean $\pm$ SEM.

enhanced Treg cell induction, providing a strong aggregate case for a Th2 cell adjuvant effect of IgE signaling in emerging food allergy responses. These results have potential implications for the design of strategies to achieve safe and effective oral desensitization in patients with peanut allergy. Although anti-IgE treatment during food desensitization has recently been applied in clinical trials, the immunomodulatory effects of such IgE blockade are not yet known.

The role of mast cells in murine disease models is challenging to assess given the limitations of each of the various mast cell deficiency models (Rodewald and Feyerabend, 2012). The fully consistent results we independently obtained with both Kit-dependent and -independent models of deficiency (*Kit*^{W-sh} and *Mcpt5*^{Cre} iDTR, respectively) provide strong converging support for a critical mast cell contribution to Th2 cell induction and Treg cell suppression. Whereas *Mcpt5*^{Cre} iDTR mice have previously been reported to have an intact mucosal mast cell compartment (based on baseline histologic analyses of the stomach), we have found that IL-4-driven and allergen-induced mucosal mast cell expansion in the jejunum is in fact suppressed in these animals, and it has been reported that MMCP-5 expression is not restricted to submucosal mast cells under Th2 cell conditions (Friend et al., 1996; Xing et al., 2011). DT-treated animals exhibited both impaired mast cell expansion and the same block in Th2 cell and IgE responses observed in IgE-deficient and *Kit*^{W-sh} mutants.

Although mast cells are not MHCII⁺ and may not function as antigen-presenting cells in this food allergy model system, we observed that they accumulate in the draining lymph nodes of sensitized animals, placing them proximal to T cell priming events. By immunofluorescent microscopy, both Treg cells and mast cells are present in the jejunal lamina propria and Peyer's patches (data not shown). Mast cells are known to produce significant amounts of the critical Th2-cell-inducing cytokine IL-4, which has the capacity both to subvert Treg cell generation, by destabilizing Foxp3 expression, and to simultaneously activate the Th2 and Th9 cell pathways via STAT6 signals (Dardalhon et al., 2008; Wang et al., 2010). We observe that when mast cells are cultured with naive T cells under Treg-cell-inducing conditions, FcεRI crosslinking inhibits TGF- β -driven expansion of Foxp3⁺ T cells. Furthermore, we find that *Il4* and *Il13* transcripts are very rapidly induced (within 1 hr) in the small intestine after allergen exposure and that these transcripts are Syk and IgE dependent, strongly implicating mast cells as cellular sources of IL-4 and IL-13 early after food ingestion. Our results from WT versus IL-4-deficient mast cell reconstitution of mice lacking mast cells demonstrate an essential role for mast-cell-derived IL-4 in promoting sensitization to ingested allergen.

Our findings that (1) peanut-specific Treg cell frequencies are higher in IgE-deficient, anti-IgE-treated, mast cell-deficient, mast cell Syk-deficient, and SYKi-treated mice and that (2) Treg cells from peanut-fed SYKi-treated mice functionally suppress the induction of peanut allergy in naive recipients identify a central mechanism whereby IgE antibodies and mast cells enhance emerging allergic sensitization: they suppress effective Treg cell responses. We and others have previously demonstrated that inherited Foxp3 deficiency leads to severe intractable food allergy and that patients with Foxp3 mutations lack

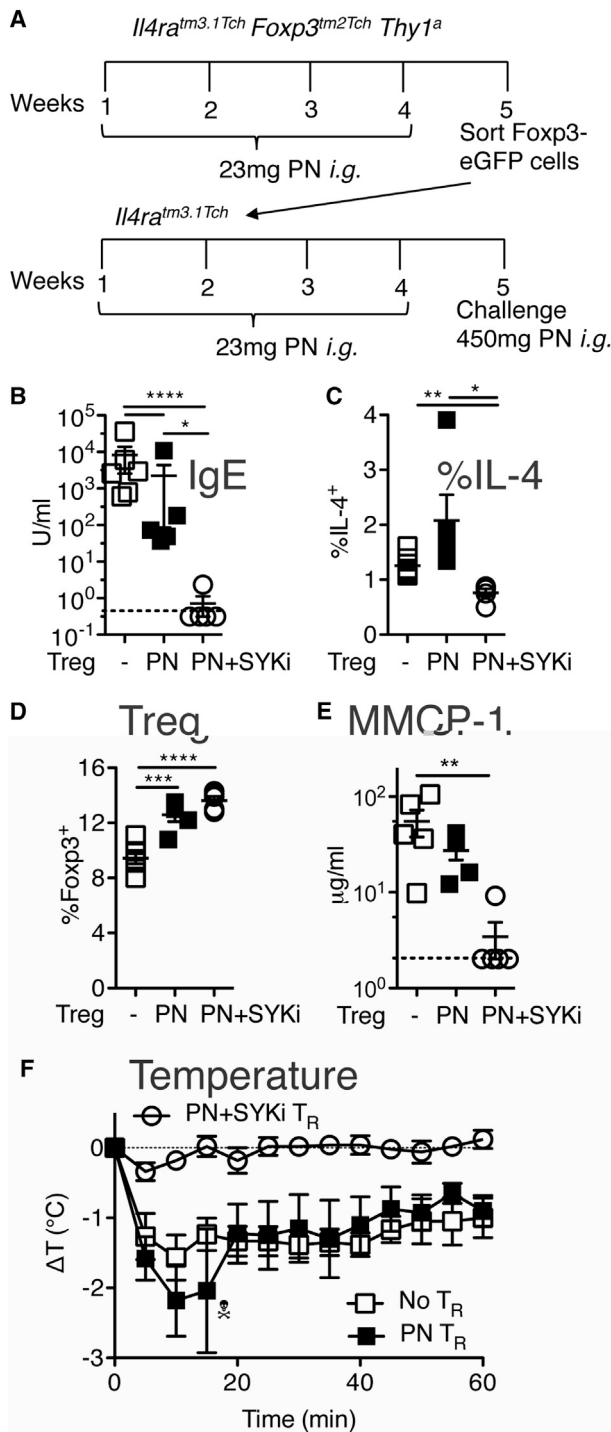


Figure 7. Treg Cells Induced while Mast Cells Are Paralyzed Effectively Control Food Allergen Sensitization

(A) Experimental schematic. IL-4 α F709 mutant Thy1.1-congenic Foxp3-eGFP (*Il4ra*^{tm3.1Tch} *Foxp3*^{tm2Tch} *Thy1*^a) donor mice ($n = 5$) were sensitized to peanut with or without SYKi for 4 weeks, at which point their CD4⁺Foxp3-eGFP⁺ Treg cells (T_R) were purified and transferred (4×10^5 /mouse) into naive IL-4 α F709 (*Il4ra*^{tm3.1Tch}) recipients (for PN and PN+SYKi T_R groups). Mice that did not receive Treg cells are included as positive controls for peanut sensitization. All recipient mice were sensitized with 23 mg PN once a week for 4 weeks and challenged with 450 mg PN by gavage ($n = 5$).

functional Treg cells (Bennett et al., 2001; Chatila et al., 2000; Wildin et al., 2001).

Syk inhibition offers an attractive pharmacologic approach for blocking IgE-induced FcεRI signaling. Previously available small-molecule Syk inhibitors have had significant off-target effects, however, especially on the closely related kinase ZAP70 in T cells. SYKi, used in this study, has >25-fold enhanced specificity for fully phosphorylated Syk versus ZAP70 (Moy et al., 2013). Although Syk is also the proximal signaling kinase for the B cell receptor (BCR), we believe that its major effects on allergic responses to peanut in our study resulted from its FcεRI inhibition. In support of this conclusion is our finding that the phenotype of peanut-fed mice in which Syk is specifically inactivated in mast cells, but not B cells, exactly mirrors that of the SYKi-treated animals. BCR activation is normal in the mice with mast-cell-targeted Syk gene deletion yet they exhibited the same impairment in Th2 cell and IgE induction and enhanced Treg cell responses to SYKi-treated mice, consistent with an FcεRI-driven effect.

In summary, our study provides clear evidence that IgE-mediated mast cell activation serves not only to elicit immediate hypersensitivity reactions but also to amplify evolving allergic sensitization to ingested proteins. We speculate that manipulations of the IgE axis might some day provide clinical benefit.

