HHS Public Access

Author manuscript

J Allergy Clin Immunol. Author manuscript; available in PMC 2024 June 07.

Published in final edited form as:

J Allergy Clin Immunol. 2023 January ; 151(1): 182–191.e6. doi:10.1016/j.jaci.2022.07.011.

IL-4 Receptor Alpha Signaling alters oral food challenge and Immunotherapy outcomes in mice

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Abstract

Background: Food allergy diagnosis and management causes a number of social an emotional challenge for individuals with food allergies and caregivers. This has led to increasing interest in the development and usage of approaches to accurately predict food allergy diagnosis, severity of food allergic reactions and treatment outcomes. However, the utility of these approaches is somewhat conflicting.

Objective: Develop and utilize a murine model that mimics the disease course of food allergy diagnosis and treatment in humans and to identify biomarkers that predict reactivity during food challenge and responsiveness during oral immunotherapy and how these outcomes are modified by genetics.

Methods: Skin sensitized Intestinal IL-9Tg (IL9Tg) and IL9Tg mice backcrossed onto the IL4Rα^{Y709F} background received single intragastric exposure of egg antigen (OVA), underwent oral food challenge (OFC) and oral immunotherapy (OIT) and food allergy severity, mast cell activation and OVA-specific IgE levels were examined to determine the predictability of these outcomes in determining reactivity and treatment outcomes.

Results: We show that s.c. sensitization and a single intragastric allergen-challenge of egg antigen to BALB/c IL-9Tg mice and IL4Rα^{Y709F} IL9Tg induced a food allergic reaction. We show that enhanced IL-4Rα-signaling altered the symptoms induced by the first oral exposure, decreased the cumulative antigen dose and increased severity of reaction during OFC and altered side effect frequency and OIT outcomes. Analysis of the biomarkers following the first oral exposure revealed that only the severity of the initial reaction significantly correlated with the cumulative dose of the OFC.

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Conclusion: Collectively, these data indicate that SNPs in IL-4Rα can alter clinical symptoms of food allergic reactions, severity and reactive dose during food challenge and OIT and that severity of first reaction can predict the likelihood of reaction during a food challenge in mice with IL-4Rα gain of function.

Capsule Summary:

Genetics alters OFC and OIT outcomes and efficacy of OIT.

Introduction

Exposure to specific foods in some individuals can cause a reaction affecting multiple organ systems including gastrointestinal (GI), cutaneous, respiratory and cardiovascular[1]. When food allergy is suspected, individuals will see a health care provider or allergist who will take a clinical history of the food reaction, perform testing (skin testing (SPTs) and radioallergosorbent tests (RASTs) to obtain food-specific serum IgE values) and possibly perform oral food challenge (OFC) to determine whether the individual had an allergic reaction and determine the exact food responsible. If the individual is diagnosed with food allergy the current standard of care for treatment is avoidance of the allergen, prescribed epinephrine, and possible participation in oral immunotherapy (OIT) treatment. One of the challenges of food allergy diagnosis is that the diagnostic modalities for food allergy (SPTs and sIgE and OFC) possess several limitations. OFCs are time consuming and involve the risk of acute allergic reaction of unpredictable severity. Positive cutoff SPT or sIgE levels are useful, however diagnostic cutoff values vary widely due to differences in patient populations and it is not uncommon for individuals with elevated SPT and sIgE to tolerate foods.

The reported significantly higher concordance rate of peanut allergy among identical twins (64.3%) compared with that among dizygotic twins (6.8%) suggested that genetic factors play a role in FA[2]. Consistent with this, GWAS and candidate-gene studies of food allergy have identified genetic loci associated within the functional categories of skin barrier integrity, vascular and endothelial cell factors, innate and adaptive immunity and immune modulation and regulation[3, 4]. The IL-4Ra/STAT6-signaling pathway plays an important role in $CD4^+$ type-2 immune responses, IgE-class switching, basophil / MC and vascular endothelial functionality and is thought to be a central pathway that underlies the food allergic phenotype. Indeed, genetic analyses have identified association been genetic variants in these molecules in allergic diseases including food allergy[5–10]. Notably, genetic variants in STAT6 have been associated with severity of allergy[11]. Furthermore, gain-of-function mutations in the IL-4Rα chain (E375A, S478P, or Q551R) have been linked with enhanced allergic inflammatory responses including increased MCs and elevated IgE levels[5–10]; Consistent with these observations, murine-based studies have revealed that mutations in the IL-4Rα that promote gain-of-function ($IL\text{-}4R\alpha^{Y709F}$, $IL\text{-}4R\alpha^{Q576R}$, and $IL\text{-}4R\alpha^{Y500F}$) leads to enhanced allergic inflammatory phenotypes including asthma, IgE-mediated responses, and severity of food allergy[12–16].

We have previously generated intestinal IL-9 transgenic mice that constitutively expresses IL-9 within the intestinal epithelial compartment of the small intestine (SI). We have shown

that a single enteral exposure of these mice to food antigen will induce an IgE-mediated food allergic response that possesses GI and systemic symptoms involving cutaneous, respiratory, and cardiovascular system[17]. Utilizing this experimental model system, we examined biomarkers that may predict diagnosis and severity of food allergic reactions and the impact of genetics on food challenge and OIT outcomes. We show that egg antigen sensitized mice that undergo a single food exposure experience a food allergic reaction. Measurement of biomarkers including severity, egg antigen specific IgE and mast cell activation following the first oral exposure and food challenge identified that the level of mast cell activation correlates with the severity of the first oral exposure and that these dependent variables can predict failure of food challenge. Modelling the disease course of food allergy diagnosis and treatment in mice revealed that gain-of-function mutations in the IL-4Rα chain can increase severity of the first oral exposure, the reactive dose and severity of reaction of the OFC and course of OIT outcomes.

Materials and Methods

Animals.

6-8-week-old BALB/c wild type (WT) mice were obtained from the National Cancer Institute (Bethesda, MD, USA) and bred in-house at the University of Michigan (UM) (Ann Arbor, MI, USA). Intestinal IL-9 transgenic (IL9Tg, BALB/c) mice were generated as previously described[18]. IL9Tg mice were bred to the IL4Rα^{Y709F} (BALB/c) background to derive IL4Rα^{Y709F}IL9Tg mice. All mice used in the described studies were on a BALB/c background. Age-, sex-, weight-matched littermates were used as controls in all experiments. The mice were maintained and bred in a clean barrier facility and were handled under an approved Institutional Animal Care and Use Committee protocols at University of Michigan animal facility.

