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TITLE:    Mechanisms of Oral Tolerance Breakdown in Food Allergy

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**ABSTRACT**

Aim 1: Th2 and mast-cell mediated suppression of allergen-specific iTR cell response. The hypothesis is that the prevailing Th2 environment in II4raF709 mice suppresses the generation of allergen-specific iTR cells. Blockade of Th2 and mast cell pathways may not only inhibit anaphylaxis but also promote tolerance.

Aim 2: Capability of mast cell depletion to restore oral tolerance in established allergic sensitization. The hypothesis is that mast cell expansion perpetuates oral intolerance to allergen, and that their acute depletion enables tolerance induction in established food allergy. Aim 3: allergen-specific TR cell therapy in the treatment of established oral sensitization. The hypothesis is that allergen-specific iTR cells of WT but not II4raF709 mice would rescue established oral allergic sensitization and suppress the Th-2 skewing and mast cell expansion.

**SUBJECT TERMS** - none provided
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1. **Introduction.** Our studies have focused on elucidating mechanisms by which oral tolerance is corrupted in food allergy, resulting in the emergence of allergic responses to food allergens. These studies have led to the identification of a critical role for a skewed Th2 environment and mast cell dysregulation in impairing the function of regulatory T cells in food allergy.

2. **Keywords:** Regulatory T (Treg) cells, food allergy, mast cells, Th2, IgE, Foxp3

3. **Accomplishments**

3A. **Overall Project Summary:**

A. **Specific Tasks.** We have proposed to carry out the following tasks:

   **Task 1. Th2 and mast-cell mediated suppression of allergen-specific iTreg cell response.** The purpose of this task is to determine mechanisms by which allergic pathways prevent the acquisition of oral tolerance.

   **Task 2. Capacity of Mast Cell Depletion to restore oral tolerance in established allergic sensitization.** The purpose of this task is to determine whether acute mast cell depletion enables the restoration of oral tolerance in mice with established allergic sensitization.

   **Task 3. Allergen-specific Treg cell therapy in the treatment of established oral sensitization.** The purpose of this task is to determine whether allergen-specific iTreg cells of WT, but not Il4raF709, mice would rescue established oral allergic sensitization.

3B. **Task-specific results:**

   **Task 1 Th2 and mast-cell mediated suppression of allergen-specific iTreg cell response.** The purpose of this task is to determine mechanisms by which allergic pathways prevent the acquisition of oral tolerance.

   **Task 1: Results:** Under **Task 1**, we have proposed to test the hypothesis that the skewed Th2 environment present in the gut of food allergic mice suppresses the development of an effective induced regulatory T (iTreg) cell response, and consequently subverts the induction of oral tolerance to allergens. The Il4raF709 mice are genetically prone to develop food allergy upon oral sensitization with allergen due to a gain of function mutation in the IL-4 receptor alpha chain (IL-4Rα) [tyrosine (Y) 709 to Phenylalanine (F)] that results in enhanced signaling via the IL-4R. We found that deletion of FcerIa in Il4raF709 mice inhibited anaphylaxis in response oral to ovalbumin (OVA) sensitization, as evidenced by failure to manifest a drop in core body temperature in the sensitized mice in response to OVA challenge (Figure 1A). Consistent with the dependence of the food allergic response in these mice on IgE/mast cells (Figure 1A). Other stigmata of allergic sensitization and anaphylaxis, including increased total and OVA-specific IgE, increased release of the mast cell protease 1 (MCP1) into the blood stream upon allergen challenge and small intestinal tissue mastocytosis, were all found dependent on intact FcεRI expression, as were severely inhibited by concurrent FcεRIa deficiency (Figure 1B-D). Thus, the mast cells were demonstrated as requisite for the anaphylaxis and, more broadly, for an effective food allergen-directed Th2 response (as reflected by IgE production) in Il4raF709 mice. Similar results were found for IL-4-deficient Il4raF709 mice (data not shown). Studies on iTreg cell production revealed that iTreg induction is increased in Il4raF709/FcerIa double
mutant mice (Figure 1E). Whereas gut CD4⁺Foxp3⁺ Treg cells of OVA-sensitized Il4raF709 mice exhibited evidence of Th2 cell-like reprogramming with increased expression of the transcription factors GATA3 and IRF4 and the cytokine IL-4, associated with Th2 cells, this reprogramming was abolished in Il4raF709/Fcer1a⁻/⁻ double mutant mice ((Figure 1F-H). These results establish a primary role for mast cell activation in the pathogenesis of in food allergy by virtue of their promotion of the Th2 cell response and suppression of the Treg cell response. The studies shown in Figure 1 have been published in part as Supplementary Figure 5 in the following publication (1): Noval Rivas M, Burton OT, Charbonnier LM, Wise P, Gregoriev P, Oettgen HC, Rachid R, Chatila TA. Regulatory T Cell Reprogramming toward a Th2-Cell-like Lineage Impairs Oral Tolerance and Promotes Food Allergy. Immunity 2015;42(3):512-23. doi: 10.1016/j.immuni.2015.02.004. PubMed PMID: 25769611; PubMed Central PMCID: PMC4366316.

