

AWARD NUMBER: W81XWH-15-1-0517

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

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REPORT DATE: JULY 2019

TYPE OF REPORT: FINAL

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

DISTRIBUTION STATEMENT: Approved for Public Release;
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REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188	
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1. REPORT DATE JULY 2019		2. REPORT TYPE FINAL		3. DATES COVERED 30 SEP 2015 - 29 MAR 2019	
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) Yui Hsi Wang, Sunil Tomar, Dana Shik, Andrew Smith Dr. Simon P. Hogan E-Mail:				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Cincinnati Children's Hospital Medical Center 3333, Burnet Ave Cincinnati OH 45229-3026				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	11	19b. TELEPHONE NUMBER (include area code)

1. Project Status

a. Accomplishments

1. The major goals of the project include:
 1. Determine how CD4⁺T_H2 cells potentiate MMC9 development and function
 2. Determine the role of the antigen/IgE/FcεRI complex in MMC9 development and function
 3. Define the biological relevance of MMC9 in human food allergy.
2. The completion of milestones under these goals are
 1. A clinical relevant experimental model of food allergy was established and optimized.
 2. Enhanced IL-4 signaling was shown to increase intestinal MMC9s induction.
 3. IL-4 signaling promotes IL9 gene expression by MMC9s.
 4. IL-4 signaling induces expression of the transcription factors BATF and Nfil3 in MMC9 cells.
 5. IL-4 and TGFβ primes MMC9 cells to be responsive to IL-33.
 6. MMC9-like cells can be detected in the duodenum of patients with food allergy. The frequency of intestinal MMC9-like cells is elevated in patients with food allergy and other allergic diseases, suggesting increased mucosal MCs in the gut may be associated with the comorbidity of allergic diseases.

b. Reportable Outcomes

1. Lee JB, Chen CY, Liu B, Mugge L, Angkasekwinai P, Facchinetti V, Dong C, Liu YJ, Rothenberg ME, Hogan SP, Finkelman FD, Wang YH. (2016) IL-25 and CD4(+) TH2 cells enhance type 2 innate lymphoid cell-derived IL-13 production, which promotes IgE-mediated experimental food allergy. *J Allergy Clin Immunol*. 2016 Apr;137(4):1216-1225.e5. doi: 10.1016/j.jaci.2015.09.019. Epub 2015 Nov 10. PMID: 26560039
2. Chen CY, Lee JB, Liu B, Ohta S, Wang PY, Kartashov AV, Mugge L, Abonia JP, Barski A, Izuhara K, Rothenberg ME, Finkelman FD, Hogan SP, Wang YH. Induction of Interleukin-9-Producing Mucosal Mast Cells Promotes Susceptibility to IgE-Mediated Experimental Food Allergy. *Immunity*. 2015 Oct 20;43(4):788-802. doi: 10.1016/j.immuni.2015.08.020. Epub 2015 Sep 22. PMID: 26410628
3. Oshea, K. and SP Hogan (2018) IgG4 in eosinophilic esophagitis: perpetrator, accomplice or bystander? *Annals of Esophagus*. doi: 10.21037/aoe.2018.09.03.
4. Kordowski, A., Reinicke, AT., Wu, D., Orinska, Z., Hagemann, P., Lang, MH., Lee, J., Wang, YH., Hogan, SP. And J Köhl (2018) C5a receptor 1^{-/-} mice are protected from the development of IgE-mediated experimental food allergy. *Allergy*. Accepted.
5. Poling HM, Wu D, Brown N, Baker M, Hausfeld TA, Huynh N, Chaffron S, Dunn JCY, Hogan SP, Wells JM, Helmrath MA, Mahe MM. (2018) Mechanically induced development and maturation of human intestinal organoids in vivo. *Nat Biomed Eng*. 2018 Jun;2(6):429-442. doi: 10.1038/s41551-018-0243-9. Epub 2018 Jun 4. PMID: 30151330
6. Dang A, Logsdon S, Hogan SP. (2018) Investigating innate immune mechanisms in early-life development and outcomes of food allergy. *J Allergy Clin Immunol*. 2018 Sep;142(3):790-792. doi: 10.1016/j.jaci.2018.08.001. Epub 2018 Aug 7. PMID: 30096392
7. Betz KJ, Maier EA, Amarachintha S, Wu D, Karnele EP, Kinder JM, Steinbrecher KA, McNeal MM, Luzader DH, Hogan SP, Moore SR. (2018) Enhanced survival following oral and systemic *Salmonella enterica* serovar Typhimurium infection in polymeric immunoglobulin receptor knockout mice. *PLoS One*. 2018 Jun 1;13(6):e0198434. doi: 10.1371/journal.pone.0198434. eCollection 2018. PMID: 29856838

8. Kordowski A, Reinicke AT, Wu D, Orinska Z, Hagemann P, Huber-Lang M, Lee JB, Wang YH, Hogan SP, Köhl J. C5a receptor 1-/- mice are protected from the development of IgE-mediated experimental food allergy. *Allergy*. 2018 Oct 19. doi: 10.1111/all.13637. PMID: 30341777
9. Poling HM, Wu D, Brown N, Baker M, Hausfeld TA, Huynh N, Chaffron S, Dunn JCY, Hogan SP, Wells JM, Helmrath MA, Mahe MM. Mechanically induced development and maturation of human intestinal organoids in vivo. *Nat Biomed Eng*. 2018 Jun;2(6):429-442. doi: 10.1038/s41551-018-0243-9. Epub 2018 Jun 4. PMID: 30151330

Progress Details

1. Specific Aim 1: Determine how CD4+TH2 cells potentiate MMC9 development and function

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

Subtask 1: MC-specific IL-4R α requirement

Subtask 2: Reconstitution approach

The data described in Aim 1 is currently unpublished. We are in preparation of drafting a manuscript describing these studies and plan to submit for publication.

To understand the effect of IL-4 on the MMC9 cell fate we have had to develop a flow cytometry detection system to identify and track MMC9 cells from the lamina propria of the SI from food allergic IL-9-GFP reporter mice. As described in Figure 1 mast cells (MC) and MMC9 cells were identified by c-kit⁺ ST2⁺ FcεRI⁺ cells. To distinguish between the mature MC, MMC9 and progenitor MC (pMC) cells we use β7 integrin expression. c-kit⁺ ST2⁺ FcεRI⁺ β7 integrin^{high} cells are identified as pMC's and they were shown to express very low levels of IL-9. c-kit⁺ ST2⁺ FcεRI⁺ β7 integrin^{low} cells which have been identified as mature mast cells were also shown to express very little levels of IL-9. In contrast the c-kit⁺ ST2⁺ FcεRI⁺ β7 integrin^{Mid}

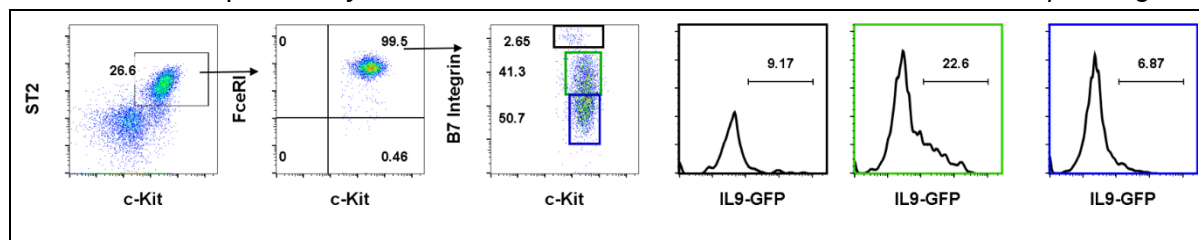
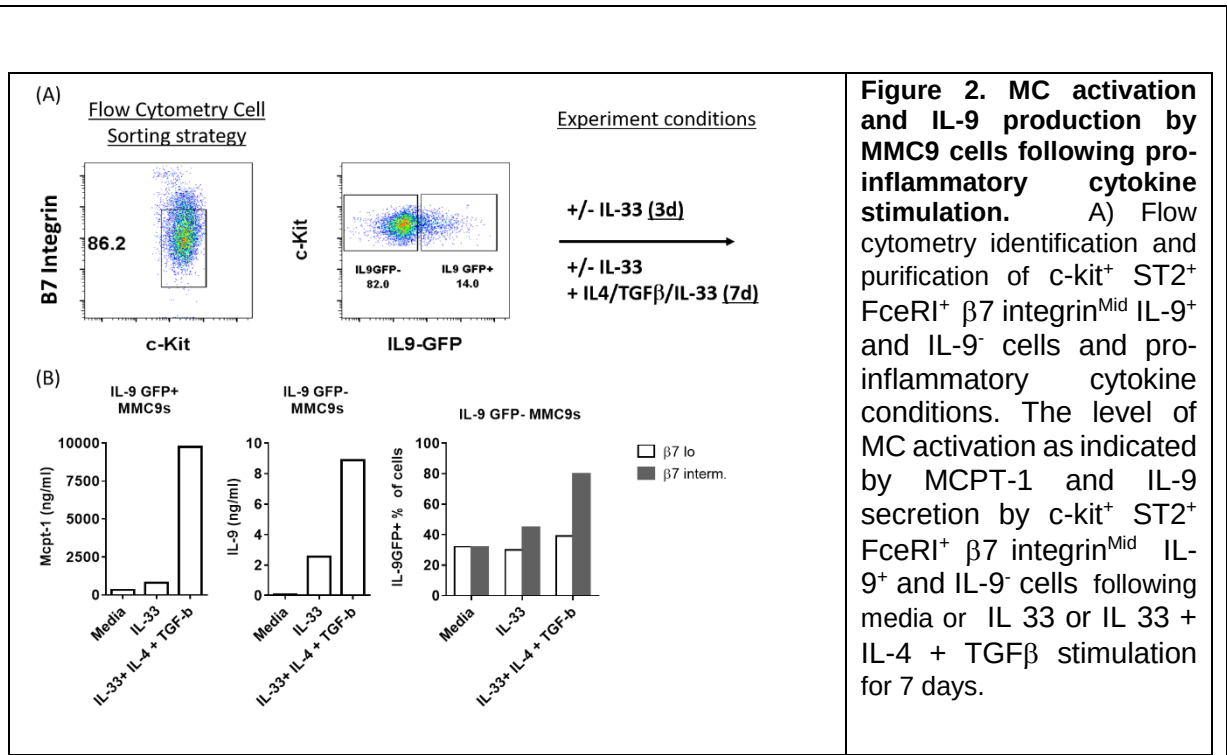