First Oral Exposure Reaction:

4-8-week-old mice were sensitized to ovalbumin (OVA) (50 μg of OVA / 1 mg of alum in sterile saline by sub cutaneous (s.c.) injection and received a single intragastric (i.g.) challenge with OVA (250 μl of OVA (50 mg) in saline or 250 μl of saline (vehicle)) as previously described[19]. Prior to each i.g. challenge, mice were deprived of food for 4-5 h. Rectal temperatures were measured prior to challenge and then every 15 min for 60 min using a rectal probe and a digital thermocouple thermometer (Model BAT-12; Physitemp Instruments, Clifton, NJ). Diarrhea was assessed by visually monitoring mice for up to 60 min following i.g. challenge and mice demonstrating profuse liquid stool were recorded as diarrhea-positive.

Open Food Challenge protocol:

Seven days after the reaction following the first oral exposure, mice received multiple intragastric challenges of increasing doses of OVA starting with the lowest dose (1.56mg, 3.625mg, 6.25mg, 12.5mg, 25mg, 50mg) every 20 minutes in 50 μl of saline (vehicle). Prior to the protocol, mice were deprived of food for 4-5 hours. Rectal temperatures were measured prior to i.g. challenge and then 20 minutes post challenge. Mice that did not lose temperature were administered the next dose of OVA dose until they demonstrated a reaction

or completed the 50mg OVA dose. Loss of temperature of ≥ 0.6°C post challenge was considered as “failing” the OFC. The cumulative OVA dose at which the mice failed OFC was recorded.

Oral Immunotherapy protocol:

Mice received i.g. OVA (initial dose 0.5mg OVA / saline 200μl) daily for 7 days and underwent dosage escalation schedule (1mg, 2.5mg, 5mg, 10mg, 25mg and 50mg) whereby they received a daily dose for seven consecutive days until a 50 mg dose was reached. Rectal temperatures were documented prior to and 30-45 minutes post i.g. Mice that demonstrated > 0.5°C temperature loss within 30 minutes post of daily dose was recorded as side effect positive. Mice that demonstrated profuse liquid stool were recorded as diarrhea-positive side effect. Mice underwent a health check prior to daily dose and if demonstrated evidence of moribund state defined as rough hair coat, hunched posture, lethargy, dehydration, hypoactivity and lack of responsiveness were removed from the protocol and euthanized.

OVA-specific IgE:

OVA-specific IgE levels were determined by means of ELISA. Plates were coated with 50 μL of Rat anti-mouse IgE (553413, 2 μg/mL; BD PharMingen, San Jose, Calif) and blocked with 200 μL of 1% FBS before adding serial dilutions of plasma samples (50 μL per well). After 2 hours of incubation, plates were washed and incubated with 50 μL of biotinylated OVA (OVA1-BN-1, 1 μg/mL; Nanocs). After 2 hours of incubation, streptavidin–horseradish peroxidase (1:40; R&D systems, Minneapolis, Minnesota) was added to each well. Before the initiation of each step, plates were washed with 0.05% Tween-20 in PBS. Finally, after a 1-hour incubation, 50 μL of substrate (TMB substrate reagent set; BD OptEIA, San Diego, Calif) was added. Colorimetric reaction was stopped with 1 mol/L H_2SO_4 and was quantified by measuring optical density with an ELISA plate reader at 450 nm. To determine concentration, the O.D value (570 nm) was subtracted from O.D value (450 nm) and the calculated concentration of OVA specific IgE was derived from O.D. of known concentrations of OVA specific IgE according to the standard curve.

MCPT-1 ELISA:

Mouse serum was collected 1 h after i.g., challenge and MCPT-1 in serum was measured by means of mouse MCPT-1 (mMCP-1) ELISA Ready-SET-Go!, according to the manufacturer’s instructions (eBioscience, San Diego, CA, USA). In short, after the 96 well plate was coated with capture antibody and blocked with ELISA diluent, serial standard or serum sample dilutions, detection antibody, Avidin-HRP and TMB Solution were added to the wells, respectively and developed at room temperature. Afterwards, the stop solution was added to each well, and the optical density (O.D) was read with an ELISA plate reader at 450 and 570 nm. To determine concentration, the O.D value (570 nm) was subtracted from O.D value (450 nm) and the calculated concentration of mMCPT-1 was derived from O.D. of known concentrations of mMCPT-1 according to the standard curve.

Statistical analysis.

Data are expressed as mean ± standard error (SE), unless otherwise stated. Statistical significance between multiple experimental groups were analyzed using the 1-way ANOVA test performed using Prism 7.0 software (GraphPad Software Inc., San Diego, CA, USA). P value of < 0.05 was considered significant. Correlation analysis (Spearman rank correlation coefficient), Linear regression and logistic regression analyses were performed using IBM SPSS Statistics V23.0 software. We first evaluated all the variables for their normal distribution and dispersion in the data. Mean and standard error of mean (SEM) were used to describe all the RWE variables; dtMax, MC degranulation and OVA IgE. Independent samples t-test was used to compare means between two groups for normally distributed variables and Mann-Whitney test was performed to check the significant difference for the variables that were failed to show normal distribution. The spearman correlation method was used to assess correlations between first oral exposure variables. Enter method was used for binary logistic regression analysis to assess the relationship between the first oral exposure variables and Food challenge outcome estimating the odds ratios (ORs) with 95% confidence intervals (CIs). The validity of the models including individual and combined first oral exposure variables were evaluated through receiver operating characteristic curve (ROC curve) analysis and the area under the ROC curve (AUC) was measured. Linear regression was used to assess the significant relationship between the first oral exposure variables and cumulative dose of food challenge. The significance level was set at $\alpha = 0.05$. Chi-square test and Fisher's exact test was used to test significance of the association of discrete variables i.e. number of reacted mice at each cumulative dose in two different genotypes.

Results

Subcutaneous sensitization and a single bolus of egg antigen induces food allergy in mice.

