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AWARD NUMBER: W81XWH-15-1-0517

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

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REPORT DATE: October 2016

TYPE OF REPORT: Annual Report

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
October 2016	Annual Report	30 Sep 2015 - 29 Sep 2016

4. TITLE AND SUBTITLE

IL-9-Producing Mast Cell Precursors and Food Allergy

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-15-1-0517

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT:
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/FcεR 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 evidences 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.

15. SUBJECT TERMS
Food allergy, mucosal mast cells, IL-9

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
Unclassified	Unclassified	Unclassified	Unclassified		19b. TELEPHONE NUMBER *(include area code)*

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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 is variable, ranging from mild cutaneous swelling and abdominal discomfort to life-threatening anaphylaxis, characterized by vomiting, diarrhea, 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 provide some protective effects or achieve short term of "sustained unresponsiveness" to food allergens for some patients (*3, 4*). However, the outcome of these food immunotherapy approaches appears ineffective in achieving complete de-sensitization or re-establishing eventual tolerance (*4*). Notably, although some subjects may obtain some levels of protective effects after repeated exposures of food allergens, others often develop adverse gastrointestinal (GI) symptoms and increased risk of systemic adverse reactions (*4*). Likewise, it has been a conundrum that why some food allergic individuals exhibit a mild discomfort characterized by pruritis of lips or uritcaria, but others experience life-threatening anaphylactic reactions after consumption of the same food allergen. These observations underline the fact that food immunotherapy for food allergy has not yet been ready for clinical practice and highlight that our knowledge of immunological mechanisms underpinning the development of allergic reaction in the GI tract remains to be established. Recent studies point to the involvements of IL-9 in regulating the pathogenesis of allergic disorders. 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 proteases 1 (chymase) and 6 (tryptase) (*5*). MMC9s are a novel 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 intestines of immunologically naïve mice and expand considerably after repeated ingested antigen exposures. 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 focus of this proposal is to understand the factors that regulate MMC9 development and function and to elucidate the pathogenic roles of MMC9 in promoting 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(*5*). 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/FcεR 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 FcεRαI; 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 30%)

(i) **Major Task 1:** Determine whether IL-4 signaling contributes directly to MMC9 development and function **(completion 50%).**

Subtask 1: MC-specific IL-4Rα requirement (completion 40%)

a) In subtask 1, we began to perform pilot experiments using newly established BALB/c Il9-IRES-GFP reporter mice (INFER) in the OVA/alum-sensitized model of experimental food allergy. We detected a fraction of MMC9s in small intestine of INFER mice that developed food allergy. These MMC9s expressed significant levels of GFP, indicative of the activation of *Il9* gene locus. These findings support our previous findings showing that MMC9s are the principle IL-9 producers by producing large amounts of IL-9, compared to type-2 innate lymphoid cells (ILC2s) and $CD4^+$TH2 cells in a mouse model of experimental food allergy. Thus, INFER mice will be an invaluable tool to elucidate the factors that regulate the induction of MMC9s.

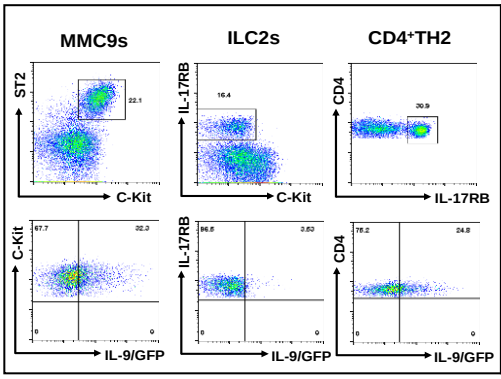

Fig. 1 Tracking MMC9s using IL-9-reporter mice in an OVA/alum model of experimental food allergy. BALB/c *Il9*-IRES-GFP reporter mice (INFER) were sensitized twice with OVA mixed with alum adjuvant within four weeks through peritoneal injection. Sensitized mice were intragastrically challenged with OVA 6 times every other day before the examination of IL-9-producing cells in the laminar propria of small intestine. MMC9s, ILC2s, and CD4$^+$TH2 cells can be detected with indicated surface markers using flow cytometry. Expression levels of GPF are indicatives of the production of IL-9 by cell subsets using flow cytometry.

b) We have established a clinical relevant model of experimental food allergy through skin sensitization routes. Mice that are sensitized with vitamin D3 analog will develop atopic dermatitis (AD)-like allergic diseases. These AD-like mice will develop symptoms of food allergy after 6 times of intragastric antigen challenge (**Fig. 2a**). As described in Specific Aim 1, we will utilize IL-4RαF709 mutant mice to test the effect of IL-4 signals

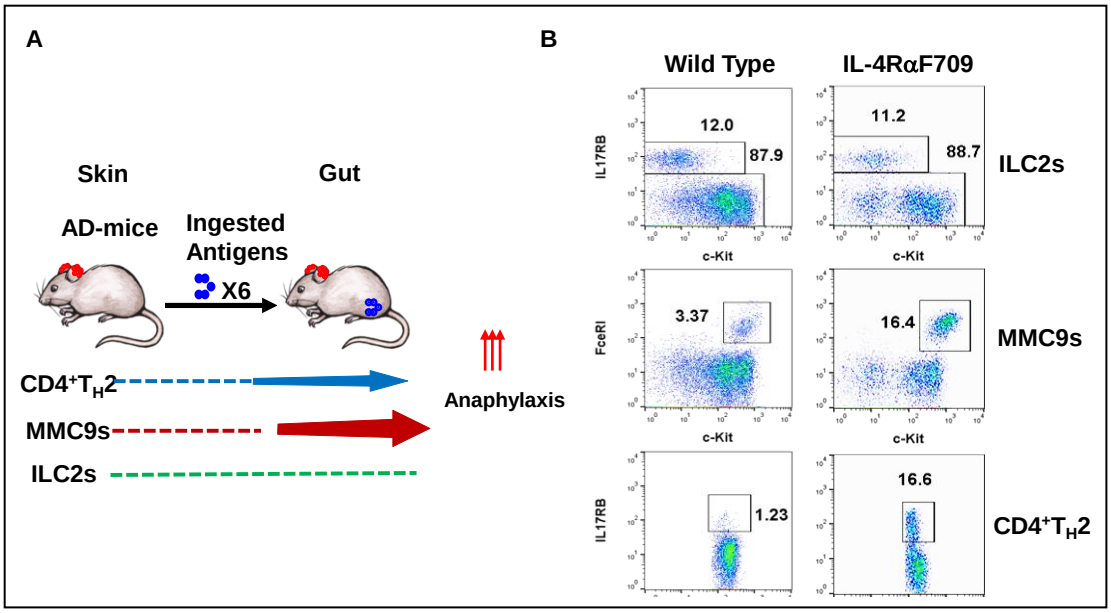

Fig. 2 Increased frequency of MMC9s and CD4+TH2 cells in the small intestine of IL-4RaF709 mutant mice in a skin-sensitized model of experimental food allergy. (**A**) BALB/c wild type or IL-4RaF709 mutant mice were sensitized with vitamin D3 analogs fourteen times daily and then challenged with OVA intragastrically every other days for six times before analysis. (**B**) Frequencies of MMC9s, CD4$^+$TH2, and ILC2s cells in the laminar propria of small intestine from wild type or IL-4RaF709 mutant mice that developed symptoms of experimental food allergy were examined and compared using flow cytometry.

on MMC9 development and function. We have established a mouse colony of IL-4RαF709 mutant mice and begin to test our hypothesis. As shown in **Fig. 2B**, our preliminary studies show that IL-4RαF709 mutant mice are more prone to generate both MMC9s and CD4$^+$TH2 cells, not ILC2s, resulting in the susceptibility to developing experimental food allergy, compared to wild type control mice.

c) To test the effect of IL-4 signals in MMC9s specifically, we are currently generating MMC9 specific Cre mice using CRISPER/CAS9 approach. We have designed, screened, and obtained 3 pairs of guide RNAs that can edit MMC9 specific *Mcpt1*

gene by inserting Cre and other regulatory elements. We are in the process of cloning the guide RNAs and other fragments into genomic fragments from *Mcpt1* gene locus. The final construct will be utilized to generate MMC9 specific mutant mice, which will be a novel and invaluable tool to address our hypothesis and beyond.

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/STAT6 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 1C). 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 $Lin^-Ly\text{-}6c^-Fc\varepsilon R^-CD41^-CD71^-FLK2^-CD150^-c\text{-}Kit^+\beta7integrin^+$ 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 4A-4C**).

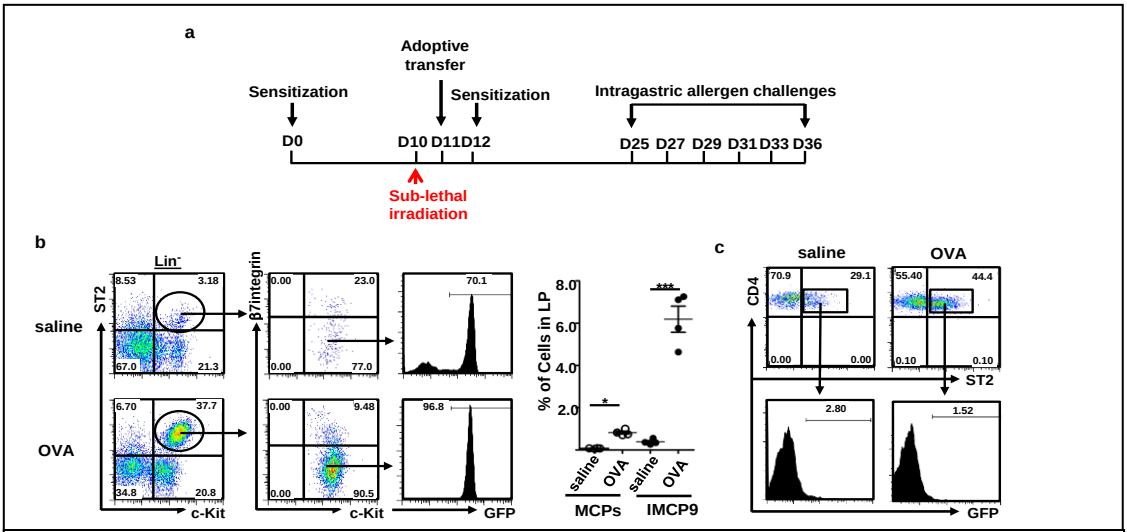

Fig. 3 Origin of intestinal MCPs, MMC9, and $CD4^+TH2$ cells in irradiated recipient mice after reconstitution in a murine model of food allergy. (**A**) Diagram of adoptive transfer protocol in the mouse model of food allergy. (**B, C**) Detection and frequency of donor-derived MMC9 ($Lin^-IL\text{-}17RB^-c\text{-}Kit^+b7integrin^{lo}$) and MCPs ($Lin^-IL\text{-}17RB^-c\text{-}Kit^+b7integrin^{hi}$) (B), and recipient-derived $GFP^-CD4^+ST2^+TH2$ cells (**C**) in the small intestine of irradiated recipient BALB/c mice reconstituted with bone marrow progenitors from IL-4-eGFP (4GET) mice after six intragastric saline or OVA challenges. Data represent one of three independent experiments (n=4 mice per group). LP, laminar propria. * $p<0.05$, *** $p<0.001$.

As proposed in the **subtask 2 in Aim 1**, we began to test requirement of STAT6, the key transcription factor downstream of IL-4 signal, for the development of MMC9s. We found that Stat6-/- BM progenitors in sensitized wild type recipients that were competent in GFP$^-$ CD4$^+$ST2$^+$TH2 and ILC2s (innate lymphoid cells) generation, and OVA-IgE production (>75 μg/mL) (**Fig. 5A and 5B**), but were impaired in MMC9 generation, resulting in considerably diminished MCPt-1 production and intestinal mastocytosis, as well as less frequent allergic diarrhea (**Fig. 5A and 5B**). In contrast, transferred Il9-/- BM progenitors generated MMC9

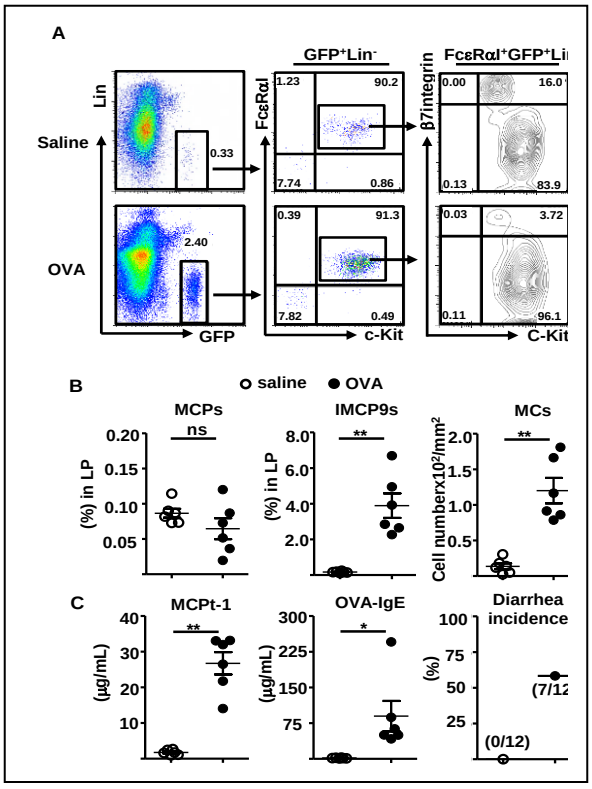

Figure 4. Bone marrow mast cell progenitors give rise to intestinal MMC9s. (**A-C**) Detection and frequency of donor-derived MMC9 (Lin$^-$GFP$^+$ST2$^+$c-Kit$^+$β7integrin^{lo}) and MCPs (Lin$^-$GFP$^+$ST2$^+$c-Kit$^+$β7 integrin^{hi}), and mast cell (MC) number in the small intestine of irradiated recipient BALB/c mice reconstituted with sorted bone marrow MCP (Lin$^-$Ly6c$^-$FcεR$^-$CD41$^-$CD71$^-$FLK2$^-$CD150$^-$c-Kit$^+$β7 integrin$^+$ cells) from IL-4-eGFP (4GET) mice (**A-C**) one day before second sensitization and six intragastric OVA challenges. Data represent one of three independent experiments (n=6 mice per group) (**A-C**) or one of two independent experiments (n=4 mice per group). LP, laminar propria. * p<0.05, ** p<0.01. NS, not significant.

efficiently in wild type recipients, the IL-9-deficent IMCP9 also had little ability to induce MCPt-1 production, intestinal mastocytosis and to drive allergic diarrhea (**Fig. 5A and 5B**).These results show that the intrinsic STAT6 are essential for MMC9 development, which drives IgE-mediated experimental food allergy. We will perform reconstitution experiments using bone marrow from IL-4RαF709 mutant mice to further demonstrate the effect of IL-4 signals in MMC9 development as proposed.

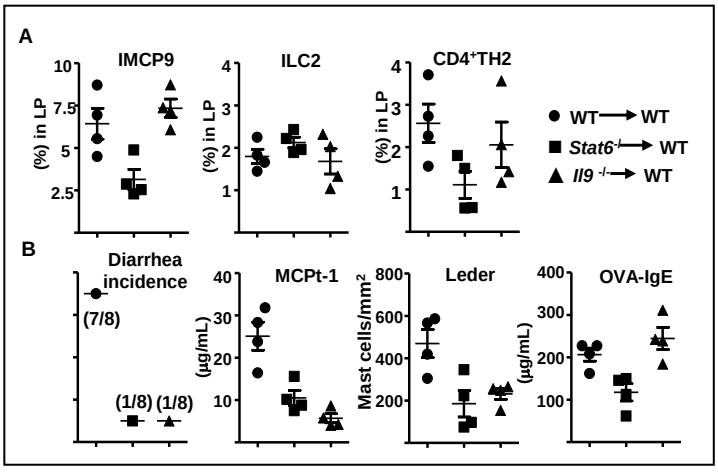

Figure 5. STAT6 signaling are required for IMCP9 induction that promotes experimental food allergy. (**A**) Frequency of IMCP9 (Lin$^-$c-Kit$^+$ST2$^+$ β7integrin^{lo}) ILC2s (Lin$^-$c-Kit-ST2$^+$IL-17RB$^+$), and CD4$^+$IL-17RB$^+$TH2 cells, and **(B)** measurement of indicated features of experimental food allergy after six intragastric OVA challenges in sub-lethally irradiated WT 4GET mice reconstituted with BM progenitors from indicated recipient strains. Data represent one of three independent experiments (n=4 mice per group). Fractions indicate incidence of allergic diarrhea (**B**).

(ii) **Major Task 2: Determine whether CD4$^+$T$_H$2 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 are currently focusing on Major Task 1. We expect to launch Major Task 2 when we begin to prepare manuscript for Major Task 1.

In summary, we have made significant progress in **Specific Aim 1 (completion 30%)** by establishing experimental systems to address the hypothesis proposed In Major Task 1. We will perform other independent experiments to obtain statistical significance for future manuscript. We will then address Major Task2 using similar approaches in the near future.

Specific Aim 2: Determine the role of the antigen/IgE/FcεRI complex in MMC9 development and function. (Completion 25%)

(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 considerable experiments to access 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 MCPt-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.

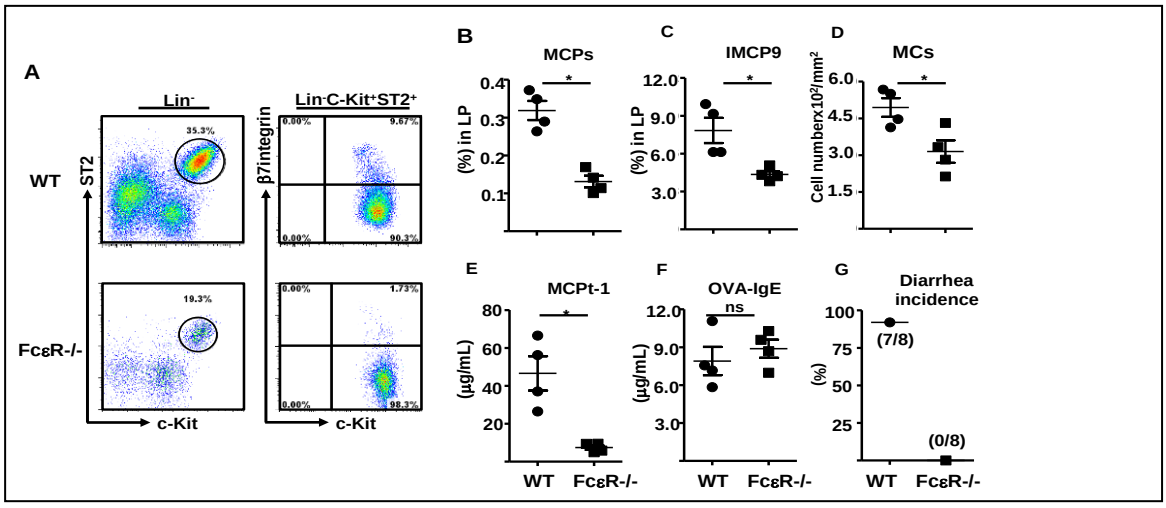

Figure 6 Diarrheal response to intragastric OVA antigen requires FcεR for food allergy. (**a**-**c**) Detection and frequency of MMC9 (Lin$^-$IL-17RB$^-$c-Kit$^+$b7integrin^{lo}) and MCPs (Lin$^-$IL-17RB$^-$c-Kit$^+$b7integrin^{hi}), intestinal mast cell (MC) numbers (**d**) in the small intestine, and titers of serum MCPT1 (e) and OVA-IgE (f) of indicated mouse strains after OVA/alum sensitization and six intragastric OVA challenges. Data represent one of two independent experiments (n=4 mice per group). LP, laminar propria. * p<0.05, NS, not significant.

Subtask 2: In vivo proliferative assay (Completion 0%)
Subtask 3: Ex vivo analysis (Completion 0%)
In this major task, we have addressed the hypothesis raised in **Subtask 1**. We will begin to address Subtask 2 and 3 in the following years.

(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 0%)**
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 10%)** 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 this Subtask 1, in collaboration with Dr. Marc Rothenberg, we have identified human MMC9-like cells based on their surface expression of C-$Kit^{+}Fc\varepsilon R^{+}ST2^{+}$, but no expression of known cell lineage markers ($CD19^{-}CD14^{-}CD3^{-}CD4^{-}CD56^{-}CD16^{-}$) using flow cytometry (**Fig. 7A**). Currently, we have analyzed 27 duodenum samples and observed elevated frequency of MMC9-like cells in the duodenum of food allergy (FA) patients with (n=5) or without eosinophilic esophagitis (EOE) (n=5)

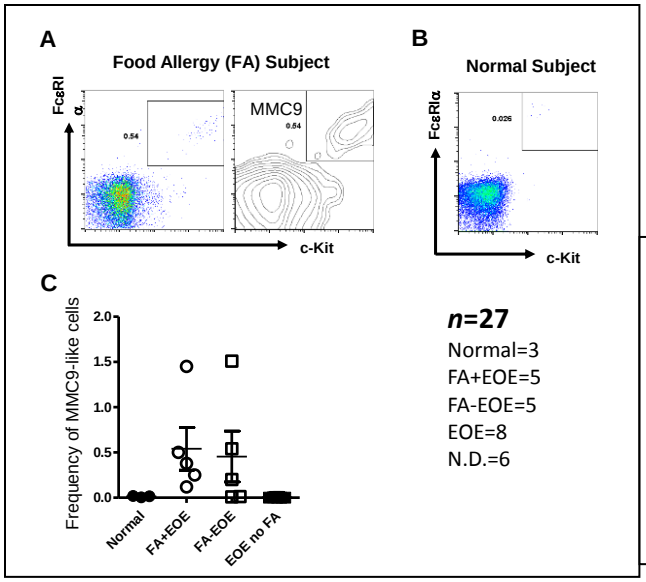

Figure 7. Detection of human MMC9-like cells in duodenum of patients with or without food allergy. Detection and frequency of MMC9-like cells ($Lin^{-}c\text{-}Kit^{+}Fc\varepsilon R^{+}$) in the small intestine of food allergic patients (**A**) or subjects without food allergy history (**B**) using flow cytometry. The frequencies of human Intestinal MMC9 cells are compared among subgroups of recruited subjects with indicated clinical characteristics (**C**). The detailed numbers of current enrolled subjects are listed (**C**).

(**Fig. 7B-7C**). By contrast, very few MMC9-like cells were detected in the small intestine of normal subject (n=3) and patients with EOE only (n=8) (**Fig. 7B-7C**). These results suggest an involvement of MMC9-like cells in the development of food allergy and other GI disorders. We will continue to collect more patient samples and analyze the proposed questions. The results will provide clinical characteristics of patients, which will address the questions raised in the Major Task 2.

Subtask 2: Cytokine production: (Completion 30%)

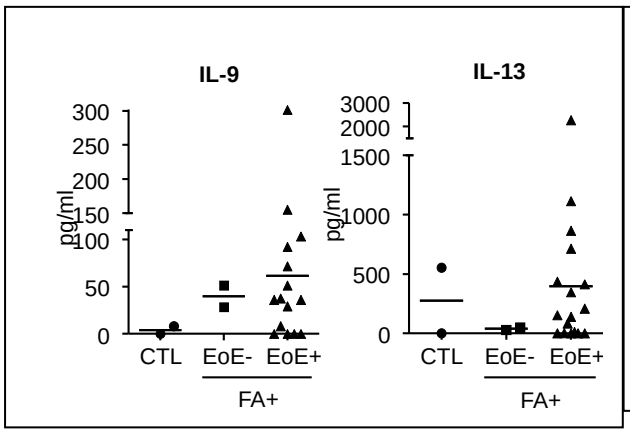

Figure 8. Detection and comparison of IL-9 and IL-13 production by human MMC9-like cells in duodenum of patients with or without food allergy. Duodenal biopsies from patients with indicated clinical characteristics were digested and cultured with cytokine sten cell factor and IL-3, plus the stimulation of IL-33.The secreted cytokines in the supernatants were collected and analyzed by ELISA method.

To address whether these intestinal MMC9-like cells 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 allery 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 sugroups. 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 additonal cytokines and continue to collect more patients samples for studying the correlation between clinincal characteristics.

Subtask 3: Gene expression: (Completion 0%)

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 begun to collect samples for proposed experiments as discussed in Major Task1. To reach statistical significance among subgroups of patients with distinct clinical characteristics, sufficient numbers of biopsies will be collected and analyzed.

In summary, we have made significant progress in Specific Aim 3, despite of the challenge in recruiting sufficient numbers of patient biopsies. We are particular 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:**

Nothing to report

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 (**Fig.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.

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:

(i) http://mykidsfoodallergies.com/food-allergy-research-discovery-could-lead-to-new-effective-treatments/

(ii) http://wvxu.org/post/newly-discovered-cell-could-improve-treatments-severe-food-allergies#stream/0

(iii) http://www.healthline.com/health-news/scientists-discover-new-cell-that-may-be-key-to-food-allergies-092215

(2) Peer-Reviewed Scientific Journals:

(i) Chen CY, Lee JB, Liu B, Ohta S, Wang PY, Burwinkel K., Izuhara K, Abonia PJ, Rothenberg ME, Finkelman FD, Hogan SP, **Wang YH**. Induction of IL-9-producing intestinal mast cell precursors promotes susceptibility to IgE-mediated experimental food allergy. 2015. Immunity. 43(4):788-802. PMCID: PMC4618257.

(ii) Sledd J, Wu D, Ahrens R, Lee J, Waggoner L, Tsai YT, **Wang YH**, Hogan SP. Loss of IL-4Ra-meidated PI3K signaling accelerates the progression of IgE/mast cell-mediated reaction. 2015. Immunity, Inflammation and Disease. 3(4):420-30. PMCID: PMC4693723.

(iii) Lee JB, Chen CY, Liu B, Luke M, Angkasekwinai P, Facchinetti V, Dong C, Liu, YJ, Rothenberg ME, Hogan SP, Finkelman FD, **Wang YH**. IL-25 and CD4+TH2 cells enhance ILC2-derived IL-13 production that promotes IgE-mediated experimental food allergy. 2016. J Allergy Clin Immunol. 137(4):1216-1225. PMCID: PMC4826796.

(3) Invited Articles:

(i) Ting W, Rothenberg ME, **Wang YH**. Hematopoietic prostaglandin D synthase: linking the pathogenic effector $CD4^{+}T_{H}2$ cells to pro-eosinophilic inflammation in gastrointestinal allergic disorders. 2016. *J Allergy Clin Immunol.* 137 (3):919-21.

(ii) **Wang, Y.-H.** Mucosal Immunity in food allergy. 2016. F1000 Faculty Reviews. 2016. *In Press*.

(iii) Shik, D., Tomar, S., Sha, W., Chen, C.-Y., Lee, J.-B., **Wang, Y.-H.** IL-9-producing cells in food allergy. 2016. *Seminars in Immunopathology. In Press*.

(4) Abstracts:

(i) Annual meeting of American Academy of Allergy, Asthma, & Immunology/AAAAI 2016.

(ii) 16th International Congress of Immunologist/ICI 2016.

b. List presentations

(i) American Academy of Allergy, Asthma, & Immunology/AAAAI 2016.

(ii) 16th International Congress of Immunologist/ICI 2016

7. **INVENTIONS, PATENTS AND LICENSES:**

Nothing to report

8. REPORTABLE OUTCOMES:

Nothing to report

9. **OTHER ACHIEVEMENTS:**

Nothing to report

10. REFERENCES:

1. R. S. Gupta *et al.*, The prevalence, severity, and distribution of childhood food allergy in the United States. *Pediatrics* **128**, e9-17 (2011).
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4. R. A. Wood, Food allergen immunotherapy: Current status and prospects for the future. *J Allergy Clin Immunol* **137**, 973-982 (2016).
5. C. Y. Chen *et al.*, Induction of Interleukin-9-Producing Mucosal Mast Cells Promotes Susceptibility to IgE-Mediated Experimental Food Allergy. *Immunity* **43**, 788-802 (2015).
6. D. J. Palmer *et al.*, Early regular egg exposure in infants with eczema: A randomized controlled trial. *The Journal of allergy and clinical immunology* **132**, 387-392 e381 (2013).
7. D. J. Hill *et al.*, Confirmation of the association between high levels of immunoglobulin E food sensitization and eczema in infancy: an international study. *Clin Exp Allergy* **38**, 161-168 (2008).
8. G. Lack *et al.*, Factors associated with the development of peanut allergy in childhood. *N Engl J Med* **348**, 977-985 (2003).

11. APPENDICES:

Attach four manuscripts.

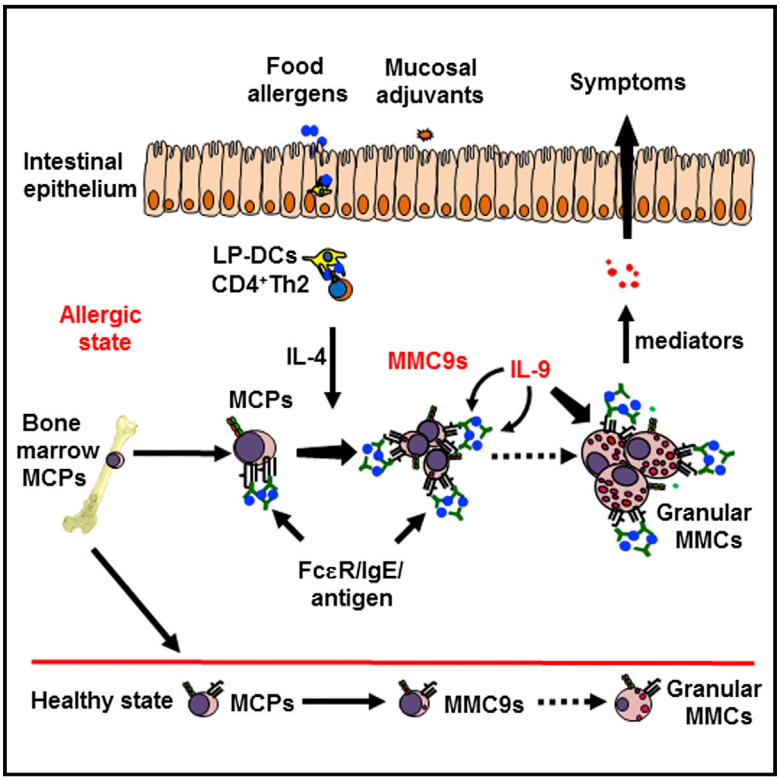
1. Chen CY, Lee JB, Liu B, Ohta S, Wang PY, Burwinkel K., Izuhara K, Abonia PJ, Rothenberg ME, Finkelman FD, Hogan SP, Wang YH. Induction of IL-9-producing intestinal mast cell precursors promotes susceptibility to IgE-mediated experimental food allergy. 2015. Immunity. 43(4):788-802. PMCID: PMC4618257.
2. Sledd J, Wu D, Ahrens R, Lee J, Waggoner L, Tsai YT, Wang YH, Hogan SP. Loss of IL-4Ra-meidated PI3K signaling accelerates the progression of IgE/mast cell-mediated reaction. 2015. Immunity, Inflammation and Disease. 3(4):420-30. PMCID: PMC4693723.
3. Lee JB, Chen CY, Liu B, Luke M, Angkasekwinai P, Facchinetti V, Dong C, Liu, YJ, Rothenberg ME, Hogan SP, Finkelman FD, Wang YH. IL-25 and CD4+TH2 cells enhance ILC2-derived IL-13 production that promotes IgE-mediated experimental food allergy. 2016. J Allergy Clin Immunol. 137(4):1216-1225. PMCID: PMC4826796.
4. Ting W, Rothenberg ME, Wang YH. Hematopoietic prostaglandin D synthase: linking the pathogenic effector $CD4^{+}T_{H}2$ cells to pro-eosinophilic inflammation in gastrointestinal allergic disorders. 2016. *J Allergy Clin Immunol.* 137 (3):919-21.

Article

Immunity

Induction of Interleukin-9-Producing Mucosal Mast Cells Promotes Susceptibility to IgE-Mediated Experimental Food Allergy

Graphical Abstract



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In Brief

Current knowledge cannot explain why only some patients and murine strains that acquire high amounts of dietary allergen-specific IgE develop life-threatening anaphylaxis. Wang and colleagues identify and characterize the IL-9-producing mucosal mast cells that amplify anaphylactic response to dietary proteins by producing large amounts of IL-9 and mast cell mediators.

Highlights

- Multi-functional MMC9s produce prodigious amounts of IL-9 and mast cell mediators
- MMC9 development increases in mice susceptible to IgE-mediated food allergy
- Induction of MMC9s requires T cells and IL-4, not IL-9, signals
- MMC9s amplify intestinal mastocytosis that drives IgE-mediated food allergy

Accession Numbers

GSE72921

Chen et al., 2015, Immunity *43*, 1–15
October 20, 2015 ©2015 Elsevier Inc.
http://dx.doi.org/10.1016/j.immuni.2015.08.020

Please cite this article in press as: Chen et al., Induction of Interleukin-9-Producing Mucosal Mast Cells Promotes Susceptibility to IgE-Mediated Experimental Food Allergy, Immunity (2015), http://dx.doi.org/10.1016/j.immuni.2015.08.020

Induction of Interleukin-9-Producing Mucosal Mast Cells Promotes Susceptibility to IgE-Mediated Experimental Food Allergy

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http://dx.doi.org/10.1016/j.immuni.2015.08.020

SUMMARY

Experimental IgE-mediated food allergy depends on intestinal anaphylaxis driven by interleukin-9 (IL-9). However, the primary cellular source of IL-9 and the mechanisms underlying the susceptibility to food-induced intestinal anaphylaxis remain unclear. Herein, we have reported the identification of multifunctional IL-9-producing mucosal mast cells (MMC9s) that can secrete prodigious amounts of IL-9 and IL-13 in response to IL-33, and mast cell protease-1 (MCPt-1) in response to antigen and IgE complex crosslinking, respectively. Repeated intragastric antigen challenge induced MMC9 development that required T cells, IL-4, and STAT6 transcription factor, but not IL-9 signals. Mice ablated of MMC9 induction failed to develop intestinal mastocytosis, which resulted in decreased food allergy symptoms that could be restored by adoptively transferred MMC9s. Finally, atopic patients that developed food allergy displayed increased intestinal expression of *Il9*- and MC-specific transcripts. Thus, the induction of MMC9s is a pivotal step to acquire the susceptibility to IgE-mediated food allergy.

INTRODUCTION

IgE-mediated food allergy is an immediate hypersensitivity reaction that can affect multiple organ systems. Clinical symptoms of food allergy patients range from a mild skin reaction to lethal shock (Boyce et al., 2010; Sicherer and Sampson, 2010). The anaphylactic response to ingested food antigens usually results from the activation of intestinal mast cells (MCs) through food-specific IgE antibodies (Finkelman, 2007; Galli and Tsai, 2012). However, it is perplexing as to why only some patients and murine strains that acquire high amounts of dietary allergen-specific IgE develop a severe immediate intestinal hypersensitivity response that can result in life-threatening anaphylaxis.

The T helper 2 (Th2) cell cytokine interleukin (IL)-4 plays key roles in promoting IgE antibody production and intestinal allergic inflammation that are required for IgE-mediated food allergy (Berin and Mayer, 2009; Brandt et al., 2009; Forbes et al., 2008; Strait et al., 2011; Vickery et al., 2011). Mice deficient in *Il4* produce little IgE antibody and are resistant to developing experimental food allergy (Brandt et al., 2009; Kweon et al., 2000). In contrast, mice harboring an activating mutation of the IL-4 receptor α chain (*Il4ra*F709) produce elevated antigen-specific IgE antibodies and are more susceptible to this disorder (Mathias et al., 2011). In addition to IL-4, recent studies point to a pivotal role for IL-9 in promoting intestinal mastocytosis and driving experimental food allergy (Forbes et al., 2008; Osterfeld et al., 2010). Repeated intragastric ovalbumin (OVA) challenge fails to increase intestinal mastocytosis, serum mast cell protease-1 (MCPt-1), the incidence of allergic diarrhea, or hypothermia in sensitized IL-9- or IL-9R-deficient mice, even though they produced OVA-specific IgE and IgG normally (Osterfeld et al., 2010). In contrast, transgenic mice that constitutively overexpress intestinal-specific IL-9 are more prone to develop experimental food allergy (Ahrens et al., 2012; Forbes et al., 2008; Osterfeld et al., 2010). Although these studies demonstrate a distinctive role for IL-9 in promoting experimental food allergy, the primary cellular source of IL-9 and the mechanisms that underlie the susceptibility to intestinal anaphylaxis have not been established. Herein, we report the identification of IL-9-producing mucosal mast cells (MMC9s) and show that the acquisition of MMC9s increases the susceptibility to IgE-mediated experimental food allergy.