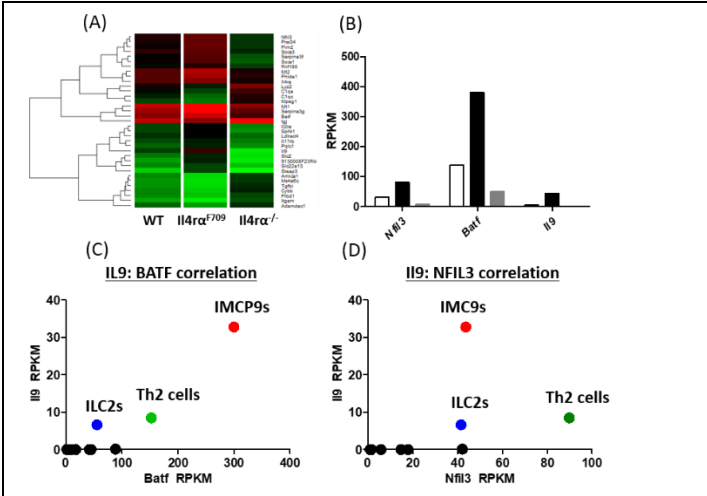
Figure 1. Phenotypic characterization of SI MMC9 cells from IL-9-GFP reporter food allergic mice. The SI of IL-9-GFP reporter food allergic mice was excised and the lamina propria on a nuclear cell population was purified as previously described in Chen et al. 2015 Immunity. The LP mast cell populations were initially distinguished by c-kit⁺ ST2⁺ FcεRI⁺ staining. The mature MC, MMC9 and progenitor MC (pMC) cells were distinguished by β7 integrin expression. c-kit⁺ ST2⁺ FcεRI⁺ β7 integrin^{high} cells are identified as pMC's (black box) and they were shown to express very low levels of IL-9. c-kit⁺ ST2⁺ FcεRI⁺ β7 integrin^{low} cells (mature mast cells) (blue box) were also shown to express very little levels of IL-9. In contrast the c-kit⁺ ST2⁺ FcεRI⁺ β7 integrin^{Mid} cells (MMC9 cells) (green box) we shown to be IL-9 positive.

cells we shown to be IL-9 positive which we have identified as MMC9 cells (**Figure 1**). These analyses identified that the MMC9 cell population is a distinct cell population from mature MCs and also pMC's.

Major Task 1. To examine the pro-inflammatory signals to stimulate MMC9 cell activation and release of IL-9 we cultured flow cytometry purified MMC9 cells and pMCs in the presence of media, IL 33 or IL 33 + IL-4 and TGFβ and examine for evidence of MMC nine cell degranulation and IL-9e secretions. We demonstrate that c-kit⁺ ST2⁺ FcεRI⁺ β7 integrin^{high} cells produced very little l'll nine following IL 33 or IL 33 + IL-4 + TGFβ stimulation. In contrast c-kit⁺ ST2⁺ FcεRI⁺ β7 integrin^{Mid} cells produced a prodigious amount of IL-9 as well as the mast cell protease MCPT-1 following IL 33 or IL 33 + IL-4 + TGFβ stimulation. Based upon these data sets we concluded that pro-inflammatory cytokine stimulation of MMC9 cells stimulates IL-9 secretion (**Figure 2**).



Subtask 1. To get insight into the molecular impact of IL-4 signaling on MMC9 development and function we have developed a flow cytometry protocol to purify MMC9 cells from reconstituted mice and submitted the samples for RNAseq analyses. The protocol requires



us to use n = 4 mice to obtain 3 x 10⁵ purified MMC9 cells and 125ng purified RNA. We required 450ng purified RNA for RNAseq analyses. We have completed n = 4 replica experiments and purified sufficient RNA for the RNAseq analyses.

We have performed initial bioinformatics analyses to determine the molecular impact of IL-4 signaling on MMC9 transcriptome (**Figure 3**

PR140927-Quarterly_Technical_Progress_Report_Yr3-QA3 2018). We have identified dysregulation of 134 genes in MMC9 cells following deletion of IL4Rα and 68 genes in hyperresponsive (IL4Y709F) MMC9 cells (**Figure 3A**). Interestingly, we show that 36 genes were significantly dysregulated by modulation of IL-4Rα signaling (24 genes upregulated in IL4Y709F and downregulated in IL4Rα^{-/-} MMC9 cells and 12 genes

downregulated in IL4Y709F and upregulated in IL4R α ^{-/-} MMC9 cells) (p < 0.05, fold change > 2.0) suggesting direct regulation by IL-4R α signaling. (**Figure 3** *PR140927-Quarterly_Technical_Progress_Report_Yr3-QA3 2018*).; Notably, many of the most highly dysregulated genes included transcription factor genes associated with Type-2 immunity including the Nuclear factor, interleukin 3 regulated (Nfil3) and Basic leucine zipper transcription factor, ATF-like (Batf) (**Figure 3A**).

We confirmed the increased expression in the transcription factors NIFL3 and BATF in the IMCP9 cells. To do this we purified IMCP9 cells from the lamina propria SI of wild type, IL-4R^{-/-} and IL-4Y709F food allergic mice using protocols previously described (*PR140927-Quarterly_Technical_Progress_Report_Yr3-QA3 2018*). Importantly we show an association between IL-4 signaling and induction of BATF, NIFL3 and IL9 mRNA expression in IMCP9 cells (**Figure 3B**). We next compared the expression of the transcription factors expressed by purified IMCP9 cells as well as other Type-2 immune cells including ILC2 and CD4⁺ Th2 cells using RNAseq analysis. We demonstrated a link between the expression of BATF and IL9 mRNA expression in ILC2s, CD4⁺ Th2 cells and IMCP9 cells (**Figure 3C**). Similarly, we demonstrated an association between NFIL3 and IL9 mRNA expression in ILC2s, CD4⁺ Th2 cells and IMCP9 cells (**Figure 3D**). Importantly we showed that the strongest association between IL9 and the transcription factors in IMCP9 cells (**Figure C and D**).

Major Task 2: Determine whether CD4⁺Th2 cells potentiate IMCP9 development through OX40/OX40L.

Subtask 1: OX40 and OX40L requirements

Subtask 2: Reconstitution approach

Subtask 3: Prophylactic approach

A component of this work is described in the following publication.

J Allergy Clin Immunol. 2016 Apr;137(4):1216-1225.e5. doi: 10.1016/j.jaci.2015.09.019.

Epub 2015 Nov 10.

IL-25 and CD4(+) TH2 cells enhance type 2 innate lymphoid cell-derived IL-13 production, which promotes IgE-mediated experimental food allergy.

Lee JB1, Chen CY1, Liu B1, Mugge L1, Angkasekwinai P2, Facchinetti V3, Dong C3, Liu YJ4, Rothenberg ME1, Hogan SP1, Finkelman FD5, Wang YH6.

PMID: 26560039 PMCID: PMC4826796 DOI: 10.1016/j.jaci.2015.09.019

3. Specific Aim 3: Define the biological relevance of IMCP9 in human food allergy.

(i) Major Task 1: Identify and characterize human IMCP9

Subtask 1: Phenotypic analysis:

Subtask 2: Cytokine production:

Subtask 3: Gene expression:

These studies are described in the publication:

Chen CY1, Lee JB1, Liu B1, Ohta S2, Wang PY3, Kartashov AV1, Mugge L1, Abonia JP1, Barski A1, Izuhara K4, Rothenberg ME1, Finkelman FD5, Hogan SP1, Wang YH6.

Immunity. 2015 Oct 20;43(4):788-802. doi: 10.1016/j.immuni.2015.08.020. Epub 2015 Sep 22. Induction of Interleukin-9-Producing Mucosal Mast Cells Promotes Susceptibility to IgE-Mediated Experimental Food Allergy.
PMID: 26410628 PMCID: PMC4618257 DOI: 10.1016/j.immuni.2015.08.020

No amendments submitted to the IRB and HRPO for review. No adverse events occurred.

2. Future Plans

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3. Problems/Issues:

a. Current Problems/Issues

We had specific challenges with generation and sustaining some of the mouse lines necessary for completion of Aim 2. No other significant problem or issues occurred.

b. Anticipated Problems/Issues

We anticipate that some murine colonies may not expand efficiently. Because of the cost of maintaining large numbers of murine colonies, we must balance the numbers of mice which are in breeding and for experiments. We will monitor status of breeding and modify our breeding strategy, if such problem occurs.

4. Financial Health

No major equipment procured in this quarter.