EXPERIMENTAL PROCEDURES

Mice

Detailed mouse information can be found in the Supplemental Experimental Procedures. All mice were bred and maintained under specific-pathogen-free conditions at Boston Children's Hospital. All animal experiments were conducted under procedures approved by the Boston Children's Hospital Institutional Animal Care and Use Committee.

Allergic Sensitization and Anaphylaxis

Mice were sensitized by enteral gavage with ball-tipped 18 g feeding needles. Peanut butter (Skippy, Hormel Foods) was gavage in 250 μ l 0.1 M sodium bicarbonate (pH 8.0). Mast cells were depleted by i.p. injection of diphtheria toxin (List Biological Laboratories) in saline over 3 days: 100 ng day 1, 500 ng day 2, and 500 ng day 3. SYKi was prepared as previously described (Moy et al., 2013) and solubilized in pH 8.0 aqueous isosmotic Tween-80 (10% w/v, Sigma). SYKi (30 mg/kg) and vehicle control were delivered by gavage in 5 ml/kg. Anti-IgE (clone R35-92, BD) was injected i.p. in tripling doses every hour starting at 50 ng, integrating to a total of 25 μ g the first day. Over the next 3 days, 25 μ g anti-IgE was injected once a day i.p., giving a total of 100 μ g anti-IgE per mouse. Core body temperature measurements taken to monitor anaphylaxis were recorded by subdermally implanted transponders (IPTT-300) and a DAS-6001 console (Bio Medic Data Systems) linked to a netbook. For sensitization protocols and further information, see Supplemental Experimental Procedures (Figure S1).

(B) PN-specific IgE production in recipient mice.

(C) Flow cytometric analysis of IL-4 in CD4⁺ T cells from the MLN.

(D) Foxp3⁺ Treg cell frequencies in the MLN of recipient mice as assessed by flow cytometric analysis of CD4⁺CD3 ϵ ⁺ cells taken directly ex vivo.

Data in (C) and (D) exclude Thy1.1⁺ donor cells from the analyses.

(E) Serum MMCP-1 release after acute enteral challenge of recipient mice with PN (450 mg).

(F) Core temperature loss after PN challenge in recipient mice ($p < 0.001$ by RM two-way ANOVA No T_R versus PN+SYKi T_R and PN+SYKi T_R versus PN T_R). The skull-and-crossbones symbol indicates a death from anaphylaxis in the PN T_R group.

Data are from a single experiment, and are shown as mean $\pm$ SEM.

Cell Culture

Bone-marrow-derived mast cells (BMMCs) were cultured as previously described (Burton et al., 2013). BMMCs were used for reconstitution experiments after 3–6 weeks of culture. Splenocytes were cultured in RPMI-1640 supplemented as previously described (Burton et al., 2013) and stimulated for antigen-specific recall with 100 µg/ml peanut extract. Endogenous allergen-specific T cells were identified by allergen-induced ex vivo proliferation, along the lines of assays developed for assessing human tetanus toxoid recall (Narendran et al., 2002). MLN cells (5×10^6) were labeled with Violet CellTrace (Life Technologies) and cultured with or without allergen (100 µg/ml peanut extract) for 5 days. Cells undergoing proliferation (dye dilution) were considered allergen specific, a conclusion supported by a lack of proliferation in the absence of allergen or in allergen-stimulated cells from unsensitized mice. We note that this approach does not provide the absolute number of Treg cells residing in the MLN but nevertheless gives a robust readout of the relative efficiency with which Treg cells are induced among experimental groups.

Mast Cell Reconstitution

Weanling *Il4ra*^{tm3.1Tch} *Kit*^{W-sh} mice were injected i.p. with 5×10^6 BMMCs and a second injection of BMMCs was performed 4 weeks later. Allergic sensitization was started 8 weeks after the initial transfer, at which point mast cell reconstitution of intestinal compartments has been shown to be evident (Grimbaldeston et al., 2005). Reconstitution was performed with matched cultures of WT C57BL/6J and IL-4-deficient *B6.129P2-Il4*^{tm1Cgn}/J (stock number 002253) mast cells prepared from age-matched female mice purchased from Jackson Laboratories.

Histology

Sections of the jejunum were taken approximately 10 cm from the pyloric sphincter and fixed overnight in 10% formalin (Sigma). Samples were stored in 70% ethanol prior to processing and toluidine blue staining by the Beth Israel Deaconess Medical Center Histology Core. Chloroacetate esterase staining, which stains mast cells with a characteristic red color, was performed as previously described (Burton et al., 2013; Friend et al., 1998). Samples were randomized and coded prior to histological processing, with scoring and staining being performed by an investigator unaware of the sample identities.

Statistics and Data Analysis

Data were graphed and analyzed with GraphPad Prism 5.0. Anaphylaxis temperature curves were analyzed by repeated-measures two-way ANOVA for overall distinction between treatment groups. Unpaired t tests were used to compute two-tailed p values for comparisons between two groups. For three or more groups, ANOVA was used, with individual p values coming from Bonferroni posttests. p values are abbreviated as *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. Because peanut-specific IgE and MMCP-1 levels varied by a few orders of magnitude, these data are presented on graphs with logarithmic y axes and were log-transformed prior to statistical analysis. Samples in which specific immunoglobulins or MMCP-1 were undetectable were assigned a nominal value corresponding to the limit of detection in the assay (0.3125 units/ml for peanut-specific IgE). Data are presented as mean ± SEM, with points representing individual mice where presented.

SUPPLEMENTAL INFORMATION

Supplemental Information include seven figures and Supplemental Experimental Procedures (including flow cytometry, cell sorting, and ELISA) and can be found with this article online at <http://dx.doi.org/10.1016/j.immuni.2014.05.017>.

AUTHOR CONTRIBUTIONS

O.T.B., H.C.O., M.N.-R., and T.A.C. designed experiments. O.T.B. and M.N.-R. performed experiments and developed experimental models. S.L.L. and J.S.Z. helped carry out experiments. K.J.K. and A.R.D. provided technical assistance and developed assays. H.H. and M.A.C. participated in the experimental design and data analysis for the SYKi experiments. A.R. provided Mcpt5^{Cre} mice and participated in the analysis of the Mcpt5^{Cre} data. O.T.B.

and H.C.O. wrote the manuscript. All authors participated in discussions of experimental results and edited the manuscript.

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Supplemental Information

Immunoglobulin E Signal Inhibition during Allergen Ingestion Leads to Reversal of Established Food Allergy and Induction of Regulatory T Cells

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Inventory of Supplemental Materials

Figure S1. Schematics of experimental food allergy procedures, related to Experimental Procedures and Figure 1.

Figure S2. Specificity of hypersensitivity reactions in *Il4raF709* mice, related to Figure 2.

Figure S3. Analysis of intestinal mast cell numbers in mast cell-deficient mice, related to Figure 3.

Figure S4. Characterization of Mcpt5-Cre mice, related to Figure 4.

Figure S5. IL-4-driven expansion of mast cells in Mcpt5-Cre/iDTR mice, related to Figure 4.

Figure S6. Effects of Syk inhibition on anaphylaxis, related to Figure 5.

Figure S7. Immunological effects of SYKi-facilitated food allergen desensitization, related to Figure 6.