We have previously demonstrated that administration of antigen-specific IgE and subsequent single oral antigen exposure of intestinal IL-9 transgenic (IL9Tg; BALB/c) mice induces a food allergic reaction with systemic and GI symptoms[17, 19]. To begin to model the course of events of food allergy in humans (reaction following first oral exposure ➔ OFC ➔ OIT), IL9Tg mice sensitized subcutaneous exposure of the egg antigen ovalbumin (OVA) to induce sensitization (OVA-specific IgE; Figure 1A and B). Fourteen days following the subcutaneous exposure, mice received a single bolus of OVA (50mg) orally and evidence of an acute food allergic reaction was examined (Figure 1A). We show that a single bolus of OVA induced a food allergic reaction in 8 / 19 (42%) Balb/c wild type (WT) mice as evidenced by greater than 0.5°C temperature loss within 30 minutes of OVA challenge (Figure 1C). In contrast a single bolus of OVA exposure to IL9Tg mice induced a food allergic reaction in 62 / 80 (77.5%) (Figure 1C). Notably the severity of the food allergic reaction was not significantly different between WT and IL9Tg mice (Maximum Temperature Loss °C: -1.66 ± 0.15 vs -1.68 ± 0.25; n = 8 vs n = 62 mice that reacted ($\Delta T > -0.5$ °C) respectively; mean ± SEM, Figure 1C). Consistent with our previous observations, the level of mast cell activation as indicated by the surrogate serum marker MCPT-1 significantly correlated with the severity (maximum temperature loss within 30

minutes of challenge) ($p < 0.05$; Table 1)[19]. Notably, OVA-specific IgE did not correlate with severity of the reaction (Table 1). Stratification of IL9Tg mice into non-reactive and reactive groups following first oral exposure revealed that OVA-specific IgE levels were not significantly different between groups (OVA-specific IgE (μg/ml) 1.51 ± 0.48 vs 1.4 ± 0.15; non-reactive vs reactive IL9Tg mice n = 18 vs n = 58; mean ± SEM, $p = 0.16$).

Food challenge of egg antigen reactive mice

To mimic the course of events that would occur in humans, mice that experienced a food allergic reaction during the first oral exposure underwent a food challenge (FC) protocol seven days following the first oral exposure reaction. The FC involved oral exposure to the eliciting antigen OVA, starting at a low dose (1.56mg) with doubling doses of the egg antigen every 20 minutes up to a cumulative total of 50 mg OVA (Figure 2A). To identify the threshold temperature loss that would indicate a reaction to a single bolus of egg antigen during the FC challenge we performed the FC on non-reactive unsensitized IL9Tg mice. We show that doubling doses of the egg antigen every 20 minutes up to a cumulative total of 50 mg OVA did not induce > - 0.5°C temperature change in response to a single dose of egg antigen (Supplementary Figure S1A and B). We therefore used the cutoff threshold of greater than > −0.5°C temperature change as evidence of a reaction to an eliciting dose. We show that 48 of 58 IL9Tg mice that received an OFC failed the food challenge reacting at accumulative doses of 1.56 – 98.44mg OVA (average 10.9mg) (Figure 2A and B). Notably, we observed no significant difference in the severity of the reaction between eliciting doses (Figure 2C). OFC of OVA-reactive WT mice identified that all n = 8 WT BALB/c mice reacted at an accumulative dose of > 23.44mg OVA (results not shown).

First oral exposure biomarkers predict FC outcomes.

We next examined whether the biomarkers food-specific IgE, mast cell activation, or shock (Temperature loss) measured following first oral exposure reaction were associated with FC outcome. We show that no first oral exposure outcome was independently associated with either pass or fail of the FC (Supplementary Table S1). Logistic regression analysis to predict whether there was an association between first oral exposure biomarkers with FC outcomes identified that individually the parameters (Temperature loss, MC activation and OVA-specific IgE) did not predict FC outcome, however multivariable analyses revealed that the dependent variables together predict pass or fail FC outcome ($p = 0.04$; Table 2). Linear regression analysis to predict whether there is an association between the first oral exposure biomarkers and the cumulative eliciting dose during OFC did not identify any biomarker individually or combined that predicted the cumulative eliciting dose of reaction at OFC (Supplementary Table S2).

Egg oral immunotherapy (OIT) in mice

The IL9Tg mice that failed the egg OFC next underwent an oral immunotherapy protocol (OIT), where mice received daily oral exposure to a low concentration of egg antigen with weekly dose escalation to determine whether mice could become desensitized and tolerate a dose of egg antigen that previously induced a reaction (50mg) (Figure 2D). IL9Tg mice (n = 11) that failed the OFC received daily doses of OVA at 0.5mg, 2.5mg. 5mg, 10mg, 25mg and 50mg for seven consecutive days. We examined temperature loss in mice within

30 minutes of dose administration and considered > −0.5°C temperature loss as evidence of side-effects of OIT. We show that IL9Tg mice that received daily doses of 0.5 and 1.0 mg OVA tolerated antigen exposure and demonstrated no side effects or evidence of reactivity (GI symptoms (diarrhea) or Temperature loss > −0.5°C) (Fig 2E and F). Notably, up dosing of OVA to 2.5 and 5mg induced significant side effects with > 60% of the IL9Tg mice demonstrating > −0.5°C Temperature loss following administration of dose (Fig 2E and F). Notably, over the course of the seven-day daily consumption of 5mg OVA, we observed decreasing incidence of side effects in the mice where by Day 7, 95% of the mice tolerated 5mg OVA (Fig 3B). Notably, each proceeding dose escalation (10 - 50mg) was associated with side effects (Temperature loss > −0.5°C) that was generally more severe and over the course of the 7 days the severity of the side effect diminished (Fig 2F). At the completion of the OIT protocol we observed 3 / 11 mice that could successfully tolerate 50mg OVA which was > 16.2-fold increase in egg antigen tolerant dose. Notably, analyses of serum OVA-specific IgE and evidence of MC activation (MCPT-1) did not significantly decline over the OIT protocol (Supplementary Figure S2A and B). Examination of biomarkers obtained following the first oral exposure reaction and OFC (Temperature loss, MC activation, OVA-specific IgE, OFC failure dose) did not reveal any association with OIT outcomes (results not shown).

IL-4Rα chain gain of function mutation increased severity and sensitivity of food allergy following first oral exposure.

To begin to determine the impact of genetics on the food challenge and OIT outcomes in mice, we examined the impact of IL-4Rα gain of function mutation (IL-4Rα^{Y709F}) on food challenge and OIT outcomes. We show that subcutaneous sensitization to OVA in the IL-4Rα^{Y709F} IL9Tg mice induced an OVA-specific IgE response (Figure 1B). The level of IgE was significantly greater than that observed in IL9Tg and WT mice (Fig 1B). First oral exposure of egg antigen to IL-4Rα^{Y709F} IL9Tg mice induced a food allergic reaction in 90.5% (19 / 21) of the mice (Fig 1C - E). Analysis of the biomarkers obtained following the first oral exposure revealed that the level of MC activation and OVA-specific IgE while not significant, were greater in the reactive IL-4Rα^{Y709F} IL9Tg mice than that observed in the nonreactive IL-4Rα^{Y709F} IL9Tg mice (Table 3). Notably, the frequency of reactions within the IL-4Rα^{Y709F} IL9Tg mice (90.5%, n = 19 / 21) was significantly higher than that observed in the IL9Tg mice (72.5%, n = 62 / 80) indicating that gain of function mutation in IL-4Rα increased likelihood of a reaction (Fischer's-Exact Test, $p < 0.001$). Furthermore, the first oral exposure induced diarrhea in 15 / 19 IL-4Rα^{Y709F} IL9Tg mice as compared to 10 / 62 IL9Tg mice suggesting that these mice also had heightened GI symptoms (Fig 1D). In the IL-4Rα^{Y709F} IL9Tg mice, the biomarkers measured following the first oral exposure (MC degranulation and food-specific IgE) correlated with severity of the first oral exposure reaction in IL-4Rα^{Y709F} IL9Tg mice ($r = −0.547$, $p = 0.01$ and $r = −0.491$, $p = 0.02$ respectively, Table 4). These studies reveal that genetics alters reactivity to eliciting antigens and indicates that antigen specific IgE can predict reactions in these individuals.