As part of Task 1, we examined the proposition that Th2 cytokine production by allergen specific Treg cells plays a central role in tolerance breakdown in food allergy, and that their specific deletion in Treg cells prevents food allergy induction in Il4raF709 mice. Accordingly, we employed Il4raF709 mice with targeted deletion of both Il4 and Il13, encoding the Th2 cytokines IL-4 and IL-13, respectively, in Treg
cells by using a floxed Il4-Il13 gene cassette and a Foxp3-directed Cre recombinase (Il4raF709Foxp3EGFPCreIl4-Il13∆/∆ mice) (Figure 2A). Results revealed that compared to Il4raF709Foxp3EGFPCre control mice, OVA-SEB-sensitized Il4raF709Foxp3EGFPCreIl4-Il13∆/∆ mice were protected against anaphylaxis after OVA challenge (Figures 2B and 2C). Furthermore, Foxp3-directed deletion of Il4 and Il13 corrected the deficit in CD4+Foxp3+ Treg cells in sensitized Il4raF709 mice and reversed their Th2 cell reprogramming, as assessed by GATA-3 and IRF-4 expression (Figures 2D and 2E). It also reduced IL-4 production by Tconv Th2 cells (Figure 2F). Collectively, these results indicate that Il4raF709 Treg cells are reprogrammed to Th2-like cells after oral allergic sensitization and contribute to disease pathogenesis through Th2 cell cytokine expression.

![Figure 2.](image)

**Figure 2.** Th2 cell cytokine production by Th2-reprogrammed Treg cells is critical to the food allergic response. A. Real time PCR analysis of Il4 mRNA transcripts in conventional T (Tconv) cells (CD4+Foxp3+) and regulatory T (Treg) cells (CD4+Foxp3+) sorted from spleens of Il4raF709 and Il4raF709 Foxp3EGFPCreIl4-Il13∆/∆. B. Core body temperature changes following OVA challenge of OVA-SEB sensitized Il4raF709 and Il4raF709 Foxp3EGFPCreIl4-Il13∆/∆ mice. C. Serum total IgE, OVA-specific IgE and MMCP-1 concentrations post anaphylaxis of mice from panel (B). D).Percentages and numbers of CD4+Foxp3+ Treg cells in the MLN of OVA-SEB-sensitized Il4raF709 and Il4raF709 Foxp3EGFPCreIl4-Il13∆/∆ mice. E. Frequencies of GATA-3+ or IRF-4+ Treg cells isolated from the MLN of OVA-SEB-sensitized Il4raF709 and Il4raF709 Foxp3EGFPCreIl4-Il13∆/∆ mice. F. Percentages (top panel) and numbers (bottom panel) of CD4+ Tconv cells producing IL-4 in the MLN of OVA-SEB-sensitized Il4raF709 and Il4raF709 Foxp3EGFPCreIl4-Il13∆/∆ mice. Results are representative of 2 independent experiments. N=3-18 mice/group; *p<0.05, **p<0.01 and ***p<0.001 by 1-and 2-way ANOVA with post-test analysis and Student's unpaired two tailed t-test.

The studies shown in Figure 2 have been published in part as Figure 5 in the following publication (1):


**Task 1: mice used**: We have budgeted 240 mice under this task. All budgeted mice under this task have been utilized.

**Task 2. Capacity of Mast Cell Depletion to restore oral tolerance in established allergic sensitization.** The purpose of this task is to determine whether acute mast cell depletion enables the restoration of oral tolerance in mice with established allergic sensitization.
**Task 2: Results:** Under Task 2, we have proposed to either sham sensitize *Il4raF709* and *Il4raF709/Mcpt5-Cre/iDTR* mutant mice or to subject them to oral sensitization with OVA. Subgroups of mice were to be treated with PBS or diphtheria toxin (DT) delivered intraperitoneally (i.p.) concurrently with the oral sensitization. In collaboration with Dr. Hans Oettgen at the BCH (co-author on references 1), we found that DT-mediated deletion of mast cells in *Il4raF709/Mcpt5-Cre/iDTR* mice resulted in the suppression of allergic sensitization and the promotion of allergen-specific Treg cell formation (Figure 3A-D). In a similar vein, deletion of the tyrosine kinase Syk in mast cells of *Il4raF709* mice using a floxed Syk allele also resulted in suppression of the Th2 response (IgE, IL-4) and promotion of the Treg cell response (Figure 3A-D). These studies have now been published as Figure 4 in the following publication (2): Burton OT, Noval Rivas M, Zhou JS, Logsdon SL, Darling AR, Koleoglou KJ, Roers A, Houshyar H, Crackower MA, Chatila TA, Oettgen HC. Immunoglobulin E signal inhibition during allergen ingestion leads to reversal of established food allergy and induction of regulatory T cells. Immunity. 2014;41(1):141-51. doi: 10.1016/j.immuni.2014.05.017. PubMed PMID: 25017467; PubMed Central PMCID: PMC4123130.