Supplemental Experimental Procedures

Supplemental References

Supplemental Figure Legends

Supplemental Materials

Supplemental Figures

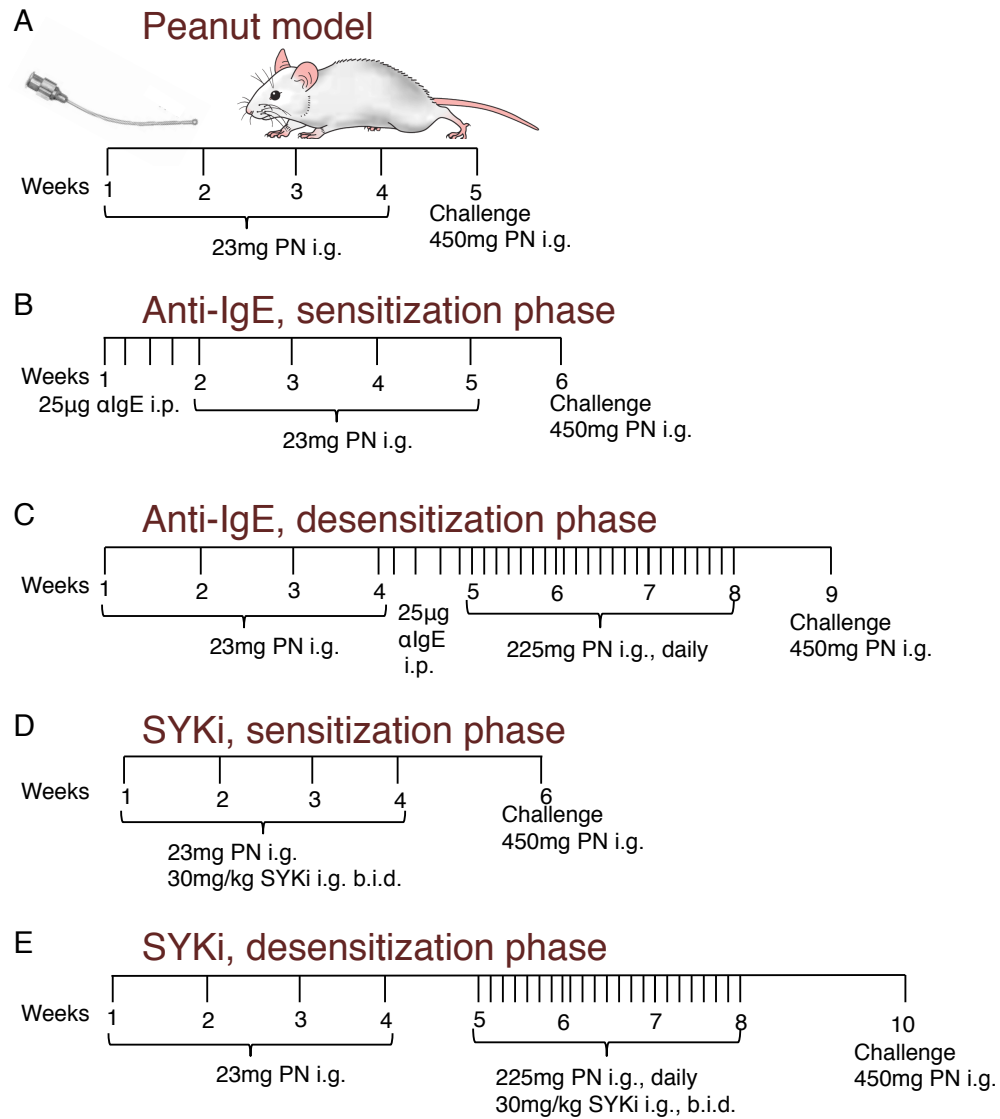


Figure S1, related to Experimental Procedures. Schematic of experimental food allergy procedures. A) Basic four-week peanut sensitization model in IL-4 α F709 mutant (*Il4ra*^{tm3.1Tch}) mice. Mice receive 23mg peanut butter (5mg protein) in 250 μ l 0.1M sodium bicarbonate pH 8.0 by oral gavage once a week over four weeks. Mice are challenged with an enteral bolus dose of 450mg peanut butter (~100mg protein) diluted with water 7-10 days after the final sensitization dose, and core body temperature readings are taken. **B)** Anti-IgE prevention therapy. To neutralize endogenous IgE, mice receive 100 μ g anti-IgE over the course of four days. On the first day, anti-IgE is injected *i.p.* in doubling or tripling doses every 90min starting at 50ng, and reaching a total of 25 μ g. On the second through fourth days, 25 μ g anti-IgE is injected *i.p.* in a single shot. Peanut sensitization is then accomplished as in A, starting three days after the completion of anti-IgE treatment. **C)** Anti-IgE assisted allergen desensitization model. Mice are sensitized to peanut as in A, and then treated with anti-IgE as in C. Desensitization therapy then begins, with mice receiving gavage doses of 225mg peanut butter (50mg protein) daily for three weeks. After 7-10 days of rest, mice are challenged with 450mg peanut butter (100mg protein) to assess anaphylaxis. **D)** SYKi-mediated allergy prevention model. Mice are sensitized to peanut as in A (or OVA as in B), but also receive two daily gavage doses of SYKi (30mg/kg) or vehicle (10% Tween-80 pH 8.0, 5ml/kg), the first 15min prior to allergen exposure, the second 10-12hrs later. SYKi is only administered once a week, in conjunction with allergen. Challenge occurs 10 days after the final sensitization dose, in the absence of SYKi. **E)** SYKi desensitization therapy.

Mice are sensitized to peanut. One week after the final sensitization dose, mice are subjected to daily dosing of 225mg peanut (50mg protein) and SYKi or vehicle (dosing as in E) for three weeks. Challenge occurs 10 days after the final desensitization dose, in the absence of SYKi.

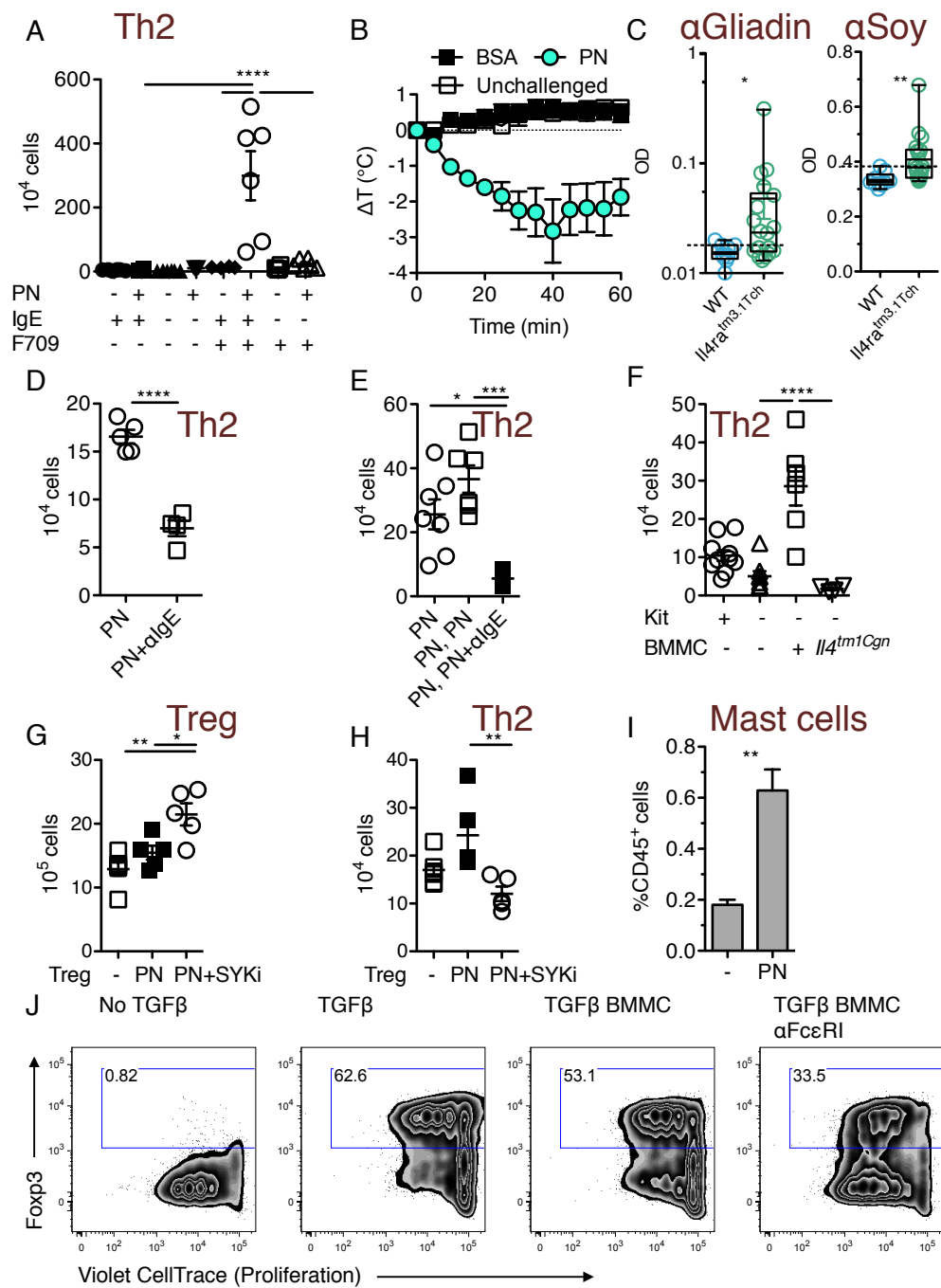


Figure S2, Related to Figures 1, 2, 3 and 7. Spontaneous sensitization to chow components and specificity of food hypersensitivity reactions in IL-4 α F709