5. Personnel Effort

Personnel	Role	Percent Effort
Rothenberg, ME	Principle Investigator	30%
Finkelman, Fred	Co-investigator	5%
Hogan, Simon P	Co-investigator	1%
Tomar, Sunil	Postdoc Fellow	100%
Ganesan, Varsha	Research Assistant	50%

6. Protocol and Activity Status

a. Human Use Regulatory Protocols

TOTAL PROTOCOLS:

One human subject research protocols will be required to complete the Statement of Work.

PROTOCOLS:

Protocol [HRPO Assigned Number]: A-18868

Title: Eosinophils and Inflammation, an Expanded Study

Target required for clinical significance: 90

Target approved for clinical significance: 90

Submitted to and Approved by:

Provide bullet point list of protocol development, submission, amendments, and approvals (include IRB in addition to HRPO).

- HRPO was approved on 13-01-2016.

- IRB (2008-0090) was approved on 14-05-2015

Status:

- Nothing to report.

(b) Use of Human Cadavers for RDT&E, Education or Training

No RDT&E, education or training activities involving human cadavers will be performed to complete the Statement of Work (SOW).

(c) Animal Use Regulatory Protocols

TOTAL PROTOCOLS:

One animal use research protocols will be required to complete the Statement of Work.

PROTOCOLS:

Protocol [ACURO Assigned Number]: PR140927

Title: Regulation and Function of Inflammatory Leukocytes in Allergic Disease

Target required for statistical significance: 2346

Target approved for statistical significance: 2346

Submitted to and Approved by:

ACURO protocol PR140927 entitled, "Regulation and Function of Inflammatory Leukocytes in Allergic Disease was approved on 26-08-2015

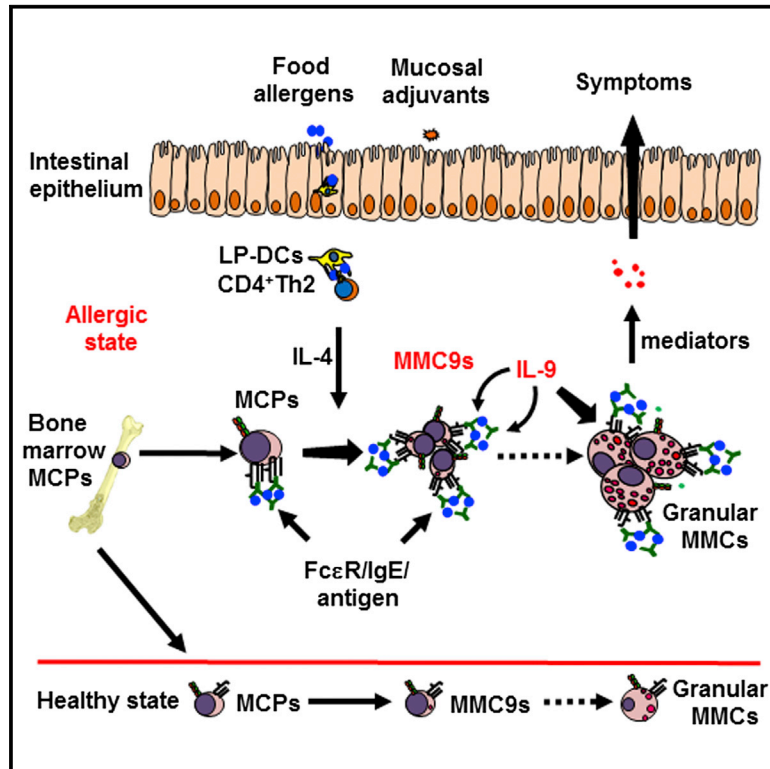
IACUC protocol number 2013-0143 entitled, "Regulation and Function of Inflammatory Leukocytes in Allergic Disease was approved on 26-08-2015

Status:

- Nothing to report in this quarter.

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

Graphical Abstract



Authors

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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



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 (*Il4raF709*) 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.

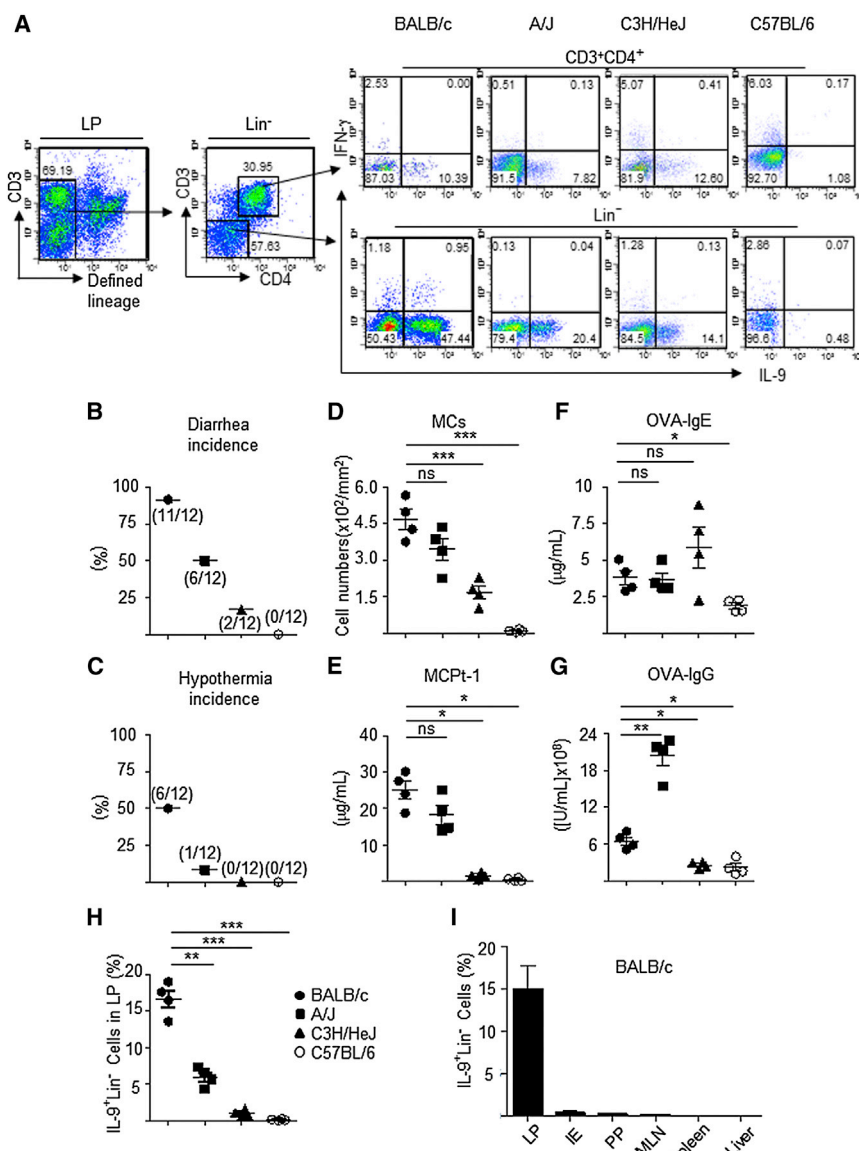


Figure 1. Lin⁻ IL-9-Producing Cell Frequency Correlates Positively with Susceptibility to Experimental Food Allergy

(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 $\pm$ SEM. *p $\leq$ 0.05; **p $\leq$ 0.01; ***p $\leq$ 0.001. NS, not significant. See also Figure S1.

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% $\pm$ 2.0%, mean $\pm$ 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% $\pm$ 1.1% and 1.0% $\pm$ 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

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

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 1). Together, these results suggest an association of Lin[−] IL-9-producing LP cells with the susceptibility to experimental food allergy.