**Figure 3.** Mast cell depletion or their inhibition by deletion of Syk prevents peanut (PN) sensitization. A. PN-specific serum IgE levels. Mast-cell-directed induction of the diphtheria toxin receptor (iDTR) or inactivation of Syk tyrosine kinase gene in *Il4raF709* mice was achieved by Mcpt5cre-driven gene expression. Mice expressing iDTR on mast cells (*Il4raF709 Mcpt5creiDTR*) or with mast-cell-targeted Syk deletion (*Il4raF709 Mcpt5creSykfl/fl*) (n = 6–11) were sensitized once a week for 4 weeks with 23 mg PN by gavage. Mast cells were depleted from Mcpt5cre 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 iDTreg DT “+”). B. ELISA analysis of PN-specific IL-4 secretion in splenocyte cultures. C. Foxp3⁺ Treg (Treg) cell frequencies among PN-responding CD3ε⁺CD4⁺ T cells from the MLN. *p<0.05; ****p<0.0001 by 1-way ANOVA with post-test analysis. D. Temperature curves from PN-treated *Il4raF709* mice after enteral challenge with high-dose PN (450 mg). p < 0.001 by repeat measures 2-way ANOVA Mcpt5cre versus Mcpt5cre iDTR and Mcpt5cre versus Mcpt5creSykfl/fl.

**Task 2: mice used:** We have budgeted 120 mice under this task. We have used all the budgeted mice.

**Task 3. Allergen-specific Treg cell therapy in the treatment of established oral sensitization.** The purpose of this task is to determine whether allergen-specific iTreg cells of WT, but not *Il4raF709*, mice would rescue established oral allergic sensitization.

**Task 3: Results:** Decisive progress have been made on Task3 of the proposal, We have now completed this task, showing that allergic sensitization and anaphylaxis in the *Il4RaY709F* mutant mice, which are genetically prone to food allergy, can be prevented by therapy with allergen-specific WT but not *Il4raF709* Treg cells. In the process, we have established the cause of Treg cell failure to control food allergy in *Il4raF709* mice. Our results have been published in two separate reports (1, 3).

To establish whether allergen-specific iTreg cells suppress food allergy, we determined the capacity of OVA-specific iTreg cells to reverse established food allergy in OVA-SEB-sensitized *Il4raF709* mice. WT- or *Il4raF709-DO11.10⁺Foxp3EGFP⁺* iTreg cells were derived that expressed the OVA 323-339
peptide-specific T cell receptor (TCR) transgene DO11.10 and also carried a Foxp3 reporter allele (Foxp3EGFP+) to enable identification of Treg cells by virtue of their expression of the enhanced green fluorescent protein (EGFP). WT- or Il4raF709-DO11.10*Foxp3EGFP+ iTreg cells were differentiated in vitro from naïve CD4+ T cells isolated from the respective mouse strain, further purified by cell sorting based on their Foxp3EGFP expression and given intravenously (2.5 x 10^6 cells/mouse) to sensitized Il4raF709 mice. The recipient mice were further sensitized for 4 additional weeks and then orally challenged. Administration of a single dose of WT DO11.10+ iTreg cells suppressed the anaphylactic response of sensitized Il4raF709 mice challenged with OVA (Figure 4A). This suppression was associated with inhibition of total and OVA-specific IgE responses as well as mast cell expansion and activation, indicative of disease remission (Figure 4B). In contrast Il4raF709 DO11.10+Foxp3EGFP+ iTreg cells failed to suppress anaphylaxis or to inhibit the aforementioned disease parameters (Figures 4A and 4B). Transferred WT and Il4raF709 DO11.10+Foxp3EGFP+ iTreg cells were retrieved at similar numbers in the spleens and MLN of recipient mice, confirming that the Il4raF709 iTreg cells were functionally defective in suppressing disease (data not shown).