mutant (Il4ra^{tm3.1Tch}) mice. A) Effect of IgE genotype on total numbers of Th2 cells in the MLN. Refer to Figure 1C. **B)** Challenge of peanut-sensitized IL-4 α F709 mutant *Il4ra^{tm3.1Tch}* mice ($n=5$) with an equivalent mass (100mg) of an irrelevant protein (BSA). $P=0.0041$ by repeated measures two-way ANOVA for BSA vs. PN challenge. **C)** Optical density (OD) values for IgE against gliadin (wheat) and soy in sera from 16-20 week old WT and *Il4ra^{tm3.1Tch}* mice. The dashed line indicates the background without serum. $*P<0.05$, $**P<0.01$ by unpaired t test on log-transformed values. **D)** Effect of α IgE treatment prior to sensitization on numbers of Th2 cells in the MLN. Refer to Figure 2B. **E)** Effect of α IgE prior to desensitization on Th2 cell numbers. Refer to Figure 2C. **F)** Effect of mast cells on Th2 cell numbers using *Il4ra^{tm3.1Tch} Kit^{W-sh}* mice. Refer to Figure 3B. **G)** Endogenous Thy1.2⁺ Treg cell numbers in the MLN of peanut-sensitized *Il4ra^{tm3.1Tch}* mice receiving Thy1.1⁺ Treg cells from SYKI-treated peanut-sensitized *Il4ra^{tm3.1Tch}* mice. Refer to Figure 7D. **H)** Th2 cell numbers in Treg cell recipients as in panel G. Refer to Figure 7C. **I)** Mast cell frequencies in the MLN following peanut sensitization. $**P<0.01$ by unpaired t test. **J)** Influence of mast cell activation on Treg cell induction *in vitro*. Violet CellTrace-labeled DO11.10 Rag-deficient Foxp3-reporter CD4⁺ T cells were cultured on plate-bound α CD3 and α CD28 for 5 days with TGF β . BMMC were added at a 1:1 ratio and activated by plate-bound α Fc ϵ RI. Foxp3 (eGFP) expression was measured by flow cytometry. Data in C are from a single cohort, while the others were replicated in at least one additional experiment. Where summarized, data are presented as mean $\pm$ SEM.

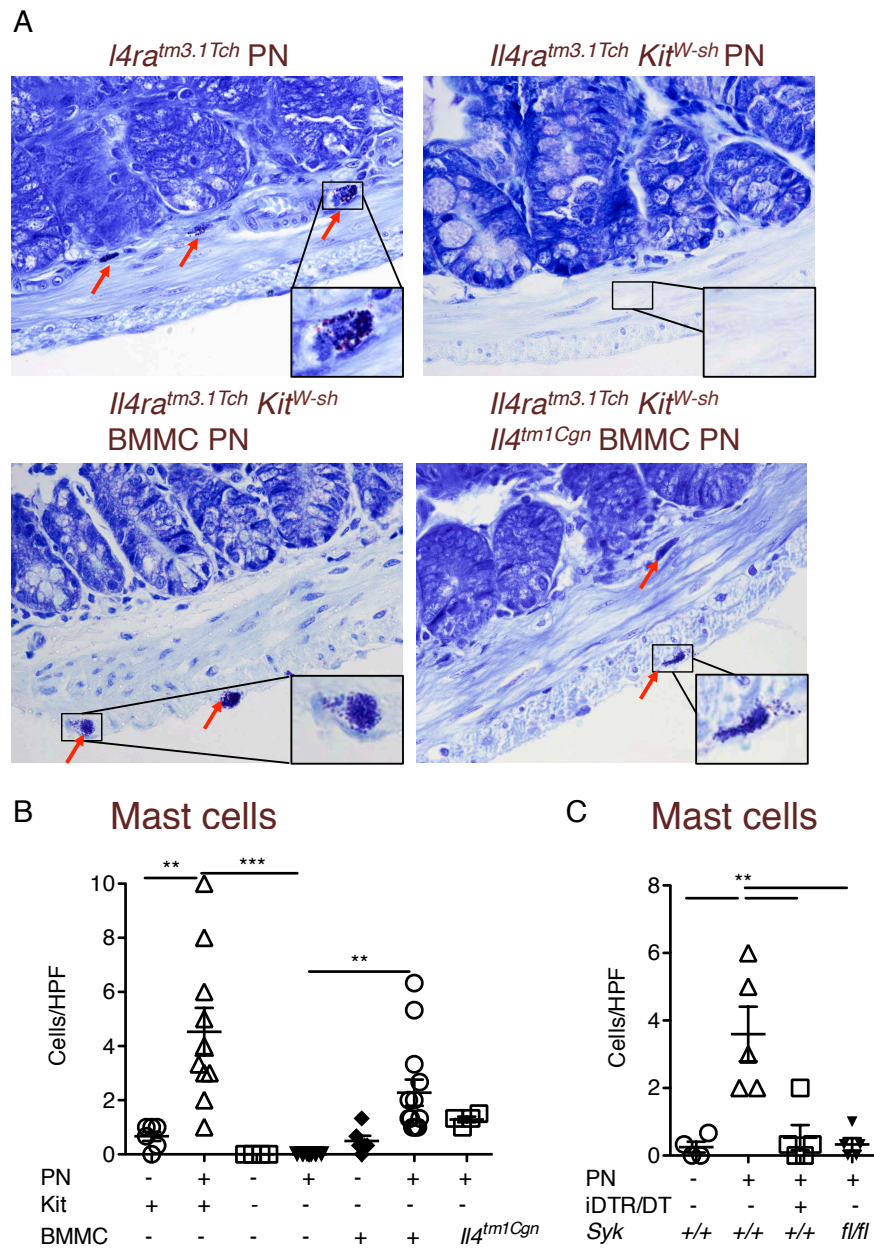


Figure S3, related to Figures 3 and 4. Analysis of intestinal mast cells in mast cell-deficient mice. A) Representative toluidine blue-stained sections of jejunum

with red arrows showing mast cells. Insets show selected mast cells at higher power. The inset for the unreconstituted IL-4 α F709 mutant *Il4ra*^{tm3.1Tch} *Kit*^{W-sh} shows a random section of jejunum without a mast cell. **B)** Mast cell counts from toluidine blue-stained sections of jejunum. Mast cell reconstitution and PN sensitization were performed as in Figure 3. **C)** Mast cell counts in chloroacetate esterase-stained sections of jejunum from mice with the Mcpt5^{cre} transgene. Diphtheria toxin (DT) was injected *i.p.* into *Il4ra*^{tm3.1Tch} Mcpt5^{cre} iDTR mice one week prior to sensitization, and the mice were sensitized to PN for four weeks as in Figure 4. Note that mast cells begin to recover from depletion after approximately three weeks (Dudeck et al., 2011; Scholten et al., 2008), and this analysis is performed five weeks post-depletion. Data in B are pooled from three experiments, while the data in C are from one of three experiments. Data are presented with mean $\pm$ SEM in B and C.