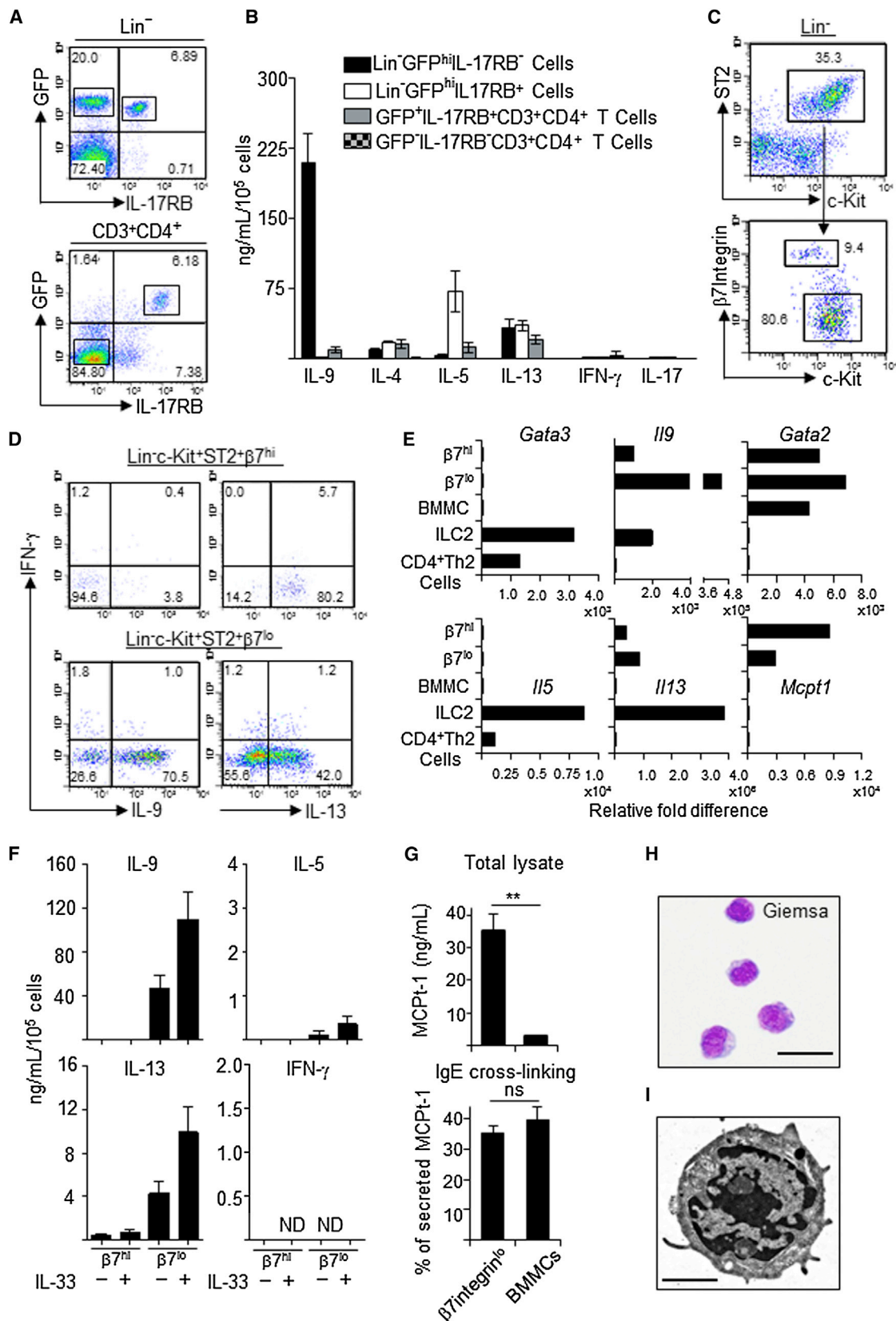
Innate MMC9s Are the Principal IL-9 Producers of Mast Cell Lineage

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, Fc ϵ R α , ST2, Thy1.2, major histocompatibility complex class II (MHCII), and CD86, but not IL-2R α , IL-7R α , Sca-1, CD23, CCR3, Siglec-F, or IL-3R α (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 β 7integrin, 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 β 7integrin^{lo} subset of Lin[−]GFP^{hi}IL-17RB[−]c-Kit⁺ST2⁺ cells, although both β 7integrin^{hi} and β 7integrin^{lo} subsets could produce IL-13 (Figure 2D). Consistently, both cell subsets expressed *Il13* transcript (>10⁴-fold), but only β 7integrin^{lo}Lin[−]GFP^{hi}IL-17RB[−]c-Kit⁺ST2⁺ cells expressed very large amounts of *Il9* transcript (>10⁵-fold), compared to naive CD4⁺ T cells (Figure 2E). Similar to bone-marrow-derived mast cells (BMMCs), both β 7integrin^{hi} and β 7integrin^{lo} subsets of Lin[−]GFP^{hi}IL-17RB[−]c-Kit⁺ST2⁺ cells expressed *Gata1* (>10³-fold), *Gata2* (>10³-fold), and *Mcpt1* (>10³-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 β 7integrin^{lo}, but not the β 7integrin^{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 β 7integrin^{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 Fc ϵ R α complex crosslinking (Figures 2G and S2C). Cytology and electron microscopy revealed that both the β 7integrin^{hi} and β 7integrin^{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 meta-chromatic granules (Figures 2H and 2I; Chen et al., 2005; Gurish et al., 2001). Notably, only the β 7integrin^{hi}, not β 7integrin^{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 β 7integrin^{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 β 7integrin^{hi} Lin[−]IL-17RB[−]c-Kit⁺ST2⁺ cells are the intestinal MCPs (Gurish and Austen, 2012) and that β 7integrin^{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-7R α , IL-2R α , Thy1.2, MHCII, CD86, and ICOS, but not c-Kit or Fc ϵ R (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* (>10³-fold), *Il13* (>10⁶-fold), and transcription factor *Gata3* (>10³-fold) and *Rora* (>10⁴-fold), and moderate amounts of *Il9* (<10³-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



(legend on next page)

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 sub-lethal 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-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-6c⁻FcεRIα⁻CD41⁻CD71⁻FLK2⁻CD150⁻c-Kit⁺β7integrin⁺ 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 β7integrin^{hi} MCP recruitment by anti-α4β7integrin 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 β7^{hi} MCPs had the potential to develop into MMC9s that displayed β7^{lo}c-Kit⁺ST2⁺FcεRIα⁺ phenotype and upregulated *I19*, *I113*, 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 β7^{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 β7integrin^{hi} MCPs of BM origin have the potential to proliferate and develop into β7integrin^{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-Kit^{lo}FcεRIα⁺DX5⁺IL-3Rβ⁺). 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-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-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-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-17RB⁻c-Kit⁺ST2⁺β7integrin^{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-17RB⁻ and IL-17RB⁺, and GFP⁺IL-17RB⁺ and GFP⁻IL-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) β7integrin^{lo} (β7^{lo}) and β7integrin^{hi} (β7^{hi}) subsets of Lin⁻GFP^{hi}IL-17RB⁻c-Kit⁺ST2⁺ cells. (E) Expression of the indicated genes by purified intestinal β7integrin^{lo} and β7integrin^{hi} subsets of Lin⁻GFP^{hi}IL-17RB⁻c-Kit⁺ST2⁺, in-vitro-generated bone-marrow-derived mast cells (BMMCs), ILC2s, and CD4⁺GFP⁺IL-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 β7integrin^{lo} and β7integrin^{hi} subsets of Lin⁻GFP^{hi}IL-17RB⁻c-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-17RB⁻c-Kit⁺ST2⁺β7integrin^{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-17RB⁻c-Kit⁺ST2⁺β7integrin^{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 ≤ 0.01. NS, not significant. See also [Figures S2 and S3](#).

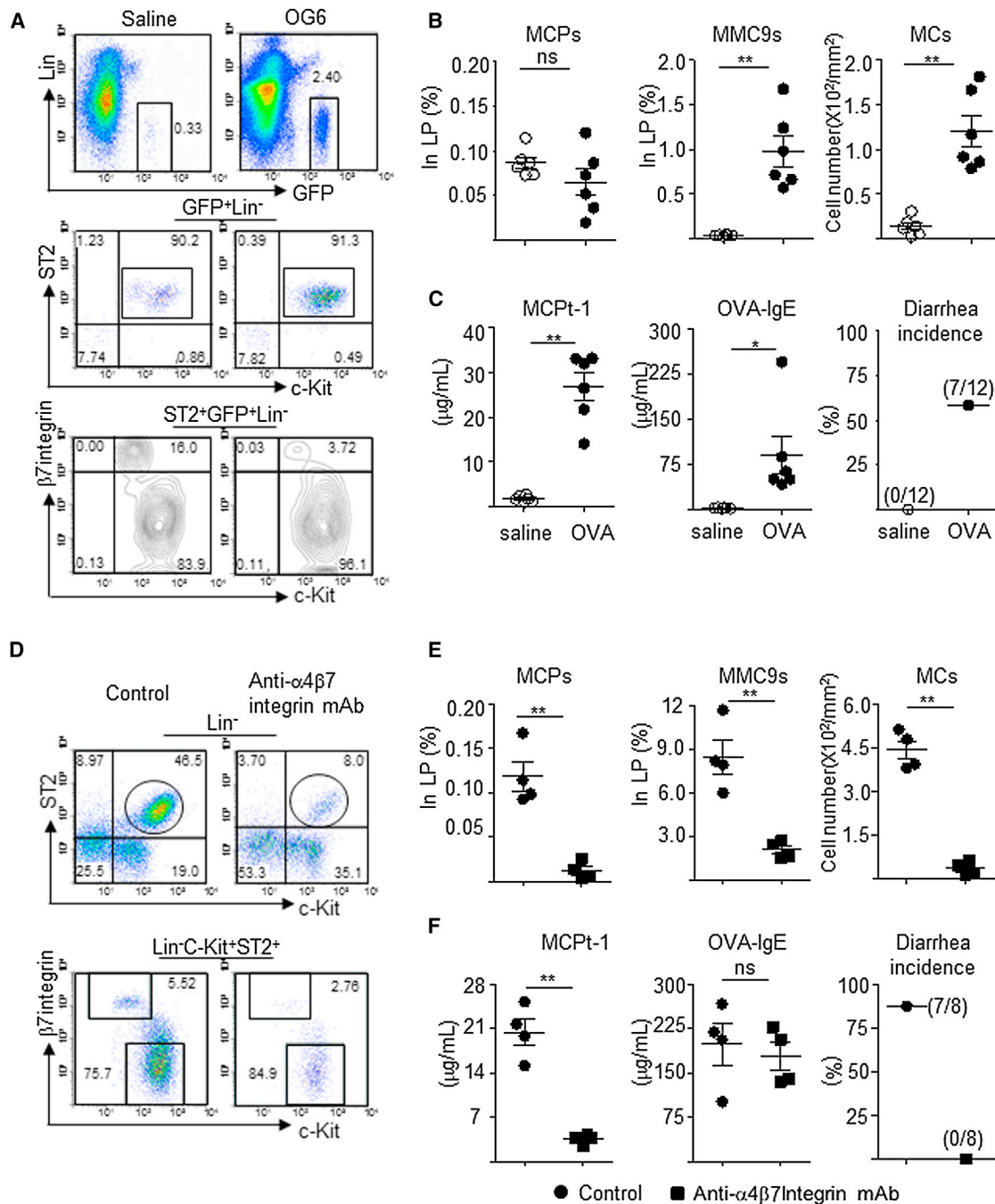


Figure 3. Blockade of Intestinal $\beta 7^{\text{hi}}$ MCP Recruitment from Bone Marrow Results in the Failure of MMC9 Induction

