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TITLE: IL-9-Producing Mast Cell Precursors and Food Allergy

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Food allergy is a harmful immune reaction driven by uncontrolled type-2 immune responses. Current knowledge provide limited insights into why only some, rather than all food allergic individuals are prone to develop life-threatening anaphylaxis. We have identified a novel multi-functional IL-9-producing mucosal mast cells (MMC9s) that produce large amounts of IL-9, IL-13, and mast cell mediators. The objective of this proposal is to identify the factors that regulate MMC9 induction, which represents the key cellular checkpoint to develop food-induced anaphylaxis. The central hypothesis is that signals induced by IL-4 and antigen/IgE/FcεRI complex crosslinking act together to induce mast cell (MC) progenitors to develop into the pathogenic MMC9s, which amplify anaphylactic response to dietary allergens. We have established genetically modified murine strains, a new reconstitution model of experimental food allergy, and the system to acquire duodenal biopsy samples from food allergic patients. Preliminary evidence show that both IL-4 and antigen/IgE/FcεRI complex are essential for MMC9 development. The findings provide a plausible view that the combinatorial signals from atopic status and dietary allergen ingestions can induce aberrant MMC9 development, resulting in the susceptibility to life-threatening anaphylaxis. The impact from these studies may facilitate the discovery of biomarkers and therapeutic targets for diagnosing, preventing, and treating food allergy.
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1. INTRODUCTION:

IgE-mediated Food allergy is an adverse immune response occurred shortly after ingestion of food. For reasons unknown, the prevalence of food allergy has increasing significantly over the past decade, affecting 3-4% of adult population, with even higher rates of 4-8% within children in the United States (1). After exposures to the causal food allergens, the symptoms of food-induced anaphylactic reaction are variable, ranging from mild cutaneous swelling and abdominal discomfort to life-threatening anaphylaxis, characterized by hypotension, and cardiovascular collapse (2). Although still less common, food allergy-induced life-threatening anaphylaxis is responsible for approximately 30,000 E.R. visits and causing approximately 150 deaths in the U.S. a year. Recent clinical studies demonstrate that food immunotherapy provides some protective effects and can achieve short term of “sustained unresponsiveness” to food allergens for some patients (3, 4). Currently, the outcome of these food immunotherapy approaches have not managed to achieve complete de-sensitization or food tolerance (4). These limitations and that immunotherapy approaches for food allergy are not currently ready for clinical practice highlights the need for a better understanding of the immunological mechanisms underpinning the development of food allergic reactions. Recent studies by us and others have identified an important contribution for IL-9 in the regulation of the important immune cells in food allergy, mast cells. Indeed, we recently published seminal studies describing a novel population of IL-9-producing mucosal mast cells (MMC9s) that produce high levels of IL-9, IL-13 and mast cell proteases 1 (chymase) and 6 (tryptase) (5). MMC9s are a type of multi-functional mucosal MCs and exhibit the following characteristics: i) a phenotype of MMC lineage (Lin-c-Kit+ST2+β7integrinlo); ii) secreting prodigious amounts of IL-9 (~2.0 pg/mL per cell) and other TH2 cytokines, including IL-4 and IL-13, in lesser amounts; iii) exhibiting a small innate helper cell-like morphology with few metachromatic granules in their scanty cytoplasm; iv) secreting mast cell proteases and histamine. MMC9s are scarce in the small intestine of immunologically naïve mice and expand considerably after repeated ingested food allergen exposure. Mice ablated of MMC9s become resistant to develop symptoms of experimental food allergy, which can be restored by adoptively transferred MMC9s. Given their anatomical location, characteristics, and function, MMC9s may serve as a key player that bridges the crosstalk between skin and gut by perpetuating allergic reactions and amplifying anaphylactic responses to dietary proteins. The primary goal of this proposal is to define the important immune signaling pathways in the regulation of the development and function of MMC9 cells and to elucidate the contribution of this cell population to susceptibility to life-threatening IgE-mediated food allergy. The results obtained from this proposal will provide new insights into the design of biomarkers and/or therapeutic targets for the diagnosis, prevention, and treatment of food allergy.
2. **KEYWORDS:**
   Food allergy, anaphylaxis, IL-9–producing mucosal mast cells (MMC9s), TH2 cells, IgE, IL-4, FcεR, mast cells.
3. **OVERALL PROJECT SUMMARY:**