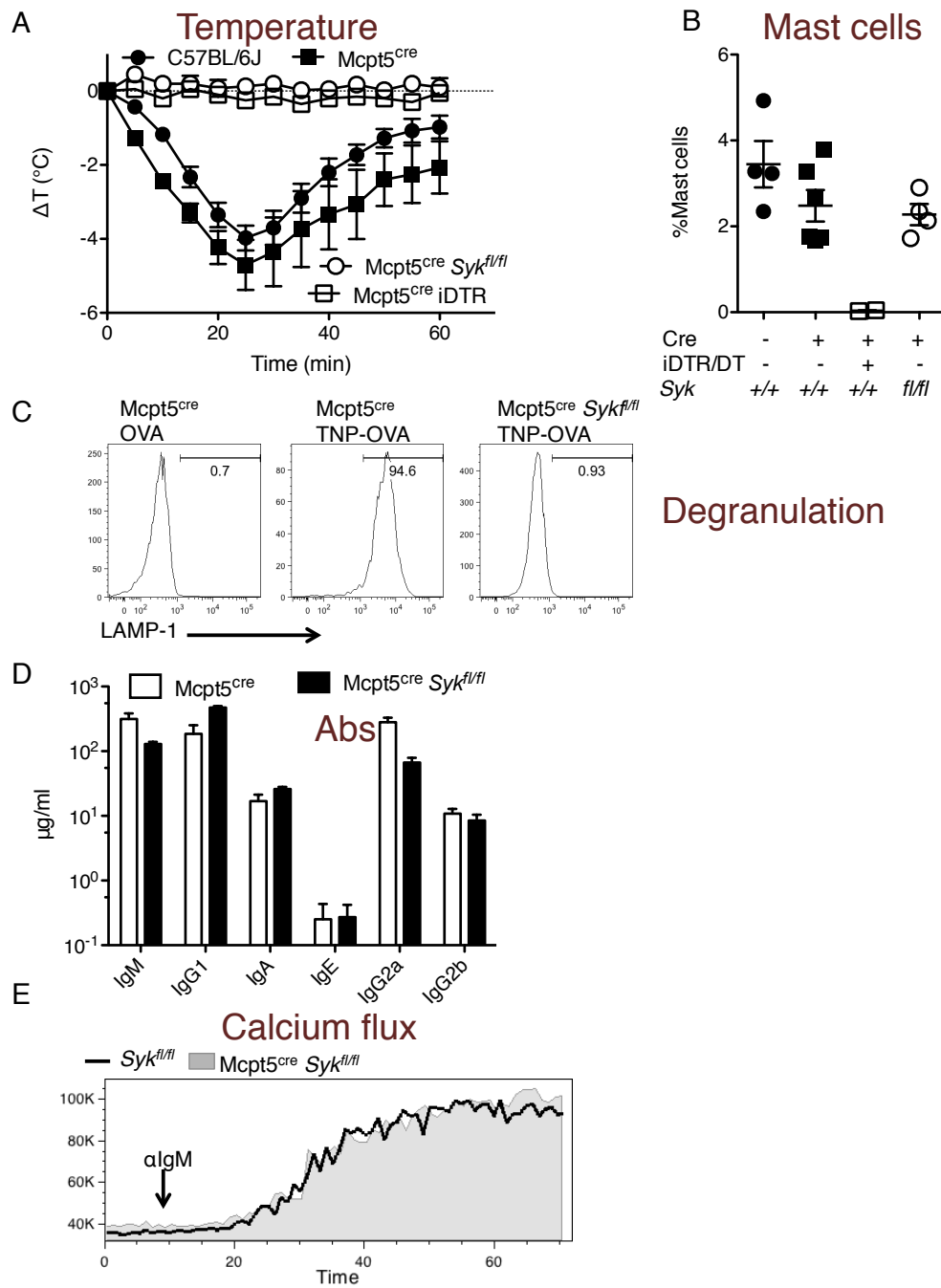


Figure S4, related to Figure 4. Characterization of *Mcpt5^{cre}* mice. A)

Temperature responses to passive anaphylactic challenge. Wild-type C57BL/6J,

Mcpt5^{cre}, Mcpt5^{cre} Syk^{fl/fl} and Mcpt5^{cre} iDTR mice ($n=4-6$) were injected *i.p.* with 2µg IgE anti-TNP, and challenged *i.p.* with 20µg TNP-OVA four days later. Mcpt5^{cre} iDTR mice were treated with DT in the interim to deplete mast cells as in Figure 4. Core body temperature was measured using subcutaneously implanted transponders. Data are presented as mean±SEM. **B)** Frequencies of mast cells in peritoneal lavage fluid. Mast cells (c-Kit⁺FcεRIα⁺) were detected by flow cytometry. **C)** Flow cytometric analysis of mast cell degranulation by LAMP-1 staining. Mast cells were expanded from peritoneal lavage cells by culture for two weeks in IL-3 (10ng/ml), SCF (20ng/ml) and IgE anti-TNP (100ng/ml). Mast cells were stimulated for ten minutes with OVA-TNP (100ng/ml) in the presence of anti-LAMP-1-PerCP-e710 to detect degranulation and co-stained with anti-c-Kit-PE. Unconjugated OVA (100ng/ml) was used as a negative control for manipulation. Non-viable cells were excluded using eFluor780 fixable viability dye. **D)** ELISA analysis of basal immunoglobulin levels in Mcpt5^{cre} and Mcp5^{cre} Syk^{fl/fl} mice ($n=4$). **E)** Kinetics of calcium flux in B cells from Mcpt5^{cre} and Mcpt5^{cre} Syk^{fl/fl} mice upon stimulation through the BCR (anti-IgM). Similar data were obtained in an additional set of experiments. Data in A and B are mean ±SEM, while in D the mean +SEM is presented.

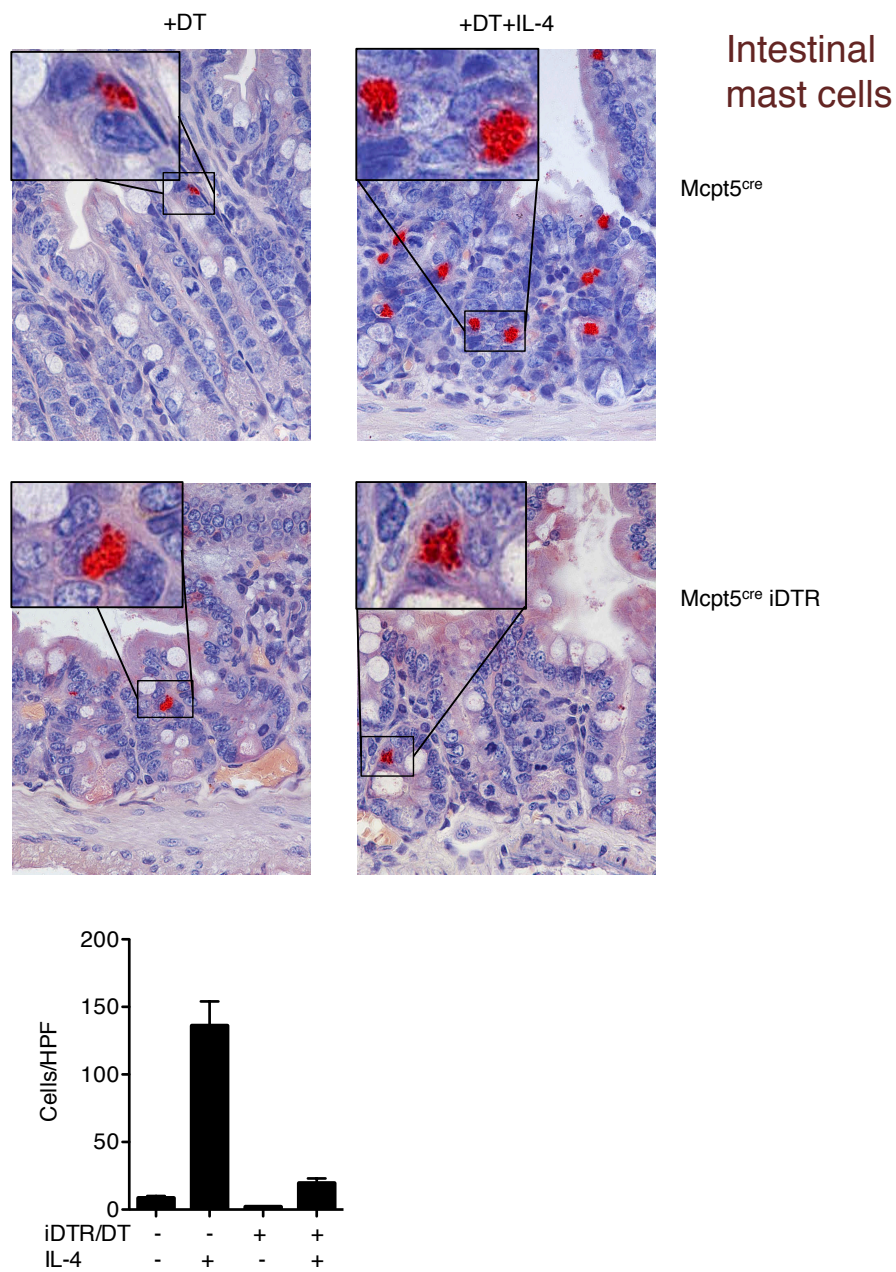


Figure S5, related to Figure 4. Representative chloroacetate esterase staining of jejunal sections showing mast cells in red and counts from three mice. Mice

were treated with DT (100ng day 1, 500ng day 2, 500ng day 3) to deplete mast cells, and IL-4:anti-IL-4 complexes (2µg IL-4 plus 10µg anti-IL-4, days 1, 4) to promote mast cell growth. Mice were sacrificed for analysis on day 7. Data derive from one of three experiments. The bar chart shows the mean +SEM.