The IL-4Rα^{Y709F} IL9Tg mice that reacted following the first oral exposure underwent a food challenge. 94.7% (18 / 19) of the IL-4Rα^{Y709F} IL9Tg mice failed the OFC. Notably the dose at which the IL-4Rα^{Y709F} IL9Tg mice reacted during the OFC was significantly lower

than that observed in the IL9Tg mice with > 84.2% of the mice reacting an accumulative dose of 10.93 mg OVA ($p = 0.005$) (Table 5). Furthermore, the severity of the reaction following the first dose of OFC was significantly greater in the IL-4Rα^{Y709F} IL9Tg mice compared with the IL9Tg mice (Maximum Temperature Loss °C: −1.76 ± 0.17 vs −1.08 ± 0.12; n = 5 vs n = 12 mice that reacted (ΔT > - 0.5°C) respectively; mean ± SEM, $p < 0.05$; Table 5). Linear regression analyses to predict whether any biomarkers measured following the first oral exposure were associated with the cumulative dose of FC failure identified that severity (Temperature loss) of the first oral exposure reaction was significantly associated with OFC failure dose in IL-4Rα^{Y709F} IL9Tg mice ($p = 0.048$; Supplementary Table S3). Collectively these studies indicate that gain-of-function mutations in the IL-4Rα chain increases the severity of a food reaction and significantly lowers the dose at which an individual reacts during an OFC. Furthermore, these studies indicate that severity of the first oral exposure reaction can predict OFC outcome.

IL-4Rα^{Y709F} IL9Tg mice have reduced sensitivity to Oral Immunotherapy (OIT)

To determine whether gain of function mutation in the IL4Ra alters OIT outcomes, IL-4Rα^{Y709F} IL9Tg mice that failed the egg oral food challenge underwent OIT. OIT in IL-4Rα^{Y709F} IL9Tg mice induced significant side effects at the first dose of the OIT protocol (0.5mg) (Fig 3A–C). Notably, dose escalation of OVA to 1mg induced significant side effects in greater than 80% of the IL-4Rα^{Y709F} IL9Tg mice. Not only was the frequency of side effects in the IL-4Rα^{Y709F} IL9Tg mice greater at a lower dose, the severity was also significantly greater than that observed in IL9Tg mice (Fig 3 B and C). Intriguingly, IL-4Rα^{Y709F} IL9Tg mice frequently demonstrated evidence of diarrhea during OIT; a side effect we did not observe in IL9Tg mice (Fig 3C). During the dosing OIT regime side effects in IL-4Rα^{Y709F} IL9Tg mice became so frequent and severe at a dose of 5mg that we were unable to complete the dose escalation OIT protocol due to moribund state (Fig 3D). The increased incidence and severity of side effects in IL-4Rα^{Y709F} IL9Tg mice during OIT was associated with increased evidence of MC activation and OVA-specific IgE (Supplementary Figure S3).

Discussion

Herein employing a mouse model of the disease course of food allergy in humans (reaction following first oral exposure, OFC and OIT), we show that 1) level of MC activation correlated with first oral exposure reactivity; 2) combination of the biomarkers, Temperature loss (shock), mast cell activation and antigen-specific IgE following the first oral exposure predicts FC outcome and 3) OIT was associated with side effects following escalation dosing and the side effects diminished during the maintenance dose. Employing this model, we show that IL-4Rα chain gain of function mutation 1) altered the types of symptoms and increased severity of food allergy following the first oral exposure; 2) decreased the dose at which mice reacted during FC and increased the severity of the response; 3) decreased the dose at which mice demonstrated side effects from OIT and increased severity of the side effects. Finally, we showed that the combined variables, level of mast cell activation and antigen-specific IgE following the first oral exposure predicted food challenge outcome and the cumulative dosage that the mice react during FC. Collectively these studies establish

a mouse-based model to define markers of and mechanisms that regulate reactivity during OFC and OIT and establish that genetics can alter OFC and OIT outcomes.

Employing this mouse system, we demonstrate that the independent variables, antigen-specific IgE, mast cell activation and the severity of the reaction following first oral exposure alone did not predict food challenge outcome, however, combination of these variables can predict FC outcome. Of these three parameters, antigen-specific IgE appeared to have the strongest independent interaction with FC outcome. Current diagnostic tests including food-specific IgE antibodies have been reported to predict outcome (pass or fail) of OFC[20–25]. While diagnostic decision points for food-specific IgE antibodies and SPT have excellent specificity, they have poor sensitivity in predicting FC outcomes. Furthermore, the eliciting antigen and age of patient can impact cutoff threshold levels and predictive value of these diagnostic tests[21, 26–29]. For example, the diagnostic value for 95% probability of failing a cow's milk oral challenge in children less than 1 year old was 5.8 KU_A/L milk-specific IgE and ~10-fold (57.3 KU_A/L) higher in 2 years or older[28]. Furthermore, the predictive value of food-specific IgE is restricted to specific foods as specific immunoglobulin E (IgE)/total IgE can predict FC outcomes for cow's milk, hen egg and wheat but not soy[30].