Considerable evidences have documented a pivotal role of mast cells, IgE, and TH2 cytokines in mediating food hypersensitivity; however, current knowledge of why only some, rather than all, individuals who have high levels of dietary allergen–specific serum IgE develop food-induced anaphylaxis remains limited. We recently published our findings describing a novel population of IL-9-producing mucosal mast cells (MMC9s) in mice that produce high levels of IL-9, IL-13 and mast cell mediators. The objective of this proposal is to identify the factors that regulate the development and function of a novel IL-9–producing mucosal mast cells (MMC9s), which may function as a key cellular checkpoint for the development of anaphylactic response to food allergens (This novel cell type was originally named as IMCP9 in the proposal). The central hypothesis is that signals of IL-4 and antigen/IgE/FcεRI complex crosslinking act together to induce mast cell (MC) progenitors to develop into the pathogenic MMC9s, which amplify the intestinal anaphylactic response to dietary allergens. We formulated this hypothesis on the basis of our recent observations including: 1) The multifunctional MMC9s secrete prodigious amounts of IL-9 and IL-13 in response to IL-33, and mast cell mediators in response to antigen/IgE complex crosslinking; 2) Repeat food ingestions induce concomitant MMC9 and CD4+TH2 cell accumulations that correlate positively with increased symptoms of experimental food allergy; 3) Mice ablated of T cells or deficient of IL-4/STAT6 fail to develop MMC9s; 4) Mice ablated of MMC9 developmental pathway fail to develop ingested antigen-induced anaphylaxis, which can be restored by adoptively transferred MMC9s; 5) Much fewer MMC9s are developed in mice lacking FcεRI; 6) Aberrant MMC9 development occurs preferentially murine strains susceptible to develop experimental food allergy; 7) Increased duodenal MMC9 frequency and expression levels of Il9 and MC-specific transcripts are associated with atopic patients who developed food allergy. The rationale for this proposal is that understanding the underlying mechanisms regulating MMC9 development and function will result in the definition of the pathogenic roles of this novel cell type and provide new insights into the design of immunotherapeutic approach for IgE-mediated food allergy.
The major goals of the project are:

Specific Aim 1: Determine how CD4+TH2 cells potentiate MMC9 development and function (completion 80%)

(i) Major Task 1: Determine whether IL-4 signaling contributes directly to MMC9 development and function (completion 90%).

Subtask 1: MC-specific IL-4Rα requirement (completion 90%)

(A). To address the question in subtask 1, we have examined the susceptibility of IL-4RF709 mutant mice to experimental food-induced anaphylaxis. Compared to wild type (WT) mice, skin sensitized IL-4RF709 mutant mice were more prone to develop food-specific IgE, evidence of mast cell activation, heightened GI CD4+ Th2 responses and experimental food allergy (Figure 1 and 2). Consistent with our previous studies and with increased food allergy, IL-4RF709 mutant mice generated much more MMC9s in their small intestine (SI) (Fig. 2). By contrast, the frequency of intestinal ILC2s was similar in mice carrying IL-4RαF709 mutant that generates constitutively activated IL-4R signals. These data suggest that IL-4R signal plays an important role in regulating MMC9 development.

One limitation of these investigations is the alteration of IL-4Ra on both the hematopoietic and non-hematopoietic compartment. In subtask 2 we proposed to address the requirement of IL-4Ra deficiency on hematopoietic compartment on IMMC9 development and induction of food allergic responses.
Subtask 2: Reconstitution approach (completion 60%).

To address the subtask 2, we have established a reconstitution model of experimental food allergy to determine the role of IL-4/IL-4Ra signal in the induction of MMC9 by adoptively transferring bone marrow (BM) cells from 4GET mice into OVA-sensitized BALB/c mice one day after sub-lethal irradiation (protocol diagramed in Figure 3A). After the second sensitization and repeated intragastric OVA challenge, transferred BM cells replenished the majority of MCPs and MMC9s, which were marked with GFP, in the irradiated recipients (Figure 3B). In contrast, most CD4+IL-17RB*ST2+TH2 cells were derived from the sensitized recipients as shown by their lack of GFP expression (Figure 3C). Compared to reconstituted mice that were challenged with saline only, repeated intragastric OVA challenge triggered a substantial increase in donor-derived (>95% GFPhi) IMCP9 and recipient-derived (>98% GFP-) CD4+TH2 cells (Figure 3B and 3C).