Detection and frequency of donor-derived MMC9s ($\text{Lin}^{-}\text{GFP}^{+}\text{ST2}^{+}\text{c-Kit}^{+}\beta 7^{\text{integrin}}^{\text{hi}}$) and MCPs ($\text{Lin}^{-}\text{GFP}^{+}\text{ST2}^{+}\text{c-Kit}^{+}\beta 7^{\text{integrin}}^{\text{hi}}$), 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 ($\text{Lin}^{-}\text{Ly-6c}^{-}\text{FcεRI}\alpha^{-}\text{CD41}^{-}\text{CD71}^{-}\text{FLK2}^{-}\text{CD150}^{-}\text{c-Kit}^{+}\beta 7^{\text{integrin}}^{+}$ cells) from IL-4-eGFP (4GET) mice (A–C) or received anti- $\alpha 4\beta 7$ integrin (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, lamina propria. Error bars denote mean $\pm$ 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,

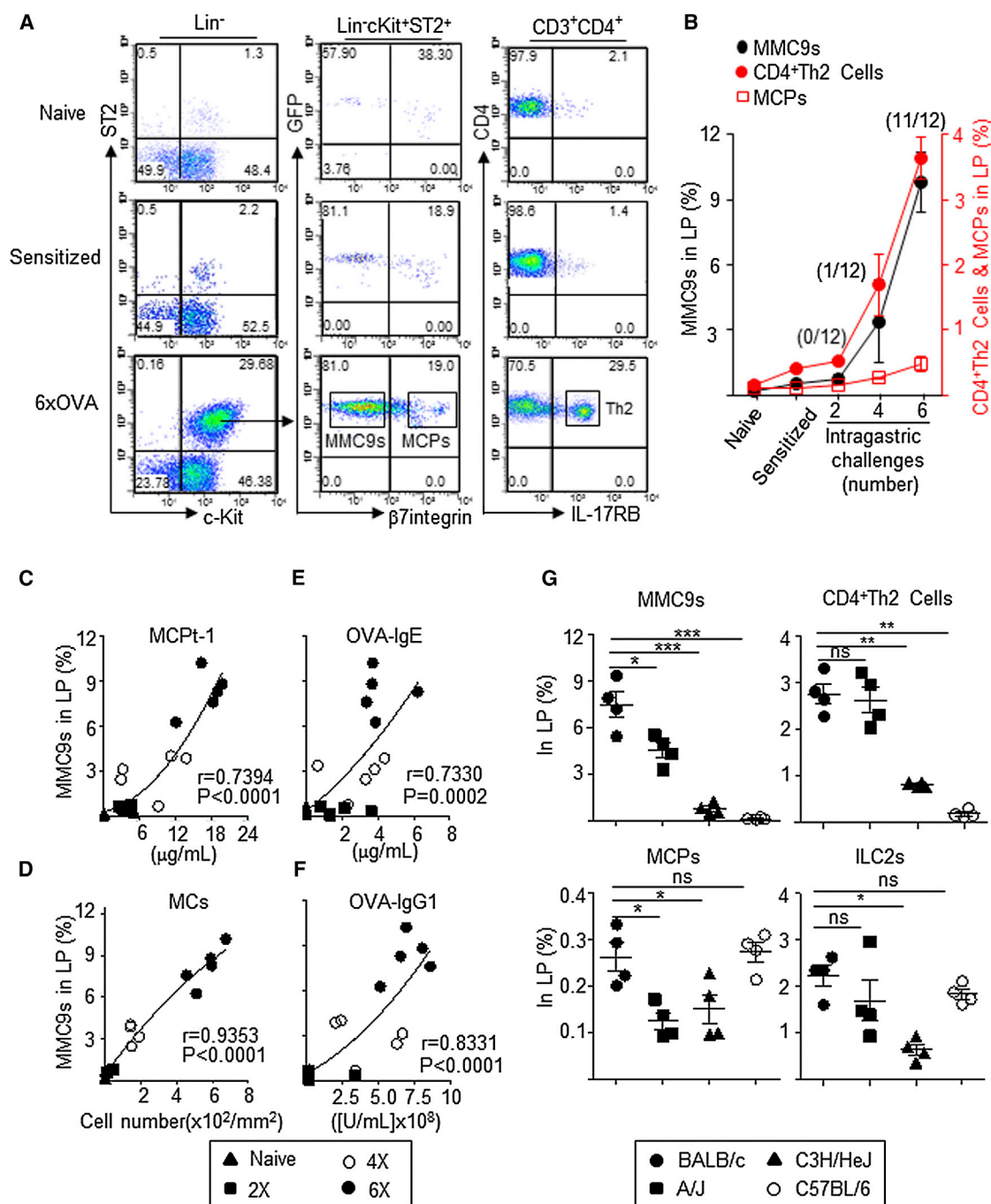


Figure 4. MMC9s and CD4⁺ Th2 Cell Frequencies Correlate Positively with Susceptibility to Experimental Food Allergy

(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 $\pm$ SEM. *p $\leq$ 0.05; **p $\leq$ 0.01; ***p $\leq$ 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-4R α ⁻, 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-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 develop-

ment 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-Fc ϵ R1 α mAb before and during the course of repeated intragastric OVA challenge. Anti-Fc ϵ R1 α 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-Fc ϵ R1 α 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 Fc ϵ R-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 Fc ϵ R complex cross-linking by antigens. Given their anatomical location, characteristics, and function, MMC9s might be a key player that amplifies intestinal allergic

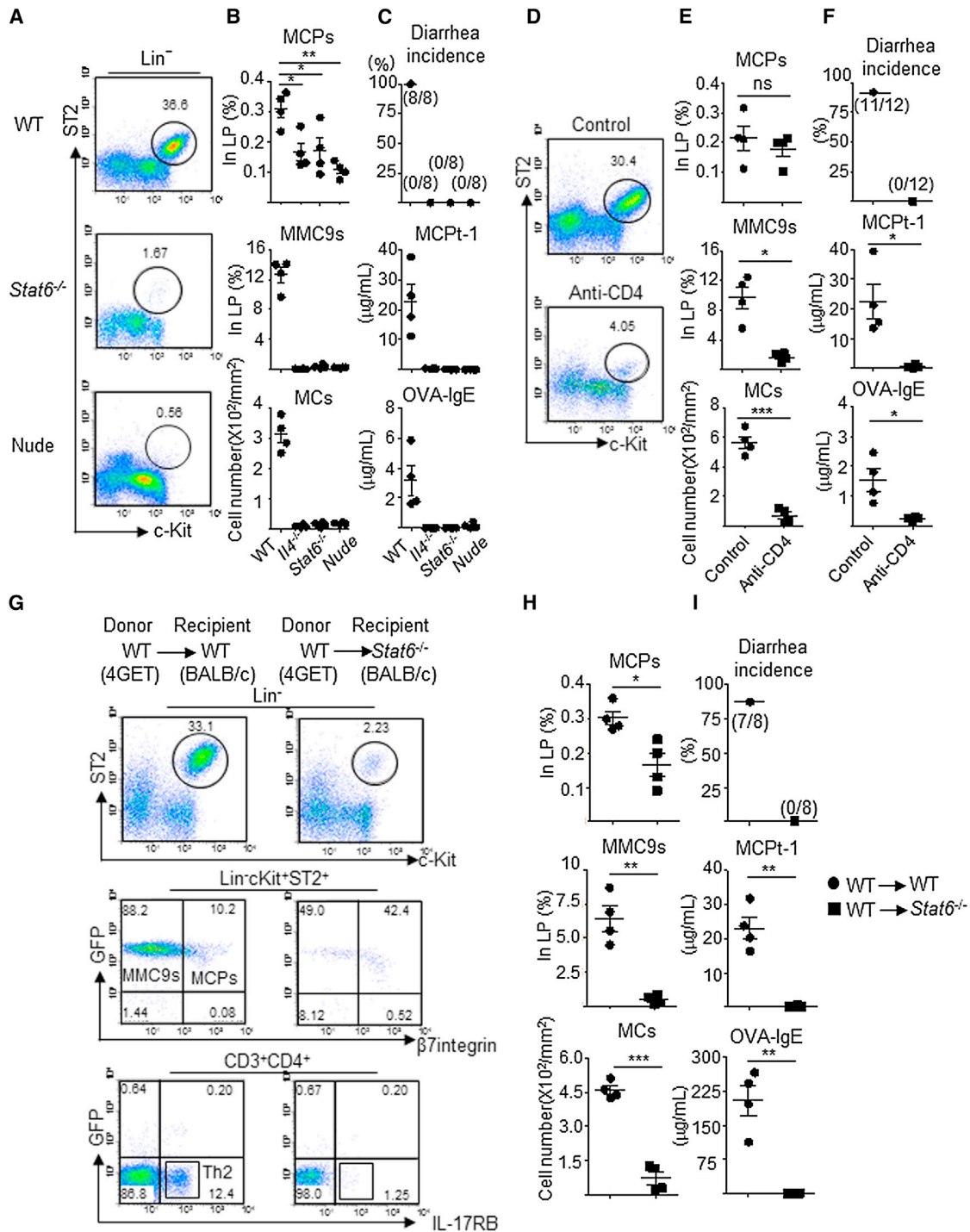


Figure 5. STAT6 Signaling and T Cells Are Required for MMC9 Induction that Promotes Experimental Food Allergy

(A–F) Detection (A, D) and frequency (B, E) of MMC9s (Lin⁻ c-Kit⁺ ST2⁺ β7 integrin^{hi}) and MCPs (Lin⁻ c-Kit⁺ ST2⁺ β7 integrin^{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 < 0.05; **p < 0.01; ***p < 0.001. NS, not significant.