We show that a gain of function mutation in the IL-4Ra alters the clinical symptoms of the first oral exposure reaction. Moreover, ~80% IL-4Rα^{Y709F} IL9Tg mice developed diarrhea during first reaction, whereas 16% IL-4Rα^{WT} IL9Tg mice developed diarrhea. Mechanism-based studies have revealed that IL-4-IL-4Rα signaling can alter IgE-mediated reactions through regulation of the hematopoietic compartment driving IgE-isotype switch and stimulating $CD4^+$ Th2 and mast cell responses[13, 31–33]. While OVA-specific IgE was significantly higher in IL-4Rα^{Y709F} IL9Tg mice as compared to IL-4Rα^{WT} IL9Tg mice, the level of MC activation following antigen exposure was comparable indicating that increased incidence of diarrhea cannot be explained by enhanced MC activation. Previous studies have demonstrated that intestinal $CD4^+$ Th2 cells through elevated IL-4 and IL-13 play a central role in allergic diarrhea[34]. We have previously demonstrated that IL-13 alone is sufficient to increase intestinal epithelial CFTR mRNA and protein expression and drives diarrhea formation via an IL-4Rα-dependent mechanism[35]. Furthermore, we and others have previously demonstrated that IL-4Rα^{Y709F} mice develop a more severe food allergy phenotype as increased evidenced by diarrhea which was associated with increased $CD4^+$ Th2 cells[36].

We also demonstrate that gain of function mutation in the IL-4Rα can also lower the threshold of the FC outcome and increase the severity of the reaction. Clinical studies have reported increased levels of IL-4 and histamine in the serum of human patients with severe anaphylaxis[37]. However, only histamine levels were associated with severe disease[38] suggesting a possible role for these molecules in expression of the severe disease phenotype. IL-4 and IL-4Rα signaling is known to amplify the IgE-mediated histamine-induced fluid extravasation through priming of the vascular endothelial compartment[39]. Indeed, pretreating IL-4Rα^{Y709F} mice with IL-4 induces a significantly enhanced histamine-induced hypovolemic shock as compared with WT mice[39]. It is likely that the heightened IL-4 signaling in the IL-4Rα^{Y709F} IL9Tg mice increases the vascular endothelial compartment sensitivity to vasoactive mediators such as histamine leading to the presentation of

symptoms (e.g. shock) at a lower dose during food challenge and a significantly more severe phenotype. Consistent with this concept, increased plasma histamine concentrations have been observed in children within 1 hour of FC[40–42]. The demonstration that IL-4Rα^{Y709F} IL9Tg mice had reduced dose threshold of reactivity during food challenge suggests that genetics may not only influence risk of a food allergy diagnosis but may also alter the FC threshold outcomes in humans. Previous studies have identified that the STAT6 SNPs, rs1059513 and rs324015 can alter clinical sensitivity. Van Ginkel and colleagues revealed that the A alleles STAT6 SNPs, rs1059513 and rs324015 were associated with a greater eliciting dose during double-blind placebo-controlled food challenge[43]. While in this case the STAT6 SNPs were associated with higher expression of STAT6 and reduced clinical sensitivity, it highlights those polymorphisms within the IL-4-IL4Rα-signaling pathway can alter the eliciting dose during FC.

We show that OIT caused significant systemic side effects in mice and that dose escalation was often associated with the most severe adverse events. Egg, milk and peanut OIT in humans is associated with adverse events including GI and systemic symptoms[44–49]. For example, 46.1% of patients experienced chronic gastroenteric symptoms and 15.3% acute asthma following milk intake during OIT[45] and 18-100% of patients on active peanut OIT have reported an allergic reaction with 0.8 - 43% of doses causing adverse symptoms[47–49]. Furthermore, adverse reactions in humans following dose escalation is not uncommon[50]. The observed increased OIT-induced adverse events and inability to achieve clinical unresponsiveness in IL-4Rα^{Y709F} IL9Tg mice suggests the presence of an underlying genetic component in OIT tolerance and responsiveness. OIT is effective in achieving clinical unresponsiveness in most subjects, however treatment failure does occur in 1 in 15 subjects[46]. Furthermore, not all subjects receiving OIT reach maintenance OIT doses. Collectively, these studies suggest that evaluation of various risk SNPs in food allergic individuals may be of value in predicting food challenge and OIT outcomes.

The reduction in adverse events during daily OIT suggests loss of mast cell reactivity and desensitization. The mechanism by which mast cells become unresponsive to allergen during OIT remains unclear. Experimental evidence suggests that mast cell desensitization can achieved by disruption of actin cytoskeleton activity and Ca^{2+} immobilization[51]. Furthermore, Burton et al. identified an IgG-FcγRIIb-dependent mechanism in suppression of IgE-mast cell degranulation[52]. Analyses of the surrogate marker for mast cell degranulation (MCPT-1) throughout our OIT protocol revealed increasing concentrations of systemic MCPT-1 in the mice suggesting that reduction in adverse events may not be related to loss of MC reactivity. Our outcome analyses were primarily restricted to measurement of the hypothermic response in mice following OIT and the hypothermic response is driven via hypovolemia as a consequence of vascular endothelial leak[53]. Given the decrease in evidence of hypothermia in the mice in the absence of any decrease in MC degranulation evokes an interesting concept that the subthreshold dosing of OVA during OIT may promote VE desensitization and reduction in side effects during OIT.

Currently we do not fully understand why we were only able to achieve clinical unresponsiveness in ~ 25% of the mice that completed the OIT protocol. Food allergy in BALB/c WT mice is driven by an IL-9-dependent amplification of mucosal mast cells

within the small intestine (SI)[54]. The IL9Tg mice used in these studies were generated using the intestine-specific promoter of the rat fatty acid–binding protein (iFABPp) gene which is predominantly expressed in enterocytes of the SI[55, 56]. The constitutive overexpression of IL-9 in the intestinal epithelial compartment drives SI mast cell amplification[17]. Previous studies by us and others have revealed that the SI mast cell amplification during the food allergic condition is predominantly driven by $CD4^+$ Th2 and MMC9 cells[36, 57, 58]. Therefore, any impact of OIT on the $CD4^+$ Th2 cells and MMC9 cell compartment is not likely to impact IL9 expression or MC frequency in IL9Tg mice and may limit the ability to achieve clinical unresponsiveness.

Recent studies have identified a collaborative cross (CC) mouse strain (CC027) that is genetically susceptible to peanut allergy and prone to severe reactions following oral food challenge[59]. Consistent with these observations, Matsushita et al., showed that CC027 mice possess potent mast cell activity and demonstrate strong passive cutaneous anaphylaxis responses[60]. The potent mast cell activity in these mice is attributed to the NOD/ShiLtJ-specific missense SNPs within the Speckled protein (SP) family member *Sp140* gene[60]. Examination of these mice as well as others that possess genetic variants in proteins involved in mast cell function and IgE responses will be of value to determine the impact of these types of mutations on food challenge and OIT outcomes.