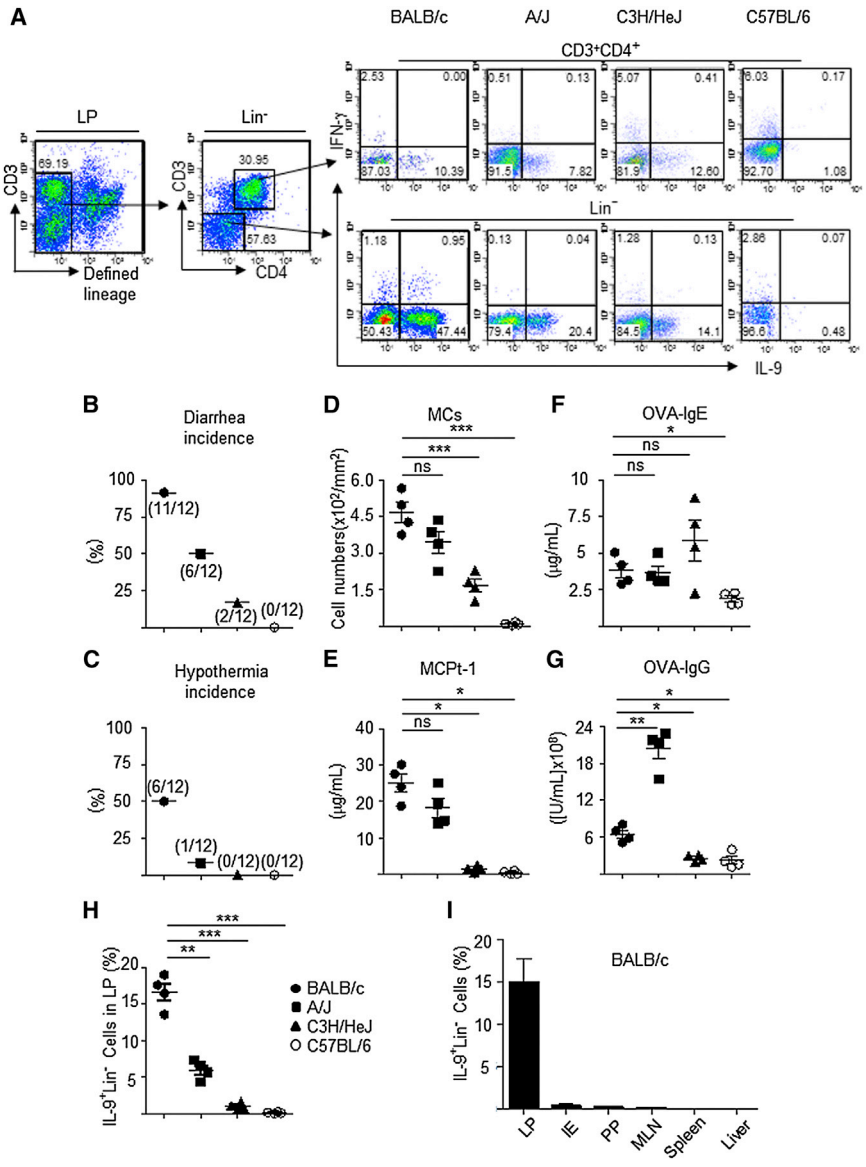


Figure 1. Lin⁻ IL-9-Producing Cell Frequency Correlates Positively with Susceptibility to Experimental Food Allergy

(A, H, and I) Flow cytometric analysis of intracellular IL-9 and IFN-γ production by $CD3^+CD4^+$ T or Lin^- cells (A) or frequencies of IL-9-producing cells in LP of small intestine of indicated mouse strains (H) or in the Lin^- fraction in the indicated tissues from BALB/c mice (I) after six intragastric OVA challenges.

(B–G) Indicated mouse strains were sensitized and challenged six times intragastrically with OVA before measuring the indicated features of experimental food allergy: incidences of allergic diarrhea (B) and hypothermia (C), intestinal mast cell numbers (D), MCPt-1 production (E), and concentration of OVA-specific IgE (F) and OVA-specific IgG (G).

Data in (A)–(I) are representative of three independent experiments (n = 4 mice per group). Fraction indicates incidences of allergic diarrhea (B) and hypothermia (C). Error bars denote mean ± SEM. *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$. NS, not significant. See also Figure S1.

RESULTS

Mice Susceptible to Food Allergy Have Increased Lineage⁻ IL-9-Producing Cells

Just as allergen-sensitized patients have varying degrees of susceptibility to food allergens, OVA-sensitized murine strains differ in their susceptibility to anaphylactic responses to ingested OVA (Helm and Burks, 2002). Intestinal IL-9 production is important for the development of experimental food allergy (Forbes et al., 2008; Osterfeld et al., 2010), so we first examined and compared the frequency of IL-9-producing cells among lamina propria (LP) mononuclear cells in the small intestine of different immunized murine strains. Although some $CD3^+CD4^+$ T cells produced IL-9, most of the IL-9-producing LP cells in BALB/c, A/J, and C3H/HeJ mice did not express cell lineage markers (Lin^-) (Figure 1A). Among the examined murine strains, sensitized BALB/c mice were the most likely to develop experimental food allergy after six intragastric OVA challenges, with BALB/c mice exhibiting higher incidences of allergic diarrhea and hypothermia (91.7%, 50.0%) as compared to A/J (50.0%, 8.3%), C3H/HeJ (12.5%, 0.0%), and C57BL/6 (0.0%, 0.0%) mice (Figures 1B and 1C). Notably, murine strains that were more susceptible to experimental food allergy, as manifested by allergic diarrhea and hypothermia, also exhibited higher amounts of intestinal mastocytosis (Figure 1D) and serum MCPt-1 (which reflects mast cell degranulation) (Figure 1E), whereas all murine strains were capable of producing considerable amounts of serum OVA-specific IgE or IgG (Figures 1F and 1G). BALB/c mice had the highest frequency of intestinal Lin^- IL-9-producing LP cells (16.7% ± 2.0%, mean ± SEM) and were also the most susceptible strain to experimental food allergy, whereas C57BL/6 mice lacked the Lin^- IL-9-producing cell population and failed to develop experimental food allergy (Figures 1A and 1B–1H). Immunized A/J and C3H/HeJ strains had 6.0% ± 1.1% and 1.0% ± 0.3%, Lin^- IL-9-producing LP cells, respectively, and were less susceptible to developing experimental food allergy (Figures 1A and 1B–1H). Similarly, a higher frequency of Lin^- IL-9-producing LP cells (4.5%) was induced in sensitized CBA mice than those in DBA2 (1.8%) and 129 (2.0%) mice after repeated challenges (Figure S1A). Immunized CBA mice also displayed more pronounced intestinal mastocytosis, produced more MCPt-1, and were more susceptible to experimental food allergy, while producing comparable amounts of OVA-specific IgE among these murine strains (Figures S1B–1E). Anatomically, these Lin^- IL-9-producing cells resided primarily within the LP, not

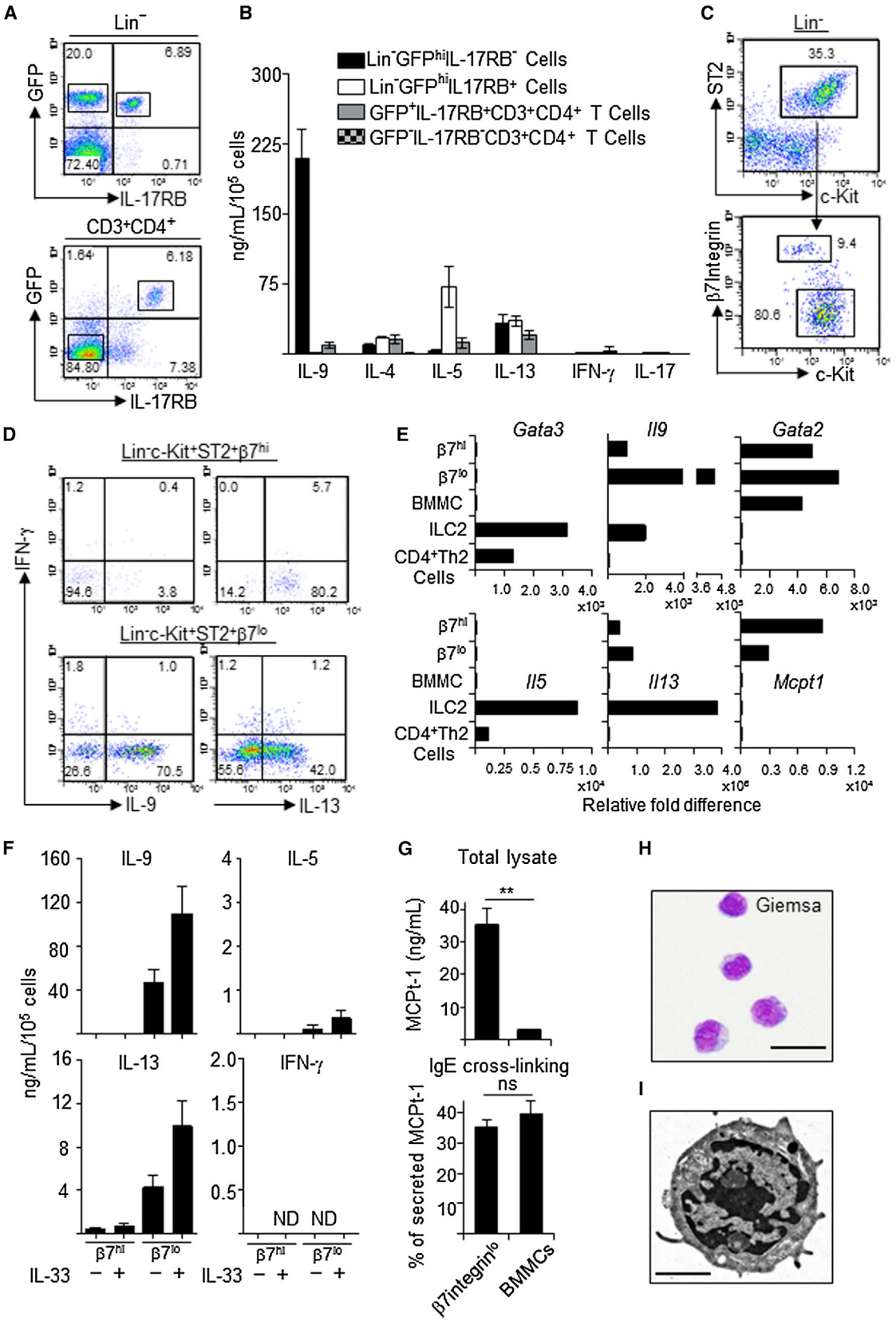
the intraepithelium of the small intestine, and very few of these cells resided in the Peyer's patch (PP), mesenteric lymph nodes (MLNs), spleen, lung, or liver (Figure 1I). Together, these results suggest an association of Lin$^-$ IL-9-producing LP cells with the susceptibility to experimental food allergy.

Innate MMC9s Are the Principal IL-9 Producers of Mast Cell Lineage

A previous report demonstrated that type 2 innate lymphoid cells (ILC2s) lack cell lineage markers and have the potential to produce IL-9 (Wilhelm et al., 2011). To determine whether these intestinal Lin$^-$ IL-9-producing cells were ILC2s, we generated a monoclonal antibody against an IL-25 receptor component, IL-17RB, the characteristic marker for ILC2s (Neill et al., 2010) and employed BALB/c IL-4-eGFP (4GET) mice that can be used to track ILC2s by their GFP expression in our murine model of food allergy. Two dominant Lin$^-$GFP^{hi} cell populations were identified: Lin$^-$GFP^{hi}IL-17RB$^-$ cells and Lin$^-$GFP^{hi}IL-17RB$^+$ cells (Figure 2A). ELISA analyses revealed that purified Lin$^-$GFP^{hi}IL-17RB$^-$ cells were the population responsible for secreting prodigious amounts of IL-9 (~2.0 pg/ml per cell) and other Th2-cell-associated cytokines, including IL-4 and IL-13, in lesser amounts after PMA plus ionomycin stimulation (Figure 2B). In contrast, the Lin$^-$GFP^{hi}IL-17RB$^+$ cell population produced considerable amounts of IL-5 (~0.8 pg/ml per cell), as well as lesser amounts of IL-13 and IL-4, but little IL-9 (< 0.01 pg/ml per cell) (Figure 2B). Both Lin$^-$GFP^{hi} cell populations produced undetectable interferon-γ (IFN-γ) and IL-17 (Figure 2B). In addition, intestinal CD3$^+$CD4$^+$ Th2 cells that displayed increased expression of IL-17RB, ST2 (IL-33 receptor), and GFP, but not CD3$^+$CD4$^+$IL-17RB$^-$ST2$^-$GFP$^-$ T cells, could also produce moderate amounts of IL-9 (~0.05 pg/ml per cell) as well as IL-4, IL-5, and IL-13 (Figures 2A and 2B and data not shown). These results demonstrate that Lin$^-$GFP^{hi}IL-17RB$^-$, not Lin$^-$GFP^{hi}IL-17RB$^+$ cells or CD4$^+$ Th2 cells, are the principal IL-9 producers in mice susceptible to experimental food allergy.

To better understand the lineage of these Lin$^-$GFP^{hi} cells, we performed detailed cellular and molecular characterization. The purified IL-9-producing Lin$^-$GFP^{hi}IL-17RB$^-$ cells were found to express c-Kit, FcεRIα, ST2, Thy1.2, major histocompatibility complex class II (MHCII), and CD86, but not IL-2Rα, IL-7Rα, Sca-1, CD23, CCR3, Siglec-F, or IL-3Rα (CD123) (Figure S2A and data not shown), implying a MC lineage phenotype (Kitamura, 1989). Notably, the majority (>90%) of Lin$^-$GFP^{hi}IL-17RB$^-$c-Kit$^+$ST2$^+$ cells did not express surface β7integrin, an adhesion molecule that characterizes previously described intestinal mast cell progenitors (MCPs) (Figure 2C; Chen et al., 2005; Gurish et al., 2001). Intracellular cytokine analysis demonstrated that the principle IL-9 producers were the β7integrin^{lo} subset of Lin$^-$GFP^{hi}IL-17RB$^-$c-Kit$^+$ST2$^+$ cells, although both β7integrin^{hi} and β7integrin^{lo} subsets could produce IL-13 (Figure 2D). Consistently, both cell subsets expressed *Il13* transcript (>10^4-fold), but only β7integrin^{lo}Lin$^-$GFP^{hi}IL-17RB$^-$c-Kit$^+$ST2$^+$ cells expressed very large amounts of *Il9* transcript (>10^5-fold), compared to naive CD4$^+$ T cells (Figure 2E). Similar to bone-marrow-derived mast cells (BMMCs), both β7integrin^{hi} and β7integrin^{lo} subsets of Lin$^-$GFP^{hi}IL-17RB$^-$c-Kit$^+$ST2$^+$ cells expressed *Gata1* (>10^3-fold), *Gata2* (>10^3-fold), and *Mcpt1* (>10^3-fold), not *Rora*, *Gata3*, *Mcpt8*, and *Mcpt11* transcripts (Figure 2E and data not shown). Notably, stimulation with IL-33 plus stem cell factor (SCF) and IL-3 ex vivo triggered the β7integrin^{lo}, but not the β7integrin^{hi}, subset of Lin$^-$IL-17RB$^-$c-Kit$^+$ST2$^+$ cells to produce large amounts of IL-9 and IL-13, with considerably less IL-5 and no IFN-γ (Figure 2F). Purified IL-9-producing β7integrin^{lo}Lin$^-$IL-17RB$^-$c-Kit$^+$ST2$^+$ cells produced comparable amounts of histamine as BMMCs and contained ~10-fold more intracellular MCPt-1 than did BMMCs, while possessing similar efficacy of MCPt-1 secretion as BMMCs in response to IgE-bound FcεRIα complex crosslinking (Figures 2G and S2C). Cytology and electron microscopy revealed that both the β7integrin^{hi} and β7integrin^{lo} subsets of Lin$^-$IL-17RB$^-$c-Kit$^+$ST2$^+$ cells resembled mucosal MCs in their small size, large nuclei, scanty cytoplasm, and small number of metachromatic granules (Figures 2H and 2I; Chen et al., 2005; Gurish et al., 2001). Notably, only the β7integrin^{hi}, not β7integrin^{lo}, subset of Lin$^-$IL-17RB$^-$c-Kit$^+$ST2$^+$ cells vigorously expanded (>450-fold) during 15 days of culture with IL-3 and SCF (Figure S2B). Furthermore, the agranular IL-9-producing β7integrin^{lo}Lin$^-$IL-17RB$^-$c-Kit$^+$ST2$^+$ cells rapidly developed into granular MCs and acquired β-hexoaminidase activity but lost robust IL-9-producing capability after IL-3 plus SCF culture (Figures S2D–S2F). These findings suggest that β7integrin^{hi} Lin$^-$IL-17RB$^-$c-Kit$^+$ST2$^+$ cells are the intestinal MCPs (Gurish and Austen, 2012) and that β7integrin^{lo}Lin$^-$IL-17RB$^-$c-Kit$^+$ST2$^+$ cells are the intestinal IL-9-producing MCs, which we term IL-9-producing mucosal mast cells or MMC9s.

In contrast to MMC9s, Lin$^-$GFP^{hi}IL-17RB$^+$ cells expressed characteristic ILC2 markers, including ST2, IL-7Rα, IL-2Rα, Thy1.2, MHCII, CD86, and ICOS, but not c-Kit or FcεR (Figure S3A). Purified Lin$^-$GFP^{hi}IL-17RB$^+$ cells underwent an expansion and produced large amounts of IL-5 and IL-13, along with a small amount of IL-9 after culture with IL-25 and/or IL-33 in the presence of IL-7; in contrast, MMC9s, which lacked IL-17RB, did not respond to these stimuli and perished (Figure S3B and data not shown; Wilhelm et al., 2011). Moreover, systemic IL-25 administration induced an expansion of Lin$^-$GFP^{hi}IL-17RB$^+$c-Kit$^-$ cells but not MMC9s (Figures S3C and S3D). Purified Lin$^-$GFP^{hi}IL-17RB$^+$c-Kit$^-$ cells displayed the characteristic ILC2 molecular profile, including large amounts of *Il5* (>10^3-fold), *Il13* (>10^6-fold), and transcription factor *Gata3* (>10^3-fold) and *Rora* (>10^4-fold), and moderate amounts of *Il9* (<10^3-fold) transcripts (Figure 2E and data not shown). In this regard, the Lin$^-$GFP^{hi}IL-17RB$^+$c-Kit$^-$ cells detected in our murine model of food allergy appeared identical to the intestinal IL-25-responding ILC2s that have previously been shown to elicit protective immunity against intestinal worm infection (Moro et al., 2010; Neill et al., 2010; Price et al., 2010; Saenz et al., 2010). In contrast, MMC9s did not respond to IL-25 stimulation in vitro and in vivo and are distinct from both ILC2s and the previously reported IL-25-elicited c-Kit^{int}-GFP$^+$ multipotent progenitor (MPP^{type2}) (Saenz et al., 2010). Furthermore, RNA-seq analyses and comparison of purified hematopoietic cell lineages revealed that in addition to MMC9-specific genes (1), MMC9s expressed MC lineage-associated transcripts (2) that were clustered closely with those by MCPs, mature MCs, and BMMCs as well as Th2-cell-mediated immune response-associated transcripts (3) expressed by intestinal CD4$^+$ Th2 cells and ILC2s (Figure S3E). Thus, distinct from ILC2s, MMC9s are the type 2



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cytokine-producing innate cells with MC-lineage molecular signatures.

Bone Marrow MCPs Give Rise to Intestinal MMC9s

To examine the MC lineage origin of intestinal MMC9s in vivo, we first established a reconstitution model of experimental food allergy by adoptively transferring bone marrow (BM) cells from 4GET mice into OVA-sensitized BALB/c mice 1 day after sublethal irradiation (protocol diagramed in Figure S4A). 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 S4B). In contrast, most $CD4^+IL\text{-}17RB^+ST2^+$ Th2 cells were derived from the sensitized recipients as shown by their lack of GFP expression (Figure S4C). Compared to reconstituted mice that were challenged with saline only, repeated intragastric OVA challenge triggered a substantial increase in donor-derived (>95% GFP^{hi}) MMC9s and recipient-derived (>98% GFP^-) $CD4^+$ Th2 cells (Figures S4B and S4C). This is probably because donor-derived hematopoietic progenitors will reconstitute innate MC lineage within 2 weeks, whereas de novo T cell generation after thymus engraftment requires >2 months (Spangrude and Scollay, 1990). Indeed, WT BM progenitors from $Thy1.1^+$ BALB/c mice could reconstitute most of intestinal MMC9s that expressed congenic marker $Thy1.1^{hi/lo}$ (>95%) in the irradiated WT $Thy1.2^+$ BALB/c recipients, in which most of intestinal $CD4^+$ T cells expressed Thy1.2 (>98%) after repeated intragastric OVA challenge (Figure S4D). Next, we showed that purified BM MCPs (defined as $Lin^-Ly\text{-}6c^-Fc\varepsilon RI\alpha^-CD41^-CD71^-FLK2^-CD150^-c\text{-}Kit^+\beta7integrin^+$ cells) (Franco et al., 2010) 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 (Figures 3A–3C). Furthermore, the blockade of intestinal $\beta7integrin^{hi}$ MCP recruitment by anti-$\alpha4\beta7$integrin mAb significantly reduced intestinal MCP and MMC9 frequency and MC number. Consequently, these reconstituted recipients failed to develop experimental food allergy, despite normal OVA-specific IgE production (Figures 3D–3F). Additionally, sorted single intestinal $\beta7^{hi}$ MCPs had the potential to develop into MMC9s that displayed $\beta7^{lo}c\text{-}Kit^+ST2^+Fc\varepsilon RI\alpha^+$ phenotype and upregulated *Il9*, *Il13*, and *Mcpt6*, while maintaining *Gata2* expression after culture with SCF, IL-4, TGF-β, and IL-33 (Figure S2G and data not shown). Colony-forming cell assays showed that all of their progeny colonies derived from single MMC9s displayed a mature MC phenotype and morphology, although MMC9s had reduced capacity (<10%) to generate compact colonies, compared to $\beta7^{hi}$ MCPs (>40%) (Figure S2H and data not shown). Given the cellular and molecular characteristics of MCPs, MMC9s, and MCs (Figures 2, 3, and S2–S4), these results suggest that intestinal $\beta7integrin^{hi}$ MCPs of BM origin have the potential to proliferate and develop into $\beta7integrin^{lo}$ MMC9s, which can mature into mucosal MCs with increased granular enzyme activity but reduced IL-9 production.

Ingested Antigens Induce Concomitant MMC9 and $CD4^+$ Th2 Cell Accumulation

We compared the occurrence in the LP of the principal cell types that may be involved in the development of experimental food allergy: MCPs, MMC9s, $CD4^+$ Th2 cells, ILC2s, and basophils ($c\text{-}Kit^{lo}Fc\varepsilon RI\alpha^+DX5^+IL\text{-}3R\beta^+$). Although MCPs and MMC9s represented only ~0.3% and ~0.5% of total mononuclear cells in the small intestine of naive or intraperitoneally sensitized BALB/c mice, respectively, repeated intragastric OVA challenge induced a considerable accumulation of MMC9s (~9%) in sensitized mice, whereas MCPs remained a minor population (Figures 4A and 4B). Concurrently, $CD4^+IL\text{-}17RB^+$ Th2 cells also increased (~4%) compared to naive mice (~0.5%) (Figures 4A and 4B). Importantly, the concomitant increase of MMC9s and $CD4^+IL\text{-}17RB^+$ Th2 cells positively correlated with increased features of experimental food allergy: allergic diarrhea, intestinal MC number, serum MCPt-1, OVA-specific IgE and IgG, and histamine (Figures 4A–4F, and data not shown). Furthermore, analysis of seven murine strains showed that the concomitant increase of MMC9 and $CD4^+IL\text{-}17RB^+$ Th2 cells was much greater in murine strains that were prone to develop experimental food allergy (i.e., BALB/c, A/J, and CBA) than in those that were resistant to developing experimental food allergy (i.e., C3H/HeJ, DBA2, 129, and C57BL/6) (Figures 1A–1H, 4G, and S1). In contrast, the frequencies of ILC2s (~2.0%) and basophils (<0.1%) remained constant even after the examined murine strains were subjected to stimuli that induce intestinal anaphylaxis (data not shown). Similarly, skin-sensitized mice by vitamin D3 analog (Calcipotriol) also developed strong MMC9 (>3.5%) and $CD4^+$ Th2 (>2%) immune response, resulting in pronounced intestinal mastocytosis and increased MCPt-1 production after

Figure 2. $Lin^-GFP^{hi}IL\text{-}17RB^-c\text{-}Kit^+ST2^+\beta7integrin^{lo}$ Cells Are the Multi-functional IL-9-Producing Mucosal Mast Cells

(A and B) Detection of (A) and measurements of (B) indicated cytokines secreted by two dominant Lin^-GFP^{hi} cell fractions that are $IL\text{-}17RB^-$ and $IL\text{-}17RB^+$, and $GFP^+IL\text{-}17RB^+$ and $GFP^-IL\text{-}17RB^-$ subsets of $CD3^+CD4^+$ T cells in LP of small intestine of IL-4-eGFP (4GET) mice after six intragastric OVA challenges.

(C and D) Detection of (C) and flow cytometric analysis of indicated intracellular cytokines by (D) $\beta7integrin^{lo}$ ($\beta7^{lo}$) and $\beta7integrin^{hi}$ ($\beta7^{hi}$) subsets of $Lin^-GFP^{hi}IL\text{-}17RB^-c\text{-}Kit^+ST2^+$ cells.

(E) Expression of the indicated genes by purified intestinal $\beta7integrin^{lo}$ and $\beta7integrin^{hi}$ subsets of $Lin^-GFP^{hi}IL\text{-}17RB^-c\text{-}Kit^+ST2^+$, in-vitro-generated bone-marrow-derived mast cells (BMMCs), ILC2s, and $CD4^+GFP^+IL\text{-}17RB^+$ Th2 cells from mice with active allergic diarrhea was analyzed by quantitative real-time PCR using primers referenced in the Experimental Procedures.

(F) Measurement of indicated cytokine production by purified intestinal $\beta7integrin^{lo}$ and $\beta7integrin^{hi}$ subsets of $Lin^-GFP^{hi}IL\text{-}17RB^-c\text{-}Kit^+ST2^+$ after 3 days culture with SCF plus IL-3 in the presence or absence of IL-33.

(G) Measurement of intracellular MCPt-1 within purified $Lin^-IL\text{-}17RB^-c\text{-}Kit^+ST2^+\beta7integrin^{lo}$ or bone-marrow-derived mast cells (BMMCs) and their MCPt-1 secretion after IgE crosslinking.

(H and I) Giemsa staining (H) and electron microscopic analysis (I) of purified $Lin^-IL\text{-}17RB^-c\text{-}Kit^+ST2^+\beta7integrin^{lo}$ cells.

Gene expression data are expressed as relative fold difference (E). Scale bars represent 20 μm (H) or 2 μm (I). Data in (A)–(G) are representative of three independent experiments. Error bars denote mean ± SEM. $^{**}p \leq 0.01$. NS, not significant. See also Figures S2 and S3.

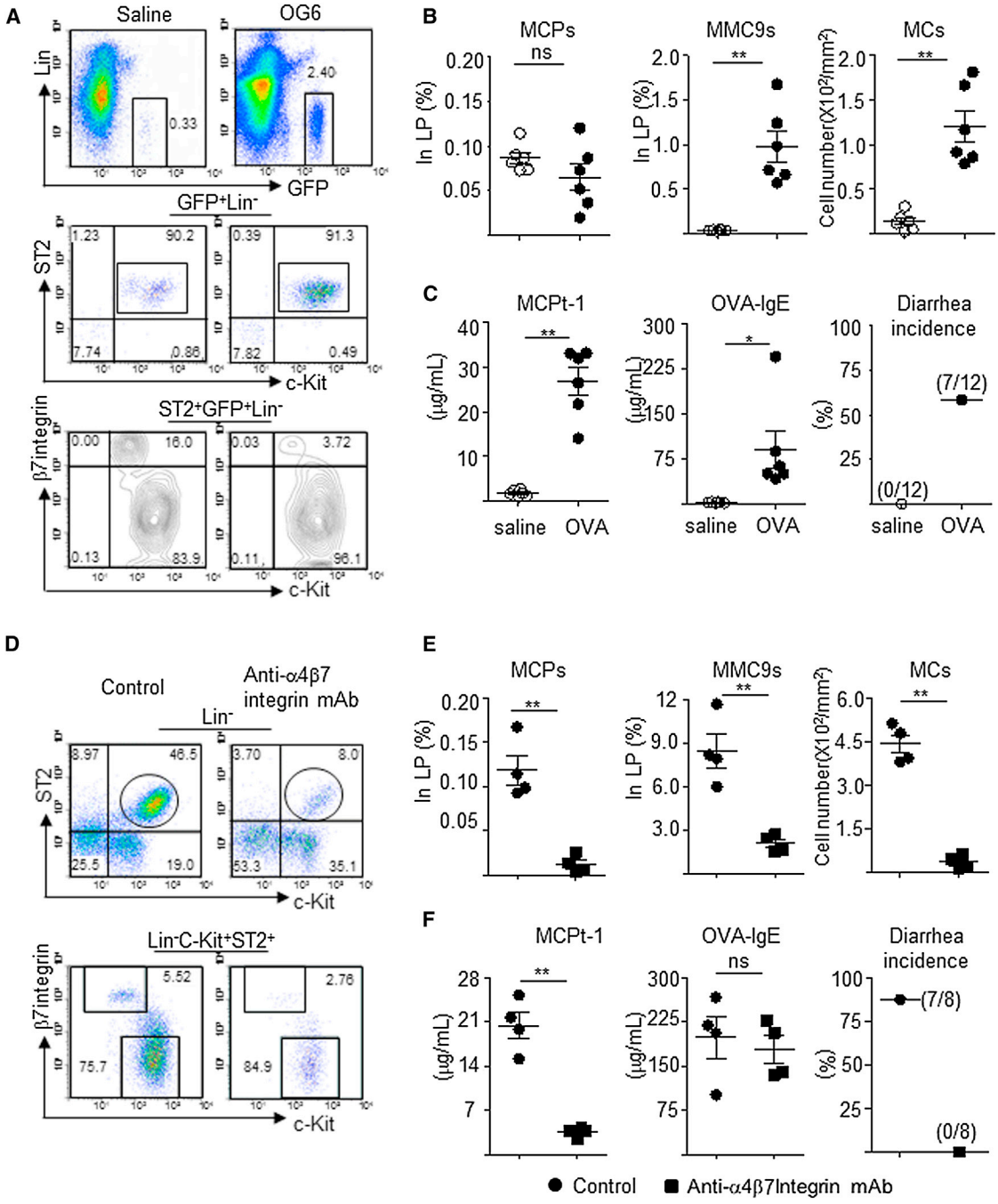

Figure 3. Blockade of Intestinal β7^{hi} MCP Recruitment from Bone Marrow Results in the Failure of MMC9 Induction

Detection and frequency of donor-derived MMC9s (Lin$^-$GFP$^+$ST2$^+$c-Kit$^+$ β7integrin^{lo}) and MCPs (Lin$^-$GFP$^+$ST2$^+$c-Kit$^+$β7integrin^{hi}), and mast cell (MC) number (A, B, D, E), and serum MCPt-1 and OVA-IgE titers, and diarrhea incidence (C, F) of irradiated recipient BALB/c mice reconstituted with sorted bone marrow mast cell progenitors (Lin$^-$Ly-6c$^-$FcεRIα$^-$CD41$^-$CD71$^-$FLK2$^-$CD150$^-$c-Kit$^+$β7integrin$^+$ cells) from IL-4-eGFP (4GET) mice (A–C) or received anti-α4β7integrin (LPAM-1) or isotype-matched mAb and reconstituted with wild-type bone marrow progenitors from IL-4-eGFP (4GET) mice (D–F) 1 day before second sensitization and six intragastric OVA challenges. Data represent one of three independent experiments (n = 6 mice per group) (A–C) or one of two independent experiments (n = 4 mice per group) (D–F). LP, laminar propria. Error bars denote mean ± SEM. *p < 0.05, **p < 0.01. NS, not significant. See also Figure S4.

repeated intragastric OVA challenge (Figures S5A–S5D). However, we failed to detect significant induction of MMC9s (<1%) and CD4$^+$ Th2 cells (<1%) in cholera toxin (CT)-sensitized C3H/HeJ or BALB/c mice that displayed few intestinal MC numbers and produced low MCPt-1 titer after repeated intragastric OVA challenge (Figures S5E–S5G). These results demonstrate that repeated oral antigen challenge triggers substantial accumulations of MMC9s and CD4$^+$ Th2 cells, but not MCPs,

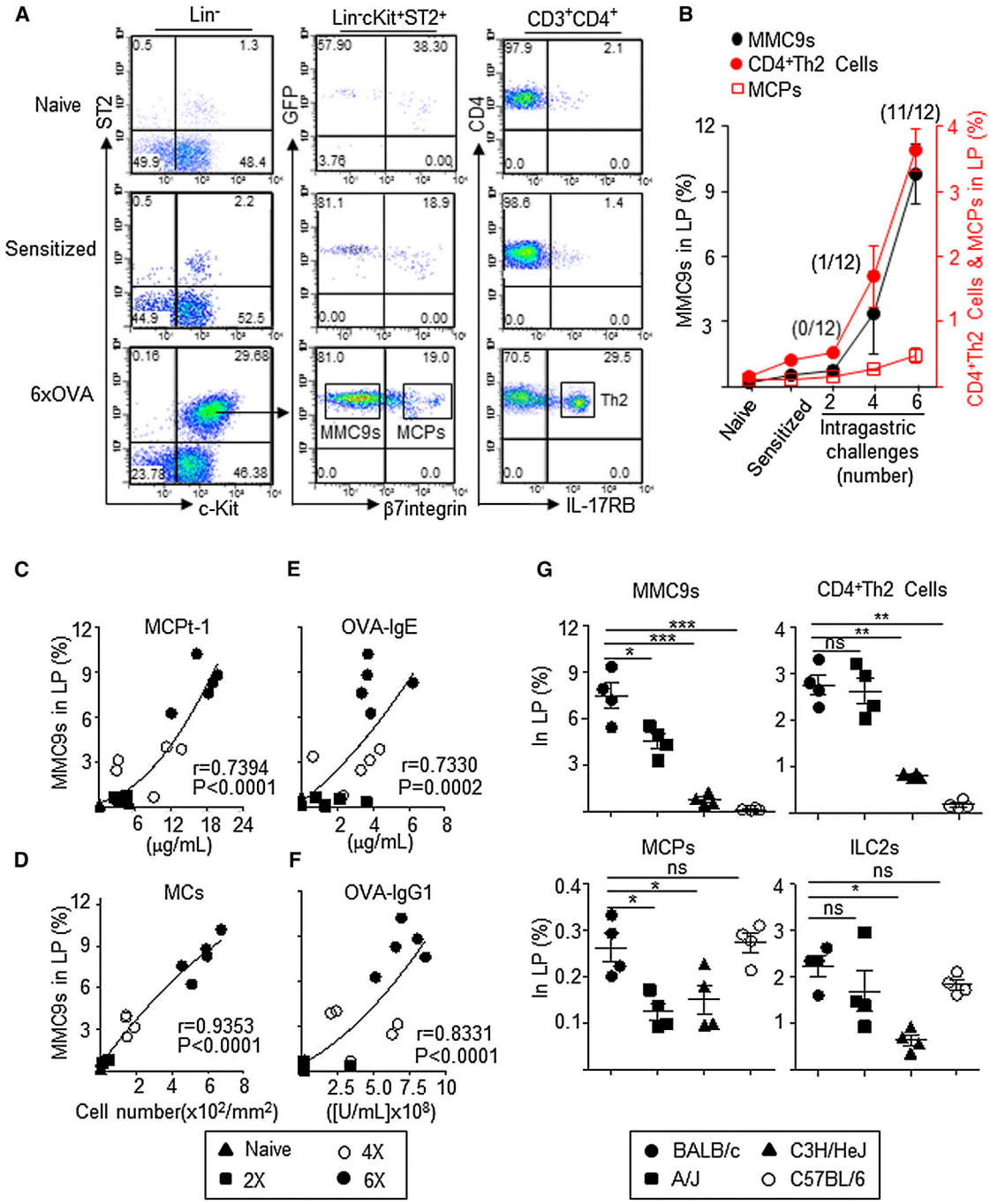

Figure 4. MMC9s and CD4⁺ Th2 Cell Frequencies Correlate Positively with Susceptibility to Experimental Food Allergy

(A–F) Detection (A) and frequency of MMC9s (A-F), MCPs (A, B), and CD4⁺IL-17RB⁺ Th2 cells (A, B) in LP of small intestine of naive, sensitized, or BALB/c mice after indicated times of intragastric OVA challenge. Serum samples (C, E, F) or intestinal tissue (D) were collected for measurement of indicated features of experimental food allergy, including allergic diarrhea incidence (B), MCPt-1 production (C), intestinal mast cell numbers (D), and titers of OVA-specific IgE (E) and OVA-specific IgG (F).