To determine whether defective suppression of oral allergic sensitization was also an attribute of in vivo–derived allergen-specific Il4raF709 Treg cells, isolated DO11.10+ Treg cells from RAG-sufficient WT and Il4raF709 DO11.10+Foxp3EGFP mice were employed in a Treg cell transfer model of enforced tolerance (Noval Rivas et al., 2013). WT but not Il4raF709 DO11.10+Foxp3EGFP Treg cells were found effective in preventing OVA-induced sensitization and anaphylaxis in Il4raF709 mice (Figures 4C and 3D). To determine whether Treg cell dysfunction in Il4raF709 mice resulted from excessive IL-4R/STAT6 signaling, we examined the capacity of Treg cells derived from STAT6-deficient WT and Il4raF709 DO11.10+Foxp3EGFP mice to suppress food allergy in sensitized Il4raF709 mice. Unlike STAT6-deficient Il4raF709 Treg cells, STAT6-deficient Il4raF709 Treg cells were equivalent to their WT counterparts in suppressing sensitization and anaphylaxis (Figures 4C and 4D). All transferred DO11.10+ Treg cell populations were retrieved at similar frequencies and numbers in recipient mice, indicating that the failure of Il4raF709 DO11.10+Foxp3EGFP Treg cells to suppress food allergy reflected an intrinsic functional defect.

The studies shown in Figure 4 below have been published in part as Figure 3 in the following publication (1): Noval Rivas M, Burton OT, Charbonnier LM, Wise P, Gregoriev P, Oettgen HC, Rachid R, Chatila TA. Regulatory T Cell Reprogramming toward a Th2-Cell-like Lineage Impairs Oral Tolerance and Promotes Food Allergy. Immunity 2015;42(3):512-23. doi: 10.1016/j.immuni.2015.02.004. PubMed PMID: 25769611; PubMed Central PMCID: PMC4366316.
Figure 4. OVA-specific Il4raF709 Treg cells fail to suppress food allergy. A. Core body temperature changes following OVA challenge of OVA-SEB-sensitized Il4raF709 mice that had received in vitro generated WT- or Il4raF709 DO11.10’Foxp3EGFP iTreg, as described in Figure S3A. (B) Total and OVA-specific serum IgE concentrations, MMCP-1 release and small intestinal mast cell counts in mouse groups shown in panel (A). (C) Core body temperature changes following OVA challenge of OVA-SEB-sensitized Il4raF709 mice that were either left untreated or given either WT DO11.10’Foxp3EGFP+ Treg cells or Il4raF709DO11.10’Foxp3EGFP+ STAT6-sufficient or deficient Treg cells. (D) Total and OVA-specific serum IgE and serum MMCP-1 concentrations post anaphylaxis of the mouse groups from panel (C). N=5-17 mice per group, pooled from two different experiments. *p<0.05; **p<0.01; ***p<0.001, 1- and 2-way ANOVA with post-test analysis.

Overall, our studies in Figures 2 (Task 1) and Figure 4 (Task 3) establish that Th2 reprogramming of Treg cells results in their failure to enforce oral tolerance in Food allergy, and suggest that measures that overcome such reprogramming such as anti-IgE therapy to suppress mast cell activation (Task 1, Figure 1) or anti-IL-4R therapy may be useful to re-establish tolerance.

Task3: Mice Utilized: We have originally budgeted a total of 180 mice. All the mice budgeted under this task have been utilized.

3C. Key Research Accomplishments:

Our studies, discussed in part in a recent review in the Journal of Allergy and Clinical Immunology, have established the capacity of therapy with allergen-specific Treg cells to prevent and cure food allergy (4). Overall, we have made the following accomplishments:

1. Demonstrated efficacy of immunotherapy for the prevention of food allergy and for curing established food allergy.
2. Identified a profound defect in the capacity of Th2 reprogrammed Treg cells (those carrying the Il4raF709 mutation) to mediate oral tolerance to food allergens.
3. Identified strategies to overcome food allergy in the context of a severely skewed Th2 environment that may reprogram the Treg cells. These include mast cell depletion or neutralization of the Th2 environment with anti-cytokine-cytokine receptor approaches.
4. Impact/Conclusions

1) Impact on the development of the principal discipline of the project

Our studies on experimental murine models of food allergy have established a critical role for impaired Treg cell function in food allergy. These insights will now be carried forward to human clinical studies that investigate the capacity of anti-IL-4/IL-4R antibodies, which neutralize the Th2 environment, to promote tolerance in food allergy.

2) Impact on other disciplines

Nothing to report

3) Impact on technology transfer

Nothing to report

4) Impact on society beyond science and technology

Food allergy is a societal problem in that it affects a large number of individuals, both children and adults, and is associated with significant morbidity as well as fatal episodes of food reactions. Our studies clarify the mechanisms by which food allergy may evolve, and will impact the development of therapies that affect the impact of disease on society.

5. Changes/Problems: Not applicable.

6. Products

6A. Publications


6B. Inventions, Patents and Licenses: Not applicable

6C. Reportable outcomes: See publication list.
6D. Other Achievements: Not applicable.

7. Participants:

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<tr>
<th>Name</th>
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<td>Talal Chatila</td>
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8. Special Reporting Requirements: Not applicable

9. Appendices: Not applicable.

References