Collectively, using a BALB/c mouse model system we show that biomarkers at the first oral exposure and reaction can predict failure of OFC. We show that gain-of-function mutations in the IL-4Rα chain increase the severity of reaction of the first oral exposure to antigen and the reactive dose and severity of the food challenge and OIT outcomes. These studies support both deeper assessment of biomarkers during reactions in the community and exploring genetics in determining OFC and OIT outcomes and efficacy of OIT. Such work is likely to increase patient safety and improve current management of patients with food allergy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Conflict of Interests:

This work was supported by National Institutes of Health grants DK073553, DK090119, AI138177, and AI112626; Food Allergy Research & Education (FARE); Department of Defense grant W81XWH-15-1-051730; M-FARA; and the Mary H. Weiser Food Allergy Center supported (to S.P.H.).

SPH receives research grant support from Regeneron Pharmaceuticals. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Abbreviations:

s.c	sub cutaneous
IgE	Immunoglobulin E
FC	Food Challenge
SI	Small Intestine
OVA	ovalbumin
GI	Gastrointestinal
SNP	Single Nucleotide Polymorphism

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Clinical Implications:

These studies support deeper assessment of biomarkers following first oral exposure reaction and genetics in determining OFC and OIT outcomes and efficacy of OIT.

Key messages:

- Biomarkers at the first oral exposure predicts food challenge outcomes in mice.
- IL-4Rα chain gain of function mutation altered the types of symptoms and increased severity of food allergy following first oral exposure in mice.
- IL-4Rα chain gain of function mutation increases sensitivity and severity of food challenge reactions in mice.
- IL-4Rα chain gain of function mutation increases the frequency and severity of side effects during OIT in mice.

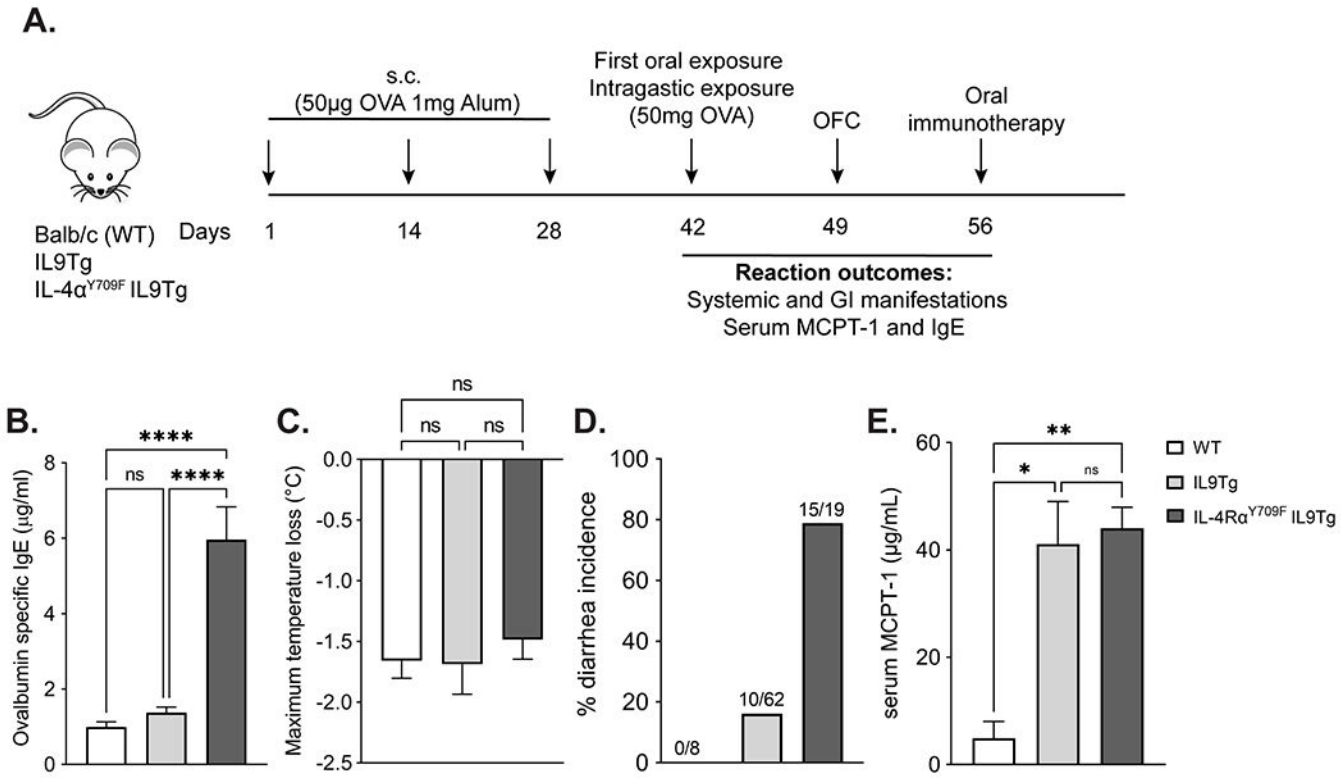

Figure. 1. Skin sensitization and single oral antigen challenge induces a Food allergic reaction.

(A) Experimental regimen, (B) OVA-specific IgE, (C) maximum body temperature change, (D) diarrhea occurence (%), and (E) serum MCPT-1 levels in OVA-sensitized and challenged WT BALB/c, IL-9Tg and Il4ra^{Y709F} IL9Tg mice. Data are represented as the mean ± SEM; n = 8 - 62 mice per group. **** $p < 0.0001$, ** $p < 0.01$, * $p < 0.05$, ns > 0.05.