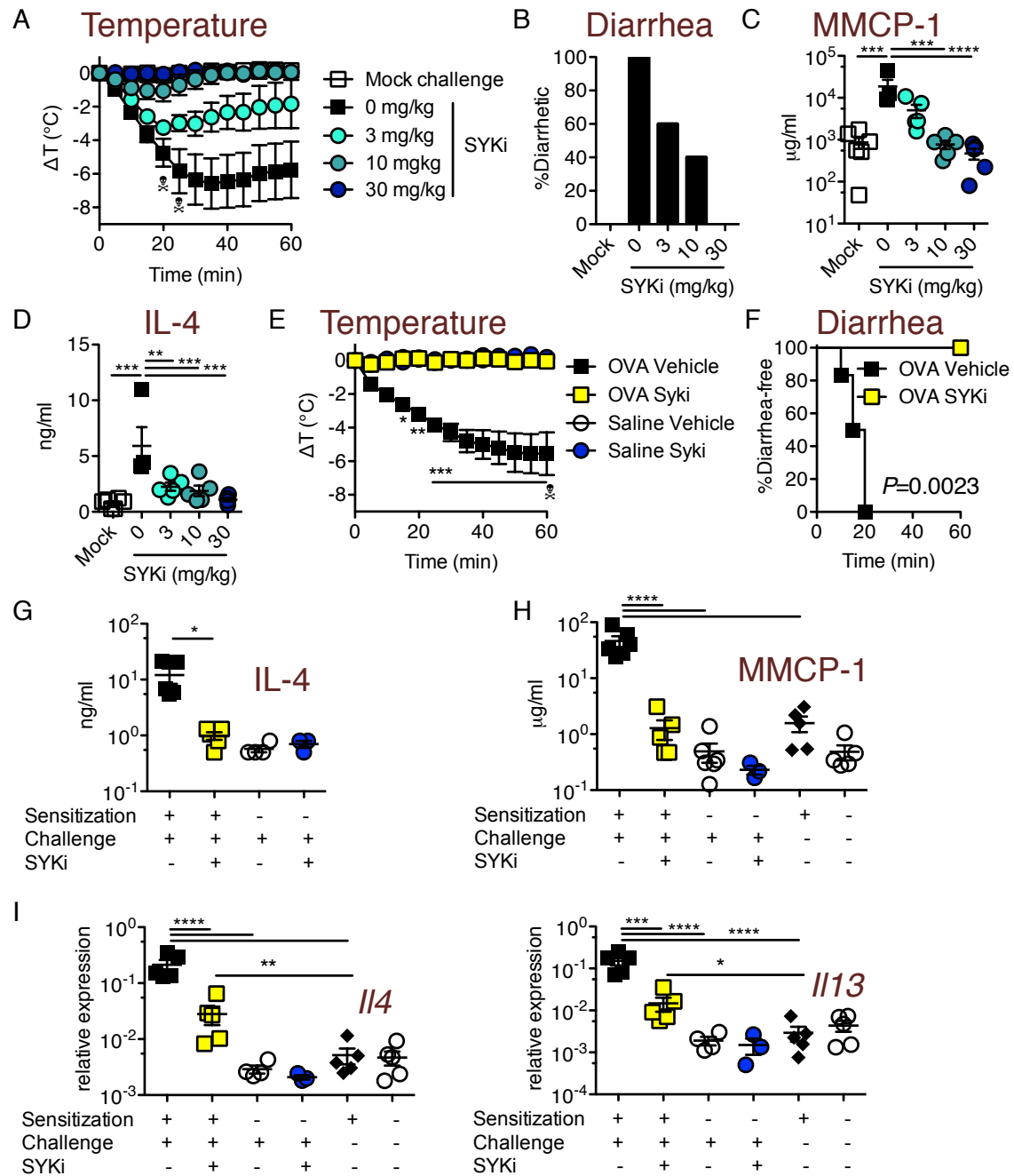


Figure S6, related to Figure 5. Effects of Syk inhibition on anaphylaxis. A)

Effects of escalating doses of SYKi on core temperature loss and lethality

following IgE-mediated anaphylaxis. IgE-deficient IL-4 α F709 mutant *Il4ra*^{tm3.1Tch} mice were sensitized with 20 μ g IgE anti-DNP (SPE-7) by intravenous injection into the retro-orbital sinus, and then challenged with 500 μ g DNP-BSA *i.p.* 16hrs later ($n=5-6$ per group). SYKi or vehicle was administered by oral gavage 15min prior to challenge. **B)** Effects of Syk inhibition on diarrhea onset ($n=5-6$). **C)** ELISA analysis of MMCP-1 release into serum ($n=4-6$). Serum was not available from mice that died during anaphylaxis, so the data for the vehicle group have effectively been stripped of the high responders. **D)** Serum IL-4 levels after IgE-mediated anaphylaxis as measured by *in vivo* cytokine capture assay. Only mice that survived the anaphylaxis were analyzed. Biotinylated anti-IL-4 (10 μ g) was injected intravenously 16hrs prior to challenge. Statistical analysis in C and D by ANOVA with Bonferroni post-tests. **E)** Effects of SYKi on acute food allergen-induced anaphylaxis. Temperature curves following enteral challenge with OVA. *Il4ra*^{tm3.1Tch} mice were sensitized for three weeks with 250 μ g OVA and 20 μ g cholera toxin or mock-sensitized with saline, and then subjected to oral challenge with 150mg OVA. SYKi (30mg/kg) or vehicle was administered 15min prior to challenge. **F)** Kinetics of diarrhea onset following acute food allergen challenge. **G)** Serum IL-4 levels as determined by IL-4 *in vivo* cytokine capture assay. **H)** MMCP-1 levels in serum of sensitized or sensitized and challenged mice. **I)** Induction of mRNA transcripts for IL-4 and IL-13 in the jejunum following acute food allergen challenge. Error bars show the mean $\pm$ SEM. Data are from a single experiment.