(G) Frequency of indicated cell populations in LP of small intestine of indicated mouse strains after six intragastric OVA challenges.

Spearman's rank coefficients and two-tailed p values were used to quantify the correlations between the indicated features of experimental food allergy and frequency of MMC9s (C–F). Fractions indicate incidence of allergic diarrhea (B). Data in (A)–(G) are representative of three independent experiments (n = 4 mice per group). Error bars denote mean ± SEM. *p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001. NS, not significant. See also Figure S5.

ILC2s, or basophils in the small intestine of sensitized mice and that the concomitant increase of MMC9s and $CD4^+$ Th2 cells correlates positively with susceptibility to and severity of experimental food allergy.

MMC9 Induction Requires $CD4^+$ Th2 Cells

To understand the requirements for MMC9 induction, we first analyzed genetically modified mice that were resistant to developing experimental food allergy. Compared to WT mice, sensitized BALB/c IL-4-, IL-4Rα-, STAT6-, or Rag-2-deficient mice and T-cell-deficient nude mice were less competent to recruit MCPs, failed to develop MMC9s, and subsequently were resistant to develop features of food allergy after repeated intragastric OVA challenge (Figures 5A–5C and data not shown). Anti-CD4 mAb ablation of T cells before challenge suppressed MMC9 induction but not MCP recruitment in sensitized mice and prevented the development of experimental food allergy (Figures 5D–5F). Analysis of the reconstitution model of experimental food allergy revealed that the reconstituted, immunized wild-type recipients, which acquired significant accumulations of GFP^{hi} MMC9s and GFP^-CD4^+ Th2 cells, eventually developed features of experimental food allergy (Figures 5G–5I). In contrast, *Stat6*$^{-/-}$ recipients, which lacked recipient-derived GFP^-CD4^+ $IL\text{-}17RB^+$ Th2 cells, recruited fewer donor-derived GFP^{hi} MCPs and failed to develop GFP^{hi} MMC9s and any characteristics of experimental food allergy (Figures 5G–5I). Collectively, these results demonstrate that the induction of MMC9s requires T cells and IL-4 signals and that an intact Th2 cell microenvironment, provided by $CD4^+$ Th2 cells, induces MMC9s that promote experimental food allergy.

IL-9 Signals Promote MMC9 Expansion and Intestinal Mastocytosis

IL-9 plays an important role in MC development and growth (Goswami and Kaplan, 2011), so we examined the requirements of IL-9 and IL-9R in the MMC9 induction. Although the MCP and MMC9 accumulations occurred normally, many fewer sensitized IL-9- and IL-9R-deficient mice developed a diarrheal response and had significantly reduced intestinal MC number and serum MCPt-1, despite normal OVA-specific IgE titers (Figures 6A–6E). The failure to develop experimental food allergy in *Il9*$^{-/-}$ and *Il9r*$^{-/-}$ mice could be restored after the reconstitution with WT BM cells (Figures S6A–S6C), because BM MCPs purified from 4GET mice could reconstitute GFP^+ MMC9s that promoted intestinal mastocytosis in *Il9*$^{-/-}$ recipients (Figure S6D). In contrast, IL-9- or IL-9R-deficient BM cells failed to restore the susceptibility to developing experimental food allergy in the irradiated 4GET recipients, despite significant generations of GFP^- MMC9s deficient of IL-9 or IL-9R (Figures 6F–6I). Additionally, IL-9R-deficient BM cells were less efficient than WT or IL-9-deficient BM cells to reconstitute GFP^- MMC9s (Figures 6F–6I), possibly due to alternative IL-9 sources produced by recipient's $CD4^+$ Th2 cells and/or ILC2s that were sufficient to enhance MMC9 expansion (Figures 6F–6H, 1A, and 2E). Finally, reconstitution with purified WT, not IL-9-deficient, MMC9s could directly promote intestinal mastocytosis and restore the propensity to develop experimental food allergy in sensitized recipients that lacked an alternative source of IL-9 (Figure S6E). These results suggest that IL-9 and IL-9R are dispensable for MMC9 development but are necessary for effective expansion of MMC9s that promote intestinal mastocytosis and susceptibility to experimental food allergy in an IL-9-dependent autocrine manner.

MMC9 Induction Augments Intestinal Mastocytosis that Drives Experimental Food Allergy

To directly demonstrate that MMC9 induction is a key step for the amplification of intestinal mastocytosis that promotes the development of experimental food allergy, we treated OVA-sensitized BALB/c WT mice with anti-FcεRIα mAb before and during the course of repeated intragastric OVA challenge. Anti-FcεRIα mAb treatment efficiently ablated MCPs and MMC9s as evidenced by a ~90% decrease in Lin^- IL-9-producing cells (Figures 7A and 7B). The loss of MCPs and MMC9s was associated with decreased MC number and goblet cell hyperplasia, and serum MCPt-1, and the failure to develop allergic diarrhea after repeated intragastric OVA challenge (Figures 7B–7D). Importantly, transfer of purified MMC9s to mice that had been treated with anti-FcεRIα mAb accelerated restoration of the ability to develop intestinal mastocytosis and promoted their development of allergic diarrhea after repeated intragastric OVA challenges (Figures 7E and 7F). Compared to WT mice, much fewer intestinal MCPs and MMC9s were induced in sensitized *Fcer1a*$^{-/-}$ mice, which consequently exhibited less intestinal mastocytosis, produced fewer MCPt-1, and failed to develop food allergy (Figures S7A–S7C). These results further demonstrate that MMC9s drive intestinal mastocytosis to promote the susceptibility to experimental food allergy in an FcεR-dependent manner.

Food Allergy Patients Display Increased Expression of *Il9* and Mast Cell-Specific Transcripts

To evaluate a biological relevance of MMC9 induction in human food allergy, we analyzed the expression of MMC9 signature transcripts in the duodenum of patients who had defined food allergy characteristics (Table S1). Notably, expression of *Il9* and *Il13* and mast cell transcripts, such as carboxypeptidase A3 (*Cpa3*), tryptase, and chymase, were significantly increased in the duodenum of food allergy patients than those in control subjects (Figure 7G). These results suggest that increased expression of both *Il9*- and MC-specific transcripts in the small intestine might be associated with atopic patients that developed food allergy.

DISCUSSION

The prevalence of food-induced allergic disorders has increased substantially in industrialized countries over the past decade (Gupta et al., 2011). Current knowledge cannot explain why only some individuals who have high titers of dietary allergen-specific serum IgE develop life-threatening intestinal anaphylaxis. In this study, we have reported the identification of IL-9-producing mucosal mast cells, MMC9s, which were preferentially induced in murine strains susceptible to food allergy. MMC9s secreted large amounts of IL-9 and IL-13 in response to IL-33, and MCPt-1 protein and histamine in response to IgE-bound FcεR complex cross-linking by antigens. Given their anatomical location, characteristics, and function, MMC9s might be a key player that amplifies intestinal allergic

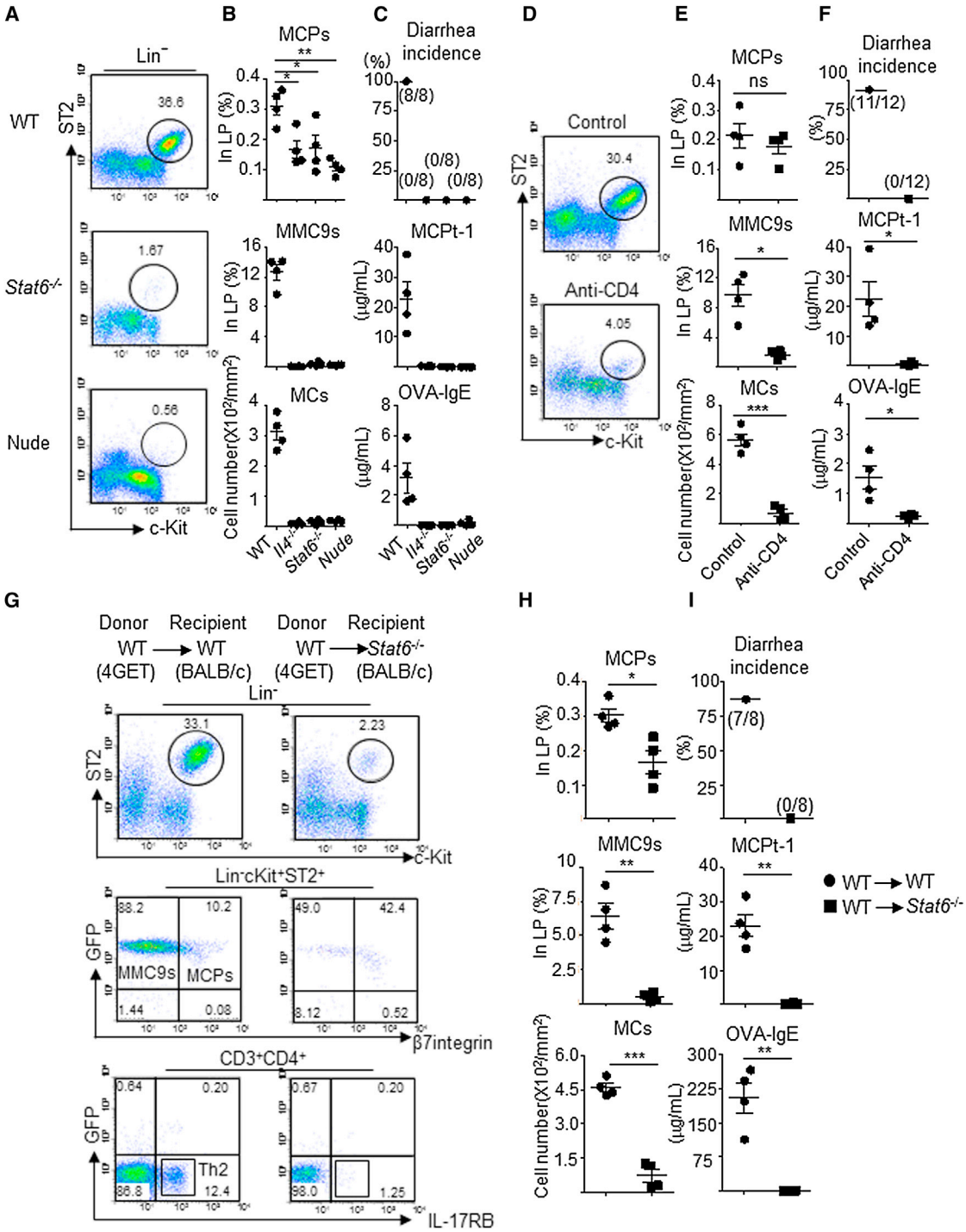

Figure 5. STAT6 Signaling and T Cells Are Required for MMC9 Induction that Promotes Experimental Food Allergy

(A–F) Detection (A, D) and frequency (B, E) of MMC9s ($Lin^-c\text{-}Kit^+ST2^+\beta7integrin^{lo}$) and MCPs ($Lin^-c\text{-}Kit^+ST2^+\beta7integrin^{hi}$) and measurement of indicated features of experimental food allergy after six intragastric OVA challenges (B, C, E, F) in wild-type BALB/c or indicated gene-deficient murine strains (A–C) or sensitized wild-type BALB/c treated with anti-CD4 or isotype control mAbs 1 day before first and fourth intragastric OVA challenges (D–F).

(G–I) Detection (G) and frequency (H) of MMC9s, MCPs, and $CD4^+$ Th2 cells and measurement of indicated features of experimental food allergy (H, I) in indicated sub-lethally irradiated recipient strains reconstituted with BM progenitors from WT 4GET mice.

Data in (A)–(I) represent one of three independent experiments (n = 4 mice per group). Fractions indicate incidence of allergic diarrhea (C, F, I). Error bars denote mean ± SEM. *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$. NS, not significant.

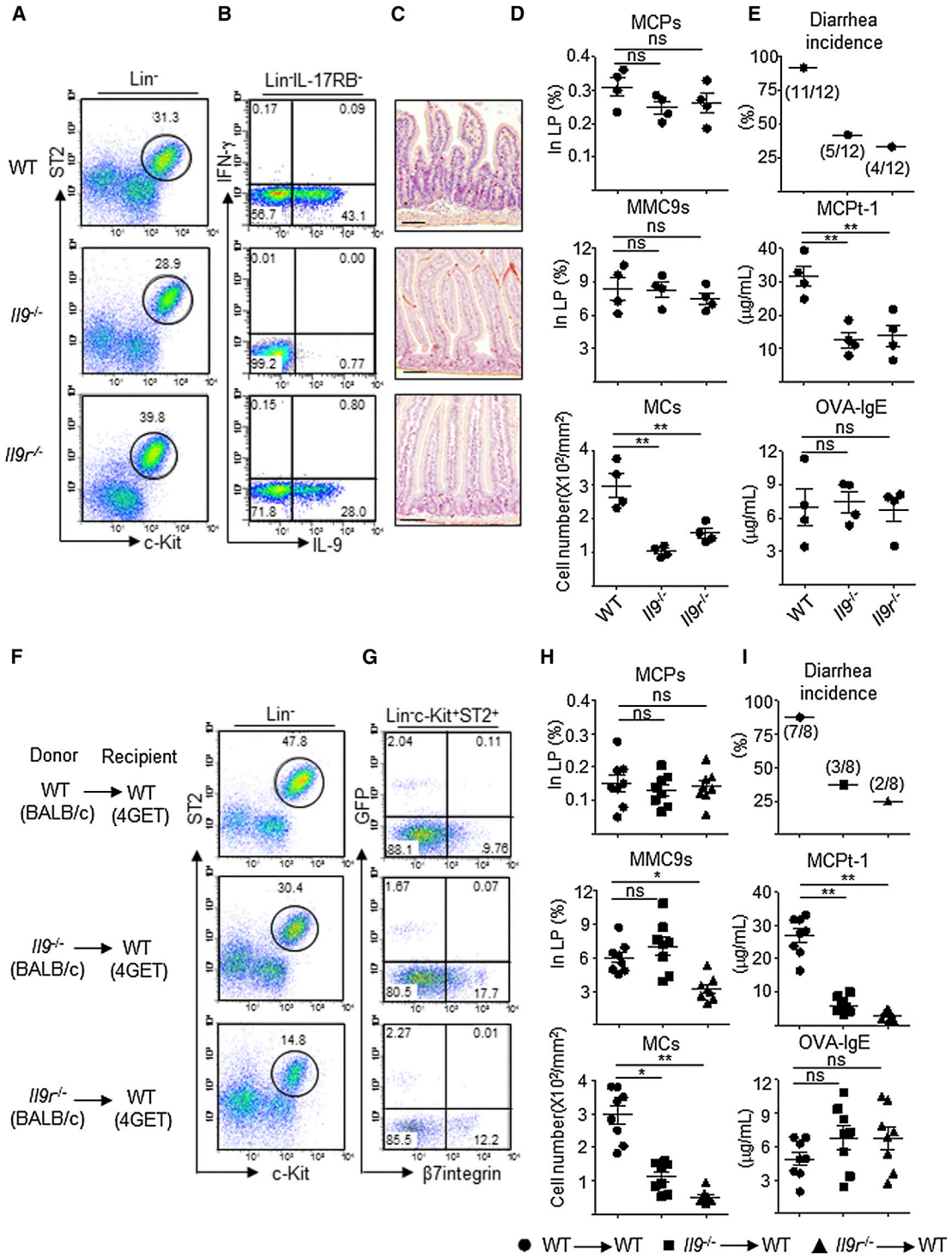

Figure 6. MMC9s Can Be Induced in IL-9- or IL-9R-Deficient Mice but Fail to Promote Experimental Food Allergy

(A–E) Detection of Lin$^-$IL-17RB$^-$c-Kit$^+$ST2$^+$ cells (A), flow cytometric analysis of indicated intracellular cytokine production by Lin$^-$IL-17RB$^-$ cells (B), staining for intestinal mastocytosis (C), and frequency of MMC9s (Lin$^-$c-Kit$^+$ST2$^+$β7integrin^{lo}) and MCPs (Lin$^-$c-Kit$^+$ST2$^+$β7integrin^{hi}) (D) and measurement of indicated features of experimental food allergy (D, E) in wild-type BALB/c or indicated gene-deficient murine strains after six intragastric OVA challenges.

(F–I) Detection (F, G) and frequency (H) of donor-derived MMC9s (Lin$^-$c-Kit$^+$ST2$^+$β7integrin^{lo}GFP$^-$) and MCPs (Lin$^-$c-Kit$^+$ST2$^+$β7integrin^{hi}GFP$^-$), and measurement of indicated features of experimental food allergy (H, I) in sub-lethally irradiated 4GET recipients reconstituted with BM progenitors from indicated murine strains.

Data represent one of three independent experiments (n = 4 or 8 mice per group). Scale bars represent 20 μm (C). Error bars denote mean ± SEM. **p ≤ 0.01. NS, not significant. See also Figure S6.

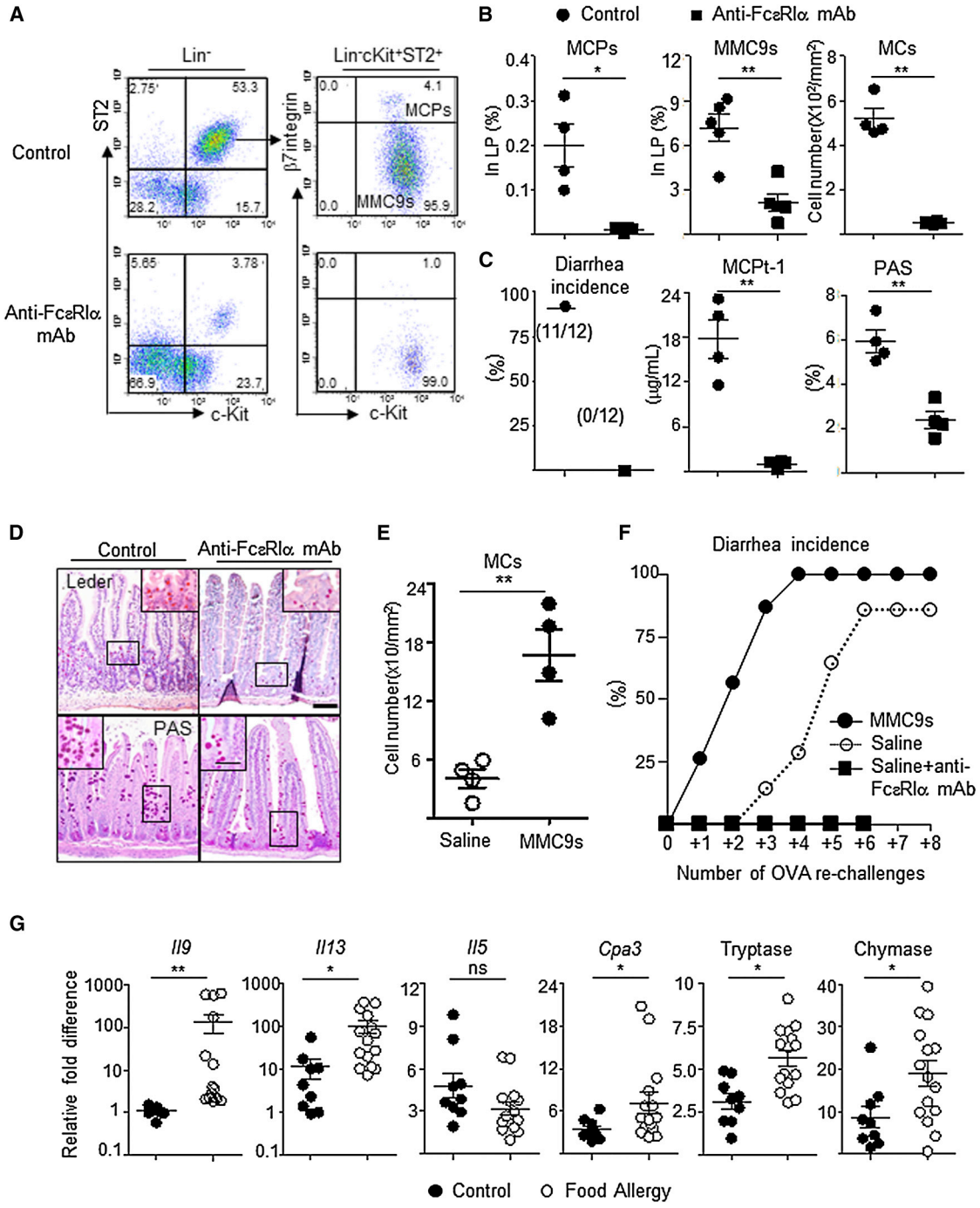

Figure 7. MMC9s Drive Intestinal Mastocytosis and *Il9* Transcript Expression Is Increased in Food Allergy Patients

(A–D) Detection (A) and frequency (B) of MMC9s and MCPs, measurement of indicated features of experimental food allergy (B, C), staining of intestinal mastocytosis and goblet cell hyperplasia (D), from mice treated with anti-FcεRIα or isotype control mAbs.

(E and F) Numbers of intestinal mast cells (E) and incidence of allergic diarrhea (F) in anti-FcεRIα antibody-treated mice reconstituted with purified MMC9s or saline only, or treated with saline plus anti-FcεRIα before re-challenge with OVA intragastrically twice (E) or indicated times (F) (12 mice per group).

(G) Expression of the indicated genes by duodenal biopsies from control and food allergy subjects was analyzed by quantitative real-time PCR using primers referenced in the methods.

Data in (A)–(D) represent one of three independent experiments (n = 4 mice per group). Gene expression data are expressed as relative fold difference as described in (G). Error bars denote mean ± SEM. Scale bars represent 20 μm (D) and 100 μm (D, the corner insets). *$p \leq 0.05$; **$p \leq 0.01$. See also Figure S7.

inflammation and perpetuates anaphylactic response to allergenic dietary proteins.

Several observations support our view that MMC9s are multi-functional and possess unique characteristics of mucosal MCs. (1) MMC9s display strong expression of *Gata1*, *Gata2*, and the mucosal MC-related chymase transcript, *Mcpt1* and *Mcpt4*, and the connective tissue MC-related tryptase transcript, *Mcpt6* (Miller and Pemberton, 2002; Welle, 1997). (2) MMC9s originate from bone marrow MCPs. (3) MMC9s exhibit a small progenitor-like morphology with few metachromatic granules in their scanty cytoplasm, the defining mucosal MC characteristics (Beaven, 2009). (4) MMC9s mature into granular MCs, but possess very poor proliferative capacity. (5) MMC9s produce prodigious amounts of IL-9, the key cytokine for the proliferation, maturation, and activation of MCs (Goswami and Kaplan, 2011; Renauld et al., 1995). (6) MMC9s are distinct from other IL-4- and IL-13-producing non-lymphoid cells (MCs, basophils, and eosinophils), which develop normally in STAT6- or IL-4Rα-deficient mice (Gessner et al., 2005), inasmuch as MMC9 development requires the IL-4 and STAT6 signals. (7) IL-9 and IL-9R signals are important to establish pulmonary and intestinal mastocytosis (Osterfeld et al., 2010; Townsend et al., 2000) but are dispensable for MMC9 induction. Thus, MMC9s might represent Th2 cytokine-producing innate cells that follow the common myeloid progenitor (CMP) and granulocyte-macrophage progenitor (GMP) developmental pathway, paralleling ILC2s, a member of ILC family that develops through the common lymphoid (CLP) pathway (Arinobu et al., 2005).

The interplay between MMC9s and $CD4^+$ Th2 cells appears to be a key mechanism that governs the susceptibility to and severity of experimental food allergy. Among seven examined murine strains, the frequencies of intestinal $\beta7^{hi}$ MCPs (~0.2%) and $\beta7^{lo}$ MMC9s (~0.1%) were initially comparable before sensitization, except C57BL/6 mice that fail to develop MMC9s. After repeated challenge, MMC9 and $CD4^+$ Th2 cell frequencies were concomitantly higher in sensitized BALB/c, A/J, and CBA mice than those in DBA2, 129, and C3H/HeJ mice. Consequently, BALB/c, A/J, and CBA mice exhibited increased intestinal MC numbers, serum MCPt-1 titers, and the propensity to develop experimental food allergy, despite the fact that murine strains DBA2, 129, and CBA share the same MHC H2 haplotype with BALB/c ($H2^d$), C57/B6 ($H2^b$), and C3H/HeJ ($H2^k$), respectively. Finally, mice ablated of or deficient in T cells or IL-4 signaling had reduced capacity to accumulate intestinal MCPs and failed to develop MMC9s. Thus, the ability of murine strains to mount the intestinal $CD4^+$ Th2-cell-mediated immune response to ingested dietary antigens, not their MHC H2 haplotype, correlated positively with MMC9 frequency, and consequently, susceptibility to food allergy. Whether our findings of MMC9s and its dependence on $CD4^+$ Th2 cells pertain to helminth-induced T-cell-dependent mucosal MCs and IL-9 dependence of the protective response to *Trichinella spiralis* infection remains to be investigated (Angkasekwinai et al., 2013; Dillon and MacDonald, 1986; Liu et al., 2013; Ruitenberg and Elgersma, 1976; Urban et al., 2000).

Several murine models have been developed in an attempt to understand the immunological mechanisms underlying the susceptibility of food allergy. In addition to adjuvant alum that induces a strong allergic sensitization, administration of CT intragastrically could also break oral tolerance and induce elevated histamine, IgE, and IgG1 titers, resulting in systemic anaphylactic responses to ingested allergen, which occurred selectively in C3H/HeJ strain (Li et al., 1999; Morafo et al., 2003). In contrast to sensitization via alum adjuvant, repeated intragastric OVA challenge failed to induce strong MMC9 (<1%) and $CD4^+$ Th2 (<1%) immune responses in CT-sensitized C3H/HeJ or BALB/c mice, despite inducing a strong humoral immune response. Consequently, much fewer intestinal MC numbers and serum MCPt-1 titers (~15 cells/mm^2 and ~2 μg/ml) were induced in CT-sensitized C3H/HeJ or BALB/c mice than those (~400 cells/mm^2 and ~20 μg/ml) detected in alum-sensitized BALB/c mice (Li et al., 1999; Morafo et al., 2003). Additionally, a significant increase of intestinal MMC9 frequency, mucosal MC number, and serum MCPt-1 titer could be observed in the vitamin D3 analog (Calcipotriol)-, not vehicle-, sensitized mice after repeated intragastric OVA antigen challenge. These findings suggest that MMC9 induction can occur in mice sensitized with "atopic-promoting" adjuvants, such as alum and vitamin D3 analog, which preferentially prime Th2 cell immune response. Importantly, the finding that *Fcer1a*$^{-/-}$ mice exhibited reduced intestinal MCP recruitment, MMC9 induction, and MC number and failed to develop food allergy further underscores that the alum-sensitized model of food allergy is dependent on the classical IgE-mediated MC and FcεR pathway (Ahrens et al., 2012; Osterfeld et al., 2010). In contrast, the genetic susceptibility (Morafo et al., 2003), *Tlr4* mutation (Bashir et al., 2004), and/or the alternative pathway mediated by IgG and FcγR complex (Smit et al., 2011) have been implicated in contributing to the susceptibility of C3H/HeJ strain to systemic anaphylaxis after CT sensitization.

Because MMC9s have limited proliferative potential, it is plausible to propose that crosslinking of IgE-bound FcεR complexes by dietary antigens results in a strong proliferation of BM-derived MCPs, which then develop into MMC9s in the presence of intestinal $CD4^+$ Th2 cells induced by ingested food allergens. These induced MMC9s might later mature into granular MCs or alternatively promote MCP maturation directly, in an IL-9-dependent manner. Additionally, our findings also suggest that MMC9s amplify intestinal mastocytosis by producing IL-9 in response to alarmin IL-33 and by releasing proteases in response to dietary antigen crosslinking of IgE-bound FcεR complexes; consequently, they serve as innate myeloid helper cells to promote the development of IgE-mediated food allergy. Indeed, the association between increased numbers of duodenal IgE-positive MCs and food allergy has been observed in clinical studies (Bengtsson et al., 1991; Caffarelli et al., 1998). Furthermore, the expression of both Th2 cytokine *Il9* and *Il13* and mast-cell-specific *Cpa3*, tryptase, and chymase transcripts were significantly increased in the duodenum of atopic patients that developed food allergy. Understanding the factors that predispose murine strains to break oral tolerance and to initiate collaboration between innate MMC9s and adaptive $CD4^+$ Th2 cells in response to dietary antigens should provide insights into the design of therapeutic strategies for IgE-mediated food allergy in humans.

EXPERIMENTAL PROCEDURES

Food Allergy Patient Characteristics

Duodenal biopsies were obtained from patients with food allergy defined by (1) clinical history of food anaphylaxis (FA), (2) a positive skin prick test with the food allergens, and/or (3) a positive IgE-RAST to food allergens. Additionally, the patient samples of both control and FA groups were selected when their endoscopy biopsies did not meet the criteria for active eosinophil-associated gastrointestinal disorders (EGID) based on the histological definition (>15 peak eosinophils/HPF in the esophagus, >30 eosinophils in five separate HPF in the stomach, and >normal values in the duodenum). All patients and/or family consented for research participation and the study was approved by the Cincinnati Children's Hospital Medical Center (CCHMC) Institutional Review Board.

Mice

BALB/c, A/J, C3H/HeJ, C57BL/6J, *Il4*$^{-/-}$ (stock number 002496), *Il4ra*$^{-/-}$ (stock number 003514), *Stat6*$^{-/-}$ (stock number 002828), athymic nude mice (stock number 000711), *Rag2*$^{-/-}$ (stock number 008338), *Fcer1a*$^{-/-}$ (stock number 005629), and IL-4-IRES-eGFP (4GET) (stock number 004190) mice were purchased from the Jackson Laboratory. *Il9*$^{-/-}$ and *Il9r*$^{-/-}$ mice were provided by Andrew McKenzie and Jean-Christophe Renauld, respectively. All genetically modified mice were backcrossed to BALB/c background for more than ten generations. All experimental procedures were approved by the Institutional Animal Care and Use Committee of Cincinnati Children's Hospital Medical Center.

Lamina Propria Mononuclear Cell Isolation

Small intestines were cut longitudinally and incubated in HBSS with 5 mM EDTA at 4°C for 30 min before vortexing to remove epithelial cells. The remaining tissues were minced and digested with 2.4 mg/ml collagenase A (Roche) and 0.2 mg/ml DNaseI (Roche) at 37°C for 30 min. After removal of tissue debris, liberated cells suspended in 44% Percoll were loaded above 67% Percoll before centrifugation. LP cells were collected from the interface between 44% and 67% Percoll.

Flow Cytometric Analysis and Cell Sorting

LP cells from small intestines of 4GET or BALB/c mice were first stained with phycoerythrin (PE)-conjugated anti-β7integrin (M293), allophycocyanin (APC)-conjugated anti-IL-17RB, APC-Cy7-conjugated anti-c-Kit (2B8), PE-Cy7-conjugated anti-CD3e (145-2C11), Horizon V500-conjugated CD4 (RPA-T4), and biotinylated anti-T1/ST2 (DJ8) antibodies. Subsequently, cells were counterstained with PerCP-Cy5.5-conjugated monoclonal antibodies against lineage (Lin) markers (CD11b [M1/70], CD11c [HL3], CD8 [53-6.7], B220 [RA3-6B2], Gr-1 [RB6-8C5], and CD335 [NKP46, 29A.4]) and with Brilliant Violet 421-labeled Streptavidin (Biolegend) before analysis with a FACSCanton II (BD Bioscience) or cell sorting with a FACSAria II (BD Bioscience). For intracellular cytokine analyses, Lin^{+} LP cells from BALB/c mice were labeled with microbeads conjugated with monoclonal antibodies against CD4, CD11b, CD8α, and B220 and then depleted with a MACS column (Miltenyi Biotech). After re-stimulation with PMA and ionomycin and treatment with Golgi blocker, enriched Lin^{-} LP cells were stained with PE-anti-β7integrin, APC-Cy7-conjugated anti-c-Kit, and biotinylated anti-T1/ST2 antibodies. Subsequently, cells were counterstained with PerCP-Cy5.5-conjugated monoclonal antibodies against Lin markers (including CD3 and CD4) and with Brilliant Violet 421-labeled streptavidin. Stained cells were fixed and permeabilized for intracellular cytokine staining using FITC-anti-IFN-γ (XMG 1.2) and APC-anti-IL-9 (RM9A4) or APC-anti-IL-13 (eBio13A).

IgE-Mediated Experimental Food Allergy

Mice were sensitized twice within a 2-week interval by intraperitoneal (i.p.) injection with 100 μg OVA and 1 mg alum and then orally gavaged with OVA (50 mg in 250 μl saline) six times within 2 weeks. In prophylactic experiments, sensitized BALB/c mice were injected intraperitoneally with 500 μg anti-FcεRIα (MAR-1) antibody (Biolegend) or hamster isotype control antibody (Bio X Cell) or with 500 μg of anti-CD4 (GK1.5) antibody (Bio X Cell) or rat isotype control antibody (Bio X Cell) twice 1 day before the first and fourth intragastric OVA challenges. Some antibody-treated mice were rested for 2 weeks after the sixth OVA challenge before reconstitution with 1 × 10^{6} purified MMC9s by retro-orbital injection. At 1 day after adoptive transfer, mice were re-challenged with OVA intragastrically, then re-challenged every other day until they developed allergic diarrhea.

Measurement of Parameters of Food Allergy

Serum samples were analyzed with ELISA kits for OVA-specific IgE (MD Bioproducts), MCPt-1 (eBioscience), and OVA-specific IgG1 (alpha diagnostic international). For intestinal histological analyses, duodenal tissue was fixed in 10% formalin and processed by standard histological techniques. 5-μm tissue sections were stained with Leder stain for chloroacetate esterase (CAE) activity in intestinal mast cells or periodic acid-Schiff (PAS) for mucins in goblet cells. Stained cells were quantified as previously described (Brandt et al., 2003). Assessment of diarrhea and measurement of hyperthermia (rectal temperature drop > 2°C) were performed as previously described (Osterfeld et al., 2010).

Measurement of Cytokines and Mediators

Purified MCPs, MMC9s, and ILC2s (5 × 10^{4}) from mice with active allergic diarrhea were stimulated with PMA (0.1 μg/ml) and ionomycin (1 μg/ml) for 1 day or with the indicated cytokines for 3 days before the collection of supernatants. CD4^{+}GFP^{hi} Th2 cells (5 × 10^{4}) were stimulated with plate-bound anti-CD3 (5 μg/ml) and soluble anti-CD28 (1 μg/ml) for 24 hr. Secreted cytokines in supernatants were assessed by ELISA kits for IL-5 (R&D), IL-9 (Biolegend), IL-4, IFN-γ, IL-17A (BD Bioscience), and IL-13 (Antigenic American). Secreted MCPt-1 was measured by ELISA (eBioscience). BMMCs were derived and degranulation assays were performed as previously described (Arumugam et al., 2011). Secreted β-hexoaminidase activity was measured by colorimetric determination with 4-nitrophenyl-N-acetyl-β-D-glucosaminide as described previously (Arumugam et al., 2011). Histamine content was determined by ELISA (Beckman Coulter).