This is probably because donor-derived hematopoietic progenitors will reconstitute innate MC lineage within 2 weeks, whereas for de novo T cell generation after thymus engraftment require >2 months (Figure 3A-3C). This approach has allowed us to begin to address subtask 2 in Aim 1. As shown in Fig 4, purified BM MCPs (defined as Lin-Ly-6c-FcεRI-CD41-CD71-FLK2-CD150-c-Kit+β7integrin+ cells from 4GET mice gave rise to intestinal GFP-expressing MCPs and MMC9s, and repeated intragastric OVA challenge triggered a significant increase of both MMC9s and MCs in the sensitized recipients, which eventually developed symptoms of experimental food allergy (Figure 4).
We have employed this reconstitution model of experimental food allergy to determine whether IL-4R signals are directly involved in MMC9 induction. Sensitized WT recipients were reconstituted with BM progenitors from wild type, IL-4R\(\alpha\)F709, or IL-4R\(\alpha\)-/- mice before repeated intragastric challenge.

Compared to wild type BM progenitors, IL-4R\(\alpha\)F709 BM progenitors gave rise to increased frequency of MMC9s and CD4+TH2 cells, not ILC2s (Figure 4). By contrast, BM progenitors lacking IL4R\(\alpha\) produced fewer MMC9s and CD4+TH2 cells, but comparable ILC2s (Figure 4). Furthermore, significant increases of serum MCPt1 production and intestinal mastocytosis could be observed in sensitized WT recipients that were reconstituted with IL-4R\(\alpha\)F709 BM progenitors (Figure 5). In contrast, the IL-4R\(\alpha\)-deficient MMC9s had little ability to induce MCPt-1 production, intestinal mastocytosis and to drive allergic diarrhea (Figure 4 and 5). These results further substantiate a role of IL-4R signals in directly regulating MMC9 development and that the intrinsic IL-4/STAT6 signals are essential for MMC9 function to drive intestinal mastocytosis and IgE-mediated experimental food allergy.

(ii) Major Task 2: Determine whether CD4+TH2 cells potentiate MMC9 development through OX40/OX40L. (completion 0%)
Subtask 1: OX40 and OX40L requirements
Subtask 2: Reconstitution approach
Subtask 3: Prophylactic approach

In this task, we have not received OX40 knockout mice and expect to receive in the future. The breeders were delayed to be exported. To reduce the burden and cost of mouse colonies and manpower, we have focused on completing Major Task 1 and submission and publication of this study prior to advancing to Major Task 2. We anticipate submission of Major Task 1 study by the end of 2017 and will focus on completion of Major Task 2. Given that we have established all the in vivo model systems and bone marrow transplant systems we do not anticipate any major delays and completion of the Major Task 2 by the end of 2018.
Specific Aim 2: Determine the role of the antigen/IgE/FcεRI complex in MMC9 development and function. (Completion 50%)

(i) Major Task 1: Determine whether IgE/FcεRI signaling induces MCPs to directly develop into MMC9.

Subtask 1: Reconstitution approach (Completion 100%)
In this task, we utilized the established reconstitution model of experimental food allergy (Fig. 3) to examine the role of FcεR in MMC9 development as described in Subtask 1. We performed a series of experiments to assess the effect of FcεR signaling. Compared to WT mice, much fewer intestinal MCPs and MMC9s were induced in sensitized Fcer1a-/- mice, which consequently, exhibited less intestinal mastocytosis, produced fewer MCPT1-1, and failed to develop food allergy (Figure 6A-6C). These results further demonstrate that MMC9s drive intestinal mastocytosis to promote the susceptibility to experimental food allergy in an FcεR-dependent manner.

(ii) Major Task 2: Determine whether Fyn/STAT-5 signaling is required for IMCP9 function. (Completion 10%)

Subtask 1: MC-specific STAT-5 requirement (Completion 10%)
Subtask 2: Reconstitution approach (Completion 10%)
Subtask 3: Fyn/STAT-5 requirement in the epigenetic modification of Il9 CNS-20 enhancer. (Completion 10%)

In this major task, we have established STAT5^{fl/fl} mice colony and will breed with MC-specific Cre mice that we are currently under construction. We will begin to perform reconstitution experiments as proposed in Subtask 2. Additionally, we will analyze and compare the levels of H3K4me3 histone mark at Il9 CNS-20 enhancer when the MMC9-specific deletion mice are established. In summary, we have addressed the major hypothesis as proposed in the Specific Aim 2 (Completion 25%) and demonstrate that FcεR signaling is important for effective MMC9 development in vivo using murine model of food allergy. Other Subtasks which are designed to study the molecular mechanisms underlying the FcεR signaling pathway in MMC9 will be performed when the conditional knockout mice are generated. Analysis of epigenetic modification will be carried out after other Subtasks are addressed.
Specific Aim 3: Define the biological relevance of MMC9 in human food allergy.