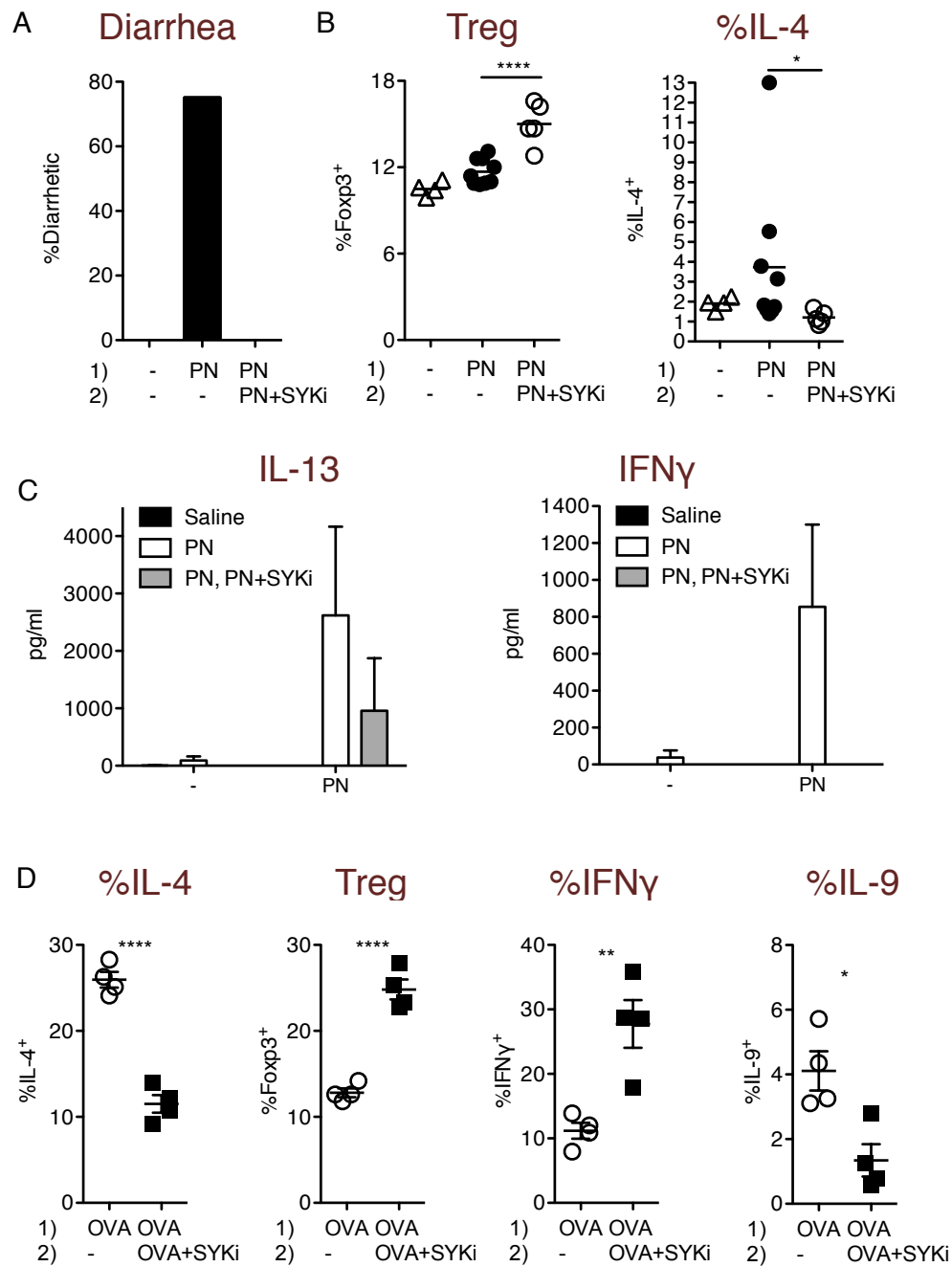


Figure S7, related to Figure 6. Immunological effects of SYKi-facilitated food allergen desensitization. A) Diarrhea incidence in PN-allergic and PN+SYKi-

desensitized mice. IL-4 α F709 mutant (*Il4ra*^{tm3.1Tch}) mice were treated as in Figure 6. **B)** Treg and Th2 cell frequencies in MLN cells following PN+SYKi desensitization therapy. Data were obtained by flow cytometric analysis of CD4⁺ T cell populations, assessing Foxp3 and IL-4 expression. **C)** ELISA analysis of IL-13 and IFN γ levels in splenocyte cell culture supernatants. Splenocytes (10⁶/ml) were cultured with PN (100 μ g/ml) for 5 days. **D)** Phenotypic analysis of CD4⁺ T cells exhibiting OVA-specific proliferative responses *in vitro*. MLN cells from *Il4ra*^{tm3.1Tch} mice ($n=4$) sensitized with OVA+CT and left untreated or desensitized with SYKi and high dose (150mg) OVA were labeled with Violet CellTrace and cultured for 5 days with 100 μ g/ml OVA. The cells were stimulated with ionomycin, phorbol 12,13-dibutyrate and brefeldin A, and then stained for intracellular cytokine and Foxp3 expression. Data shown derive from analyses of CD4⁺ viable single cells exhibiting dye dilution. * $P<0.05$, ** $P<0.01$, **** $P<0.0001$ by unpaired t test. Data are representative of three experiments, and are presented as mean +SEM in C or mean $\pm$ SEM in D.

Supplemental Experimental Procedures

Mice

The generation of IL-4 α F709 mutant (C.129X1-*Il4ra*^{tm3.1Tch}), IgE-deficient (*Igh-7*^{-/-}) and *Il4ra*^{tm3.1Tch} *Igh-7*^{-/-} mice on the BALB/c background has been previously described (Burton et al., 2013). *Kit*^{W-sh} mice (B6.Cg-*Kit*^{W-sh}/HNihrJaeBsmGlliJ, stock number 005051) were purchased from The Jackson Laboratory and crossed with B6.*Il4ra*^{tm3.1Tch} mice (N8 C57BL/6J background). B6.Tg(Mcpt5^{cre})

mice (Scholten et al., 2008) were interbred with B6.*Il4ra*^{tm3.1Tch} mice, and then crossed with iDTR mice (C57BL/g-Gt(*ROSA*)26Sor^{tm1(HBEGF)Awai}/J, stock number 007900, The Jackson Laboratory) to create *Il4ra*^{tm3.1Tch} Mcpt5^{cre} iDTR mice, and separately crossed with *Sykb*^{fl/fl} mice (B6.129P2-*Sykb*^{tm1.2Tara}/J, stock number 017309, The Jackson Laboratory) to generate *Il4ra*^{tm3.1Tch} Mcpt5^{cre} *Sykb*^{fl/fl}. For the purposes of analyzing the specificity of the Mcpt5^{cre} transgene for targeting mast cells, we generated the following Mcpt5^{cre}-transgenic mice with normal IL-4R alleles: Mcpt5^{cre} (B6.Tg(Mcpt5^{cre})), Mcpt5^{cre} iDTR (B6.Tg(Mcpt5^{cre}) Gt(*ROSA*)26Sor^{tm1(HBEGF)Awai}), Mcpt5^{cre} *Sykb*^{fl/fl} (B6.Tg(Mcpt5^{cre}) *Sykb*^{tm1.2Tara}). *Il4ra*^{tm3.1Tch}, *Syk*, *Kit* and *Igh-7* alleles were maintained in homozygous states; iDTR and Mcpt5^{cre} alleles were checked for presence or absence using PCR. Thy1.1-congenic Foxp3-eGFP-reporter IL-4rα F709 mutant mice (C.129X1-*Il4ra*^{tm3.1} *Foxp3*^{tm2Tch} *Thy1*^a) and Thy1.1 DO11.10 TCR-transgenic Foxp3-eGFP-reporter Rag-deficient mice (C.Cg-Tg(DO11.10) *Thy1*^a *Foxp3*^{tm2Tch} *Rag1*^{tm1Mom}) on the BALB/c background have been previously described (Noval Rivas et al., 2013). Wild-type BALB/cAnNTac breeders were initially purchased from Taconic Farms, and C57BL/6J breeders were originally obtained from The Jackson Laboratory. Both were then bred in house for experimental use.

Reagents

Grade V ovalbumin was purchased from Sigma (St. Louis, MO), and cholera toxin was obtained from List Biological Laboratories (Campbell, CA). BSA was obtained from Sigma (St. Louis, MO). IL-3, SCF and IL-4 were purchased from Shenandoah Biotechnology, Inc. (Warwick, PA). Anti-IL-4 (clone 11B11) was

enriched from hybridoma supernatants using a 100kDa molecular weight cut-off centrifugal filter (Pall Corporation, Northborough, MA) as previously described (Burton et al., 2013).

Cell culture

Peritoneal mast cells were enriched from peritoneal lavage by culturing them in complete RPMI supplemented with 10ng/ml IL-3, 20ng/ml SCF and 100ng/ml IgE anti-TNP (clone C38-2, BD Biosciences, Franklin Lakes, NJ) for two weeks and removing adherent cells (macrophages). BMMC were cultured as previously described (Burton et al., 2013). Bone marrow-derived dendritic cells were cultured as previously described (Zaccone et al., 2009). For BMMC and T cell co-culture experiments, 10^5 CD4⁺ T cells were cultured for 5 days with an equal number of BMMC. T cells were purified using CD4 microbeads and an AutoMACS (Miltenyi Biotec, Cambridge, MA). Cells were stimulated with 2ng/ml recombinant human TGFβ1 (Shenandoah Biotechnology, Warwick, PA), 1μg/ml αCD3 (145-2C11, Biolegend, San Diego, CA), 5μg/ml αCD28 (37.51, Biolegend, San Diego, CA) and 5μg/ml αFcεRI (MAR-1, eBioscience, San Diego, CA). Antibodies were coated overnight on 48-well plates. Cell suspensions were counted using a hemocytometer, and Th2 and Treg numbers were calculated using frequencies of IL-4⁺ or Foxp3⁺ cells among CD4⁺CD3ε⁺ T cells as determined by flow cytometry.