Statistical Analysis

Data are presented as the mean ± SEM. Power analysis was performed based on preliminary datasets to determine the sample sizes, which are large enough to obtain adequate statistical significance. The inclusion and exclusion criteria for murine studies were pre-established. For all murine studies, mice were assigned at random in groups. For the measurement of parameters of food allergy, studies were performed in a blinded fashion. For comparisons between groups, statistical significance was determined with a nonparametric, two-tailed Mann-Whitney t test or unpaired Student's t test. Correlation analysis was performed with a nonparametric Spearman correlation (sensitivity analyses was performed), and a linear regression of the data is displayed. Statistical tests used for all data are justified as deemed appropriate. Results are considered significant at $p \leq 0.05$. F test was used to compare variances within each group of data, and Welch's correction was used, if the variances were significantly different between groups. All data were analyzed with Prism (Graphpad Software).

ACCESSION NUMBERS

The RNA-seq data reported in this paper have been deposited under accession number GEO: GSE72921.

SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures, one table, and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.immuni.2015.08.020.

AUTHOR CONTRIBUTIONS

C.-Y.C., J.-B.L., and Y.-H.W. performed and designed experiments, B.L. and L.M. assisted with experimental model of food allergy, P.-Y.W. performed adoptive transfer experiments, S.O., K.I., F.D.F., and S.P.H. provided reagents, M.E.R. and J.P.A. provided human biopsies, A.V.K. and A.B. performed RNA-seq analysis, Y.-H.W. conceived the project, and Y.-H.W., J.-B.L., and C.-Y.C. wrote and edited the manuscript with input from F.D.F. and S.P.H.

ACKNOWLEDGMENTS

We thank D.A. Hidleman, C. Karp, and A. Munitz for critical reading of the manuscript and S. Hottinger for editorial assistance. This work is supported by the NIH (AI090129-1 to Y.-H.W., A1073553 to S.P.H.), the Digestive Health Center (P30 DK078392, Pilot and Feasibility Award to Y.-H.W.), Campaign Urging Research For Eosinophilic Diseases (CURED) Foundation, the Buckeye Foundation, the Food Allergy Research Education Fund to M.E.R., and VA Merit Award to F.D.F.

Received: September 10, 2014
Revised: May 30, 2015
Accepted: August 20, 2015
Published: September 22, 2015

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Immunity

Supplemental Information

Induction of Interleukin-9-Producing Mucosal Mast Cells Promotes Susceptibility to IgE-Mediated Experimental Food Allergy

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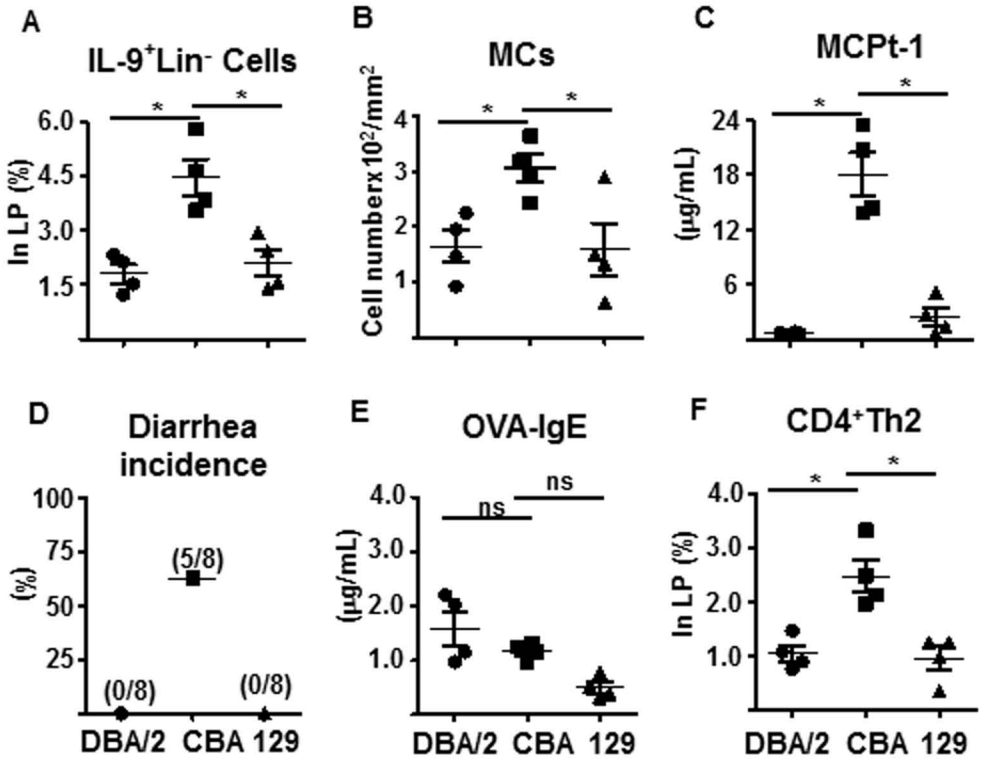

Figure S1. Frequencies of Intestinal IL-9-producing Lin⁻ and CD4⁺Th2 Cells Correlate Positively with the Susceptibility to Developing IgE-mediated Experimental Food Allergy. (related to Figure 1 and Figure 4) (**A**) Frequency of IL-9⁺Lin⁻ and (**F**) CD4⁺ST2⁺IL-17RB⁺ cells, (**B**) intestinal mast cell (MC) number, (**C**) serum MCPt-1 and (**E**) OVA-specific IgE production, and (**D**) allergic diarrhea incidence of indicated murine strains after sensitization and six intragastric OVA challenges. Data represent one of two independent experiments (n=4 mice per group). Fraction indicates incidence of allergic diarrhea. LP, laminar propria. * $p<0.05$. NS, not significant.

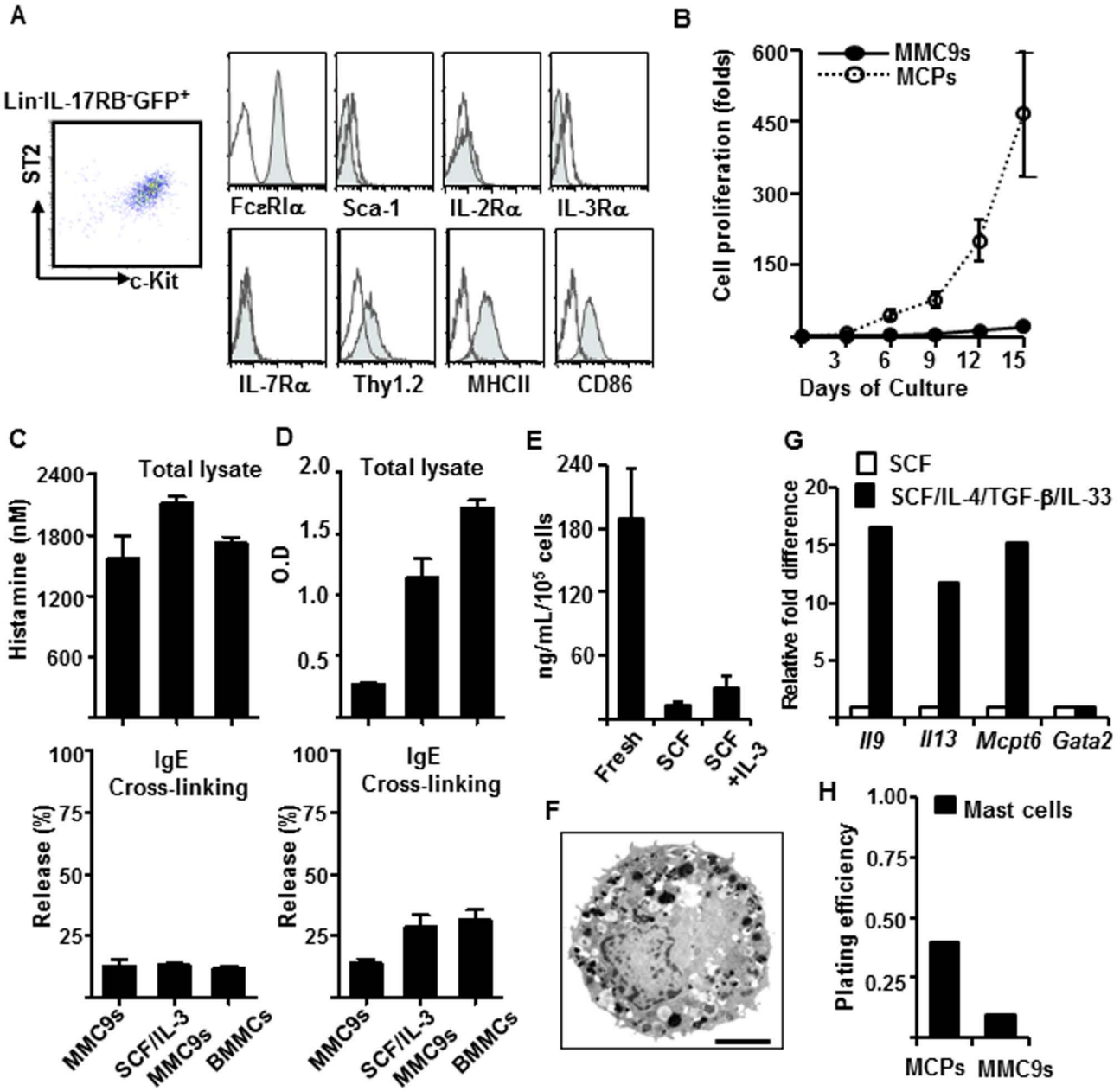

Figure S2. Characteristics of Intestinal MCPs and MMC9s. (related to Figure 2) (**A**) Phenotypic analysis of intestinal Lin$^-$GFP^{hi}IL-17RB$^-$c-Kit$^+$ST2$^+$ cells from BALB/c mice with active allergic diarrhea. (**B**) Comparison of proliferative response to SCF plus IL-3 between MCPs (Lin$^-$ IL-17RB$^-$c-Kit$^+$ST2$^+$ β7integrin^{hi} cells) and MMC9s (Lin$^-$IL-17RB$^-$c-Kit$^+$ST2$^+$β7integrin^{lo} cells) *in vitro*. (**C-E**) Measurements of intracellular or released histamine (**C**), β-hexoaminidase activity (**D**), and IL-9 production (**E**) within or by freshly isolated MMC9s, differentiated MMC9s cultured with indicated cytokines for 12 days, and bone marrow-derived mast cells (BMMCs) (**C**, **D**). (**F**) Electron microscopic analysis of MMC9s after 7 days of culture with SCF plus IL-3. (**G-H**) Expression of indicated genes by (**G**) and colony forming efficiency of (**H**) sorted single MCP (**G, H**) and MMC9s (**H**) cultured with indicated cytokines (**G**) or Methocoult GF medium (**H**). Filled histograms represent the staining of indicated markers on Lin$^-$GFP^{hi}IL-17RB$^-$c-Kit$^+$ST2$^+$ cells; open histograms represent isotype control (**A**). Scale bars are 20 μm (**F**). Gene expression was analyzed by quantitative real-time PCR using primers referenced in the methods (**G**). Data represent one of three independent experiments. Error bars denote mean ± S.E.M.

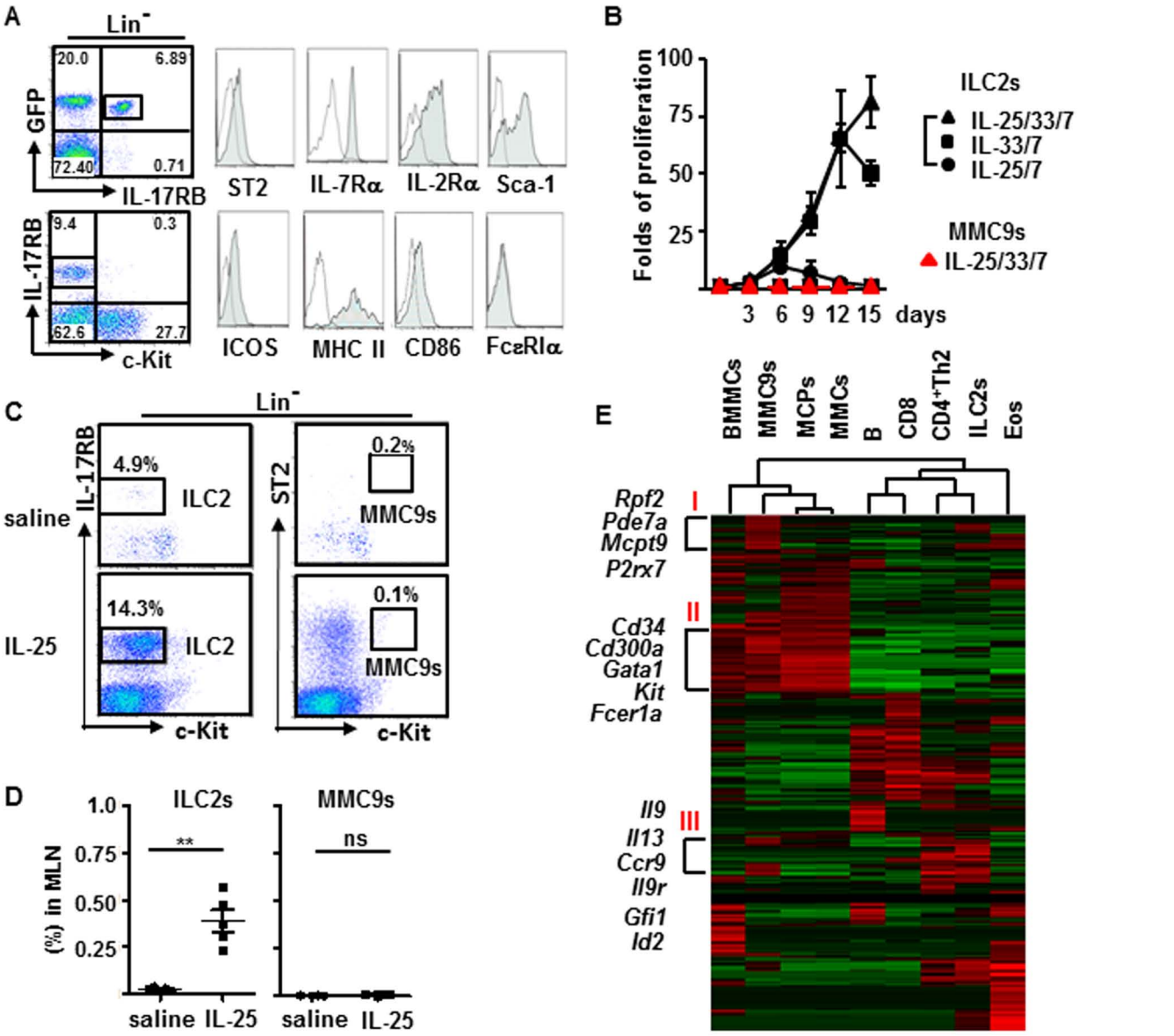

Figure S3. Characterizations of Intestinal ILC2s and MMC9s. (related to Figure 2)(**A**) Detection and phenotypic analysis of Lin$^-$GFP^{hi}IL-17RB$^+$c-Kit$^-$ cells. (**B**) Proliferative response of purified MMC9s (Lin$^-$GFP^{hi}IL-17RBc-Kit$^+$β7integrin^{lo} cells) and ILC2s (Lin$^-$GFP^{hi} IL-17RB$^+$c-Kit$^-$IL-7Rα$^+$) from 4GET mice with active allergic diarrhea after culture with indicated cytokines for the indicated number of days. (**C, D**) Detection (**C**) and frequency (**D**) of ILC2s and MMC9s in mesenteric lymph node of BALB/c mice injected with IL-25 (0.5 μg) for 4 consecutive days. (**E**) RNA sequencing data obtained from 2 independent samples of indicated hematopoietic cell lineages were centered, and 3,138 genes were grouped by similarity in expression patterns via hierarchical clustering. Clustered image accompanies a dendrogram showing the relationships among tested samples. Color reflects the magnitude of their RPKM (reads per kilobase of transcript per million reads mapped) value. Cluster I-III and selected genes are shown. Genes with higher expression in MMC9s, MC lineages, and type-2 immune cells (ILC2s and CD4$^+$Th2 cells) are shown in cluster I, II, and III, respectively. BMMCs, bone marrow–derived mast cells; MCPs, mast cell progenitors; MMCs, mature mast cells from peritoneal; Eos, eosinophils. Filled histograms represent the staining of indicated markers on Lin$^-$GFP^{hi}IL-17RB$^+$c-Kit$^-$ cells; open histograms represent isotype control. Data (**A**-**D**) represent one of three independent experiments (n=5 mice per group). Error bars denote mean ± S.E.M. **p≤ 0.01. NS, not significant.

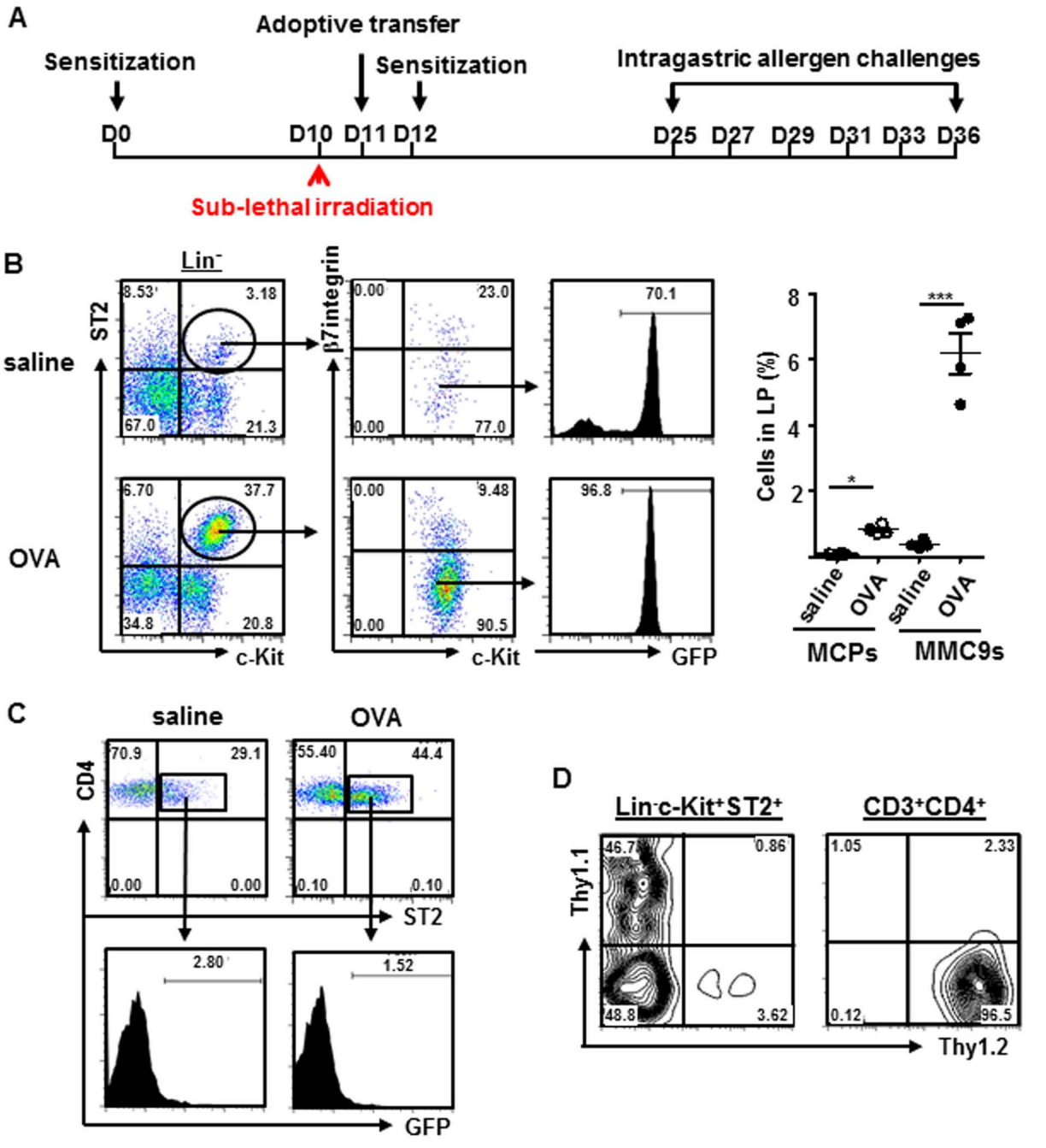

Figure S4. Origin of Intestinal MCPs, MMC9s, and CD4⁺Th2 Cells in a Reconstitution Model of Experimental Food Allergy. (related to Figure 3) (**A**) Diagram of adoptive transfer protocol in the mouse model of food allergy. (**B-D**) Detection and frequency of donor-derived MMC9s ($Lin^-IL\text{-}17RB^-c\text{-}Kit^+\beta7integrin^{lo}$) and MCPs ($Lin^-IL\text{-}17RB^-c\text{-}Kit^+\beta7integrin^{hi}$) (**B**), recipient-derived $GFP^-CD4^+ST2^+$Th2 cells (**C**), or indicated cell subsets (**D**) in the small intestine of $Thy1.2^+$ BALB/c recipient mice reconstituted with bone marrow progenitors from IL-4-eGFP (4GET) mice (**A-C**) or $Thy1.1^+$ (**D**) BALB/c mice after six times of intragastric saline or OVA challenges. Data represent one of three independent experiments (n=4 mice per group). LP, laminar propria. * $p<0.05$, *** $p<0.001$.

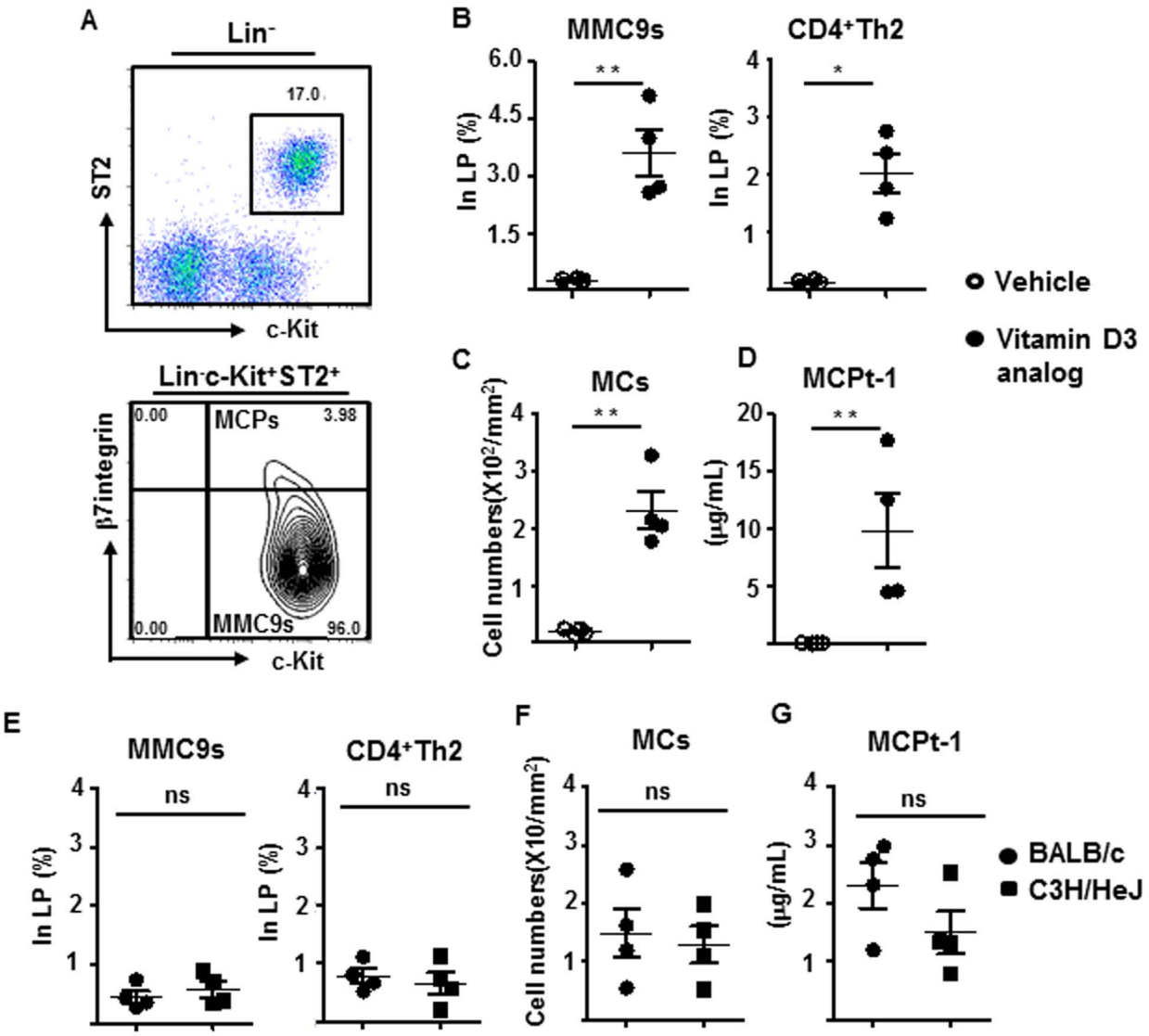

Figure S5. Induction of MMC9s and CD4⁺Th2 cells and Intestinal Mastocytosis in the Cholera Toxin- or Skin-sensitized Model of Experimental Food Allergy. (related to Figure 4) (**A**) Detection and (**B, E**) frequency of MMC9s ($Lin^-IL\text{-}17RB^-c\text{-}Kit^+\beta7integrin^{lo}$), and $CD4^+IL\text{-}17RB^+Th2$, (**C, F**) intestinal mast cell (MC) number, and (**D, G**) serum MCPt-1 titers of indicated murine strains after sensitization with vitamin D3 analog (Calcipotriol) (**A-D**) or cholera toxin (**E-G**) and six intragastric OVA challenges. Data represent one of two independent experiments (n=4 mice per group). LP, laminar propria. * $p<0.05$, ** $p<0.01$, NS, not significant.

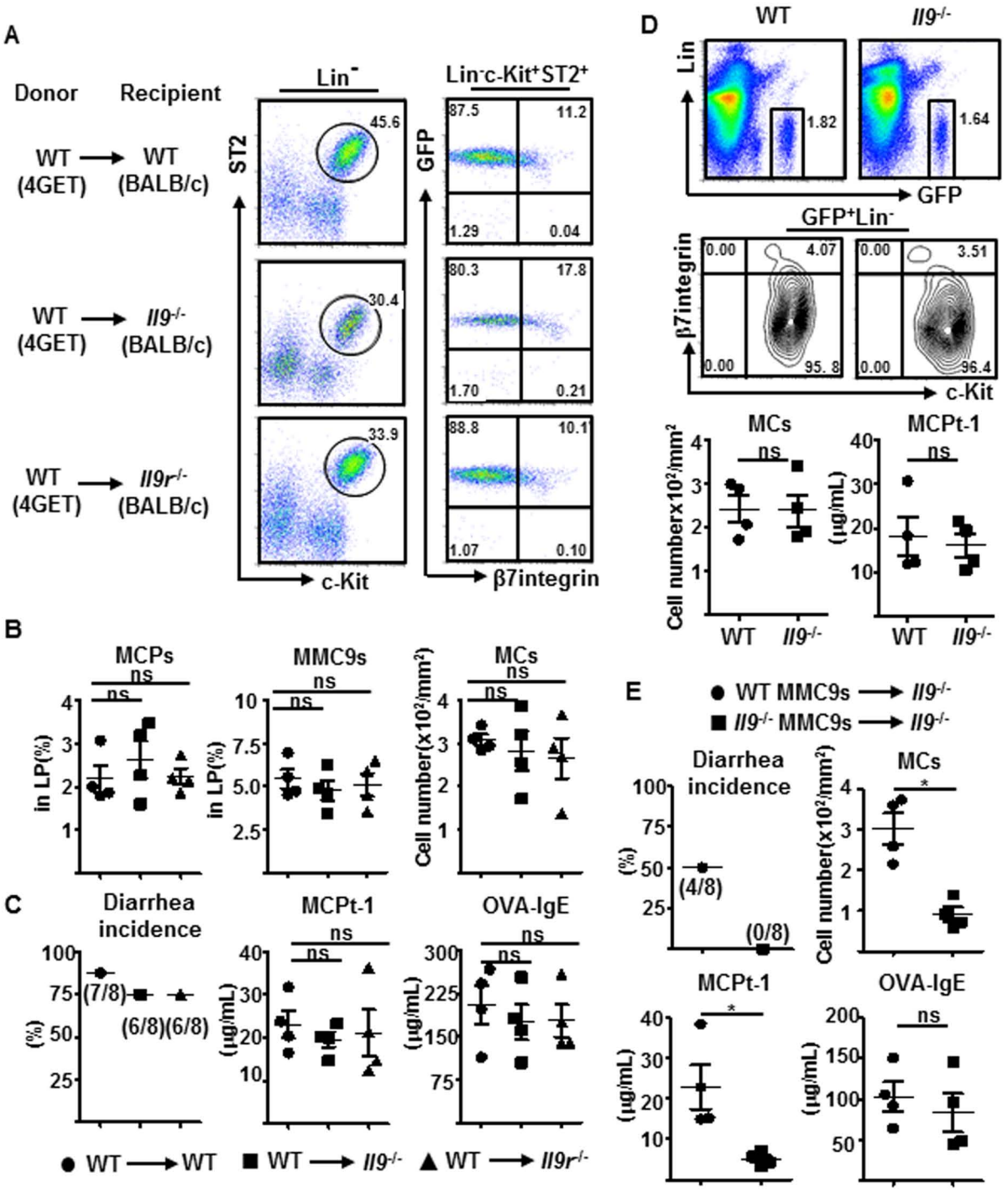

Figure S6. IL-9 Produced by MMC9s Promotes the Susceptibility to Experimental Food Allergy. (related to Figure 6) (**A**,**D**) Detection and **(B-E)** frequency of MCPs (Lin^-IL-17RB$^-$c-Kit$^+$β7integrin^{hi}) and MMC9s (Lin^-IL-17RB$^-$c-Kit$^+$β7integrin^{lo}), intestinal mast cell (MC) number, diarrhea incidence, and serum MCPt-1 and OVA-IgE titers of indicated murine strains reconstituted with wild type bone marrow (BM) cells (**A-C**) or reconstituted with sorted BM MCPs isolated from IL-4-eGFP (4GET) mice (**D**), or reconstituted with MMC9s isolated from indicated murine strains (**E**). Data represent one of two independent experiments (n=4 mice per group). LP, laminar propria. * $p<0.05$, NS, not significant.

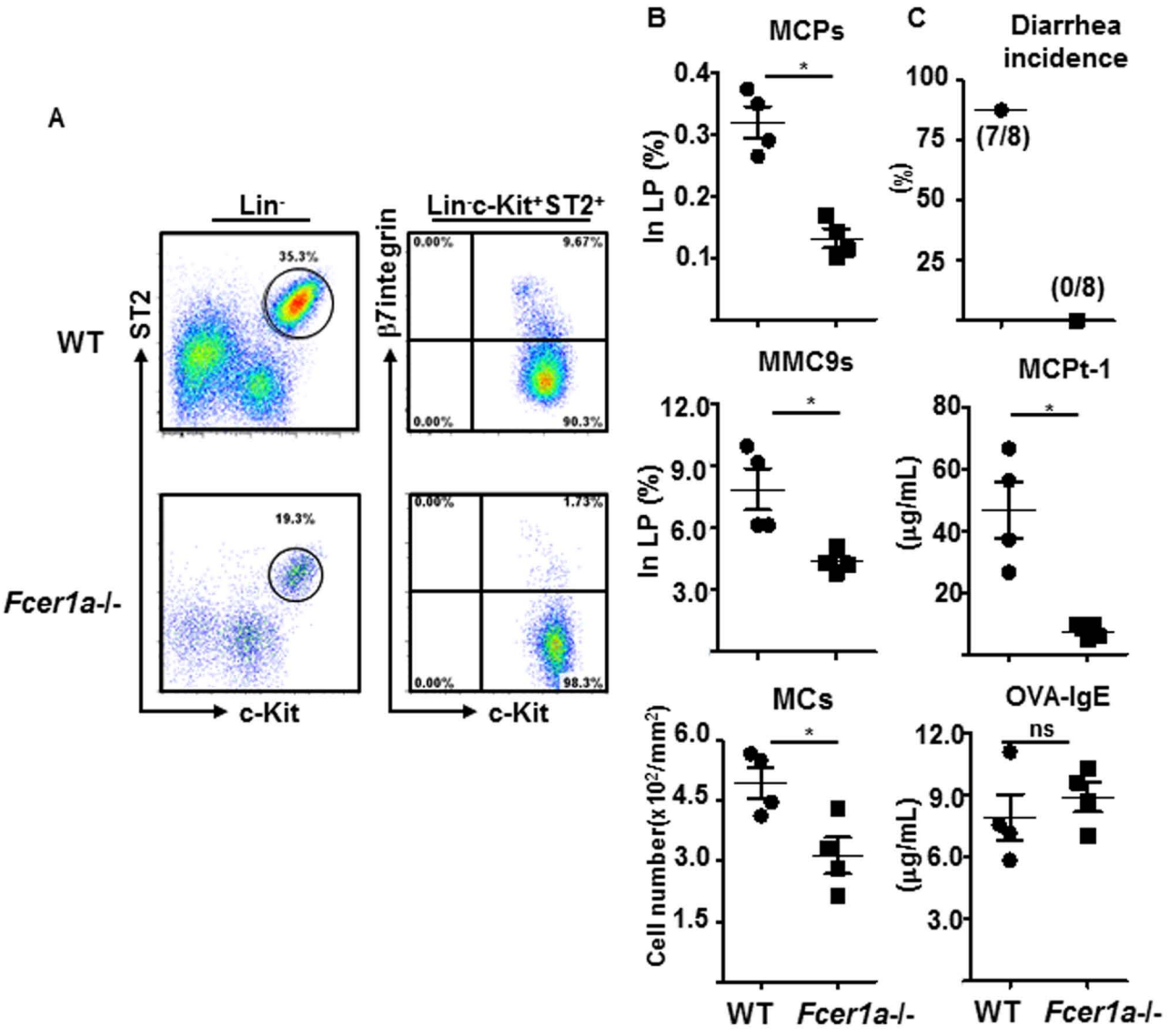

Figure S7. Diarrheal Response to Intragastric OVA Antigen Requires FcεRαI in a Murine Model of Food Allergy. (related to Figure 7) (**A**) Detection and (**B**) frequency of MCPs ($Lin^-IL\text{-}17RB^-c\text{-}Kit^+\beta7integrin^{hi}$) and MMC9s ($Lin^-IL\text{-}17RB^-c\text{-}Kit^+\beta7integrin^{lo}$), and intestinal mast cell (MC) numbers and (**C**) diarrhea incidence, and titers of serum MCPt-1 and OVA-IgE of indicated mouse strains after OVA/alum sensitization and six intragastric OVA challenges. Data represent one of two independent experiments (n=4 mice per group). LP, laminar propria. * $p<0.05$, NS, not significant.

Table S1 (related to Figure 7). Summary of patient characteristics

	CTL (n=9)	FA (n=15)	*P* value[a]
Age (*y*, mean ± SD, [range])	23.9 ± 19.3 (11-57)	17.4 ± 13.7 (4-46)	0.0789
Male (*n*)	4	10	1.0000
Female (*n*)	5	5	1.0000
History of allergy (*n*)	1	15	<0.0001
History of asthma (*n*)	3	6	1.0000
History of Allergic Rhinitis (*n*)	4	11	0.1793
History of Eczema (*n*)	0	12	<0.0001
History of Urticaria (*n*)	1	10	0.0094
Total serum IgE	11 (IU/mL, n=1[b])	374.44 (165-1958 IU/mL)	N/A
SPT (*n*)	N.D.	15	N/A
RAST (*n*)	N.D.	15	N/A

CTL, control; FA, food allergy; SPT, skin prick test; RAST, radioallergosorbent test; N.A., not available; N.D., not determined

[a]P value for parameter comparison between control and FA groups. For age, unpaired t test was used to determine significance; for other characteristics, Fisher's exact test was used to evaluate the statistical significance of the difference in frequency.