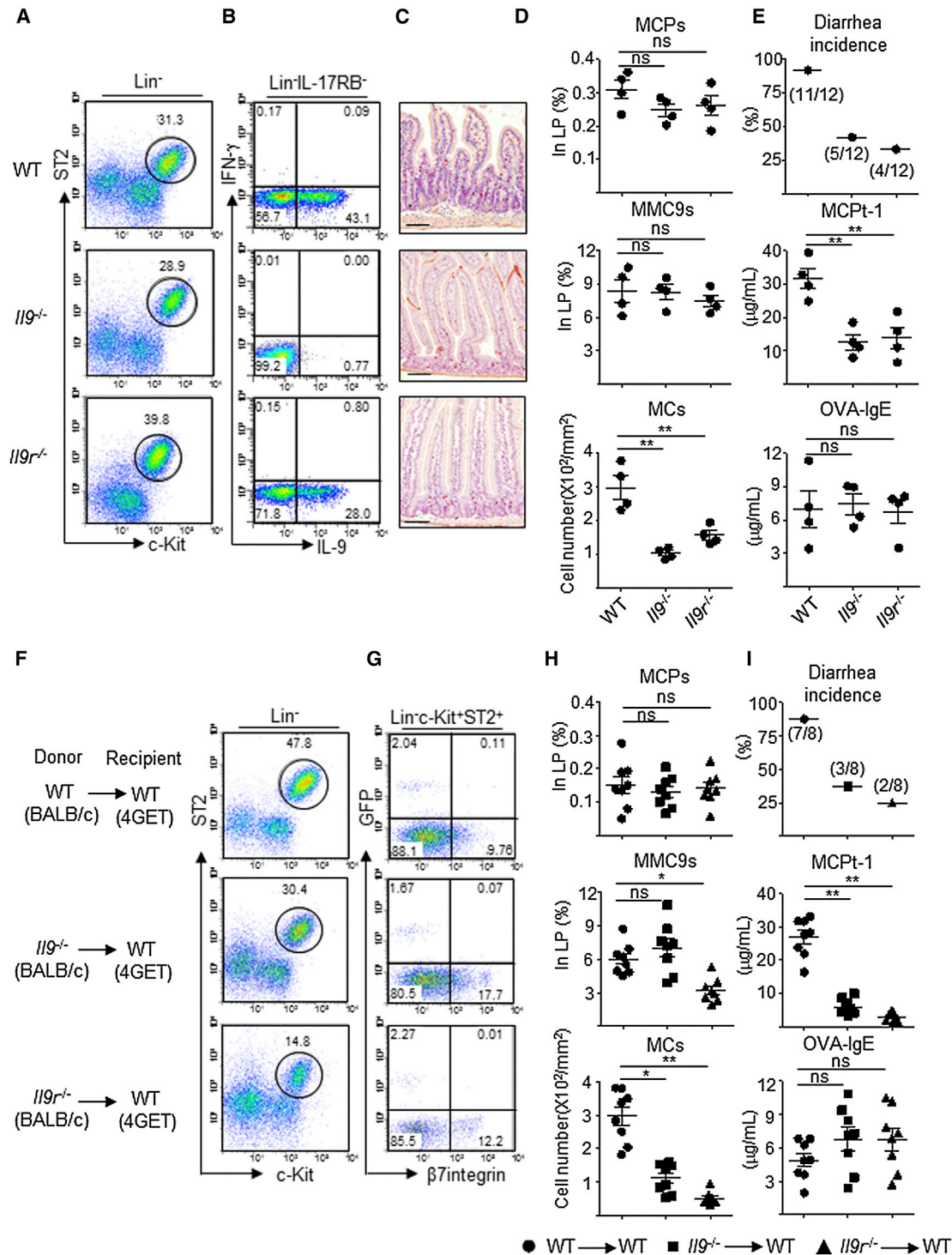


Figure 6. MMC9s Can Be Induced in IL-9- or IL-9R-Deficient Mice but Fail to Promote Experimental Food Allergy

(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⁺ β 7 integrin^{lo}) and MCPs (Lin⁻ c-Kit⁺ ST2⁺ β 7 integrin^{hi}) (D) and measurement of indicated features of experimental food allergy (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⁺ β 7 integrin^{lo} GFP⁺) and MCPs (Lin⁻ c-Kit⁺ ST2⁺ β 7 integrin^{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 μ m (C). Error bars denote mean $\pm$ SEM. **p $\leq$ 0.01. NS, not significant. See also Figure S6.

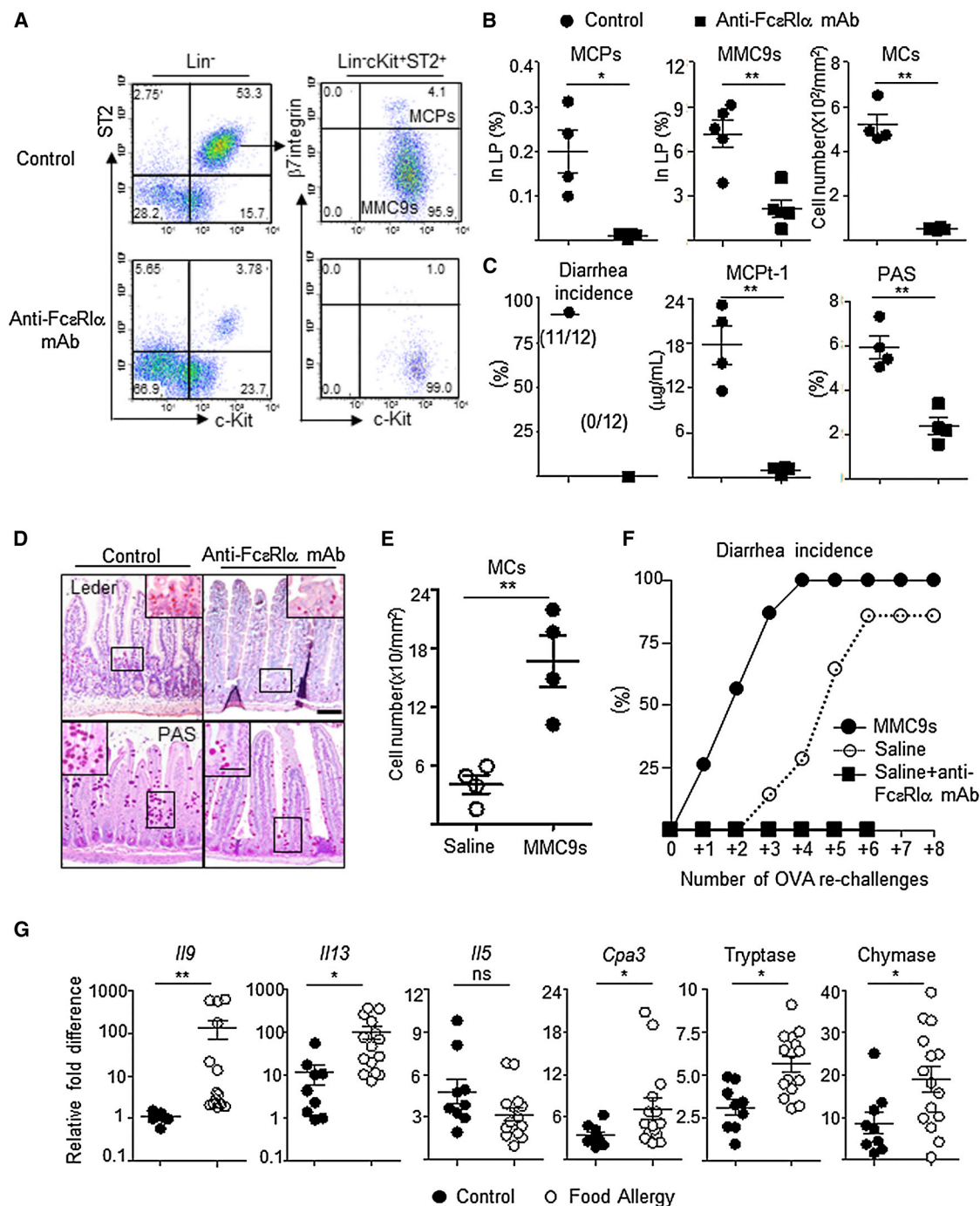


Figure 7. MMC9s Drive Intestinal Mastocytosis and I19 Transcript Expression Is Increased in Food Allergy Patients

(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 < 0.05; **p < 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-4R α -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 $\beta 7^{\text{hi}}$ MCPs (~0.2%) and $\beta 7^{\text{lo}}$ 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 (Angkasekwina et al., 2013; Dillon and MacDonald, 1986; Liu et al., 2013; Ruitenbergh 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/mm² and ~2 μ g/ml) were induced in CT-sensitized C3H/HeJ or BALB/c mice than those (~400 cells/mm² and ~20 μ g/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 Fc ϵ R 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 Fc γ R 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 Fc ϵ R 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 Fc ϵ R 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-β7 integrin (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 FACSCanto 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 Biotec). After re-stimulation with PMA and ionomycin and treatment with Golgi blocker, enriched Lin⁺ LP cells were stained with PE-anti-β7 integrin, 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 × 10⁶ 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 × 10⁴) 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 × 10⁴) 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-hexosaminidase 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 ≤ 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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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 AI073553 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 Kinneer 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