(i) Major Task 1: Identify and characterize human MMC9 (Completion 30%)

Subtask 1: Phenotypic analysis: (Completion 50%)

In the subtask 1, we have identified MMC9-like human cells in human duodenal biopsies from food allergic individuals (Figure 7). We have observed the C-Kit+ FcεR+ cells in the duodenum of food allergy patients that also developed other allergic diseases, as comorbidity of allergic diseases (CADs) (Fig. 7). We have analyzed 22 duodenum samples by this flow cytometry panel approach we have developed and strikingly, observed elevated frequency of MMC9-like cells in the duodenum of CAD patients with (n=13). In contrast, very few MMC9-like cells were detected in the duodenal biopsies of normal subject (n=9) (figure 7). These results indicate the presence of IMMC9 cells in human gastrointestinal tissue and that levels are significantly elevated in food allergic individuals. We are still collecting more samples to obtain statistical significance of symptomatic parameters.

Subtask 2: Cytokine production: (Completion 30%)

To address whether these newly identified intestinal MMC9-like cells from biopsy samples can produce IL-9 and IL-13 cytokines, duodenal biopsies of subjects with food allergy plus EOE (FA*EOE*), food allergy without EOE (FA*EOE*), or without both food allergy and EOE (FA*EOE*) were collected and compared. Notably, we observed markedly elevated IL-9 and IL-13 production by intestinal cells from patients with food allergy plus EOE, compared to other subgroups. These intriguing observations support our overall hypothesis and highlight the importance of detailed analysis of intestinal immune components in patients with gastrointestinal allergic disorders. We will analyze additional
cytokines and continue to collect more patients samples for studying the correlation between clinical characteristics.

Subtask 3: Gene expression: (Completion 25%)
We will focus on addressing the questions raised in the subtask 1 and 2 by recruiting sufficient numbers of patient samples to reach the statistical significance. We will then begin to perform proposed experiments in Subtask 3, which required additional biopsies.

(ii) Major Task 2: Define the correlation between human IMCP9 frequency and clinical food allergy. (Completion 30%)
Subtask 1: MMC9 frequency vs clinical characteristics:
Subtask 2: MMC9 transcript expression vs clinical characteristics:
We have detected increased expression of MMC9-associated transcripts, such as TH2 cytokine, IL9, IL13, and mast cell transcripts, CPA3, Tryptase, and Chymase in patients with food allergy or CADs (Fig. 9).

Summary Specific Aim 3:
We have begun to collect samples for proposed experiments as discussed in Major Task 1. The initial datasets look consistent with our hypotheses. To reach statistical significance among subgroups of patients with distinct clinical characteristics, sufficient numbers of biopsies will be collected and analyzed. We have made significant progress in Specific Aim 3, despite of the challenge in recruiting sufficient numbers of patient biopsies. We are excited in the results obtained in these human studies, which are novel and have strong potential for clinical applications in the field of food allergy.
4. KEY RESEARCH ACCOMPLISHMENTS:

We demonstrate that;

1. IL-4R signal plays an important role in regulating MMC9 development in mouse model system of food induced anaphylaxis.
2. IL-4/STAT6 signals are essential for MMC9 function to drive intestinal mastocytosis and IgE-mediated experimental food allergy.
3. Identified MMC9-like human cells in human duodenal biopsies from food allergic individuals.
4. We have detected increased expression of MMC9-associated cytokines IL9 and IL13 from duodenal biopsy samples from patients with food allergy or CADs.
5. We have detected increased expression of MMC9-associated mRNA transcripts, such as TH2 cytokine, IL9, IL13, and mast cell transcripts, CPA3, Tryptase, and Chymase in patients with food allergy or CADs.
5. CONCLUSION:

Food allergy is a harmful immune reaction driven by uncontrolled type-2 immune responses. Considerable evidences demonstrate the key roles of mast cells, IgE, and TH2 cytokines in mediating food allergy. However, these information provide limited insights into why only some, rather than all food allergic individuals are prone to develop life-threatening anaphylaxis. We have identified the novel IL-9–producing mucosal mast cells (MMC9s) and suggest that MMC9 induction may represent a key cellular checkpoint in acquiring susceptibility to developing an anaphylactic response to ingested antigens. Our proposal to determine the factors that govern the development and function of MMC9s represents a new and substantive departure from the current paradigm by linking atopic status (IL-4) and the interactions between dietary antigens and the IgE/FcεR complex with MMC9 biology and food hypersensitivity. We have made significant progress in each Major Task, specifically:

1) A recent clinical finding shows that infants with atopic eczema are prone to be sensitized to egg at only 4 months of age (6), suggesting that some patients with atopic dermatitis (AD) in early life may have a higher risk of developing food allergy (7, 8). To provide the clinical relevance, we have established an experimental food allergy model by inducing allergic sensitization via skin route (Figure 2).

2) We have developed a reconstitution model of experimental food allergy (Fig. 3). By employing this model and IL4RαF709 mutant mice, we demonstrate that IL-4 signals are key for the induction of MMC9 development (Fig 2-5). We are currently constructing a novel tool of MMC9-specific Cre murine strain, which will allow us to target IL4 signaling pathway in MMC9 directly.

3) We have demonstrate that FcεR signal is essential for effective MMC9 expansion using FcεR deficient mice (Fig. 6). The cellular and molecular mechanisms underlining the FcεR signaling pathway will be addressed in the following years.

4) We have identified human ortholog of MMC9s in the duodenal biopsies of patients with food allergy using flow cytometry. We have collected sufficient numbers of patient biopsies to obtain important preliminary results, showing that frequency of MMC9s and the levels of their secreted IL-9 and IL-13 cytokines are increased in patients with food allergy (Fig 7-9).

In conclusion, we have established important murine models and an infrastructure to collect human biopsies to address the hypothesis raised in this proposal. The new findings are significant in the field of allergy and will have strong impacts on our understanding of human food allergy.

Our future plan is to construct the proposed novel murine strains that target MMC9 specifically. By employing the new tool, we will then address other questions raised in each sub tasks. We will continue to collect and analyze duodenal biopsies in order to address the correlation between the frequency and function MMC9 and clinical reactivity of patients with food allergy. Our tentative goal is to have two manuscripts prepared for submission in the coming year.
6. PUBLICATIONS, ABSTRACTS, AND PRESENTATIONS:

a.  

(1) Lay Press:

https://www.facebook.com/HoganLab/

(2) Peer-Reviewed Scientific Journals:


(3) Abstracts:

1. Noah TK, Knoop KA, McDonald KG, Gustafsson JK, Waggoner L, Vanonia S, Batie M, Mahe M, Helmthar M, Newberry R, Hogan SP: Intestinal secretory epithelial cell antigen passages (IntSAPs) sample food allergens and control the onset of food-induced anaphylactic reactions, 18th International Congress of


3. Noah T, Knoop K, McDonald KG, Gustafsson J, Waggoner L, Batie M, Mahe M, Helmthar M, Newberry R, Hogan SP: IL-13-Induced Goblet Cell Antigen Passages (GAP’s) are Required for the Acute Onset of a Food-Induced Anaphylactic Reaction, AAAAI Annual Meeting, Atlanta, GA,


(4) List presentations

1. Emerging concepts of non-hematopoietic cell involvement in induction and severity of food-induced anaphylaxis, University of Arizona, Department of Basic Medical Sciences, April 2017, Phoenix, AZ

2. Emerging concepts of non-hematopoietic cell involvement in induction and severity of food-induced anaphylaxis, University of Michigan, Department of Pathology, June 2017, Ann Arbor, MI


4. Food Allergy Research Education Research Retreat, Symposium, April 2016, McLean, VA

5. Non-IgE mediated Food Allergies in Children and Adults, American Academy of Allergy, Asthma, and Immunology Annual Meeting, November 2016, Los Angeles, CA

6. Gastrointestinal Secretory Epithelial cells passage food allergens and drive the onset of Food-induced Anaphylaxis, 10th Biennial Banff Inflammation Workshop, January 2017, Banff Centre for Arts and Creativity, Banff, Alberta, Canada
   a. Animal models of Asthma and Food Allergy, American Academy of Allergy, Asthma, and Immunology Annual Meeting, March 2017, Atlanta, GA
7. INVENTIONS, PATENTS AND LICENSES:

Nothing to report
8. REPORTABLE OUTCOMES:

Nothing to report
9. OTHER ACHIEVEMENTS:
   Nothing to report
10. REFERENCES:

11. APPENDICES:

none