[b]After chart review, only one patient in the control group was examined for the serum IgE titer.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Mice BALB/c, C3H/HeJ, DBA2, 129, CBA, *Stat6*$^{-/-}$ (stock number 002828), *Fcer1a*$^{-/-}$ mice (stock number 005629), and IL-4-IRES-eGFP (4GET) mice (stock number 004190) were purchased from the Jackson Laboratory. *Il9*$^{-/-}$ and *Il9r*$^{-/-}$ mice were provided by Andrew McKenzie and Jean-Christophe Renauld, respectively. All genetically modified mice were backcrossed to BALB/c background for more than ten generations. All experimental procedures were approved by the Institutional Animal Care and Use Committee of Cincinnati Children's Hospital Medical Center.

Generation of rat anti-human/mouse IL-17RB monoclonal antibody (mAb) (clone SS13B) His-tagged extracellular domain of human IL-17RB (His-hIL-17RB) proteins were induced and purified from culture supernatant of Drosophila S2 cells that were co-transfected with plasmids of the extracellular domain of human *Il17RB* cDNA inserted into the pMT/Bip/V5-His expression vector and of pAc-hygro vector following manufacturer's protocol (Invitrogen, Carlsbad, CA). Anti-IL-17RB mAbs were produced by immunizing a Wister rat with purified His-hIL-17RB proteins with a standard protocol. After initial screening for the reactivity with His-hIL-17RB proteins by ELISA, hybridoma clones were further characterized by flow cytometry for their reactivity with Ba/F3 transfectant cell lines that expressed surface FLAG-tagged human or murine IL-17RB. The anti-IL-17RB mAb (clone SS13B) was selected for its cross-reactivity with both human and mouse IL-17RB.

IgE-mediated experimental food allergy Mice were sensitized twice within a two-week interval by intraperitoneal (i.p.) injection with 100 μg OVA and 1 mg alum. Sensitized mice were orally gavaged with 50 mg of OVA in 250 μl saline six times within two weeks and subsequently examined for food allergy symptoms (Brandt et al., 2003). For CT-sensitized food allergy model, mice were sensitized and intragastrically challenged as previously described (Li et al., 1999). For skin sensitization food allergy model, mice were first sensitized by applying 20 μl of MC903 (0.1 μM Calcipotriol, TOCRIS Bioscience) and 1 μl of OVA (200mg/ml) to the ear for 14 days

consecutively and then orally gavaged with OVA (50 mg in 250 μl saline) six times within two weeks. Following this application, solution was added directly over the site of the MC903 application for a total of 100 μg of OVA. In reconstitution experiments, sensitized mice (first injection only) were intravenously injected with $5x10^6$ BM cells one day after sub-lethal irradiation (9.5 Gy), then subjected to a second sensitization injection and intragastric challenges per the procedure above.

Measurement of parameters of food allergy Serum samples were analyzed using ELISA kits for OVA-specific IgE (MD Bioproducts), MCPt-1 (eBioscience), and OVA-specific IgG1 (alpha diagnostic international). For intestinal histological analyses, duodenal tissue was fixed in 10% formalin and processed by standard histological techniques. 5-μm tissue sections were stained with Leder stain for chloroacetate esterase (CAE) activity in intestinal mast cells or periodic acid-Schiff (PAS) for mucins in goblet cells. Stained cells were quantified as previously described(Brandt et al., 2003). Assessment of diarrhea and measurement of hyperthermia (rectal temperature drop > 2^oC) were performed as previously described (Osterfeld et al., 2010).

***Ex vivo* cell culture and CFU assay** Purified IMCP9 or Ba/MCPs were cultured with RPMI plus 10% fetal bovine serum in the presence of SCF (100 ng/mL) plus IL-3 (100 ng/mL) or seeded on methylcellulose medium (100-200 cells/plate, Methocult GF M3434; Stem Cell Technologies). Their proliferative responses were compared in liquid culture. Fold expansion was determined in three independent experiments by dividing the final cell number by the initial cell number. In CFU assay, colonies were scored after 10 days of culture, and colony-forming cells were cytospun for Wright-Giemsa staining.

Quantitative real-time PCR analysis RNA from sorted cell populations was isolated with an RNeasy Plus Mini kit (Qiagen), and cDNA templates were synthesized with iScript reverse transcriptase (BioRad).Quantitative real-time PCR analyses were performed with SYBR Green

Chemistry (Applied Biosystems) or Taqman Assays in an ABI Prism 7900 detection system using previously described primer sets (Abonia et al., 2010; Forbes et al., 2008; Kashiwakura et al., 2008; Park et al., 2008; Yamashita et al., 2006) or Taqman probes for human *Il9* and *Il13*. Expression levels of target genes were normalized to endogenous *Gapdh* transcript levels and relative quantification of samples was compared to the expression level of indicated genes in naïve $CD4^+$ T cells or normal subjects as the baseline.

RNA-Seq analysis. RNA-Seq libraries were constructed using Illumina stranded (dUTP) RNA-Seq kit at CCHMC sequencing core. Sequencing was performed on HiSeq2500 to obtain at least 10M reads for each sample. Briefly, fastq files from Illumina pipeline were aligned by STAR_2.4.2a (Dobin et al., 2013) with "--outFilterMultimapNmax 1 --outFilterMismatchNmax 2". RNA-seq data analysis was performed using BioWardrobe (Kartashov and Barski, 2015). RefSeq annotation for mm10 genome obtained from UCSC genome browser (11/2012) was used for analysis (Meyer et al., 2013). The --outFilterMultimapNmax parameter is used to allow unique alignment only and --outFilterMismatchNmax parameter is used to allow no more than 2 errors. All reads from produced .bam files were assigned to isoforms in the RefSeq annotation. Then expectation maximization – based algorithm was used to estimate appropriate number of reads for each isoform and to calculate RPKMs. Clustering was performed with Cluster3 and the heatmaps was visualized in JavaTreeView (de Hoon et al., 2004; Saldanha, 2004).

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IL-25 and CD4$^+$ T_H2 cells enhance type 2 innate lymphoid cell–derived IL-13 production, which promotes IgE-mediated experimental food allergy

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Background: Food-mediated allergic reactions have emerged as a major health problem. The underlying mechanisms that promote uncontrolled type 2 immune responses to dietary allergens in the gastrointestinal tract remain elusive.
Objective: We investigated whether altering IL-25 signaling enhances or attenuates allergic responses to food allergens.
Methods: Mice of an IL-25 transgenic mouse line (iIL-25Tg mice), which constitutively overexpress intestinal IL-25, and *Il17rb*$^{-/-}$ mice, in which *Il17rb* gene expression is disrupted, were sensitized and gavage fed with ovalbumin (OVA). We assessed symptomatic characteristics of experimental food allergy, including incidence of diarrhea, incidence of hypothermia, intestinal T_H2 immune response, and serum OVA-specific IgE and mast cell protease 1 production.
Results: Rapid induction of *Il25* expression in the intestinal epithelium preceded onset of the anaphylactic response to ingested OVA antigen. iIL-25Tg mice were more prone and *Il17rb*$^{-/-}$ mice were more resistant to experimental food allergy. Resident intestinal type 2 innate lymphoid cells (ILC2s) were identified as the major producers of IL-5 and IL-13 in response to IL-25. Reconstituting irradiated wild-type mice with *Rora*$^{-/-}$ or *Il17rb*$^{-/-}$ bone marrow resulted in a deficiency or dysfunction of the ILC2 compartment, respectively, and resistance to experimental food allergy. Repeated intragastric antigen challenge induced a significant increase in numbers of CD4$^+$ T_H2 cells, which enhance IL-25–stimulated IL-13 production by ILC2s *ex vivo* and *in vivo*. Finally, reconstituted IL-13–deficient ILC2s had reduced capability to promote allergic inflammation, resulting in increased resistance to experimental food allergy.
Conclusion: IL-25 and CD4$^+$ T_H2 cells induced by ingested antigens enhance ILC2-derived IL-13 production, thereby promoting IgE-mediated experimental food allergy. (J Allergy Clin Immunol 2016;137:1216-25.)

Key words: *IL-25, type 2 innate lymphoid cells, CD4$^+$ T_H2 cells, IL-13, food allergy*

The gastrointestinal mucosa is the largest immunologic site in the body and constantly encounters a myriad of commensal microbes and dietary proteins. The epithelial lumen and gastrointestinal-associated lymphoid tissues function to combat invading microbes during development of immune tolerance to food antigens. The loss of mucosal tolerance to foods and the associated induction of adverse immune-mediated reactions can lead to development of food-induced allergic disorders. Studies in both human subjects and animals have demonstrated that the immediate hypersensitivity response to ingested food arises from the activation of intestinal mast cells (MCs) by food-specific IgE antibodies.[1] Although the prevalence of food-induced allergic disorders has increased significantly in industrialized countries over the past decade,[2] our knowledge of the underlying mechanisms that potentiate the induction of cell-mediated allergic immune responses to food allergens in the gastrointestinal tract remains limited.

IL-25 (IL-17E), a distinct IL-17 inflammatory cytokine member, is a key factor that functions to promote allergic inflammation.[3-6] Systemic administration or overexpression of IL-25 induces increased T_H2 cytokine and eotaxin production, which results in eosinophilia, increased serum IgE levels, mucus hyperplasia, and pathologic changes in lung and gastrointestinal tissues.[7-10] In contrast, administration of IL-25–neutralizing antibody significantly attenuates pulmonary allergic inflammation and prevents airway hyperresponsiveness.[11] In addition to airway and skin IL-25, endogenous intestinal IL-25 can limit T_H1- and T_H17-mediated gastrointestinal inflammation induced by commensal flora[12,13] and promote protective type 2 immunity to combat parasitic infection.[3,14] Indeed, IL-25–deficient mice infected with the gastrointestinal parasite *Trichuris muris* do not

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Supported by the National Institutes of Health (AI090129-1 to Y.H.-W. and A1073553 to S.P.H.), the Digestive Health Center (P30 DK078392, Pilot and Feasibility Award to Y.H.-W.), American Partnership For Eosinophilic Disorders (APFED; HOPE Pilot Grant to Y.H.W.), Campaign Urging Research For Eosinophilic Diseases (CURED) Foundation, the Buckeye Foundation, and the Food Allergy Research Education Fund to M.E.R. and the VA Merit Award to F.D.F.

Disclosure of potential conflict of interest: J.-B. Lee receives research support from the National Institute of Health (NIH). C. Dong has received speaking fees from Genentech and Biogen Marc Rothenberg is an Associate Editor for the *Journal of Allergy and Clinical Immunology.* S. P. Hogan receives research support from the NIH and Kinnear Pharmaceuticals. F. D. Finkelman is an Associate Editor for the *Journal of Allergy and Clinical Immunology.* Y.-H. Wang receives research support from the NIH and the American Partnership for Eosinophilic Disorders. The rest of the authors declare that they have no relevant conflicts of interest.

Received for publication September 24, 2014; revised September 10, 2015; accepted for publication September 15, 2015.

Available online November 10, 2015.

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0091-6749/$36.00

http://dx.doi.org/10.1016/j.jaci.2015.09.019

Abbreviations used
APC: Allophycocyanin
BM: Bone marrow
CT: Cholera toxin
FITC: Fluorescein isothiocyanate
4GET: *Il4* expression–driven GFP reporter
GFP: Green fluorescent protein
ICOS: Inducible costimulator
iIL-25Tg: IL-25 transgenic mouse line
ILC2: Type 2 innate lymphoid cell
IL-2Rα: IL-2 receptor α
IL-7Rα: IL-7 receptor α
LP: Lamina propria
MC: Mast cell
MCPt-1: Mast cell protease 1
OVA: Ovalbumin
PE: Phycoerythrin
WT: Wild-type

have a lymphocyte-dependent protective type 2 immunity to expel chronic parasitic infection.[14] These studies suggest that intestinal epithelium–derived IL-25 can regulate the balance of the immune response triggered by commensal microbes and dietary proteins in the gastrointestinal tract.

Recent studies demonstrate that type 2 innate lymphoid cells (ILC2s) are the early cellular source of the T_H2 cytokines IL-5 and IL-13.[3,15-19] Although lacking antigen-specific receptors, ILC2s express an array of cytokine receptors, including IL-17RB, ST-2, IL-7 receptor α (IL-7Rα), and IL-2 receptor α (IL-2Rα)[15-18] and respond to IL-25 and IL-33 stimulation in the presence of IL-7, IL-2, or both by producing large amounts of IL-5 and IL-13.[15-18] Subsequent studies reveal that ILC2s originate from common lymphoid progenitors[20,21] and express *Id2* and *Rorα*,[15,20,22] the signature transcription factors for innate lymphoid cell lineages.[23-25] Similar to $CD4^+$ T_H2 cells, ILC2s require transcription factor *Gata3* for differentiation and maintenance.[26] Parallel to their critical role in protective immunity against helminth infection, ILC2s can promote allergic asthma.[19,27,28] Whether ILC2s promote the induction of experimental food allergy has not been determined.

Herein we show that mice overexpressing intestinal IL-25 or lacking a component of the IL-25 receptor IL-17RB were prone or resistant to experimental food allergy, respectively. Our studies suggest that IL-25 and ingested antigen-induced $CD4^+$ T_H2 cells can enhance ILC2-derived IL-13 production, which promotes the development of experimental food allergy.

METHODS

Further information can be found in the Methods section in this article's Online Repository at www.jacionline.org.

IgE-mediated experimental food allergy

Mice were sensitized twice within a 2-week interval by means of intraperitoneal injection with 100 μg of ovalbumin (OVA) and 1 mg of alum. Two weeks after the second sensitization, mice underwent orally gavage with 50 mg of OVA in 250 μL of saline a total of 6 times within 2 weeks and were subsequently examined for symptomatic features in experimental food allergy.[2,3] The manifestations of systemic symptoms begin with diarrhea (profuse liquid stool), airway hyperreactivity, and then hyperthermia (rectal temperature decrease >2°C)[4,5] 30 to 45 minutes after the last challenge. Blood samples and intestinal tissues were collected from mice killed immediately after measurement of rectal temperature.

Measuring parameters of food allergy

Duodenal tissue was fixed in 10% formalin and processed by using standard histologic techniques to measure intestinal MC numbers and levels of goblet cell hyperplasia. Five- to 8-μm tissue sections were stained with Leder stain for chloroacetate esterase activity in intestinal MCs or periodic acid-Schiff for mucins in goblet cells. Stained cells were quantified, as previously described.[3] Serum samples were analyzed with ELISA kits of OVA-specific IgE (MD Bioproducts, St Paul, Minn), mast cell protease 1 (MCPt-1; eBioscience, San Diego, Calif), and OVA-specific IgG_1 (Alpha Diagnostic International, San Antonio, Tex) to measure secreted mediators. Diarrhea assessments (profuse liquid stool) and hyperthermia measurements (rectal temperature decrease >2°C) were performed, as previously described.[4]

Statistical analysis

For comparisons between experimental groups, statistical significance was determined by using the unpaired Student *t* test. Results were considered significant at a P value of .05 or less. Error bars denote means ± SDs. All data were analyzed with Prism software (GraphPad Software, La Jolla, Calif).

RESULTS

Early induction of intestinal IL-25 promotes susceptibility to experimental food allergy

Intestinal *Il25* expression was examined to determine the involvement of IL-25 in regulating food allergy. Compared with naive mice, sensitized mice that received only 2 intragastric OVA challenges had rapidly upregulated *Il25* expression (>5-fold) in the duodenal epithelium; this *Il25* expression remained increased until the onset of anaphylactic response to ingested OVA (Fig 1, *A*). Concomitantly, the expression of *Il13* (>5-fold) and chemokine genes, including thymus- and activation-regulated chemokine (*Tarc*) (>7-fold), C-X-C motif ligand 1 (*Cxcl1*) (>7-fold), and C-C motif chemokine 11 (*Ccl11*) (>20-fold; eotaxin 1), but not C-C motif ligand 24 (*Ccl24*) (eotaxin 2), was also upregulated, primarily in the small intestinal epithelium before onset of experimental food allergy.

To address whether dysregulated IL-25 signaling contributes to susceptibility to developing experimental food allergy, we generated IL-25 transgenic mouse lines (iIL-25Tg mice) that constitutively overexpress murine IL-25 driven by the promoter of intestinal fatty acid–binding protein in the small intestinal epithelium and $Il17rb^{-/-}$ mice that had disrupted *Il17rb* gene expression. Although intestinal IL-25 overexpression induced increases of intestinal *Il5* (>20,000-fold) and *Il13* (>75-fold) gene expression and MC numbers and levels of goblet cell hyperplasia in naive or sensitized iIL-25Tg mice (Fig 1, *B*, and data not shown), these mice did not have symptomatic features of IgE-mediated experimental food allergy, including allergic diarrhea, hypothermia, intestinal mastocytosis, or increased serum OVA-specific IgE and/or MCPt-1 production, the latter of which indicates MC degranulation (Fig 1, *B*). Notably, a regimen of only 4 intragastric OVA antigen challenges was sufficient to induce sensitized iIL-25Tg mice, but not their littermate control animals, to manifest systemic symptoms beginning with diarrhea (>84%, profuse liquid stool) and then hyperthermia (>60%) within 45 minutes (Fig 1, *B*). These iIL-25Tg mice also produced higher titers of serum MCPt-1 and OVA-specific IgE and exhibited more pronounced goblet

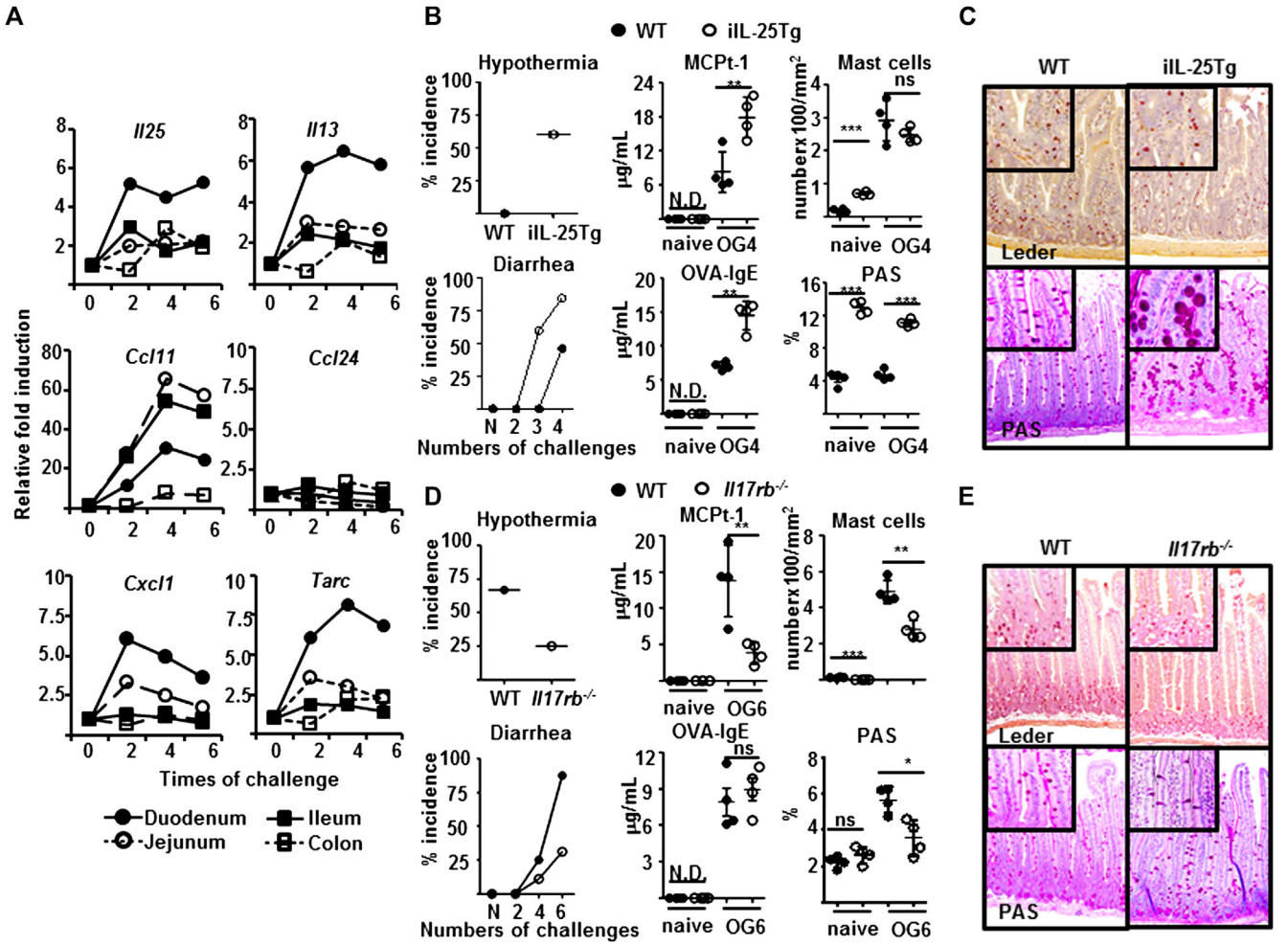

FIG 1. **A,** Expression levels of indicated genes by indicated tissues of sensitized BALB/c mice after indicated times of intragastric OVA challenge were examined and compared, as described in the Methods section. **B-E,** Indicated murine strains were sensitized and underwent oral gavage *(OG)* with OVA 4 times (Fig 1, *B* and *C*), 6 times (Fig 1, *D* and *E*), or the indicated times (Fig 1, *B* and *D*) before measuring the indicated features of experimental food allergy and staining of intestinal mastocytosis and goblet cell *(GC)* hyperplasia (Fig 1, *C* and *E*). For Fig 1, *A*, 2 independent experiments were performed (n = 4, total 8 mice per group) For Fig 1, *E* to *B*, 3 independent experiments (n = 4, total 12 mice per group) were performed in blind fashion. *N.D.*, Not detected; *ns*, not significant; *PAS*, periodic acid-Schiff. $*P \leq .05$, $**P \leq .01$, and $***P \leq .001$.

cell hyperplasia, but not intestinal mastocytosis, compared with littermate control mice (Fig 1, *B* and *C*).

In contrast, sensitized mice lacking IL-17RB were more resistant allergic diarrhea and hypothermia than wild-type (WT) BALB/c mice after 6 intragastric OVA challenges (Fig 1, *D*). These *Il17rb*$^{-/-}$ mice produced less MCPt-1 and displayed less intestinal mastocytosis and goblet cell hyperplasia while producing comparable amounts of OVA-specific IgE (Fig 1, *D* and *E*). These results suggest that intestinal epithelial *Il25* expression increases at the early phase of experimental food allergy and that alterations in IL-25 signaling can positively or negatively regulate susceptibility to experimental food allergy.

Intestinal ILC2s produce IL-5 and IL-13 in response to IL-25

Next, we searched for intestinal IL-17RB–expressing immune cells during development of experimental food allergy. Two dominant IL-17RB–expressing cell populations were identified in the lamina propria (LP) of the small intestine of mice with active allergic diarrhea, (1) $CD3^+CD4^+$IL-$17RB^+$ cells and (2) $CD3^-CD4^-$IL-$17RB^+$c-KIT^- cells, both of which expressed the hematopoietic cell lineage marker CD45, but not other known cell lineage markers (Lin^-; Fig 2, *A*, and data not shown). $CD3^+CD4^+$IL-$17RB^+$ cells expressed surface markers of T_H2 effector/memory cells, including CD44, CD69, IL-7Rα, ST-2, and Thy1.2, and produced significant amounts of the T_H2 cytokines IL-4, IL-5, and IL-13, but not IL-17 or IFN-γ, on CD3/CD28 restimulation (Fig 2, *B*, and see Fig E1, *A* and *B*, in this article's Online Repository at www.jacionline.org). Notably, the $Lin^-CD3^-CD4^-$IL-$17RB^+$c-KIT^- cells expressed Sca-I, KLRG1, IL-7Rα (CD127), ST-2, Thy1.2, inducible costimulator (ICOS), IL-2Rα (CD25), and MHC class II, the signature markers of recently described ILC2s,[29] but not FcεRαI, IL-3 receptor (CD123), and Siglec-F (Fig 2, *B*, and see Fig E2, *A*, in this article's Online Repository at www.jacionline.org). On the contrary,

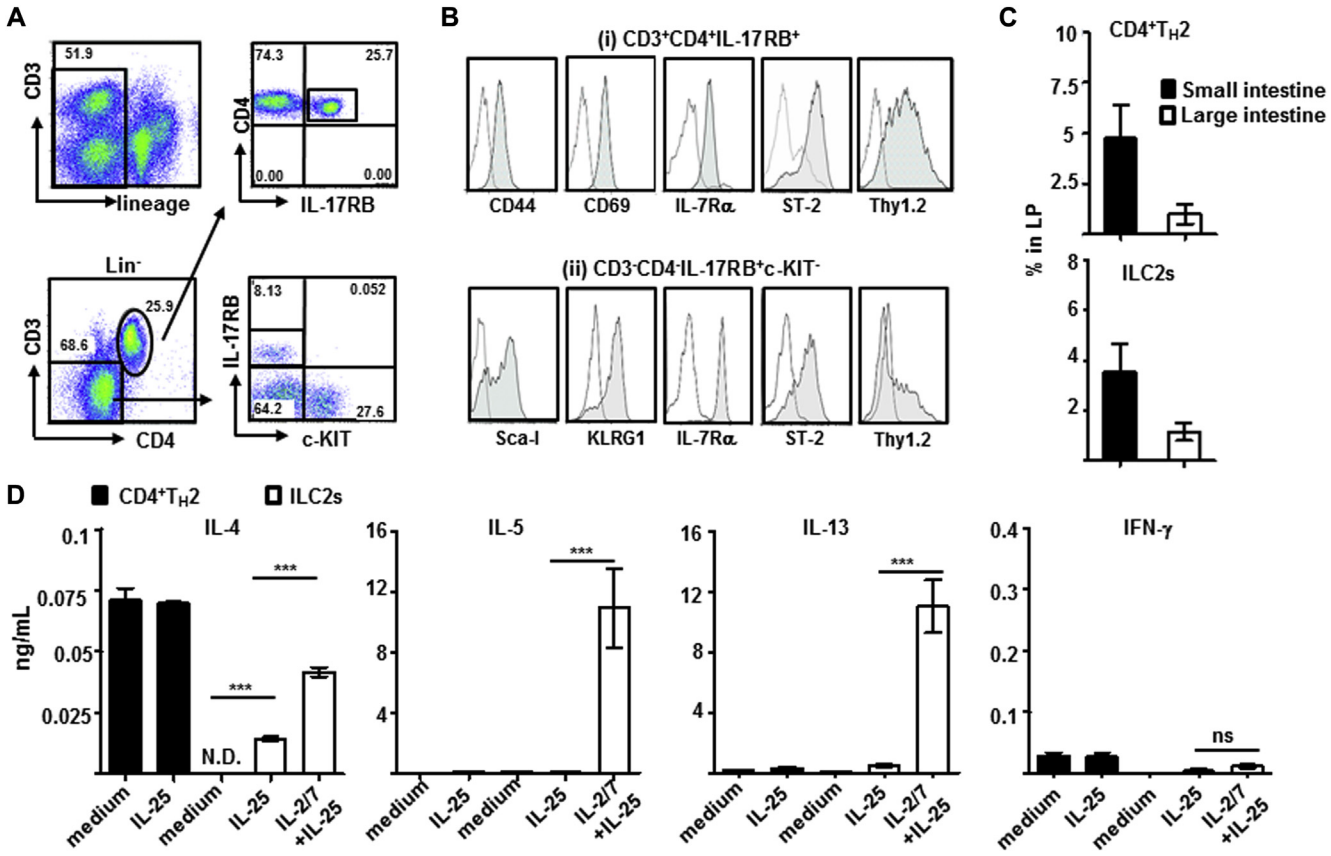

FIG 2. A-C, Detection (Fig 2, *A*), phenotypic analysis (Fig 2, *B*), and frequencies (Fig 2, *C*) of Lin$^-$CD3$^+$CD4$^+$IL-17RB$^+$ T_H2 cells and Lin$^-$CD3$^-$CD4$^-$IL-17RB$^+$c-KIT$^-$ ILC2s in mice with food allergy. **D,** Indicated cytokines produced by purified Lin$^-$CD3$^+$CD4$^+$IL-17RB$^+$ T_H2 cells and Lin$^-$CD3$^-$CD4$^-$IL-17RB$^+$c-KIT$^-$ ILC2s from mice with food allergy after stimulation with IL-25 alone or IL-25 plus IL-2 and IL-7 (IL-2/IL-7) were measured and compared. *N.D.*, Not detected; *ns*, not significant. ***$P \leq .001$.

Lin$^-$CD3$^-$CD4$^-$IL-17RB$^-$c-KIT$^+$ cells expressed surface FcεRαI, but not IL-2Rα (CD25), IL-3 receptor (CD123), or ICOS, suggesting that these cells are of the MC lineage (see Fig E2, *A*). Thus systemic administration of IL-25 proteins in mice resulted in selective expansion of Lin$^-$CD3$^-$CD4$^-$IL-17RB$^+$c-KIT$^-$ cells, but not Lin$^-$CD3$^-$CD4$^-$IL-17RB$^-$c-KIT$^+$ cells, in their mesenteric lymph nodes (see Fig E2, *B*). Comparative gene expression analyses among examined hematopoietic cell lineages revealed that both IL-17RB–expressing cell subsets expressed high levels of *Gata3* (>5 × 10^3–fold) and the T_H2 cytokine genes *Il4* (>5 × 10^3–fold), *Il5* (>1 × 10^4–fold), and *Il13* (>5 × 10^4–fold); however, only CD3$^-$CD4$^-$IL-17RB$^+$c-KIT$^-$ cells expressed ILC2 signature transcripts: *Id2* (>50-fold) and *Rora* (>3 × 10^3–fold; see Fig E1, *C*). Thus Lin$^-$CD3$^+$CD4$^+$IL-17RB$^+$ and Lin$^-$CD3$^-$CD4$^-$IL-17RB$^+$c-KIT$^-$ cell subsets represent intestinal CD4$^+$ T_H2 cells and ILC2s, respectively.

Both IL-17RB–expressing CD4$^+$ T_H2 cells (approximately 5.0%) and ILC2s (approximately 3.0%) accumulated preferentially in the small intestines of mice with experimental food allergy (Fig 2, *C*), the primary anatomic site that expressed induced *Il25* transcript (Fig 1, *A*). IL-25 alone induced very little T_H2 cytokine production by purified intestinal CD4$^+$ T_H2 cells and ILC2s; however, the addition of IL-2 and/or IL-7 potentiated ILC2s, but not CD4$^+$ T_H2 cells, to respond to IL-25 stimulation by producing large amounts of IL-5 (>10 ng/mL per 10^4 cells) and IL-13 (>10 ng/mL per 10^4 cells) and very little IL-4 (<0.05 ng/mL per 10^4 cells) and IFN-γ (<0.1 ng/mL per 10^4 cells; Fig 2, *D* and data not shown). These results suggest that both CD4$^+$ T_H2 cells and ILCs are intestinal IL-17RB–expressing cells and that ILC2s are the primary IL-5 and IL-13 producers in response to IL-25 stimulation in experimental food allergy.

IL-25–responsive ILC2s promote susceptibility to experimental food allergy

To determine the contributions of ILC2s to the development of food allergy, we developed a reconstitution model of experimental food allergy, adoptively transferring bone marrow (BM) cells from *Il4* expression–driven GFP reporter (4GET) BALB/c mice into OVA-sensitized BALB/c mice 1 day after sublethal irradiation (protocol diagramed in Fig E3, *A*, in this article's Online Repository at www.jacionline.org). After the second sensitization and repeated intragastric OVA challenge, transferred BM cells replenished the IL-17RB$^+$c-KIT$^-$IL-7Rα$^+$KLRG1$^+$ ILC2 compartment with green fluorescent protein (GFP)–marked cells in the irradiated recipients, whereas most of the CD4$^+$ST-2$^+$IL-17RB$^+$ T_H2 cells lacked GFP expression and were therefore derived from the sensitized recipients (Fig 3, *A*). Consistently, most of the replenished ILC2s were Thy1.1$^{high/low}$ cells

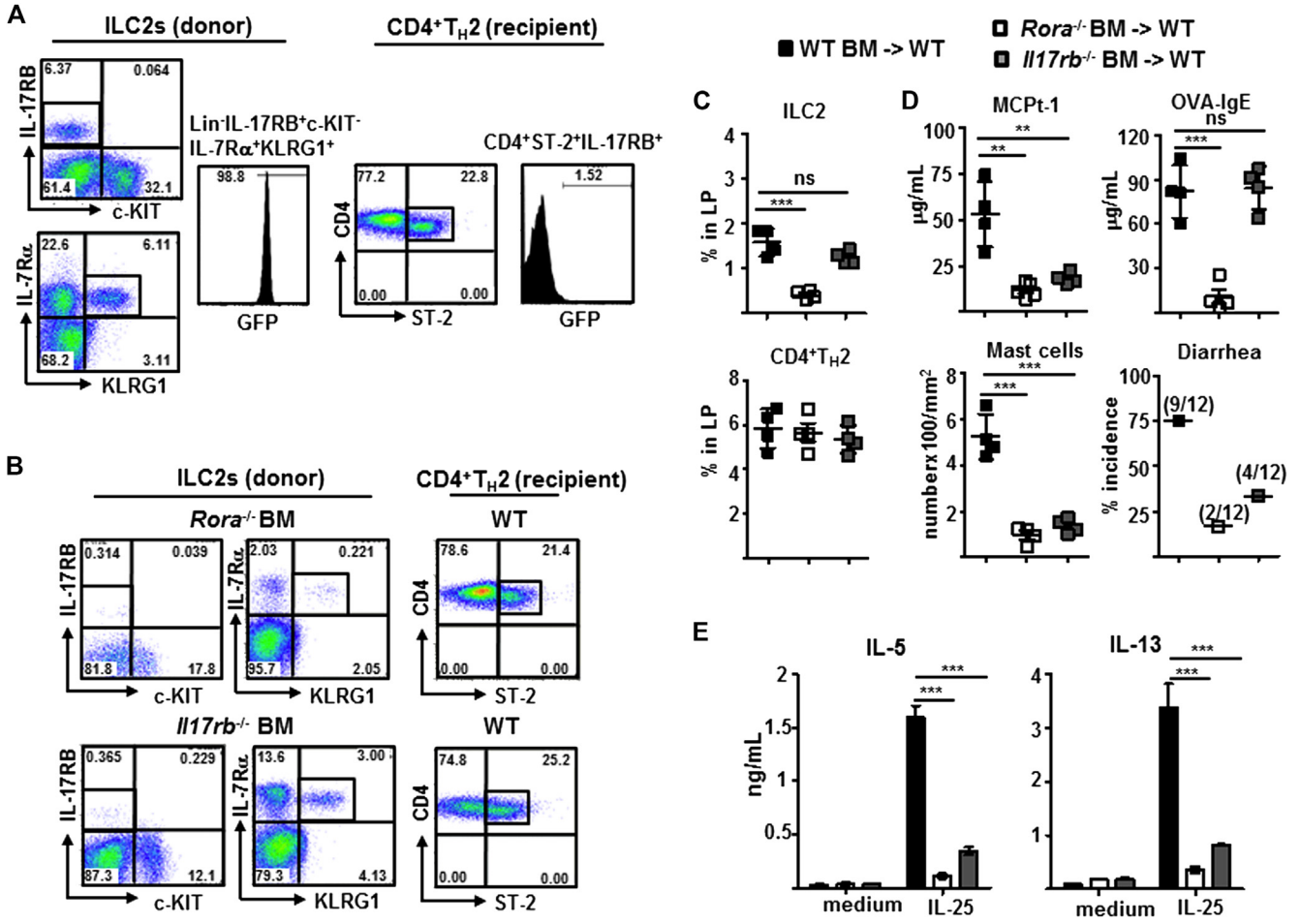

FIG 3. Detection **(A** and **B)** and frequency **(C)** of donor-derived ILC2s ($Lin^-CD3^-CD4^-IL\text{-}17RB^+c\text{-}KIT^-IL\text{-}7R\alpha^+KLRG1^+$) and recipient-derived $CD4^+$ T_H2 cells ($Lin^-CD3^+CD4^+IL\text{-}17RB^+ST\text{-}2^+$) and measurements of indicated parameters of experimental food allergy **(D)** and indicated cytokine production by IL-25–stimulated LP cells **(E)** from irradiated WT BALB/c recipients reconstituted with BM progenitors from 4GET mice (Fig 3, *A*) or WT BALB/c, *Rora*$^{-/-}$, or *Il17rb*$^{-/-}$ mice (Fig 3, *B-E*). Three independent experiments (n = 4, total 12 mice per group) were performed in blind fashion. *ns,* Not significant. $^{**}P \geq .01$ and $^{***}P \leq .001$.