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<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:	<i>Il4</i> 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-2R α :	IL-2 receptor α
IL-7R α :	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-7R α), and IL-2 receptor α (IL-2R α)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 *Rora*,^{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 μ g 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 μ L 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- μ m 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 IgG₁ (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 $\pm$ 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

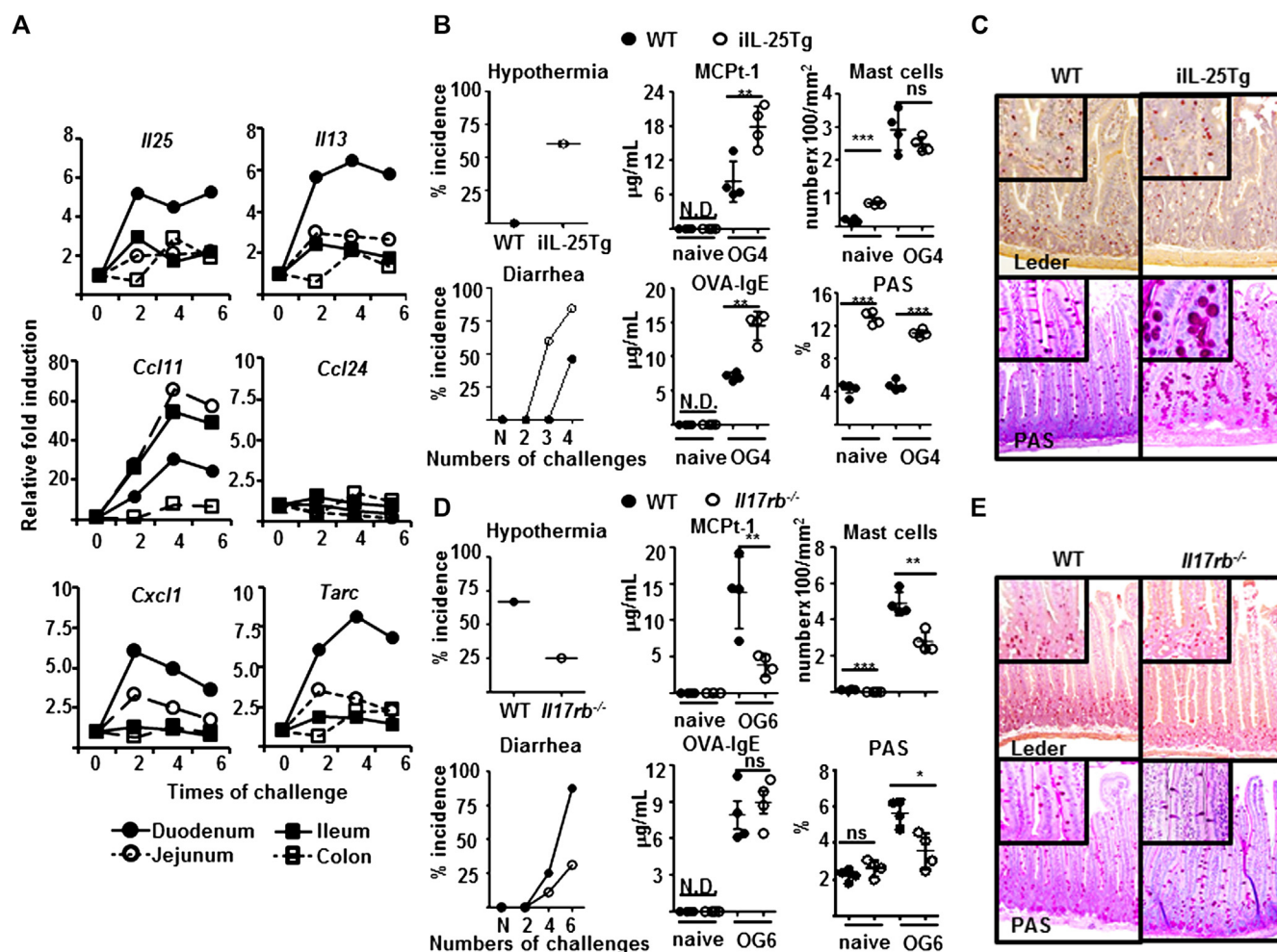


FIG 1. A, Expression levels of indicated genes by indicated tissues of sensitized BALB/c mice after indicated times of intragastric OVA challenge were examined and compared, as described in the [Methods](#) section. B-E, Indicated murine strains were sensitized and underwent oral gavage (OG) with OVA 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* ≤ .05, ***P* ≤ .01, and ****P* ≤ .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-1, 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 FcεRα1, 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,

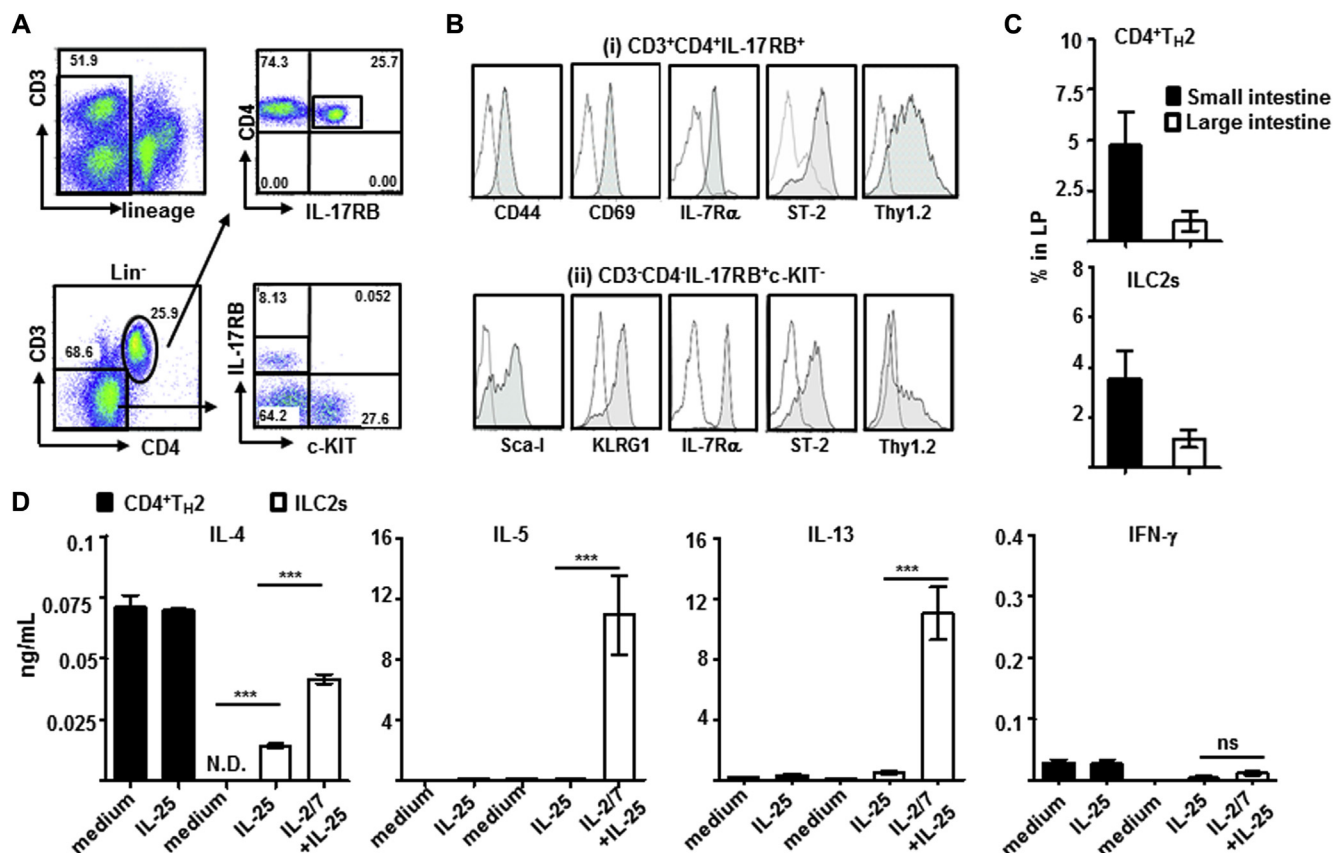


FIG 2. A–C, Detection (Fig 2, A), phenotypic analysis (Fig 2, B), and frequencies (Fig 2, C) of Lin⁻ CD3⁺ CD4⁺ IL-17RB⁺ TH2 cells and Lin⁻ CD3⁻ CD4⁻ IL-17RB⁺ c-KIT⁻ ILC2s in mice with food allergy. D, Indicated cytokines produced by purified Lin⁻ CD3⁺ CD4⁺ IL-17RB⁺ TH2 cells and Lin⁻ CD3⁻ CD4⁻ IL-17RB⁺ 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 Fc ϵ R α I, but not IL-2R α (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* ($>5 \times 10^3$ -fold) and the TH2 cytokine genes *Il4* ($>5 \times 10^3$ -fold), *Il5* ($>1 \times 10^4$ -fold), and *Il13* ($>5 \times 10^4$ -fold); however, only CD3⁻ CD4⁻ IL-17RB⁺ c-KIT⁻ cells expressed ILC2 signature transcripts: *Id2* (>50 -fold) and *Rora* ($>3 \times 10^3$ -fold; see Fig E1, C). Thus Lin⁻ CD3⁺ CD4⁺ IL-17RB⁺ and Lin⁻ CD3⁻ CD4⁻ IL-17RB⁺ c-KIT⁻ cell subsets represent intestinal CD4⁺ TH2 cells and ILC2s, respectively.