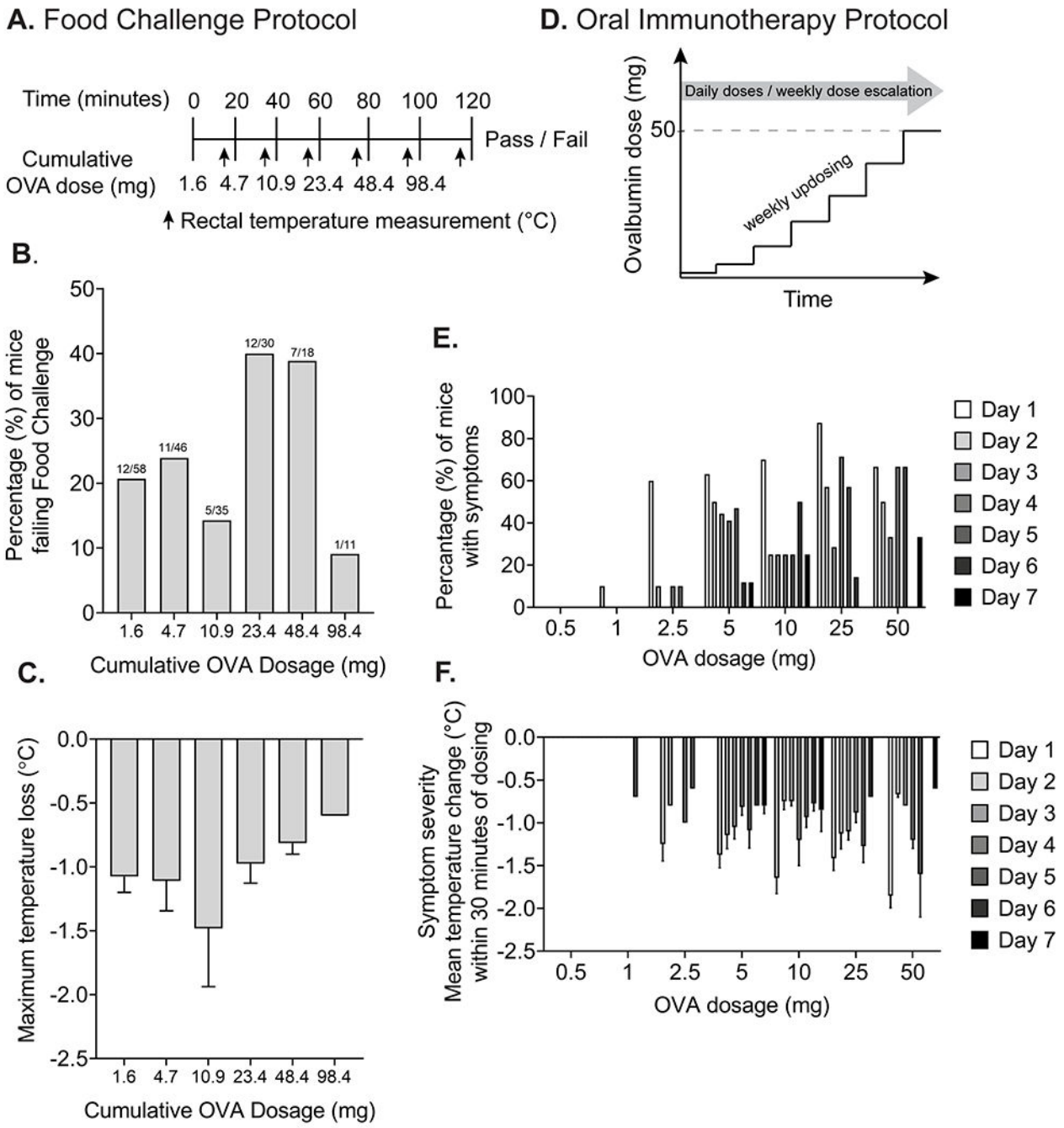

Figure. 2. Oral Food Challenge and oral Immunotherapy outcomes in food allergic IL9Tg mice.

(A) Experimental protocol, (B) percentage of mice and (C) maximum body temperature change (°C) of IL9Tg mice who reacted at specific cumulative doses of ovalbumin (1.6 – 98.4mg). (C) Experimental regimen, (D) percentage of mice with symptoms and (E) symptom severity (Maximum body temperature change °C) following daily administration of OVA during the OIT protocol in IL9Tg mice. (B). Fraction indicates number of mice out of total number of mice that reacted at that cumulative dose. (C) Data are represented as the

mean ± SEM; n = 58 mice. (F) Data are represented as the mean ± SEM; n = 11 mice per group.

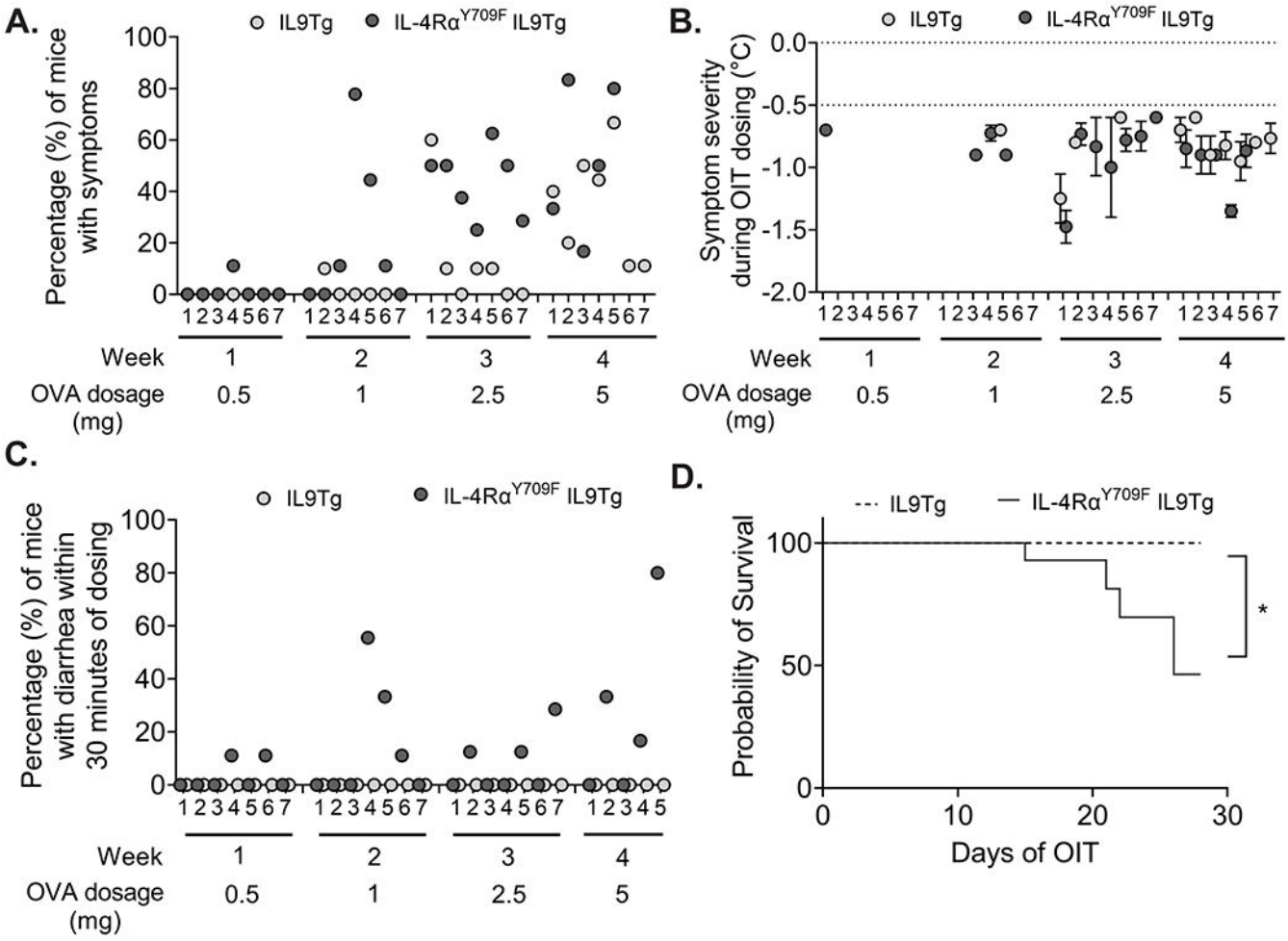

Figure. 3. Oral Immunotherapy outcomes in food allergic Il4ra^{Y709F} IL9Tg mice.