(>96%) in the irradiated Thy1.2$^+$ recipients, which generated the primarily Thy1.2-expressing CD4$^+$ T-cell compartment (>96%) after reconstitution with congenic Thy1.1$^+$BM cells. Furthermore, ILC2 frequencies were comparable (approximately 2.0%) between reconstituted WT and *Stat6*$^{-/-}$ Thy1.2$^+$ recipients (see Fig E3, *B* and *C*). Thus BM transplants will reconstitute the innate ILC2 cell lineage within 2 weeks, whereas *de novo* T-cell generation after thymus engraftment from donor-derived hematopoietic progenitors require greater than 2 months.[30] Having established a reconstitution model of experimental food allergy, we showed that irradiated mice reconstituted with BM cells that lacked the transcription factor retinoic acid receptor-related orphan receptor α (*Rora*), a transcription factor for ILC2 development,[20] did not develop ILC2s, whereas CD4$^+$ T_H2 cells developed normally (Fig 3, *B* and *C*). Consequently, irradiated mice reconstituted with WT but not *Rora*-deficient BM cells exhibited pronounced intestinal mastocytosis, produced increased MCPt-1 and OVA-specific IgE, and had allergic diarrhea after repeated antigen challenge (Fig 3, *D*). Although IL-17RB–deficient BM cells developed into ILC2s normally, these reconstituted IL-17RB–deficient ILC2s did not promote experimental food allergy in the irradiated recipients that produced OVA-specific IgE normally (Fig 3, *B-D*). Notably, LP cells from the small intestines of WT BM reconstituted mice produced significantly higher amounts of IL-5 and IL-13 than that produced by LP cells from *Rora*$^{-/-}$ or *Il17rb*$^{-/-}$ BM reconstituted recipients, which lacked ILC2s or generated dysfunctional ILC2s, respectively, in response to IL-25 stimulation *ex vivo* (Fig 3, *E*). These results demonstrate that intestinal ILC2s are the primary IL-5 and IL-13 producers in response to IL-25 stimulation and play a key role in promoting experimental food allergy *in vivo*.

Antigen-induced CD4$^+$ T_H2 cells enhance ILC2 function in response to IL-25

Recent reports suggest an interplay relationship between innate ILC2s and adaptive CD4$^+$ T cells in the protective response against helminth infection.[31,32] Frequencies of intestinal ILC2s and CD4$^+$ T_H2 cells during the development of experimental food allergy were examined to address whether the ingested antigen-induced CD4$^+$ T_H2 immune response is involved in ILC2 function. Although very few CD4$^+$ T_H2 cells (<0.1%) could be detected, a considerable pool of ILC2s resided constantly in

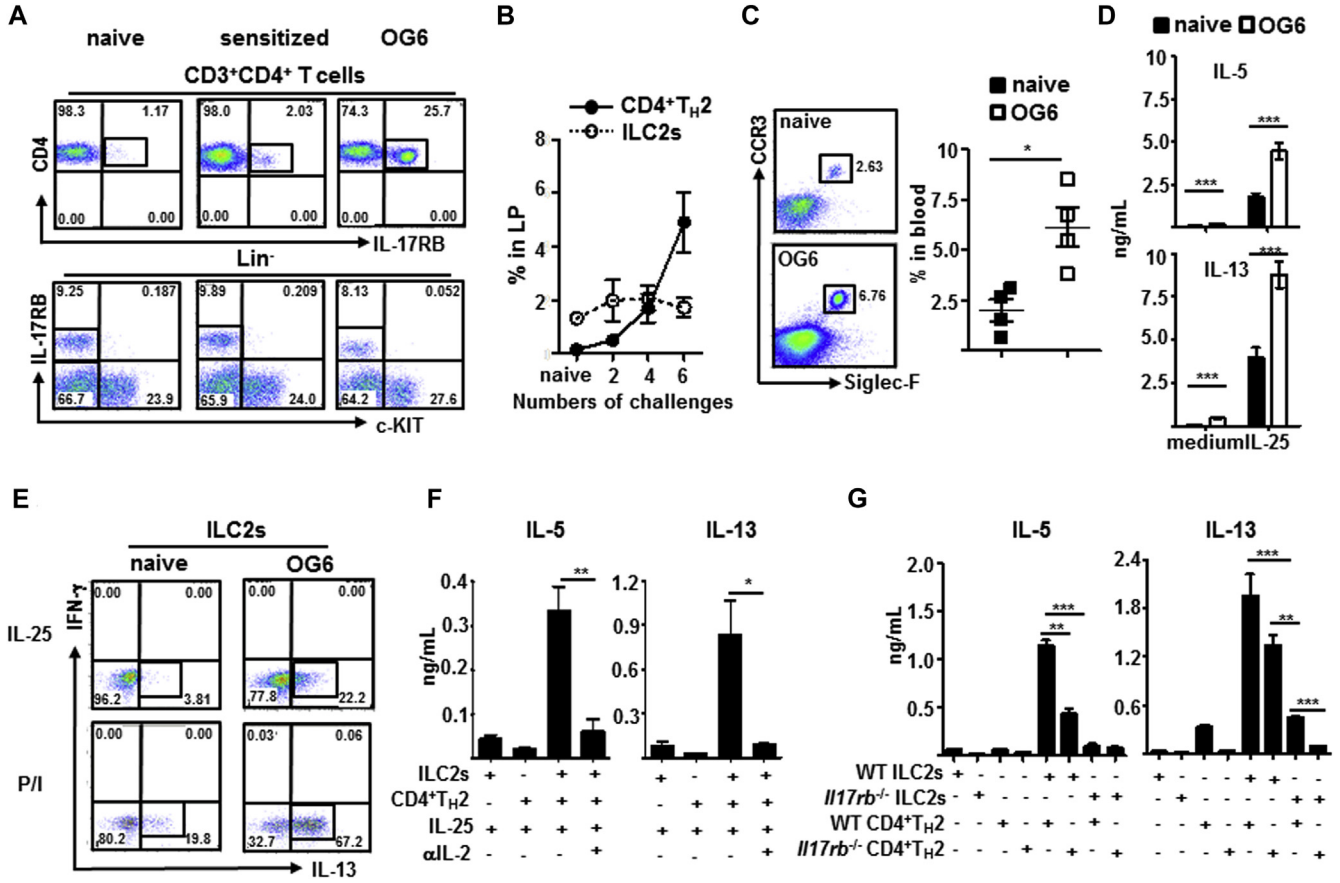

FIG 4. Detection and frequency of intestinal $CD4^+$IL-17RB$^+$ T_H2 cells and Lin^-IL-17RB$^+$c-KIT$^-$ ILC2s **(A** and **B)**, blood CCR3$^+$Siglec-F$^+$CD11b$^+$ eosinophils **(C)** and intracellular IL-13–producing ILC2s **(E)** from naive or sensitized mice undergoing oral gavage *(OG)* with OVA 6 times (Fig 4, *A*, *C*, and *E*) or indicated times (Fig 4, *B*). IL-5 and IL-13 production by medium- or IL-25–stimulated LP cells (Fig 4, *D*) or indicated cells from WT (**F** and **G**) or *Il17rb*$^{-/-}$ mice (Fig 4, *G*) OG with OVA 6 times after a 3-day coculture with IL-25 only (Fig 4, *F* and *G*) or IL-25 plus anti–IL-2 or control antibodies (Fig 4, *F*). Three independent experiments (n = 4, total 12 mice per group) were performed in blind fashion. *$P \leq .05$, **$P \leq .01$, and ***$P \leq .001$.

the small intestines of naive or sensitized mice (1.5% to 3.0%; Fig 4, *A* and *B*). Notably, repeated intragastric OVA antigen challenge induced a significant accumulation of intestinal $CD4^+$ T_H2 cells (<0.1% to >5%) but not ILC2s and an increase in peripheral eosinophil counts (Fig 4, *A-C*). Correspondingly, intestinal LP cells from rechallenged mice produced significantly higher amounts of IL-4, IL-5, and IL-13 than LP cells from naive mice after OVA peptide stimulation *ex vivo* (see Fig E4, *A*, in this article's Online Repository at www.jacionline.org). Although ILC2 frequencies were comparable among naive, sensitized, and rechallenged mice, the capability of ILC2s to produce IL-5 and IL-13 after IL-25 or phorbol 12-myristate 13-acetate/ionomycin stimulation was significantly enhanced only in rechallenged mice with a considerable pool of $CD4^+$ T_H2 cells (Fig 4, *A*, *D*, and *E*).

To directly assess whether $CD4^+$ T_H2 cells would enhance ILC2 responsiveness to IL-25 stimulation, we measured their T_H2 cytokine–producing capability in the presence or absence of $CD4^+$ T_H2 cells *ex vivo*. Indeed, coculture of *in vitro*–derived $CD4^+$ T_H2 cells significantly enhanced the responsiveness of ILC2s to IL-25 stimulation because these stimulated cocultured ILC2s produced large amounts of IL-5 and IL-13 (Fig 4, *F*, and see Fig E4, *B*). Intriguingly, the presence of OVA peptides further enhanced IL-5 production by cocultured OVA antigen-specific $CD4^+$ T_H2 cells and ILC2s in response to IL-25 stimulation (see Fig E4, *B*). Treatments with antibodies against IL-2 greatly diminished the ILC2s' enhanced responsiveness to IL-25 stimulation in $CD4^+$ T_H2 cell coculture (Fig 4, *F*). Furthermore, ILC2s purified from the small intestine of rechallenged WT or *Il17rb*$^{-/-}$ mice did not respond to IL-25 when cultured alone; however, purified WT $CD4^+$ T_H2 cells from mice with active allergic diarrhea greatly enhanced the IL-25–stimulated IL-5 and IL-13 production by cocultured WT, but not IL-17RB–deficient, ILC2s (Fig 4, *G*). Notably, IL-17RB–deficient $CD4^+$ST-2$^+$ T_H2 cells purified from *Il17rb*$^{-/-}$ mice, which were resistant to experimental food allergy, were less capable of potentiating IL-5 and IL-13 production by WT ILC2s in response to IL-25 (Fig 4, *G*). Collectively, these intriguing findings suggest that IL-17RB–expressing $CD4^+$ T_H2 cells induced by ingested antigens enhance the capability of intestinal ILC2 residents to respond to epithelium-derived IL-25 by producing prodigious amounts of IL-5 and IL-13, thereby promoting experimental food allergy.

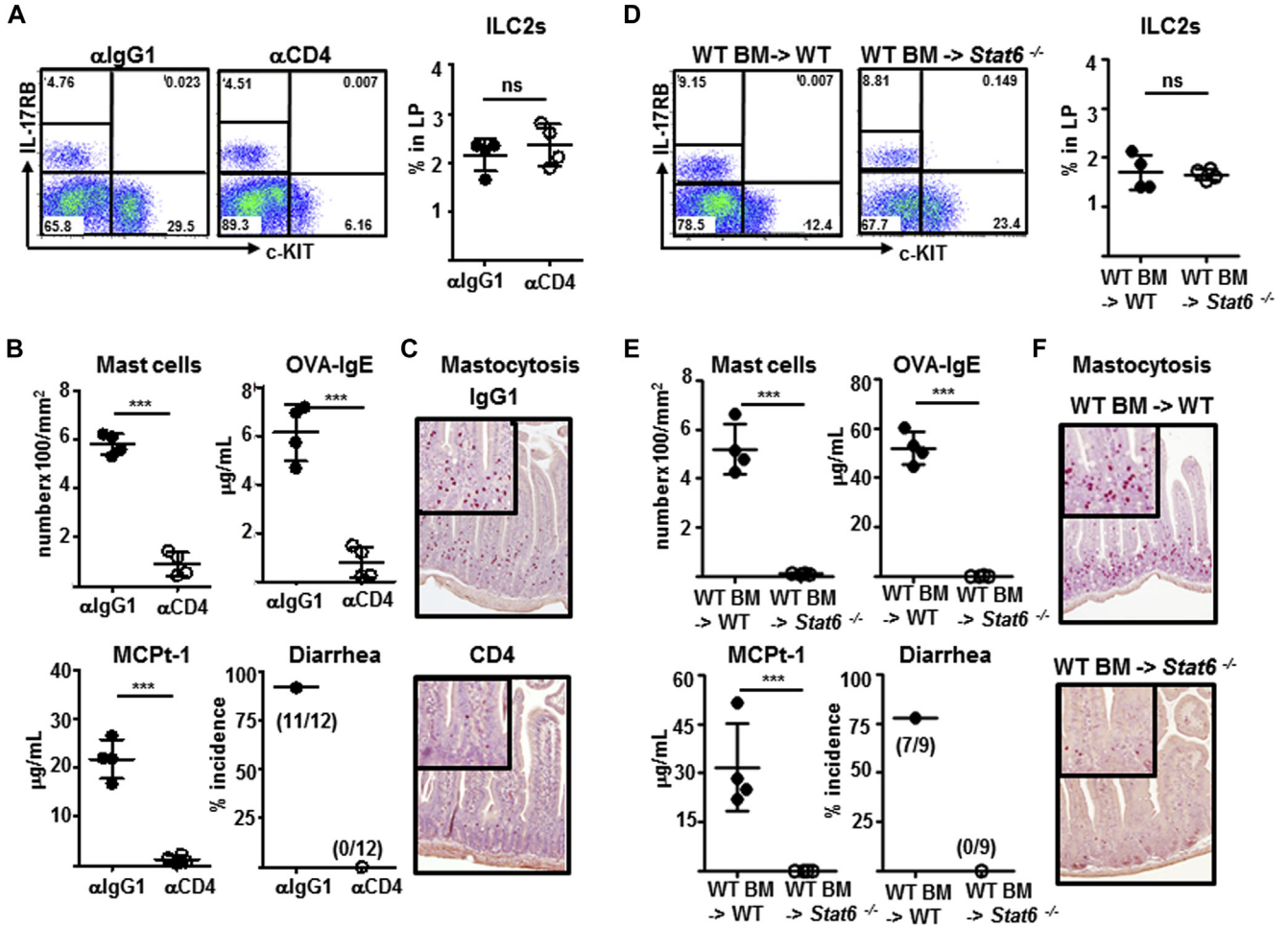

FIG 5. Detection and frequency of ILC2s **(A** and **D)**, measurement of indicated features of experimental food allergy **(B** and **E)**, and staining for intestinal mastocytosis **(C** and **F)** in sensitized WT BALB/c mice treated with indicated antibodies 1 day before the first and fourth intragastric OVA challenges (Fig 5, *A-C*) or in irradiated recipients reconstituted with BM progenitors from WT or *Stat6*$^{-/-}$ mice (Fig 5, *D-F*). Three independent experiments (n = 4, total 12 mice per group) were performed in blind fashion. *ns*, Not significant. ***$P \leq .001$.

ILC2s do not promote experimental food allergy in mice that lack CD4$^+$ T_H2 cells

Next, we addressed whether induction of CD4$^+$ T_H2 cells by ingested antigens is essential for ILC2 function in promoting experimental food allergy. Compared with mice that received isotype-matched antibodies, mice ablated of CD4$^+$ T cells after anti-CD4 antibody treatments did not have symptomatic features of experimental food allergy, despite their intestinal ILC2 compartment remaining intact (Fig 5, *A-C*). Although replenished ILC2s restored the susceptibility of irradiated WT recipients to experimental food allergy after BM reconstitution, these donor-derived ILC2s did not restore the capability of irradiated *Stat6*$^{-/-}$ recipients, which lacked CD4$^+$ T_H2 cells, to have intestinal mastocytosis, produce MCPt-1 or OVA-specific IgE, or have allergic diarrhea (Fig 5, *D-F*). These results suggest that ILC2s alone are insufficient to drive the development of experimental food allergy and that the induction of intestinal CD4$^+$ T_H2 cells by ingested antigens is a prerequisite for ILC2 function to promote experimental food allergy.

IL-13 production by ILC2s promotes experimental food allergy

To understand the mechanisms underlying the function of ILC2s in promoting susceptibility to experimental food allergy, we examined the role of IL-13, the major cytokine produced by ILC2s, which has been shown to be involved in the development of IgE-mediated experimental food allergy.[33] Although ILC2s developed normally in *Il13*$^{-/-}$ mice, fewer CD4$^+$ T_H2 cells were induced in *Il13*$^{-/-}$ mice than in WT mice after repeated intragastric OVA antigen challenge (Fig 6, *A*). Consequently, *Il13*$^{-/-}$ mice exhibited reduced levels of intestinal mastocytosis, had goblet cell hyperplasia, produced less MCPt-1 and OVA-specific IgE, and were more resistant to experimental food allergy compared with WT mice (Fig 6, *B*). Consistently, the capabilities of WT and IL-13–deficient BM cells were comparable in replenishing ILC2s in irradiated recipients that developed CD4$^+$ T_H2 cells normally (Fig 6, *C* and *D*). However, recipients reconstituted with *Il13*$^{-/-}$ BM cells exhibited less pronounced intestinal mastocytosis and goblet cell hyperplasia, produced less MCPt-1 and OVA-IgE, and were thereby more

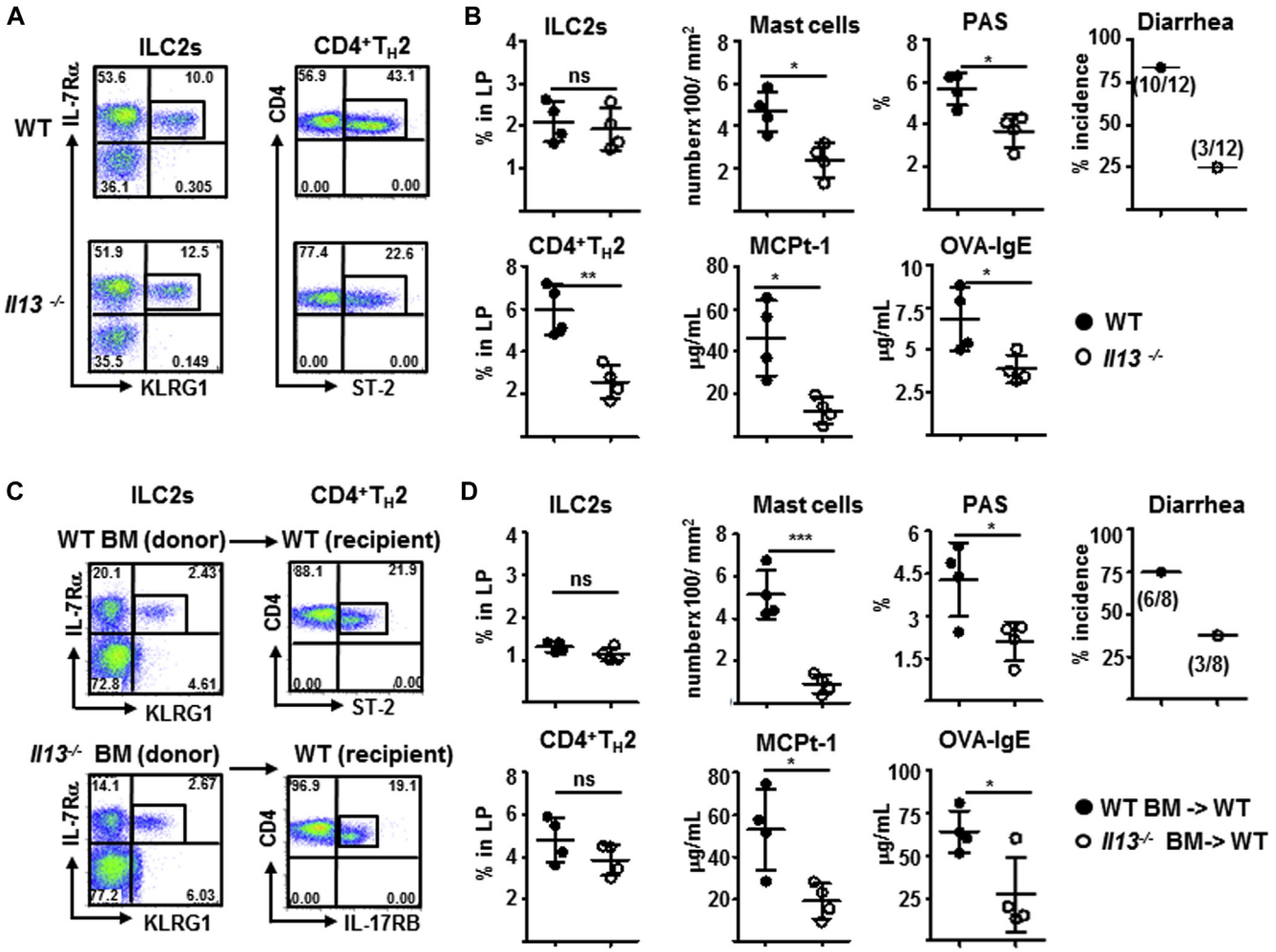

FIG 6. Detection **(A** and **C)** and frequency of $Lin^{-}IL\text{-}7R\alpha^{+}KLRG1^{+}$ ILC2s and $CD4^{+}ST\text{-}2^{+}$ T_H2 cells in and measurements of indicated parameters of experimental food allergy of **(B** and **D)**, sensitized WT or *Il13*$^{-/-}$ mice (Fig 6, *A* and *B*) or irradiated WT BALB/c recipients reconstituted with BM progenitors from WT or *Il13*$^{-/-}$ mice after 6 intragastric OVA challenges (Fig 6, *C* and *D*). For Fig 6, *A* and *B*, 3 independent experiments (n = 4, total 12 mice per group) were performed in blind fashion. For Fig 6, *C* and *D*, 2 independent experiments were performed (n = 4, total 8 mice per group) *ns*, Not significant. $*P \leq .05$, $**P \leq .01$, and $***P \leq .001$.

resistant to having an anaphylactic response to ingested OVA antigen compared with those that received WT BM cells (Fig 6, *D*). These intriguing results suggest that IL-13 elicited by ILC2s might play a key role in promoting intestinal allergic inflammation, which promotes the development of IgE-mediated experimental food allergy.

DISCUSSION

The immune response to allergenic dietary proteins depends on the balance of a complex interplay between immune and nonimmune cell interactions in the gastrointestinal tract. Mechanistically, it remains unclear how an adverse allergic reaction to ingested food can be initiated and amplified, leading to loss of oral tolerance. The epithelium-derived cytokines thymic stromal lymphopoietin, IL-33, and IL-25 have been shown to play an important role in initiating and amplifying type 2 immune responses in allergic asthma and against parasitic infection.[6,34] Thymic stromal lymphopoietin can potentiate dendritic cells to promote naive $CD4^{+}$ T cells to differentiate into $CD4^{+}$ T_H2 cells and maintain $CD4^{+}$ T_H2 memory/effector cells[35-38] and enhance allergic sensitization in the cutaneous sensitization model of food allergy.[39] Although IL-33 is responsible for the development of allergic lung diseases,[40-42] this cytokine appears to be dispensable for cutaneous allergic sensitization but essential for inducing IgE-dependent anaphylaxis.[39] Whether IL-25 is involved in regulating the dysregulated type 2 immune response to ingested food antigens has not been established. Herein we demonstrate that repeated intragastric antigen challenge induces an increase in *Il25* gene expression by intestinal epithelium before the onset of an anaphylactic response to ingested food. Studies with genetically modified mice further demonstrate that IL-25 signaling promotes susceptibility to experimental food allergy. Notably, $CD4^{+}$ T_H2 cell induction by ingested antigens appears to be a prerequisite for intestinal ILC2 residents to produce large amounts of IL-13 in response to IL-25 stimulation and to promote the susceptibility to experimental food allergy. Our studies further suggest that intestinal IL-25 signaling promotes the interplay

between $CD4^+$ T_H2 cells and ILC2s to elicit allergic reactions to ingested food.

The finding that IL-25 mediates protective immunity against gastrointestinal helminth infection, such as *Trichuris muris*, *Nippostrongylus brasiliensis*, and *Trichinella spiralis*, by promoting a T_H2 cytokine–dependent immune response[3,14,43,44] suggests a potential involvement of IL-25 in allergic reactions to ingested food antigens. Indeed, analyses of iIL-25Tg and *Il17rb*$^{-/-}$ mice reveal their susceptibility and resistance to IgE-mediated experimental food allergy, respectively, substantiating the role of IL-25 in promoting allergic reaction to ingested antigens. Although intestinal ILC2s are identified to be the primary IL-25–responding cells, ILC2s alone are insufficient to drive intestinal anaphylaxis in naive or sensitized transgenic mice overexpressing IL-25. Their capabilities to produce IL-5 and IL-13 in response to IL-25 and to promote experimental food allergy require $CD4^+$IL-17RB$^+$ T_H2 cells, which are induced after repeated intragastric OVA antigen challenge. In agreement with recent studies,[31,32] anti–IL-2 antibody treatments result in a significant reduction of IL-5 and IL-13 production by IL-25–stimulated ILC2s cocultured with $CD4^+$ T_H2 cells. Notably, IL-17RB–deficient $CD4^+$ST-2^+ T_H2 cells are less capable of enhancing ILC2s to produce T_H2 cytokine in response to IL-25, possibly because IL-17RB–deficient $CD4^+$ST-2^+ T_H2 cells produce less IL-2 after IL-25 stimulation. Our studies support the view that IL-25 enhances the concerted interactions between intestinal ILC2s and antigen-induced $CD4^+$ T_H2 cells to amplify allergic reactions to ingested food antigens.

A role of ILC2s in promoting IgE-mediated experimental food allergy has been further substantiated in a reconstitution model of experimental food allergy. Donor-derived ILC2s promote irradiated recipient mice to have experimental food allergy after WT BM reconstitution. However, irradiated recipients reconstituted with *Rora*$^{-/-}$ or *Il17rb*$^{-/-}$ BM that are defective in ILC2 development or function, respectively, are resistant to experimental food allergy. It appears that IL-13 produced primarily by ILC2s plays a key role in promoting susceptibility to experimental food allergy, possibly by inducing goblet cell hyperplasia, increasing intestinal permeability, and modulating gut barrier function.[45-47] In contrast to our findings, a recent study suggests that mice ablated of ILC2s and deficient in IL-25 or IL-13 remain capable of the splenic T_H2 immune response and systemic anaphylactic response to antigen administered intraperitoneally after sensitization by orally gavaged cholera toxin (CT).[48,49] The discrepancies might be attributable to differences in the experimental approaches, including sensitization to adjuvant (alum vs CT), murine strains (BALB/c vs C3H/HeJ or C57/B6),[33,49,50] and the anatomic locations where the anaphylaxis is induced (intestine vs peritoneum).[33,48,49] Importantly, previous studies demonstrate that the anaphylactic response to ingested antigens administered by means of intragastric inoculations after alum sensitization is dependent on the classic MC/IgE/FcεRαI pathway,[51,52] whereas anaphylaxis induced by intraperitoneal antigens after CT sensitization can be mediated by the alternative IgG/FcγR pathway.[53,54] It remains to be determined whether the differences in the genetic predisposition to allergic sensitization between the BALB/c and C57/B6 strains and/or the antigen administration routes results in this perceived discrepancy in the necessity of IL-25 signaling in experimental food allergy.

In our murine food allergy model, mice eventually have systemic manifestations pertaining to some characteristics of food allergy–induced life-threatening anaphylaxis in human subjects, including cutaneous and mesenteric vascular leak, airway hyperresponsiveness, and hypothermia,[51,55] despite the fact that mice with "food allergy" do not have skin allergic disorders that are often comorbid in some of patients with food allergy.[56] Although the initial triggers and genetically predisposing factors that initiate allergic sensitization in the gastrointestinal tract remain unclear, our findings suggest that IL-25–mediated collaborations between ILC2s and $CD4^+$ T_H2 cells can be a key step in amplifying the cascade of allergic reactions to ingested antigens at the effector phase of IgE-mediated food allergy through the MC/IgE pathway.[51,57] These studies underscore the importance of understanding the mechanisms that underlie the intestinal allergic response to ingested food, and the knowledge gained from these basic studies might eventually serve as the rationale to design innovative approaches for the care of food allergy in human subjects.

We thank S. Hottinger for editorial assistance.

Key messages

- **Alteration of IL-25 signals can positively or negatively regulate susceptibility to experimental food allergy.**
- **Intestinal ILC2s are the primary IL-5 and IL-13 producers in response to IL-25 stimulation and play a key role in promoting experimental food allergy.**
- **Ingested antigen-induced $CD4^+$ T_H2 cells potentiate the capability of ILC2s to respond to IL-25 by producing prodigious amounts of IL-13, which promotes experimental food allergy.**

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METHODS

Generation of iIL-25Tg and *Il17rb*$^{-/-}$ mice

Murine *Il25* cDNA was inserted behind intestinal fatty acid-binding protein promoter, and the expression plasmid was linearized by means of *Eco*RI digestion for generation of iIL-25Tg mice.[E1] For *Il17rb*$^{-/-}$ mice, a targeting vector was constructed to delete exons 5 to 11, resulting in loss of the transmembrane and intracellular regions, as well as the alternative splicing isoforms of IL-17RB. Three independent iIL-25Tg founders and 2 *Il17rb*$^{-/-}$ founders were screened, selected, and backcrossed onto the BALB/c background for more than 10 generations. BALB/c and 4GET (*Il4*–IRES–eGFP; stock no. 004190) mice and *Stat6*$^{-/-}$ (stock no. 002828) mice were purchased from the National Cancer Institute (Bethesda, Md) and Jackson Laboratories (Bar Harbor, Me), respectively, and maintained in our animal facility under specific pathogen-free conditions. *Il17rb*$^{-/-}$ mice were crossed with 4GET mice to generate *Il17rb*$^{-/-}$/4GET mice. *Il13*$^{-/-}$ mice were a kind gift of Dr Andrew McKenzie. All genetically modified mice used in this study were backcrossed onto the BALB/c background for more than 10 generations. All mice were used at 8 to 9 weeks of age, and all experiments used age- and sex-matched control mice to account for any variations in data sets compared across experiments. All animal experiments were approved by the Animal Care and Use Committee of Cincinnati Children's Hospital Medical Center and performed in accordance with institutional guidelines.

LP mononuclear cell isolation

Small intestines were isolated, cut, and washed before vigorous vortexing to remove epithelial and intraepithelial cells. After enzyme digestion with collagenase A (Roche, Mannheim, Germany) and DNase I (Roche) at 37°C for 30 minutes and removal of tissue debris, liberated cells were suspended in 44% Percoll and loaded on 67% Percoll for centrifugation. LP cells were collected from the interface for subsequent analysis.

Flow cytometric analysis and cell sorting

LP cells from the small intestines were first stained with phycoerythrin (PE)–conjugated anti-CD127 (A7R34), allophycocyanin (APC)–conjugated anti–IL-17RB or anti-KLRG1 (2F1/KLRG1), V500-conjugated anti-CD4 (GK1.5), PE-Cy7–conjugated anti-CD3e (145-2C11), fluorescein isothiocyanate (FITC)–conjugated anti-KLRG1 or GFP (for phenotype, anti-CD44 [IM7], CD62L [MEL-14], CD69 [H1.2F3], Thy1.2 [30-H12], ICOS [C398.4A], and Sca-I [D7]), biotinylated anti–T1/ST-2 (DJ8) or anti-IL-7Rα, and APC-Cy7–conjugated anti–c-KIT (2B8) antibodies. Subsequently, cells were counterstained with PerCP-Cy5.5–conjugated mAbs against lineage (Lin) markers (CD11b [M1/70], CD11c [HL3], CD8 [53-6.7], B220 [RA3-6B2], Gr-1 [RB6-8C5], and CD335 [NKP46, 29A.4]) and with Brilliant Violet 421–labeled Streptavidin (BioLegend, San Diego, Calif) before analyses with a FACSCanto II (BD Biosciences, San Jose, Calif) or cell sorting with a FACSAria II (BD Biosciences). After lysis of red blood cells, peripheral blood cells were stained with Brilliant Violet 421–labeled Siglec-F, FITC-conjugated CCR3, APC-conjugated CD11b, and PerCP-Cy5.5–conjugated mAbs against CD11c, CD4, CD8, B220, and Gr-1 for the analysis of blood eosinophils. For intracellular cytokine analyses, Lin^{+} LP cells from BALB/c mice were labeled with microbeads conjugated with mAbs against CD11b, CD8α, and B220 and then removed with a MACS column (Miltenyi Biotech, Bergisch Gladbach, Germany). After restimulation with phorbol 12-myristate 13-acetate/ionomycin or IL-25 and treatment with Golgi blocker, enriched Lin^{-} LP cells were stained with APC-conjugated anti–IL-17RB, APC-Cy7–conjugated anti-CD3e, V500-conjugated anti-CD4, PE-Cy7–conjugated anti-FcεRIα, and biotinylated anti–IL-7Rα antibodies. Subsequently, cells were counterstained with PerCP-Cy5.5–conjugated mAbs against Lin markers, as described above, and with Brilliant Violet 421-labeled streptavidin. Stained cells were fixed and permeabilized for intracellular cytokine staining by using FITC-conjugated anti–IFN-γ (XMG 1.2) and PE-conjugated anti–IL-13 (eBio13A).

Measuring cytokines and mediators

Sorted ILC2s (1×10^4) and CD4^{+} T_H2 cells (1×10^4) from mice with active allergic diarrhea were cultured with IL-25 (50 ng/mL) in the presence or absence of IL-2 (50 ng/mL) and IL-7 (50 ng/mL; R&D Systems, Minneapolis, Minn) for 3 days. Enriched LP cells (3×10^5 cells per well) from the small intestines of WT mice or WT mice reconstituted with WT BM, *Rora*$^{-/-}$ BM, or *Il17rb*$^{-/-}$ BM with or without active allergic diarrhea were cultured with IL-25 (50 ng/mL) in 96-well plates for 3 days before collection of supernatants to examine IL-25 responsiveness. In some experiments sorted ILC2s (1×10^4 cells/well) from WT mice that were injected daily with 0.4 μg of recombinant mouse IL-25 (R&D Systems) for 4 days were cultured with or without *in vitro*–derived CD4^{+} T_H2 cells (4×10^4 cells/well) in the presence of IL-25 or IL-25 plus αIL-2 mAbs (clone: S4B6-1; Bio X Cell, West Lebanon, NH) for 3 days before collection of supernatants. Secreted cytokines in supernatants were assessed by using ELISA kits for IL-4, IFN-γ (BD PharMingen), IL-5 (R&D Systems), and IL-13 (Antigenix America, Huntington Station, NY).

RNA isolation and quantitative real-time PCR analysis

RNA from sorted cell populations was isolated by using an RNeasy Plus Mini kit (Qiagen, Hilden, Germany) and served as templates to synthesize cDNA by using the iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, Calif). Quantitative real-time PCR analyses were performed with SYBR Green Chemistry (Applied Biosystems, Foster City, Calif) in an ABI Prism 7900 detection system by using previously described primer sets[E2-E6] or the following primer sets: *Gapdh* (forward 5′-TGCACCACCAACTGCTTAGC, reverse 5′-GGCAT GGACTGTGGTCATGAG), *Il13* (forward 5′-TGACCAACATCTCCAATTGCA, reverse 5′-TTGTTATAAAGTG GGCTA CTTCGATTT), *Il25* (forward 5′-TGTACCAGGCTGTTGCATTC, reverse 5′-CTCCACTTCAGCCACTCCTC), *Tarc* (forward 5′-CAGGAAGTTGGTGAGCTGGT, reverse 5′-GGGTCTGCACAGATGAGCTT), *Cxcl1* (forward 5′-TAGGGTGAGGACATGTGTGG, reverse 5′-AAATGTCCAAGGGA AGCGT), *Ccl11* (eotaxin 1, forward 5′-CCCAACTTCCTGCTGCTTTA, reverse 5′-AGATCTCTTTGCCCAACCTG), and *Ccl24* (eotaxin 2, forward 5′-CTCCTTCTCCTGGTAGCCTGCG, reverse 5′-GTGATGAAGATGACCCCTGCCTT). Expression levels of target genes were normalized to endogenous *Gapdh* transcript levels, and relative quantification of samples was compared with the lowest expression level as the baseline.