Flow cytometry and cell sorting

Treg cells were stained with Foxp3-APC (FJK-16s, Affymetrix eBioscience, San Diego, CA), CD4-PerCP-Cy5.5 (RM4-5, Biolegend, San Diego, CA) and CD3ε-FITC (145-2C11, BD Bioscience, Franklin Lakes, NJ) using the Foxp3 staining kit (Affymetrix eBioscience, San Diego, CA). For the Thy1.1 Foxp3-eGFP congenic experiment, cells were stained with Thy1.1-eFluor450 (HIS51, Affymetrix eBioscience, San Diego, CA), IL-17-Brilliant Violet 605 (TC11-18H10.1, Biolegend, San Diego, CA), Foxp3-FITC or eGFP (FJK-16s, Affymetrix eBioscience, San Diego, CA, or endogenous Foxp3-eGFP), IL-4-PE-CF594 (11B11, BD Biosciences, Franklin Lakes, NJ), IL-13-PerCP-eFluor710 (eBio13a, Affymetrix eBioscience, San Diego, CA), CD4-PE-Cy7 (RM4-5, Biolegend, San Diego, CA), IFNγ-APC (XMG1.2, BD Biosciences, Franklin Lakes, NJ) and IL-10-AlexaFluor700 (JES5-16E3, Affymetrix eBioscience, San Diego, CA). Robust coordinate analysis of Foxp3 and Th2 cytokines was achieved using the BD CytoFix CytoPerm kit (BD Biosciences, Franklin Lakes, NJ) and staining overnight with reduced concentrations of antibodies after a 4hr stimulation with 500ng/ml ionomycin (Sigma-Aldrich, St. Louis, MO), 500ng/ml phorbol 12,13-dibutyrate (Tocris Bioscience, Bristol, UK), and 1μg/ml brefeldin A (Sigma-Aldrich, St. Louis, MO). Dead cells were routinely excluded from analyses on the basis of staining with fixable viability dye eFluor780 (Affymetrix eBioscience, San Diego, CA), low affinity Fc receptor interactions were blocked with anti-FcγRII/III (clone 93, Biolegend, San Diego, CA) and analyses were restricted to single cells using FSC-H/W signals. Data were acquired using DIVA software on an LSRIIFortessa (BD Biosciences, Franklin Lakes, NJ) with 405nm, 488nm, 561nm

and 640nm lasers, and analyzed using FlowJo version 8.8.7 (Tree Star Inc., Ashland, OR). T cells from *Il4ra^{tm3.1Tch}* Foxp3-eGFP Thy1.1 mice were enriched under sterile conditions from pooled splenic and MLN cells using CD4 microbeads on an AutoMACS (Miltenyi Biotec, Cambridge, MA). Foxp3⁺ Treg cells were then sorted on the basis of CD4-PE-Cy7⁺Foxp3-eGFP⁺ on a FACS Aria (BD Biosciences, Franklin Lakes, NJ). Mast cells in the mesenteric lymph node were identified by staining with c-Kit-PE-Cy7 and IgE-PE, and by excluding B220-APC⁺ and non-viable eFluor780⁺ cells.

LAMP-1 degranulation assay

Mast cell degranulation was assessed by fusion of LAMP-1 (CD107a)⁺ granules the plasma membrane. IgE-sensitized mast cells were stimulated for ten minutes in RPMI containing IL-3 (10ng/ml), SCF (10ng/ml), TNP-OVA (100ng/ml, Biosearch Technologies, Petaluma, CA) and anti-LAMP-1-PerCP-eFluor710 (2µg/ml, clone eBio1D4B, Affymetrix eBioscience, San Diego, CA). Mast cells were then promptly washed twice in ice-cold FACS buffer (PBS, 0.5% BSA, 0.01% sodium azide) prior to staining with anti-c-Kit-PE-Cy7 (ACK2, Affymetrix eBioscience, San Diego, CA) and subsequent flow cytometric analysis.

Calcium flux

Splenic B cells were interrogated for calcium flux following B cell receptor stimulation as follows: single cell suspensions of splenocytes were subjected to ammonium chloride-induced erythrocyte lysis, and then loaded with Fluor-4 AM (3µM) and Fura Red AM (6µM, both Life Technologies, Carlsbad, CA) for 30min

at 37°C in HBSS containing divalent cations. B cells were rested for 20min at room temperature, and then stained with anti-B220-PE-Cy7 (RA3-6B2, Biolegend, San Diego, CA). After acquiring basal fluorescence signals for 20s, B cells were stimulated with anti-IgM (10µg/ml goat polyclonal, Southern Biotech, Birmingham, AL) to elicit calcium flux. Ionomycin (500ng/ml, Sigma-Aldrich, St. Louis, MO) was used as a control to elicit maximal calcium flux. Signals were acquired in linear set-up on an LSRFortessa, and analyzed as a kinetic of the algebraic parameter Fluo-4/Fura Red.

Anaphylaxis

Passive systemic anaphylaxis was induced by sensitizing 16-24-week old *Il4ra^{tm3.1Tch} Igh-7^{-/-}* mice overnight by i.p. injection with 20µg IgE anti-DNP (clone SPE-7, Sigma-Aldrich, St. Louis, MO), and challenging the mice the following day by i.p. injection with 500µg DNP-BSA (Biosearch Technologies, Petaluma, CA). Mice were randomly selected for treatment with SYKi or vehicle gavage, given 15min prior to challenge. A mock challenge was performed using unconjugated BSA (Sigma-Aldrich, St. Louis, MO). In some experiments, IgE anti-TNP (2µg, clone C38-2) and TNP-OVA (20µg) were used to induce anaphylaxis.

To address the immunological specificity of food allergen-induced anaphylaxis in *Il4ra^{tm3.1Tch}* mice (Figure S7B), enterally peanut-sensitized mice were sequentially monitored for temperature fluctuations over the course of an hour, followed by challenge with 100mg of BSA i.g., and then challenged with peanut.

ELISA

IL-4 was detected in cell culture supernatants by sandwich ELISA using purified 11B11 and biotinylated BVD4-24G2 (both Biolegend) followed by streptavidin-HRP (R&D) and trimethylbenzidine substrate (BD). MMCP-1 in serum was analyzed using MCPT-1 (MMCP-1) ELISA Ready-Set-Go! (Affymetrix eBioscience). Peanut-specific IgE was captured onto ELISA coated overnight with 3µg/ml anti-IgE (R35-92, BD) and detected using 10µg/ml biotinylated peanut extract. Peanut-specific IgG1 ELISAs were performed similarly using goat anti-mouse IgG1 for capture purposes (Southern Biotech). Peanut extract was prepared as previously described and biotinylated using an EZ-Link Sulfo-NHS-Biotinylation kit (Thermo Fisher) (Varshney et al., 2011). Anti-peanut serum from BALB/c mice immunized and primed with peanut extract adsorbed to alum was used as a source of peanut-specific immunoglobulins to develop a standard curve based on arbitrarily assigned units/ml.

Sandwich ELISA analysis of total immunoglobulin isotype levels was performed using highly adsorbed goat polyclonal anti-mouse isotype antibody pairs from Southern Biotech (Birmingham, AL). Purified anti-isotype antibodies were coated overnight in PBS at 2µg/ml, and biotinylated secondary antibodies were used for detection at 500ng/ml. IL-13 and IFNγ ELISAs were performed according to the manufacturer's instructions (Affymetrix eBioscience, San Diego, CA). To detect circulating IL-4 levels, mice were injected either i.p. or i.v. with 10µg biotinylated anti-IL-4 (clone BVD6-24G2, Biolegend, San Diego, CA). The biotinylated anti-IL-4:IL-4 complexes that subsequently formed were captured from serum onto plates coated with anti-IL-4 (clone 11B11, Biolegend, San Diego, CA) and

detected using streptavidin-HRP (R&D Systems, Minneapolis, MN). OVA-specific IgE was captured onto plates coated with OVA and detected using biotinylated anti-IgE (R35-72, BD Biosciences, Franklin Lakes, NJ) using IgE anti-OVA clone TO ϵ as a standard. IgE against chow components was assessed by coating plates with gliadin (MyBioSource, San Diego, CA) or soy extract at 100 μ g/ml, incubating with sera at a 1:5 dilution, and developing with the same biotinylated anti-IgE as for the OVA-IgE ELISA. Soy extract is an aqueous extract of soy flour (Bob's Red Mill, Milwaukie, OR).

Quantitative PCR

Real-time quantitative RT-PCR was performed as previously described (Mathias et al., 2011) using TaqMan probes from Life Technologies.

Supplemental References.

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