(A) percentage of mice with symptoms and (B) severity of symptoms (maximum body temperature change), (C) diarrhea occurrence (% of mice) and (D) percentage of mice survive during OIT protocol (0 – 5mg daily doses) in IL9Tg and Il4ra^{Y709F} IL9Tg mice. Mice received each dose of OVA for seven consecutive days before escalation. Data are represented as the mean ± SEM; n = 11 and 15 mice per group. * $p < 0.05$.

Table 1:

Correlation analyses between severity of the reaction, MC degranulation, OVA-specific IgE in IL9Tg mice that reacted to the first oral exposure.

		dT Max. (°C)	MC degranulation (ug/ml)	OVA sp. IgE (ug/ml)
dT Max. (°C)	Correlation Coefficient	1.000	**−0.252** *	−0.14
	Sig. (2-tailed)	-	**0.028**	0.25
MC degranulation (ug/ml)	Correlation Coefficient	**−0.252** *	1.000	0.03
	Sig. (2-tailed)	**0.028**	-	0.81
OVA sp. IgE (ug/ml)	Correlation Coefficient	−0.135	0.028	1.00
	Sig. (2-tailed)	0.246	0.809	-

*Correlation analyses were performed on n = 58 IntIL9Tg mice that reacted following first oral exposure and we were able to obtain serum from mice. Severity was measured as maximal temperature loss (°C) and indicated as dT Max (°C). MC degranulation was determined by serum MCPT-1 levels. – indicates no value.

Table 2:

Logistic regression to predict the association of first oral exposure outcomes with OFC reactivity in IL9Tg mice.

Model	Odds ratio (95%CI)	AUC	p Value
1.dtMax (°C)	1.07 (0.36 - 3.2)	0.50 (0.26-0.70)	0.99
2. MC_Degranulation	1.02 (0.99 - 1.05)	0.65 (0.47-0.83)	0.14
3. OVA IgE	2.29 (0.66 - 7.97)	0.63 (0.43-0.83)	0.20
4. Combined All. dtMAX	1.38 (0.42 - 4.53)	**0.71 (0.54-0.87)**	**0.04**
Combined All. MC-deg	1.02 (0.99 - 1.05)		
Combined All. OVA sup	2.20 (0.66 - 7.33)		

*Logistic regression analyses were performed on n = 58 IntIL9Tg mice that reacted during first oral exposure. Severity was measured as maximal temperature loss (°C) and indicated as dT Max (°C). MC degranulation was determined by serum MCPT-1 levels.

Table 3.

Severity of the reaction, MC degranulation, OVA-specific IgE in IL4Rα^{Y709F} IL9Tg mice following the first oral exposure.

Variable	Non-reactive (n = 2)	Reactive (n = 19)
dT Max (°C)	−0.1 ± 0.3	−1.48 ± 0.2
MC Degranulation (ug/ml)	10.6 ± 8.1	41.1 ± 7.9
OVA IgE	2.3 ± 1.6	6.3 ± 0.9

*Severity was measured as maximal temperature loss (°C) and indicated as dT Max (°C). MC degranulation was determined by serum MCPT-1 levels. Data are represented as the mean ± SEM.

Table 4:

Correlation analyses between severity of the reaction, MC degranulation, OVA-specific IgE in IL4Rα^{Y709F} IL9Tg mice that reacted to the first oral exposure.

		dT max. (°C)	MC degranulation (ug/ml)	OVA sp. IgE (ug/ml)
dT max. (°C)	Correlation Coefficient	1.000	−0.547	−0.491
	Sig. (2-tailed)	-	**0.01**	**0.02**
MC degranulation (ug/ml)	Correlation Coefficient	−0.547	1.000	0.195
	Sig. (2-tailed)	**0.01**	-	0.397
OVA sp. IgE (ug/ml)	Correlation Coefficient	−0.491	0.195	1.00
	Sig. (2-tailed)	**0.02**	0.397	-

*Correlation analyses were performed on n = 19 IntIL9Tg mice that reacted during first oral exposure, and we were able to obtain serum from mice. Severity was measured as maximal temperature loss (°C) and indicated as dT Max (°C). MC degranulation was determined by serum MCPT-1 levels. – indicates no value.

Table 5:

Number of mice that reacted and severity of the reaction at cumulative doses during OFC in IL9Tg and IL4Rα^{Y709F} IL9Tg mice.

	Number of mice that failed OFC by dose			Severity of Reaction (Temperature loss °C)		
Accumulative Dose	**IL9Tg$^{+/-}$ (n = 58)**	**IL9Tg IL4Rα^{Y709F} (n = 19)**	**P value**	**IL9Tg$^{+/-}$**	**IL9Tg IL4Rα^{Y709F}**	**P value**
1.56mg, n (%)	12 (20.7%)	5 (26.3%)	0.412	**−1.06 ± 0.11**	**−1.76 ± 0.17**	**0.007**
4.69mg, n (%)	23 (39.7%)	12 (63.2%)	0.064	−1.11 ± 0.23	−0.91 ± 0.13	0.542
10.93mg, n (%)	**28 (48.3%)**	**16 (84.2%)**	**0.005**	−1.49 ± 0.46	−0.83 ± 0.17	0.263
23.93mg, n (%)	40 (69.0%)	17 (89.5%)	0.066	−0.98 ± 0.15	−0.90 ± 0.0	0.894
48.93mg, n (%)	47 (81.0%)	18 (94.7%)	0.142	−0.79 ± 0.08	−0.80 ± 0.0	0.736
98.93mg, n (%)	48 (82.8%)	18 (94.7%)	0.182	−0.60 ± 0.00	-	-

*Severity of reaction as measured by Maximum body temperature change (oC) in IL9Tg and Il4ra^{Y709F} IL9Tg mice who reacted at specific cumulative dosage of ovalbumin (1.6 – 98.4mg). Data are represented as the mean ± SEM; Total of n = 19 and 58 mice per genotype. p values as indicated. – indicates no value.