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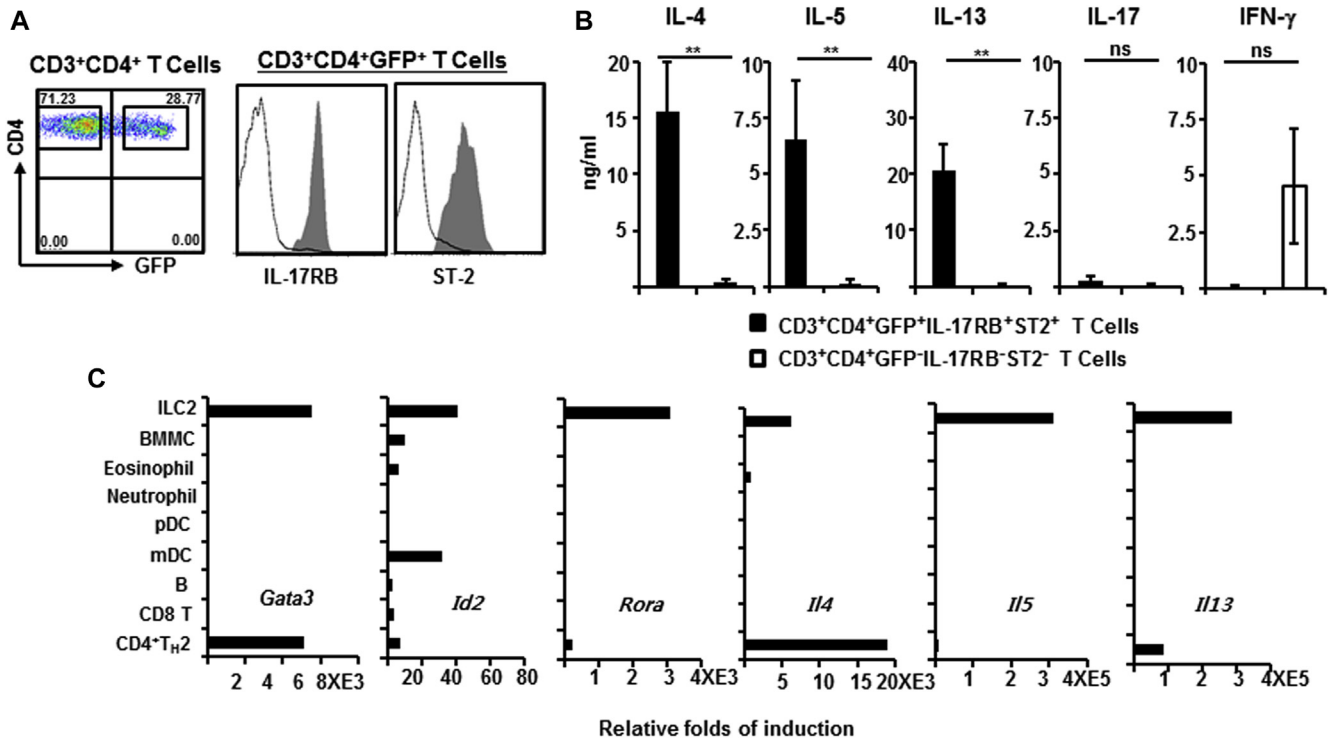

FIG E1. A, Detection of and phenotypic analysis of $CD3^+CD4^+$ T cells in LP of the small intestine of 4GET mice after 6 intragastric OVA challenges. **B,** Measurements by using ELISA of indicated cytokines secreted by sorted indicated T-cell subsets (1×10^5 cells) after stimulation with CD3 and CD28 for 24 hours. **C,** Expression of indicated genes by intestinal ILC2s, $CD4^+$ T_H2 cells, eosinophils, and a panel of other indicated hematopoietic cell lineages from spleens of mice with active allergic diarrhea, as well as by *in vitro*–generated bone marrow-derived mast cells *(BMMCs)*, was analyzed by means of quantitative real-time PCR with primers referenced in the Methods section. Data are expressed as relative fold difference and represent one of 3 independent experiments. *mDC*, Myeloid dendritic cells ($CD11c^{hi}CD11b^{hi}CD4^+$); *pDC*, plasmacytoid dendritic cells ($CD11c^{med}CD11b^-Gr\text{-}1^+B220^+$). Data represent one of 3 independent experiments. *Error bars* denote means ± SEMs. $^{**}P \leq .01$. *ns*, Not significant.

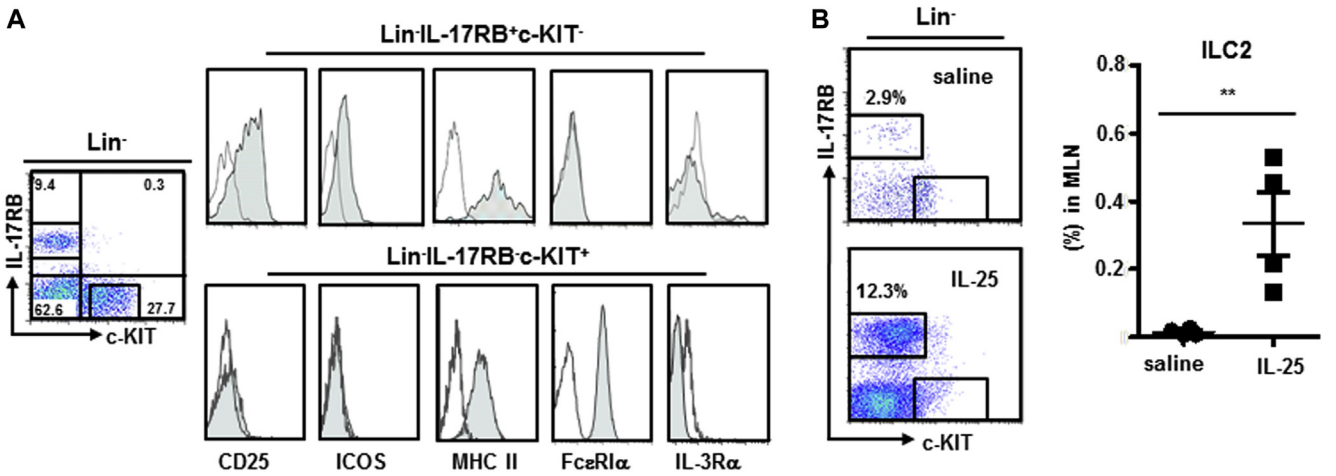

FIG E2. A, Phenotype of Lin$^-$IL-17RB$^+$c-KIT$^-$ and Lin$^-$IL-17RB$^-$c-KIT$^+$ cells in LP of the small intestine from mice with experimental food allergy was analyzed by using flow cytometry. **B,** Detection and frequency of ILC2s in mesenteric lymph nodes of BALB/c mice injected with recombinant IL-25 (0.5 mg) for 4 consecutive days. *Filled histograms* represent staining of indicated markers on indicated cell subsets (Fig E2, *A*); *open histograms* represent isotype control. Data represent one of 3 independent experiments (n = 4 mice per group). *Error bars* denote means ± SEMs. $^{**}P \leq .01$.

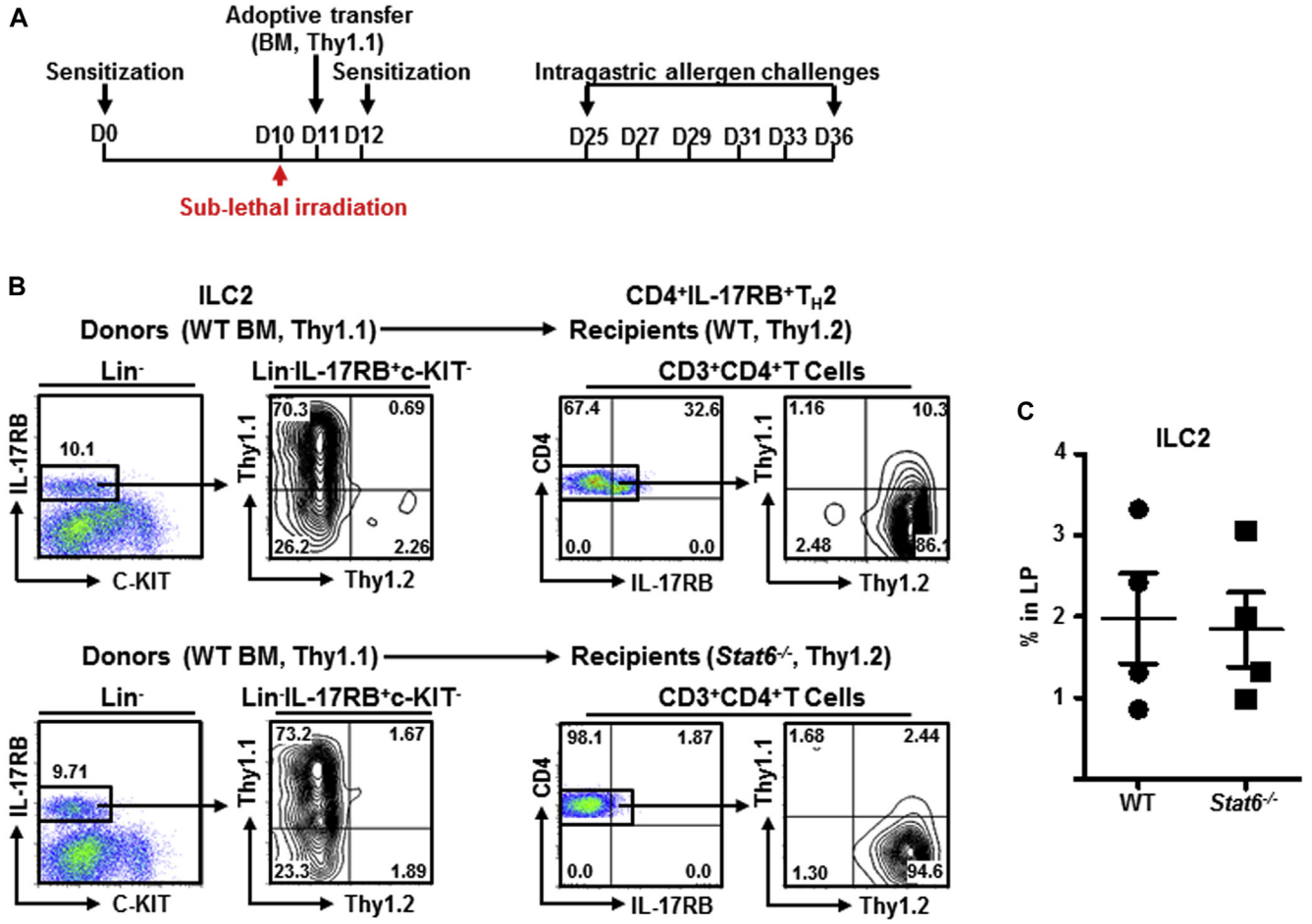

FIG E3. Origin of intestinal ILC2s and $CD4^+$ T_H2 cells in irradiated recipient mice after reconstitution in a murine model of food allergy. **A,** Diagram of adoptive transfer protocol in the mouse model of food allergy. **B** and **C,** Detection (Fig E3, *B*) and frequency (Fig E3, *C*) of donor-derived ILC2s (Lin^-IL-$17RB^+$c-$KIT^-$$Thy1.1^{lo/hi}$$Thy1.2^-$) and recipient-derived $CD4^+$$Thy1.1^-$$Thy1.2^+$ T cells in the small intestines of irradiated recipient $Thy1.2^+$ WT or *Stat6*$^{-/-}$ BALB/c mice reconstituted with BM progenitors from WT $Thy1.1^+$ BALB/c mice after 6 intragastric OVA challenges. Data represent one of 3 independent experiments (n = 4 mice per group).

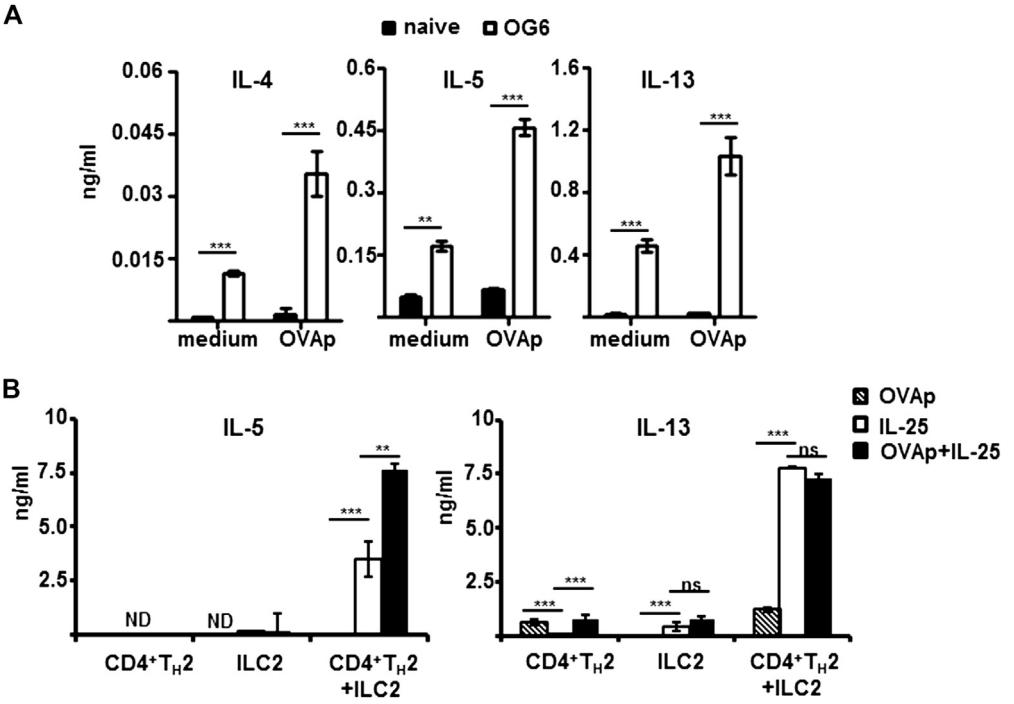

FIG E4. A, Measurement of indicated cytokines produced by LP cells of naive mice or mice with experimental food allergy after culture with medium only or $OVA_{323-339}$ peptides (10 μmol/L) for 3 days by using ELISA. **B,** Measurement of IL-5 and IL-13 production by ILC2s, $CD4^+$ T_H2 cells, or ILC2s cocultured with $CD4^+$ T_H2 cells after stimulation with $OVA_{323-339}$ peptides, IL-25, or both for 3 days by using ELISA. Data are representative of 2 independent experiments. *Error bars* denote means ± SDs. $^{**}P \leq .01$ and $^{***}P \leq .001$. *N. D.*, Not detected; *ns*, not significant.

Hematopoietic prostaglandin D synthase: Linking pathogenic effector CD4$^+$ T_H2 cells to proeosinophilic inflammation in patients with gastrointestinal allergic disorders

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Key words: *Eosinophilic gastrointestinal disorders, immunology, pathogenic effector T_H2 cells, type 2 innate lymphoid cells*

Eosinophilic gastrointestinal disorders (EGIDs) are characterized as gastrointestinal disorders associated with overproduction of local T_H2 cytokines, including IL-4, IL-5, and IL-13.[1] Although recent studies have identified multiple hematopoietic cell types to be involved in adverse allergic immune responses in the gastrointestinal tract, the primary cellular sources of T_H2 cytokines and their interactions in the pathogenesis of EGIDs remain elusive.[1] Mechanistically, IL-4 secretion results in immunoglobulin class-switching to IgE in B cells[2] and induction of alternatively activated macrophages.[3] Production of IL-5 promotes the development, function, and survival of eosinophils,[4] and IL-13 induces dysregulated expression of epithelial cell–derived genes, including eotaxin 3,[5] desmoglein-1,[6] and *LRRC31*,[7] which have been shown to be involved in eosinophil recruitment and esophageal epithelial barrier functions. In addition, epithelial cell–derived T_H2-promoting cytokines, including thymic stromal lymphopoietin (TSLP), IL-25, and IL-33, are likely to be potential mediators involved in the induction of gastrointestinal allergic diseases.[8,9]

TSLP is considered one of the key genes linked to susceptibility to eosinophilic esophagitis,[10] possibly by instructing dendritic cells to induce CD4$^+$ T_H2 cells to differentiate[11] and maintain the CD4$^+$ T_H2 central memory/effector cell pool.[12]

IL-25 or IL-17E, a distinct member of the IL-17 cytokine family, functions to amplify the type 2 immune response against parasitic infection and allergic inflammation in the airway and gastrointestinal tract[8,13] by enhancing effector function of antigen-experienced CD4$^+$ T_H2 memory/effector cells, particularly their IL-5 production.[14,15] During cellular stress and tissue injury, release of the biologically active form of IL-33, a nuclear cytokine of the IL-1 family, can also activate CD4$^+$ T_H2 cells to produce T_H2 cytokines.[9] Furthermore, these T_H2-promoting innate cytokines also have a key role in initiating allergic reactions at mucosal sites by activating the newly described type 2 innate lymphoid cells (ILC2s) to produce prodigious amounts of IL-5 and IL-13 cytokines.[16] Accumulating evidence suggests a collaborative relationship between the antigen-experienced CD4$^+$ T_H2 cells and innate ILC2s,[17-19] which are potentially involved in the pathogenesis of EGIDs. Thus, a better defined marker to identify human CD4$^+$ T_H2 cells and ILC2s in the gastrointestinal tract will be instrumental in advancing our understanding of EGIDs.

One of the key surface markers to identify human circulating CD4$^+$ T_H2 cells and ILC2s is the chemoattractant receptor-homologous molecule expressed on T_H2 cells (CRTH2),[20,21] which is also expressed by eosinophils, basophils, and some CD8$^+$ cells.[22,23] CRTH2 is a G protein–coupled receptor for prostaglandin D_2 (PGD_2) that can selectively induce chemotaxis of CD4$^+$ T_H2 cells and eosinophils.[24] Previous studies demonstrate that the frequency of CRTH2$^+$CD4$^+$ T cells is increased in the bronchial lavage fluid and blood of patients with asthma, atopic dermatitis, or both.[12,20] Notably, treatments with CRTH2 antagonists can ameliorate the characteristics of asthma and eosinophilic esophagitis in murine and/or human studies.[25-27] Building on previous findings, in this issue of the *Journal*, Mitson-Salazar et al[28] report that a subpopulation of CD4$^+$CRTH2$^+$ T_H2 cells that express high levels of surface CD161 and intracellular hematopoietic prostaglandin D synthase (hPGDS) are the *bona fide* CD4$^+$ T_H2 cytokine producers, which are termed pathogenic effector T_H2 (peT_H2) cells. Indeed, intracellular cytokine analysis reveals that these hPGDS-expressing CD4$^+$CRTH2$^+$CD161$^+$ cells are capable of producing higher levels of intracellular IL-5 and IL-13 cytokines after phorbol 12-myristate 13-acetate/ionomycin stimulation than other CD4$^+$CRTH2$^+$ subsets lacking hPGDS. Notably, most of the CD4$^+$CRTH2$^+$ cells within the antrum, duodenum, and esophagus of patients with EGIDs express hPGDS, suggesting that infiltrated CD4$^+$ T_H2 cells at

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Supported by National Institutes of Health (NIH) grant AI112626-01 and the Department of Defense (Investigator-Initiated Research Award; to Y.-H.W.) and NIH grants AI124355, R37 AI045898, and NIDDK P30 DK078392 of the Digestive Diseases Research Core Center in Cincinnati; the Campaign Urging Research For Eosinophilic Diseases (CURED) Foundation; the Buckeye Foundation; the Food Allergy Research Education Fund; the American Partnership for Eosinophilic Disorders (APFED); and the Sunshine Charitable Foundation and its supporters, Denise A. Bunning and David G. Bunning (to M.E.R.).

Disclosure of potential conflict of interest: T. Wen is an inventor of patents submitted to and owned by Cincinnati Children's Health Medical Center. M. E. Rothenberg has received a grant from the National Institutes of Health (NIH); has received payment to his institution from the CURED Foundation, the Buckeye Foundation, and Food Allergy Research and Education (FARE); has consultant arrangements with Receptos, Celsus Therapeutics, Genentech/Roche, NKT Therapeutics, and Merck; is an inventor of patents submitted and owned by Cincinnati Children's Health Medical Center; has received royalties from Teva Pharmaceuticals; has stock/stock options in Immune Pharmaceuticals, Receptos, Celsus Therapeutics, NKT Therapeutics; and has received travel support from Merck. Y.-.H Wang has received a grant from the NIH.

Received for publication November 12, 2015; accepted for publication November 20, 2015.

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J Allergy Clin Immunol 2016;137:919-21.
0091-6749/$36.00

http://dx.doi.org/10.1016/j.jaci.2015.11.032

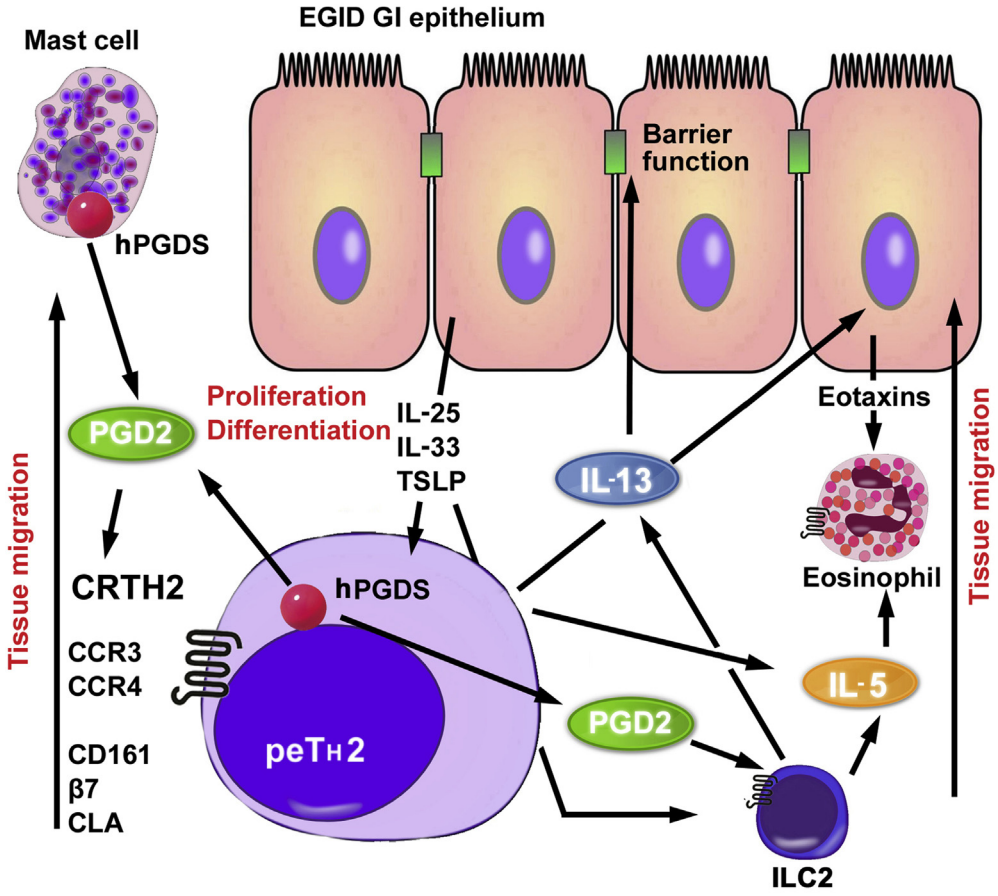

FIG 1. Proposed immunologic mechanism involved in the pathogenesis of EGIDs. Enhanced epithelial IL-25, IL-33, and TSLP production during T_H2 inflammation in tissue of patients with EGIDs triggers peT_H2 cells to initiate a dysregulated T_H2 cytokine surge. peT_H2 cells, as well as mast cells, provide ample amounts of PGD_2 through hPGDS to induce chemotaxis and activation of eosinophils and ILC2s, which produce more T_H2 cytokines and thereby form a self-enhancing loop. hPGDS bridges the mast cell–peT_H2–ILC2 axis to amplify proeosinophilic inflammation in patients with gastrointestinal allergic disorders. *β7*, β7 Integrin; *CLA*, cutaneous lymphocyte antigen; *GI*, gastrointestinal.

sites of eosinophilic inflammation are the potent T_H2 cytokine producers, peT_H2 cells. It has been well documented that PGD_2, which is primarily produced by mast cells, is a potent mediator to promote airway allergic inflammation.[29,30] A recent study further demonstrates that PGD_2 can induce the migration and activation of ILC2s to release IL-5 and IL-13 cytokines.[31] The new observations in this report point to a plausible notion that the hPGDS-expressing peT_H2 cells not only produce IL-5 to activate eosinophils but also have the potential to release PGD_2, which recruits and activates CRTH2-expressing ILC2s. Together with previous reports showing that the *TSLP* gene links to susceptibility to the most common EGID, eosinophilic esophagitis,[10] and that TSLP-activated dendritic cells induce upregulation of hPGDS expression by $CD4^+$ T_H2 memory/effector cells,[12] these intriguing findings suggest that the TSLP/CRTH2/hPGDS/PGD_2 axis might potentiate a collaborative interaction between peT_H2 cells and ILC2s to amplify eosinophilic inflammation, resulting in development of EGIDs (postulated in Fig 1).

Detailed characterizations further reveal that peT_H2 cells express increased levels of *TSLPR*, *IL17RB*, and *IL1RL1* transcripts, suggesting that these pathogenic cells might respond directly to the epithelium-derived, T_H2-promoting cytokines TSLP, IL-25, and IL-33 at the inflamed mucosal sites of patients with EGID. Indeed, purified peT_H2 cells respond to combined IL-25, IL-33, and IL-2 stimulation by expressing increased *IL5*, *IL9*, and *IL13* transcripts *ex vivo*. Consistent with previous studies in human subjects,[32] TSLPR expression is found to be upregulated in peT_H2 cells or activated $CD4^+$ T cells. Whether TSLP functions directly to induce increased T_H2 cytokine production by peT_H2 cells or acts as an IL-7–like cytokine to enhance survival of peT_H2 cells remains to be investigated. On the basis of these findings, the authors imply that the environmental cues at the inflamed mucosal sites might endow infiltrated classical $CD4^+$ T_H2 cells with the ability to develop into proallergic peT_H2 cells with functional resemblance to ILC2s by upregulating the expression of innate cytokine receptors. However, because of the limitation of the intracellular cytokine staining approach in this study, the capacity of purified peT_H2 cells to produce IL-5 and IL-13 in response to these T_H2-promoting cytokines cannot be evaluated. Thus, the current study cannot conclude whether peT_H2 cells are the major IL-5 producers in the gastrointestinal tract, especially because ILC2s are known to be the most potent IL-5 and IL-13 producers.[33] Perhaps these antigen-experienced peT_H2 cells are

responsible for induction of the allergic immune response driven by food ingestion and recruit ILC2s to amplify eosinophilic inflammation at the mucosal sites. Indeed, clinical studies demonstrate that the symptoms of EGIDs can be ameliorated after eliminating instigating foods from the diet,[34] suggesting the importance of antigen-specific peT_H2 cells in regulating the pathogenesis of EGIDs.

In summary, this study identifies hPDGS as a reliable marker to define peT_H2 cells at proeosinophilic inflammatory sites and, potentially, to be a diagnostic tool for EGIDs. These antigen-experienced peT_H2 cells are not only potent T_H2 cytokine producers but also capable producers of PGD_2, which might recruit other CRTH2-expressing cells, such as ILC2s, eosinophils, and basophils, thereby amplifying allergic eosinophilic inflammation. Future characterizations of peT_H2 cells by using the hPGDS biomarker and transcriptome and epigenetic approaches will advance our understanding of the roles of peT_H2 cells in the pathogenesis of human allergic disorders.

We thank Shawna Hottinger for editorial assistance.

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Immunity, Inflammation and Disease

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ORIGINAL RESEARCH

Loss of IL-4Rα–mediated PI3K signaling accelerates the progression of IgE/mast cell–mediated reactions

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Keywords
food-induced anaphylaxis, IgE and mast cells, interleukin 4 (IL-4) receptor (IL-4R) chain

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Funding information This work was supported by NIH R01 AI073553, R01 DK090119, P30 DK078392 (S.P.H.); U19 A1070235, Crohns Colitis Foundation of America and a Food Allergy Research Education Award.

Received: 27 March 2015; Revised: 27 July 2015; Accepted: 10 August 2015
Final version published online 17 September 2015.

Immunity, Inflammation and Disease
2015; 3(4): 420–430

doi: 10.1002/iid3.80

Abstract

Clinical and experimental evidence indicate that polymorphisms within the interleukin 4 (IL-4) receptor (IL-4R) chain are sufficient for altered strength of IL-4/IL-13 signaling, leading to an exaggerated allergic inflammatory response and increase susceptibility to allergic phenotypes. In the present study, we show that ablation of IL-4Rα–induced phosphatidylinositol 3-kinase (PI3K) activating signal by germline point mutation within the IL-4Rα motif (Y500F) did not alter susceptibility to IgE-mediated, food-induced experimental anaphylaxis. Moreover, diarrhea occurrence, antigen-specific IgE and intestinal mastocytosis were comparable between WT and IL-4Rα^{Y500F} mice. However, mice unable to stimulate IL-4Rα–mediated PI3K signaling had accelerated disease progression. Notably, the accelerated anaphylactic response was associated with more rapid histamine-induced hypovolemia. Mechanistic in vitro and in vivo analyses revealed that endothelial IL-4Rα PI3K signaling negatively regulates the histamine-induced endothelial leak response. These results define an unanticipated role for IL-4Rα–mediated PI3K signaling in negative regulation of IgE-mediated anaphylactic reactions.

Introduction

Food allergy is currently on the rise in the Western world: the prevalence of pediatric peanut allergy has doubled from 1997 to 2002 [1–4], and the Centers for Disease Control and Prevention has recently documented an 18% increase in the prevalence of reported food allergy in children from 1997 to 2007 [5]. Severe food allergy-related reactions are most often caused by peanuts (50–62%) and tree nuts (15–30%) [6], placing 2.7–5.4 million people at risk for food-induced anaphylaxis.

A food-induced anaphylactic reaction encompasses a variety of symptoms that may affect one or more target organs including gastrointestinal, cutaneous, respiratory, and cardiovascular systems [7, 8]. Clinical and experimental analyses have identified a central role for IgE/FcεR/mast cells and mast cell-derived mediators, including histamine, platelet-activating factor (PAF), serotonin, proteases (tryptase and chymase), lipid-derived mediators (prostaglandins [PGD_2] and leukotrienes [LTC_4, LTD_4, and LTE_4]), in promoting the clinical manifestations associated with food-triggered anaphylaxis [9–15]. The interleukin (IL)-4 /IL-13 signaling pathway is integral to the food allergic reaction via regulating $CD4^+$ Th2 responses, IgE synthesis and mast cell and vascular endothelial cell function [16, 17]. Indeed, targeted ablation of IL-4/IL-13 signaling alleviates IgE-mediated, food-induced allergic reactions [16].

The biological activity of IL-4 and IL-13 is regulated via receptor (R) binding: IL-4 can bind the type I (IL-4Rα chain and γ_c chain) and type II (IL-4Rα chain and IL-13Rα1 chain) IL-4R, and IL-13 can bind the type II IL-4R and type II IL-13R (IL-13Rα1 and IL-13Rα2 chains). Ligand (IL-4 and/or IL-13) interaction with the type I IL-4R and type II IL-4R induces downstream signaling including the signal transducer and activator of transcription (STAT) 6 and phosphatidylinositol 3-kinase (PI3K) pathways. Phosphorylation of Y575, Y603 and Y633 of human IL-4Rα mobilizes the transcription factor STAT-6, which induces IL-4- and IL-13-responsive genes [18–20]. Phosphorylation of Y497 of IL-4Rα, which is part of the IL-4R motif necessary for recruiting insulin receptor substrate (IRS) 1 and IRS-2, activates the PI3K and mitogen-activated protein kinase (MAPK) pathways and mediates IL-4 proliferative activity [20]. Y713 of IL-4Rα is part of an immunoreceptor tyrosine-based inhibition motif (ITIM) that binds Src homology 2 (SH2) domain-containing phosphatases, including SH2 domain-containing tyrosine phosphatase (SHP) 1 and SHP-2, and inositol phosphatases and thereby negatively regulates IL-4/IL-13 responses [21–23].

Clinical studies have identified a number of atopic susceptibility genes linking polymorphisms in the IL-4R/IL-13 axis with atopic diseases including food allergy and asthma [24, 25]. This has been supported by corroborative evidence provided by studies employing mice deficient in components of the IL-4/IL-13 signaling pathway and knock-in murine models demonstrating that disruption of individual signaling domains within the IL-4Rα in mice can amplify IgE responses and elicits enhanced allergic responses [26–28]. One such mutation is within the part of the IL-4R motif (Y497 of IL-4Rα in humans and Y500 of IL-4Rα in mouse) that regulates PI3K signaling. IL-4Rα^{Y500F} mice possess a germline mutation in the *Il4ra* gene resulting in a loss of IL-4Rα-induced PI3K signaling and leading to impaired IL-4-induced $CD4^+$ T-cell proliferation, increased allergen-induced IgE production and an allergic asthma phenotype [29]. In this study, we examined the effects of the IL-4Rα^{Y500F} mutation on susceptibility of mice to food-induced anaphylaxis. Unexpectedly, we show that loss of IL-4Rα-induced PI3K signaling did not alter susceptibility to IgE-mediated food-induced reactions but rather increased histamine-induced endothelial leak response and accelerated disease progression.

Materials and Methods

Animals

Wild-type (WT) BALB/c and IL-4Rα^{Y500F} (BALB/c) were originally provided by The Jackson Laboratory, Bar Harbor, ME, USA [29]. The mice were crossed to generate heterozygotes (F_1 IL-4R$\alpha^{Y500F/WT}$) and subsequently back-crossed to generate age-, sex-, and litter-matched IL-4Rα WT and IL-4R$\alpha^{Y500F/Y500F}$ mice as described [29]. The mice were maintained in a barrier facility, and animals were handled under Institutional Animal Care & Use Committee-approved protocols from Cincinnati Children's Hospital Medical Center.

Oral antigen-induced anaphylaxis

Six- to 8-week-old mice were sensitized subcutaneously with 50 μg of ovalbumin (OVA) (Sigma–Aldrich, St. Louis, MO, USA) in the presence of 2 mg of aluminum potassium sulfate adjuvant (alum: $AIK(SO_4)_2$-$12H_2O$) (Sigma–Aldrich) in sterile saline. Two weeks later, mice were deprived of food for 5 h and received repeated intragastric (i.g) challenge of OVA (50 mg/250 μL saline) via i.g. feeding needles (Fisher Scientific Co., Pittsburgh, PA, USA). Rectal temperature was monitored at 0, 10, 15, 30, 45, and 60 min following the sixth or seventh OVA challenge with a rectal probe (Physitemp Model BAT-12) as previously described [30]. In some experiments, mice were administered an i.v. (final volume 200 μL) injection with the histamine receptor antagonists Triprolidine (200 μg) and Cimetidine (200 μg) 30 min prior to OVA challenge.

IL-4- and histamine-induced hypothermia

Histamine biphosphate monohydrate (Sigma–Aldrich) (25 μg/1 mL saline per mouse) and/or IL-4C (recombinant, IL-4-neutralizing, anti–IL-4 monoclonal antibody [mAb] complex produced by mixing recombinant mouse IL-4 with an anti-IL-4 mAb [BVD4-1D11] at a 2:1 molar [1:5 weight] ratio, which saturates the mAb with IL-4. We have previously demonstrated that these complexes have an in vivo half-life of approximately 1 day and slowly dissociate, releasing biologically active IL-4 [31]. IL-4C or histamine was i.v. injected, and body temperature was monitored by rectal thermometry every 10 min for 60 min, as we have previously described [30].

Hematocrit

Blood was drawn from incised mouse tail veins into heparinized capillary tubes and centrifuged for 5 min at 10,000 rpm. Hematocrit (percentage of packed red blood cell [RBC] volume) was calculated as the length of packed RBCs divided by the total length of serum and red cells in the capillary tube and multiplied by 100%, as previously described [15].

Mast cell quantification

Jejunum (7–10 cm distal to the stomach) were collected and fixed in 10% formalin and processed by standard histologic techniques. Longitudinal sections (5 μm) were stained for mast cells with chloroacetate esterase (CAE) activity, as described previously [30]. At least four random sections per mouse per area examined were analyzed. Quantification of stained cells was performed by counting the number of CAE-positive cells in 5 fields for tongue, 10 fields for ear, and 20 fields for intestine (magnification 400×).

Enzyme-Linked Immunosorbent Assay measurements

Mast cell protease 1 (MCPT-1) serum levels were measured by the mouse MCPT-1 ELISA Ready-SET-Go!, according to the manufacturer's instructions (ebioscience, San Diego, CA, USA). Serum total IgE levels were determined using the ELISA MAX Deluxe SET Mouse IgE Kit (Biolegend, San Diego, CA, USA). Serum OVA-specific IgE levels were determined by means of ELISA. Plates were coated with anti-IgE antibody (EM-95; 10 μg/mL; BD PharMingen, San Jose, CA, USA) and blocked with 200 μL of 10% fetal bovine serum (FBS) before adding serial dilutions of plasma samples (100 μL per well). After overnight incubation, plates were washed and incubated with biotinylated OVA (2.5 mg/mL, 100 μL per well). After 1 h of incubation, streptavidin–horseradish peroxidase (1 mg/mL; Biosource, Camarillo, CA, USA) was added and the assay developed with 100 μL of substrate (TMB substrate reagent set; BD OptEIA, San Diego, CA, USA). 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.

In vitro permeability

The human vascular endothelial cell line EA.hy926 (ATCC, Manassas, VA, USA) was maintained in DMEM supplemented with 10% FCS, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 10 mM HEPES and 1X penicillin/streptomycin (Invitrogen, Grand Island, NY, USA) in a humidified incubator (5% CO_2, 37°C). On snap wells (12-mm diameter, 0.4-μm pore; Corning Glass, Corning, NY, USA), 5×10^5 cells were seeded and cultured for 18–21 days under maintenance media conditions as described above. Transendothelial resistance (TER) was monitored with an EVOM/Endohm (WPI Inc, Sarasota, FL, USA), and endothelial monolayers with TER >100 ohms $\cdot$ cm^2 were used for all experiments. Monolayers were mounted between the hemi-chambers of an Ussing apparatus (U2500 dual Ussing chamber, Warner Instruments, Hamden, CT, USA), and 0.112 cm^2 of tissue was exposed to 10 mL of Krebs buffer at 37°C. The transendothelial potential difference was detected with two paired electrodes that contain 4% agar in 3 M KCl. The electrodes were connected to a voltage clamp amplifier (EC-800, epithelial voltage clamp, Warner Instruments, Hamden, CT, USA). The electrode potential difference and fluid resistance were compensated before mounting tissue segments into the chamber. To establish equilibrium, potential difference was continuously monitored under open-circuit conditions for 15 min. Thereafter, the tissues were voltage-clamped at 0 mV while continuously measuring the short circuit current (I_{sc}). Voltage pulses (3-mV square waves sustained for 5 s) were delivered every 50 s to yield a current response for calculation of the resistance across a mucosa from Ohm's law. IL-4 (10 ng/mL)-, histamine (100 μM)- and vehicle-stimulated endothelial monolayers were placed in Ussing chambers in the presence and absence of DHEA (100 nM) and allowed to equilibrate for 15 min; basal I_{sc} and TER were measured as described previously [21].

Western Blot analysis

EA.hy926 cell lysates (30 μg) were loaded in 4–12% BisTris gels and transferred to a nitrocellulose membrane (Invitrogen). P85 PI3K was detected by using rabbit polyclonal anti-p85 PI3K followed by anti-rabbit peroxidase-conjugated antibody (Cell Signaling, Danvers, MA) and ECL-plus detection reagents (GE Healthcare, Pittsburgh, PA). Rabbit anti-actin (Santa Cruz Biotechnology, Santa Cruz, CA) was used as a loading control.

Statistical analysis

Data are expressed as mean ± standard deviation (SD), unless otherwise stated. In experiments comparing multiple experimental groups, statistical differences between groups were analyzed using the one-way ANOVA parametric and a Tukey's multiple comparison post-test. In experiments comparing two experimental groups, statistical differences between groups were determined using a Student's *t*-test. $P < 0.05$ was considered significant. Spearman's rank coefficients were used to quantify the relations between hemoconcentration and hypothermia. All analyses were performed with Prism 5.0 software (GraphPad Software Inc., San Diego, CA).

Results

Susceptibility of IL-4Rα^{Y500F} mice to food-induced anaphylaxis

Previous studies in the IL-4Rα^{Y500F} mice have revealed that the Y500F mutation in the IL-4Rα receptor and loss of

IL-4Rα-mediated PI3K activation increased allergic inflammation and the asthmatic phenotype [29]. To determine the effect of this mutation on susceptibility to food allergy, we assessed intestinal and systemic symptoms of anaphylaxis (diarrhea and hypothermia) in BALB/c WT and IL-4Rα^{Y500F} mice that were sensitized to OVA and then challenged with OVA via oral gavage 14 days later and then every other day for a total of seven challenges. We observed no significant difference in the occurrence of anaphylaxis between WT and IL-4Rα^{Y500F} mice (Fig. 1). After the fourth challenge, 34.6% of WT and 42.1% of IL-4Rα^{Y500F} mice demonstrated symptoms of anaphylaxis, which increased to 77.1% of WT and 84.3% of IL-4Rα^{Y500F} mice following the seventh challenge (Fig. 1A and B). Assessment of systemic symptom and disease severity (hypothermia) revealed no significant difference in the maximal shock response between WT and IL-4Rα^{Y500F} mice after the seventh challenge (Fig. 1B). During these analyses, we observed that the IL-4Rα^{Y500F} mice appeared to develop signs of anaphylaxis earlier than WT mice. Moreover, the IL-4Rα^{Y500F} mice demonstrated evidence of anaphylaxis (scratching and rubbing around the nose and head, decreased activity with an increasing respiratory rate and pilar erecti) earlier than WT mice following the seventh OVA challenge (results not shown). To quantitate these observations, we examined shock response (body temperature) of the WT and IL-4Rα^{Y500F} mice at 0, 15, 30, and 45 min after the seventh OVA oral gavage challenge. Indeed, the IL-4Rα^{Y500F} mice demonstrated a more rapid decrease in body temperature than the WT mice (Fig. 1C). Importantly, by 45 min, there was no significant difference in body temperature between groups (Fig. 1C). These datasets indicate that IL-4Rα^{Y500F} mice do not have increased susceptibility to food-induced anaphylaxis or develop a more severe disease phenotype but rather experience an accelerated disease progression.

In previous studies, we have demonstrated that antigen-specific IgE and intestinal mast cells are the critically important factors in the regulation of food-induced experimental anaphylaxis [30]. Assessing intestinal mast cell levels revealed no differences in number between WT and IL-4Rα^{Y500F} mice (Fig. 1D). Furthermore, we observed no significant difference in the level of mast cell activation

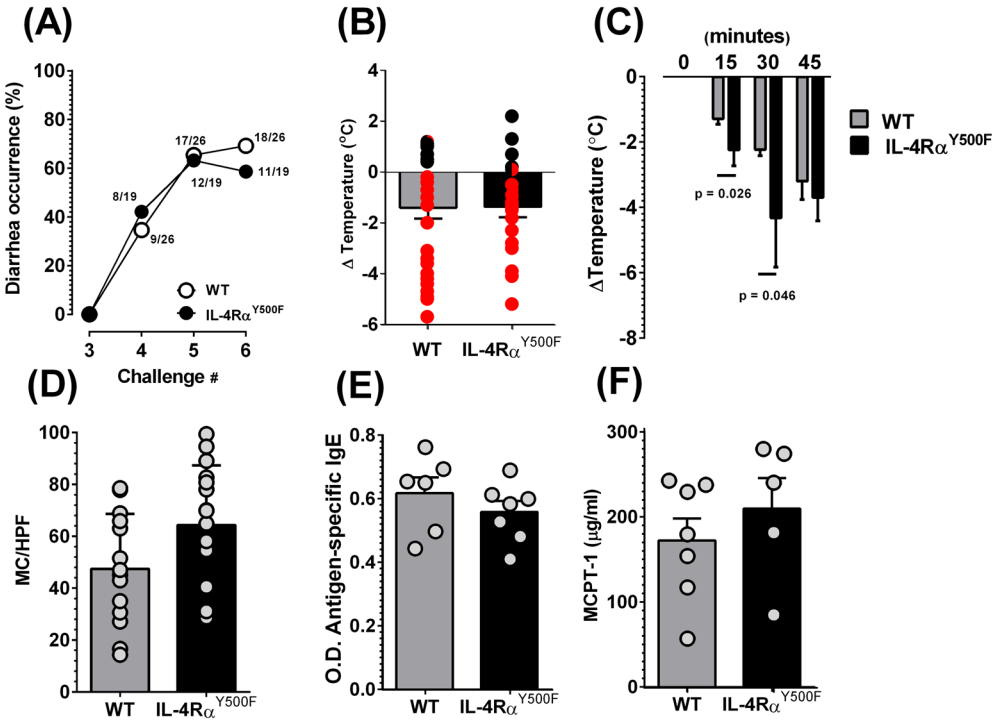

Figure 1. Loss of IL-4Rα-PI3K signaling accelerates progression of an anaphylactic reaction. A: Diarrhea occurrence in OVA-sensitized, intragastric (i.g.) OVA-challenged WT and IL-4Rα^{Y500F} mice. B: Temperature change from 0 to 60 min and C: 0, 15, 30, and 45 min following the seventh intragastric (i.g.) OVA challenge in OVA-sensitized, OVA-challenged WT and IL-4Rα^{Y500F} mice. D: Mast cell (MC) numbers per high power field (HPF) in the small intestine, OVA-specific IgE (E) and mast cell protease 1 (MCPT-1), (F) levels in the serum of OVA-sensitized, OVA-challenged WT and IL-4Rα^{Y500F} mice following the seventh challenge. A: Data are presented as a percentage of diarrhea occurrence after a number of OVA challenges. The fraction indicates the number of mice with diarrhea out of the total number of mice in that group. (B, D–F: Individual circles represent 1 mouse). B: Red circles represent identification of positive intestinal symptoms of anaphylaxis (diarrhea), and black circles represent no evidence of intestinal symptoms of anaphylaxis. B–F: Data represent mean ± SD; $n = 4$–18 mice per group; P values as indicated. O.D., optical density.

(secreted MCPT 1) or of antigen-specific and total IgE in WT and IL-4Rα^{Y500F} mice after the seventh oral gavage challenge (Fig. 1E and F and Fig. S1). We concluded from this that the observed accelerated disease progression cannot be explained by altered IgE and mast cell levels.

In mice, the shock organ is the capillary bed; IgE-mediated, mast cell-dependent anaphylaxis causes capillary bed dilatation and extravasation, leading to severe hypovolemia [32, 33]. A consequence of the hypovolemia-induced shock in mice is hypothermia [16, 34, 35]. Consistent with this concept, we show a direct relationship between hypovolemia (fluid extravasation as measured by hemoconcentration) and severity of oral antigen-induced anaphylaxis (hypothermia) in our mice ($P < 0.0001$), indicating a direct relationship between fluid extravasation and hypothermia associated with a food-induced anaphylactic reaction (Fig. S2). To determine whether the increased progression of food allergy in the IL-4Rα^{Y500F} mice is associated with hypovolemic shock, we examined hypothermia and hemoconcentration in WT and IL-4Rα^{Y500F} mice 10 min following the seventh oral antigen challenge. We observed a significantly stronger hypothermic response in the IL-4Rα^{Y500F} mice than WT mice, and the increased temperature loss was associated with increased hemoconcentration, indicating that the IL-4Rα^{Y500F} mice experience a more accelerated hypovolemic shock response (Fig. 2A and B).

Previous studies have demonstrated that the systemic manifestations of IgE/mast cell-dependent anaphylaxis, particularly the hypothermic component of shock response, is mediated by histamine, as it can be blocked by histamine H1 and H2 receptor antagonism [36]. Pretreatment of WT and IL-4Rα^{Y500F} mice with the histamine H1 and H2 receptor antagonist completely abrogated the oral antigen-induced hypothermia in both WT and IL-4Rα^{Y500F} mice (Fig. S3), suggesting that the accelerated disease progression in the IL-4Rα^{Y500F} mice is a consequence of altered histamine-induced hypothermic response. To determine whether histamine was sufficient to promote accelerated progression of the systemic manifestations of anaphylaxis, naive WT and IL-4Rα^{Y500F} mice received an i.v. injection of histamine, and hypothermia was assessed. Consistent with our OVA-induced anaphylaxis experiments, IL-4Rα^{Y500F} mice experienced an accelerated progression of hypothermia in response to histamine compared to WT mice (Fig. 3). Importantly, we show that administration of equivalent amounts of histamine (25 μg) to WT and IL-4Rα^{Y500F} mice induced a more accelerated response in IL-4Rα^{Y500F} mice, suggesting 1) that histamine is sufficient to promote accelerated progression of the shock response in IL-4Rα^{Y500F} mice and 2) that the observed accelerated response is related in part to altered histamine responsiveness and not histamine levels.

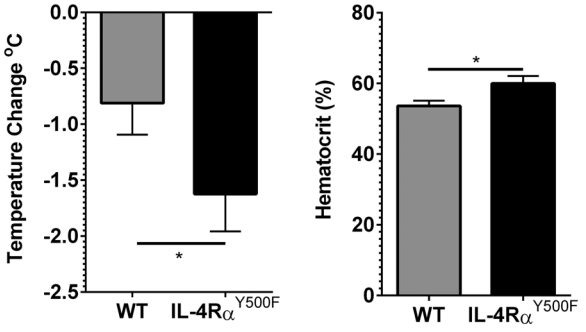

Figure 2. Loss of IL-4Rα-PI3K signaling accelerates progression of hypovolemic shock. A: Temperature change from 0 to 10 min and B: percentage hemacrit levels at 10 min following the seventh intragastric (i.g.) OVA challenge in OVA-sensitized, OVA-challenged WT and IL-4Rα^{Y500F} mice. Data represent mean ± SD; $n = 5$–8 mice per group; $^{*}P < 0.05$.

Increased rate of shock in IL-4Rα^{Y500F} mice in response to histamine

It is postulated that histamine-induced hypothermia is a consequence of vascular endothelial leak and fluid shift into the periphery. Furthermore, previous studies have demonstrated that IL-4 can modulate histamine-induced hypothermia [15]. The demonstration of 1) a direct link between fluid extravasation and hypothermic response in OVA-challenged mice; 2) that fluid extravasation and hypothermic response in OVA-challenged WT and IL-4Rα^{Y500F} mice were dependent on H1 and H2 receptor and 3) an accelerated hypothermic response in the IL-4Rα^{Y500F} mice compared to WT mice led us to speculate that the IL-4Rα/PI3K signaling pathway negatively regulated histamine-induced vascular endothelial leak. To begin to assess this possibility, we examined the effect of IL-4Rα^{Y500F} mutation on IL-4/histamine–induced vascular leak and increased hemoconcentration. WT and IL-4Rα^{Y500F} mice were primed with IL-4C and treated 24 h later with the vasoactive mediator histamine. Histamine treatment of WT mice induced a hypothermic response and increased hematocrit, with the former being amplified by pretreatment with IL-4C (Fig. 4A and B; Average difference between WT Vehicle + Histamine and WT IL-4C + Histamine: -1.05 ± 0.60 ΔTemperature (°C); mean ± SEM; indicated by gray pattern in column). Similarly, histamine treatment of IL-4Rα^{Y500F} mice induced hypothermia and increased hematocrit. The temperature change induced by histamine in the IL-4Rα^{Y500F} mice was significantly greater than that of WT mice (Fig. 4A). Importantly, combined IL-4C and histamine treatment of IL-4Rα^{Y500F} mice caused a significantly greater hypothermic response and increase in hematocrit than that observed in histamine only-treated

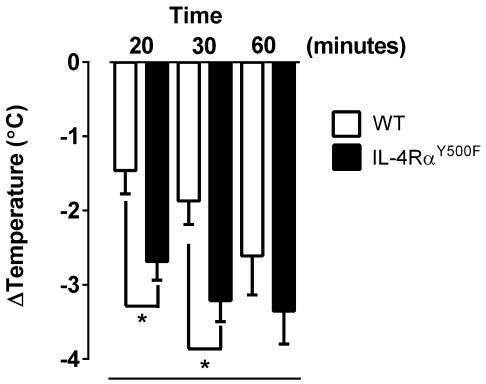

Figure 3. Loss of IL-4Rα-PI3K signaling accelerates progression of histamine-induced hypothermia. Temperature change from 0 to 30 min after i.v. administration of histamine to WT and IL-4Rα^{Y500F} mice. Data represent $n = 4$ mice per group from three independent experiments and mean ± SD; $^{*}P < 0.01$.

IL-4Rα^{Y500F} mice or combined IL-4C- and histamine-treated WT mice (Fig. 4A and B; $P < 0.05$; Average difference between IL-4Rα^{Y500F} Vehicle + Histamine and IL-4Rα^{Y500F} IL4C + Histamine: -2.7 ± 0.66; mean ± SEM; indicated by gray pattern in column). The greater hypothermic and hematocrit response in the absence of PI3K signaling (IL-4Rα^{Y500F}) suggests that IL-4Rα/PI3K signaling negatively regulates histamine-induced vascular endothelial responses.

PI3K signaling negatively regulates histamine-induced hypothermic response

The observation of accelerated progression of OVA-induced and IL-4/histamine–induced vascular leak indicate that the absence of PI3K signaling (IL-4Rα^{Y500F}) accelerates and/or enhances histamine-induced vascular endothelial responses. On the basis of these datasets, one would speculate that stimulating vascular endothelial PI3K signaling would attenuate histamine-induced vascular leak. Dehydroepiandrosterone (DHEA), an adrenal steroid that acts as a precursor in the biosynthesis of testosterone and estrogen, has also been implicated in regulating vascular endothelial cell function [37, 38]. Notably, DHEA-mediated effects are predominantly induced via G-protein coupled receptor (GPCR)-stimulated, PI3K/AKT-dependent activation of FOXO1 [37]. We therefore speculated that exposure of mice to DHEA would induce vascular endothelial cell PI3K activation and subsequently attenuate histamine-induced vascular endothelial leak. Firstly, we confirmed that histamine and DHEA stimulation of endothelial cells induce PI3K activation. To do this, we examined phosphorylation of the p85 subunit of PI3K in the human vascular endothelial cell line EA.hy926, which is derived from A549 and HUVEC cells and used as a model of systemic endothelial cells [39], following histamine and DHEA stimulation. We demonstrate increased phosphorylation of the p85 subunit of PI3K between 5 and 15 min following histamine (100 μM) and DHEA (100 nM) stimulation (Fig. S4). Next, WT and IL-4Rα^{Y500F} mice were pretreated with vehicle or DHEA and IL-4C and received i.v. histamine treatment 24 h later, after which hypothermia was evaluated. Histamine treatment induced a hypothermic response in WT and IL-4Rα^{Y500F} mice, with the response being significantly greater in the IL-4Rα^{Y500F} mice (Fig. 5; $P < 0.05$). Notably, pretreatment with DHEA did not significantly alter the level of hypothermia in histamine-treated WT mice but did significantly attenuate the level of hypothermia in histamine-treated IL-4Rα^{Y500F} mice (Fig. 5; $P < 0.05$). These data suggest that constitutive PI3K activation can attenuate the

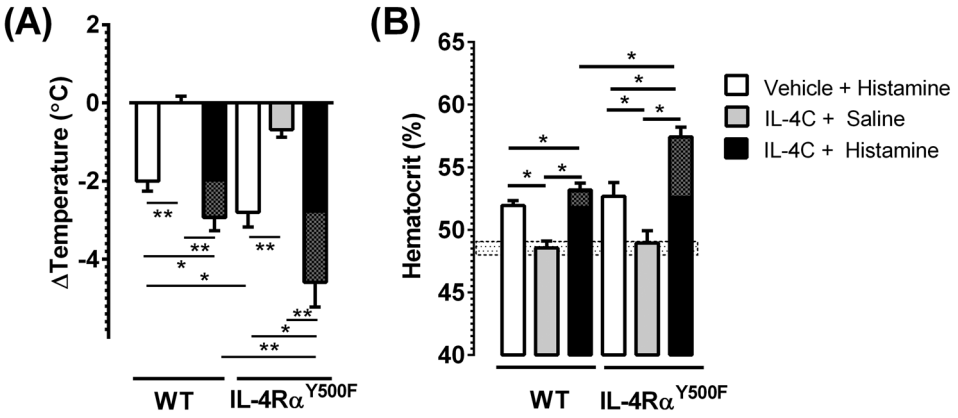

Figure 4. IL-4 attenuation of histamine-induced hypothermia is alleviated in IL-4Rα^{Y500F} mice. A: Temperature change from 0 to 30 min and B: hematocrit at 60 min after i.v. administration of IL-4C and/or histamine to WT and IL-4Rα^{Y500F} mice. B: Hatched box indicates average hematocrit level of WT BALB/c mice. Grey checkered box within columns indicates difference between Vehicle + Histamine and IL-4C + Histamine within the respective strains. Data represent mean ± SD. $n = 6$–18 mice per group. $^{*}P < 0.05$; $^{**}P < 0.01$.

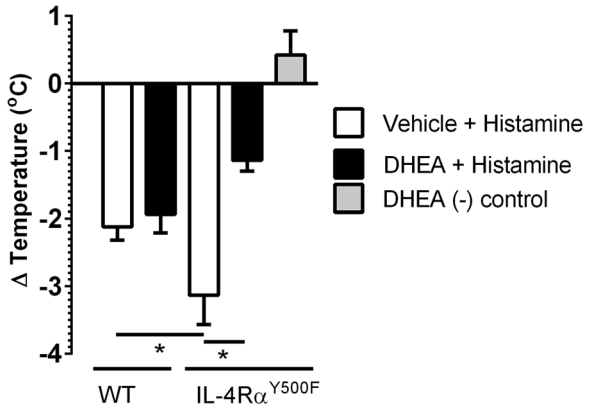

Figure 5. DHEA attenuates histamine-induced hypothermia in IL-4Rα^{Y500F} mice. Temperature change from 0 to 30 min after i.v. administration of histamine to WT and IL-4Rα^{Y500F} mice after pretreatment with vehicle or DHEA (500 μg). Data represent mean ± SD; $n = 3$–8 mice per group from $n = 2$ experiments. $^{*}P < 0.01$.

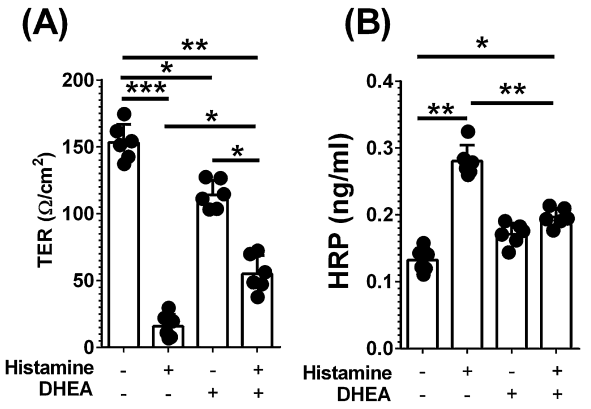

Figure 6. DHEA attenuates histamine-induced paracellular leak in human vascular endothelial cell line EA.hy926. A: Transendothelial resistance (TER) and B: HRP flux in DHEA-treated human vascular endothelial cell line (EA.hy926) after histamine stimulation. Confluent (>100 Ω/cm^2) vascular endothelial cells treated with vehicle or DHEA (100 nM) were stimulated with histamine (100 μM) for 30 min, and TER and HRP flux were determined. Data are representative of 5 wells per treatment group from two independent experiments. Individual circles represent an individual well. Column represents mean ± SD from $n = 2$ experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.005$.

histamine-induced increase in hypothermia. Furthermore, these data support the concept that IL-4–induced PI3K activation attenuates histamine-induced hypothermia.

As these experiments were performed in IL-4Rα^{Y500F} global mice, all cells of the hematopoietic and non-hematopoietic compartment were deficient in IL-4Rα–mediated PI3K activation. Thus, we cannot determine whether IL-4Rα–mediated PI3K signaling in endothelial cells directly or indirectly attenuates anaphylactic symptoms. To further elucidate the mechanism, we performed a similar experiment using the human vascular endothelial cell line EA.hy926 [39]. Histamine stimulation decreases TER of EA.hy926 cells (Fig. 6A). Notably, the decrease in endothelial TER was associated with increased flux of horseradish peroxidase (HRP) (40 kDa), indicating increased paracellular permeability and vascular endothelial leak (Fig. 6B). Stimulation of EA.hy926 cells with DHEA also induced a small decrease in TER and increase in paracellular permeability compared with unstimulated cells (Fig. 6A and B). Importantly, the histamine-induced increase in endothelial cell permeability was attenuated by pretreatment with DHEA, supporting the concept that endothelial cell PI3K signaling reduces histamine-induced endothelial permeability.

Discussion

Previous clinical and murine studies have revealed a link between gain-of-function mutations in the IL-4Rα chain and increased susceptibility to allergic inflammatory responses [26, 27]. The majority of the mutations described are thought to drive atopy susceptibility via modulation of the effects of IL-4/IL-4Rα on hematopoietic cell function. In this study, we demonstrate that loss of IL-4Rα/PI3K signaling, via a mutation in the IL-4R motif necessary for the recruitment of IRS-1 and IRS-2, does not increase severity or susceptibility in allergic disease but rather accelerates IgE/mast cell-mediated, food-induced anaphylaxis progression in mice. We show that the increased rate of symptom development was not associated with dysregulation of IgE and mast cell function but rather was due to increased sensitivity of the vascular endothelium to mast cell-derived histamine.

Clinical and murine-based evidence indicate that the symptoms of food allergy are driven by allergen/IgE/FcεRI-mediated mast cell degranulation and release of mast cell mediators that act on target cells to promote the pathophysiologic features of disease, including urticaria, diarrhea, bronchoconstriction, respiratory and cardiovascular collapse, the latter of which reflects a decrease in intravascular volume resulting in decreased vital organ perfusion and shock [10, 11, 14, 15, 40–43]. Consistent with previous reports, we show that the fluid extravasation and decreased intravascular volume (increased hemoconcentration) is dependent on histamine, as pharmacologic antagonism of H1 and H2 receptors inhibited the hypothermic component of shock response [36].

The molecular basis of histamine-mediated increase in vascular endothelial leak is not yet fully delineated. Histamine ligation to the H1 receptor leads to Gq-protein–coupled and phospholipase C (PLC) activation, inositol phospholipid hydrolysis and increased intracellular Ca^{2+} [44, 45], which can lead to 1) reduced F-actin focal attachment formation [46]; 2) destabilization of the

adherens junction VE-cadherin and catenin interactions, leading to decreased intercellular tethering resulting in reduced endothelial cell adhesiveness and increased paracellular permeability [47]. The IL-4/IL-4Rα pathway has previously been shown to magnify the histamine-mediated effector phase of anaphylaxis [15]. The mechanism by which IL-4 modulates histamine responses is not fully elucidated; however, it is postulated that IL-4 can magnify histamine responses via enhancement of histamine-induced PAF synthesis and PGE2 release via IL-4-induced upregulation of the H1 receptor expression [48].

Unexpectedly, we show that loss of IL-4Rα/PI3K signaling leads to an accelerated histamine-induced hypothermic response and anaphylaxis progression. Murine-based and in vitro studies indicate that the accelerated response could be attributed to increased responsiveness of the vascular endothelium to histamine. The molecular basis of IL-4Rα/PI3K-mediated negative regulation of histamine-induced anaphylactic shock response is unclear; however, we speculate that the mechanism is related to IL-4Rα/PI3K's negative regulation of Ca^{2+}-dependent responses. Recent investigations have reported that IL-4 can attenuate carbachol- and caffeine-induced Ca^{2+} mobilization from the sarcoplasmic reticulum (SR) in airway smooth muscle cells [49]. Notably, the IL-4–mediated inhibition of transient Ca^{2+} release was sensitive to PI3K antagonism, implicating IL-4–induced PI3K activity in intracellular Ca^{2+} release. Since carbachol- and caffeine-induced Ca^{2+} release in the SR is mediated by different Ca^{2+} release channels, the reduction in the transient Ca^{2+} release by IL-4/PI3K is not by inhibition of Ca^{2+} release channels but rather by reduction in the amount of SR-restricted Ca^{2+} levels. Importantly, in some cell types, including HUVECs, PI3K activation promotes PLCγ activation and inositol 1,4,5 triphosphate (IP3) metabolism [50], thus linking IL-4R activation to PLCγ-generated IP3 and Ca^{2+} release. In support of PI3K signaling's negative regulation of histamine-induced shock, we show that constitutive activation of the endothelial PI3K pathway by DHEA attenuated histamine-induced shock in IL-4Rα^{Y500F} mice and that DHEA reduced histamine-induced endothelial paracellular leak in vitro. Demonstrating that DHEA can also suppress histamine responses eliminates concerns with respect to the possibilities of IL-4Rα^{Y500F} mice possessing an intrinsic defect in endothelial PI3K signaling or IL-4Rα mediating suppression of Ca^{2+} channel expression or function. Interestingly, we show in vitro that DHEA alone decreased endothelial barrier function as compared with unstimulated endothelial cells. Notably, this baseline DHEA-induced effect was not related to increased paracellular leak, as there were no differences in HRP flux between vehicle-treated and DHEA only-treated cells, suggesting that DHEA was stimulating altered ion secretion.

Previous studies in IL-4Rα^{Y500F} mice have demonstrated a role for the loss of IL-4Rα/PI3K signaling in the exacerbation of allergic inflammatory responses [29]. Moreover, in a pulmonary airway inflammation model, IL-4Rα^{Y500F} mice developed a more severe asthmatic phenotype as demonstrated by increased airway hyperresponsiveness, pulmonary eosinophilia and mucus hypersecretion [29]. We did not observe increased severity of food-induced anaphylaxis but rather the accelerated rate of symptom onset in IL-4Rα^{Y500F} mice. Importantly, the features of pulmonary allergic inflammation in this particular murine asthma model are not dependent on mast cell–mediated vascular endothelial permeability and fluid extravasation. However, Blaeser et al. reported that the IL-4Rα^{Y500F} mice had increased total IgE and allergen-induced IgE production following OVA/Alum immunization [29]. In contrast, we did not observe differences in total and antigen-specific IgE following OVA/Alum immunization and challenge. One possible explanation for the observed differences between our studies and that of Blaeser et al. with respect to serum IgE is the intestinal microbial diversity [51]. Recent mouse studies indicate that absence of microbial colonization or colonization with low-diversity microbiota leads to increased serum IgE levels and enhancement of $CD4^+$ T-cell and IL-4 responses [51].

We show that though MCPT-1 levels were comparable between WT and IL-4Rα^{Y500F} mice 60 min following OVA challenge, IL-4Rα^{Y500F} mice experienced an accelerated progression of hypovolemia and hypothermia compared to WT mice, suggesting that the IL-4/PI3K pathway alters histamine-mediated responses. We cannot rule out the possibility of a more rapid activation of mast cells and increase in the level of mast cell mediators in the IL-4Rα^{Y500F} mice, which could accelerate progression of the oral antigen-induced anaphylactic symptoms. Consistent with this argument, IL-4 has been shown to amplify mast cell secretory function and release of preformed mediators such as serotonin and arachidonates [52, 53]. However, we do show that administration of 25 μg of histamine to IL-4Rα^{Y500F} mice also lead to an accelerated progression of hypovolemia and hypothermia, suggesting that the altered response in IL-4Rα^{Y500F} mice can be attributed in part to altered sensitivity of the vascular endothelium to mast cell-derived histamine.

A number of murine-based studies have revealed that additional gain-of-function mutations in IL-4Rα can enhance allergic inflammatory responses. IL-4Rα^{Y709F} mice, which have a tyrosine to phenylalanine mutation at position 709 within the ITIM of IL-4Rα, have increased susceptibility to allergen-induced airway inflammation and enhanced sensitivity to food allergens [26, 27]. Similarly, mice that possess the glutamine to arginine substitution at position 576 (Q576R) of IL-4Rα exhibited increased

allergen-induced inflammation and remodeling [28]. To the best of our knowledge, this study is the first demonstration that an IL-4Rα mutation can accelerate disease progression. Though no polymorphisms have been observed in the human equivalent tyrosine residue within the insulin: IL-4 receptor motif (Y497), a human serine proline polymorphism six amino acids downstream of Y497 and within the IL-4Rα ITIM motif (S503P) has been reported [54, 55]. The impact of this polymorphism on the function of the PI3K motif of the human IL-4Rα chain is currently unknown; however, the presence of polymorphic amino acid residues at this location (P503 and R576) are known to alter receptor polarity and secondary structure and affect IRS-1 and IRS-2 propagation of the IL-4Rα signaling [54].

In the current manuscript, we show that loss of IL-4Rα–mediated PI3K signaling accelerates the progression of oral antigen-induced anaphylactic reactions. In vitro and in vivo studies suggest that IL-4Rα PI3K signaling negatively regulates histamine-mediated vascular endothelial leak and loss of this pathway leads to accelerated histamine-mediated hypovolemic shock and hypothermia. These results define an unanticipated role for IL-4Rα–mediated PI3K signaling in negative regulation of IgE-mediated anaphylactic reactions.

Acknowledgments

We thank Dr. Patricia Fulkerson and Fred Finkelman and members of the Divisions of Allergy and Immunology and of Gastroenterology, Hepatology, and Nutrition, Cincinnati Children's Hospital Medical Center for critical review of the manuscript and insightful conversations. We would also like to thank Shawna Hottinger for editorial assistance and manuscript preparation.

Disclosures

All of the authors have declared that they have no conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article at the publisher's web-site.

Figure S1. No effect of loss of IL-4Rα-PI3K signaling on total IgE. Total IgE levels in the serum of OVA-sensitized, intragastric (i.g.) OVA-challenged WT and IL-4Rα^{Y500F} mice following the seventh challenge. Each filled circle represents an individual mouse. Data represent mean ± SD.
Figure S2. A positive relationship between vascular leak and shock response in murine oral antigen-induced anaphylaxis. Correlation between hematocrit and systemic symptoms of oral antigen-induced anaphylaxis. Spearman's rank correlation coefficient between hematocrit and temperature change from 0 to 60 min after the seventh intragastric (i.g.) OVA challenge in OVA-sensitized WT mice. Individual symbols represent 1 mouse.
Figure S3. Systemic anaphylaxis in WT and IL-4Rα^{Y500F} mice is dependent on histamine. Temperature change from 0 to 30 min in OVA-sensitized, intragastric (i.g.) OVA-challenged (A) WT and (B) IL-4Rα^{Y500F} mice following the sixth and seventh intragastric (i.g.) OVA challenge. OVA-sensitized WT and IL-4Rα^{Y500F} mice receive repeated i.g. OVA challenges, and temperature change from 0 to 30 min was determined following the sixth challenge. Prior to the seventh challenge, mice were administered the histamine Type 1 and type 2 receptor antagonists Triprolidine (200 μg) and Cimetidine (200 μg) intravenously (i.v.) (200 μL final volume) 30 min prior to OVA challenge. Each filled circle represents an individual mouse. Data represent the temperature change from 0 to 30 min following the sixth and seventh challenge; *P* values as indicated.
Figure S4. Histamine and DHEA-induced PI3K activation in human vascular endothelial cell line EA.hy926. Representative Western blot analyses probing for PI3K p85 full-length protein and actin in protein lysates from human vascular endothelial cell line EA.hy926 following 0, 1, 5, 15, 30 and 60 min stimulation with histamine (20 nM) or DHEA (100 nM).