Both IL-17RB⁻ expressing CD4⁺ TH2 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 TH2 cytokine production by purified intestinal CD4⁺ TH2 cells and ILC2s; however, the addition of IL-2 and/or IL-7 potentiated ILC2s, but not CD4⁺ TH2 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⁺ TH2 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 diagrammed 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-7R α ⁺ KLRG1⁺ ILC2 compartment with green fluorescent protein (GFP)-marked cells in the irradiated recipients, whereas most of the CD4⁺ ST-2⁺ IL-17RB⁺ TH2 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

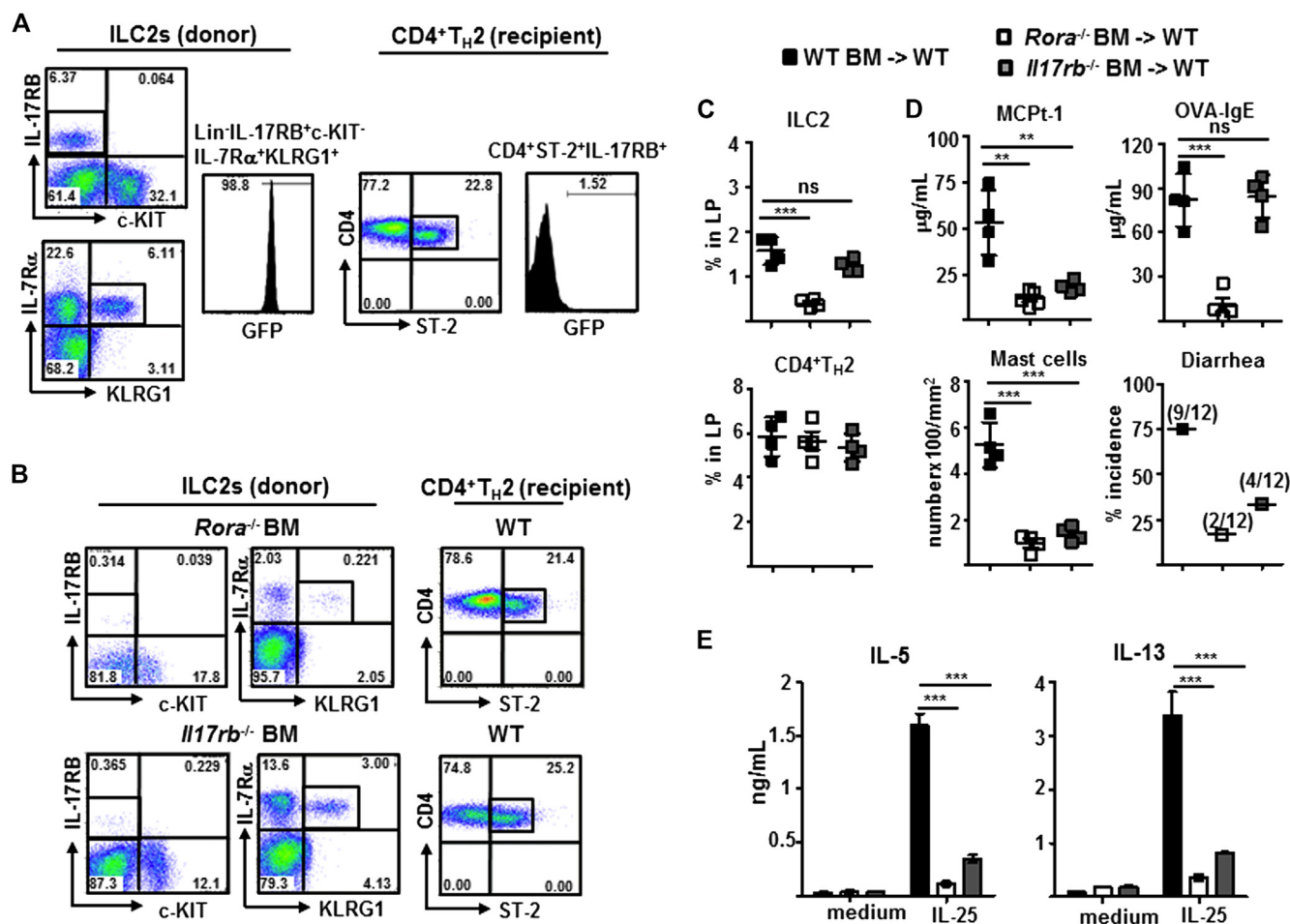


FIG 3. Detection (A and B) and frequency (C) of donor-derived ILC2s (Lin⁻CD3⁻CD4⁺IL-17RB⁺c-KIT⁻IL-7Rα⁺KLRG1⁺) and recipient-derived CD4⁺T_H2 cells (Lin⁻CD3⁺CD4⁺IL-17RB⁺ST-2⁺) and measurements of indicated parameters of experimental food allergy (D) and indicated cytokine production by IL-25-stimulated 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 < .01 and ***P < .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

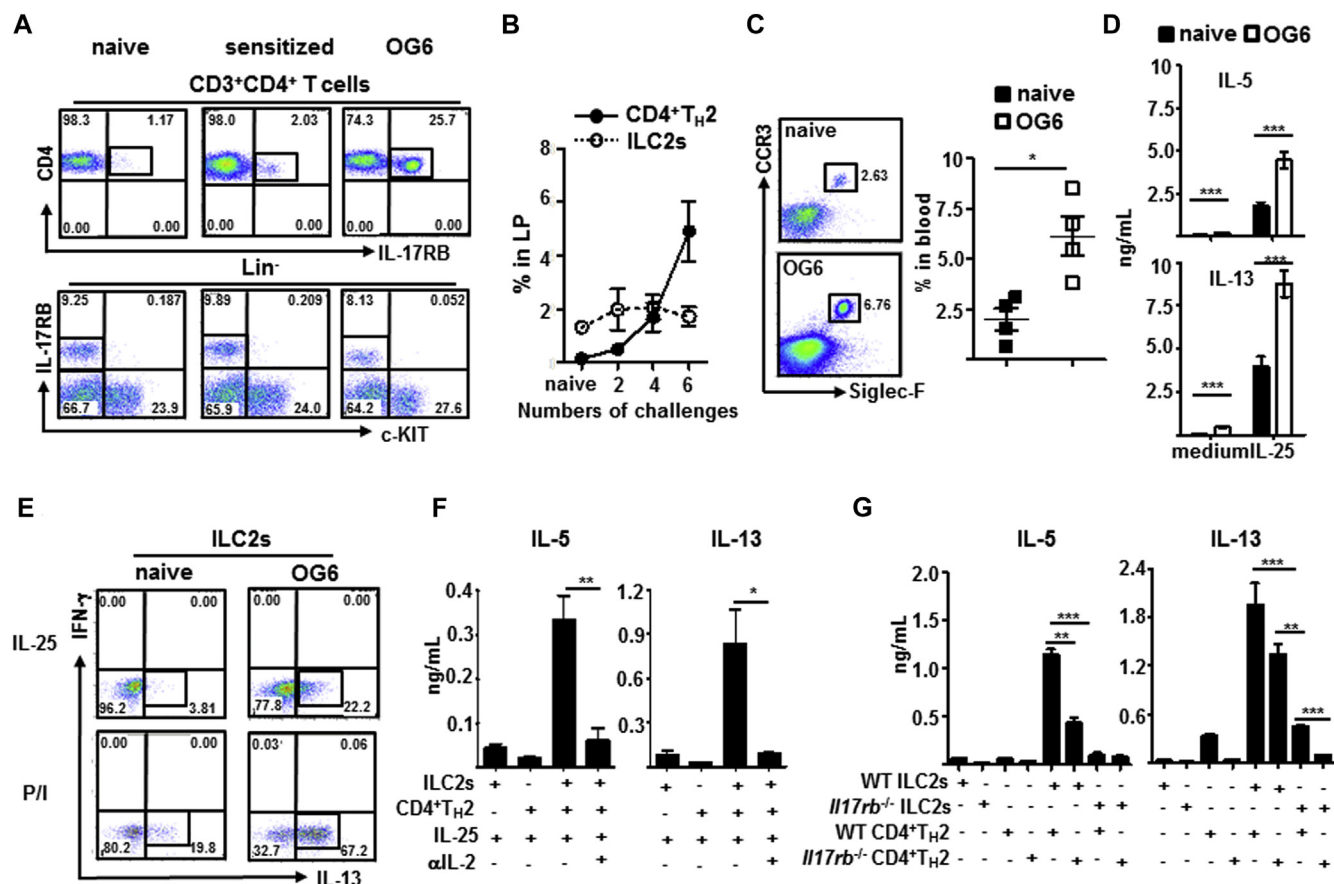


FIG 4. Detection and frequency of intestinal CD4⁺IL-17RB⁺ T_H2 cells and Lin⁻IL-17RB⁺c-KIT⁻ ILC2s (A and B), blood CCR3⁺Siglec-F⁺CD11b⁺ eosinophils (C) and intracellular IL-13-producing ILC2s (E) from naive or sensitized mice undergoing oral gavage (OG) with OVA 6 times (Fig 4, A, C, and E) or indicated 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 ≤ .05, **P ≤ .01, and ***P ≤ .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.

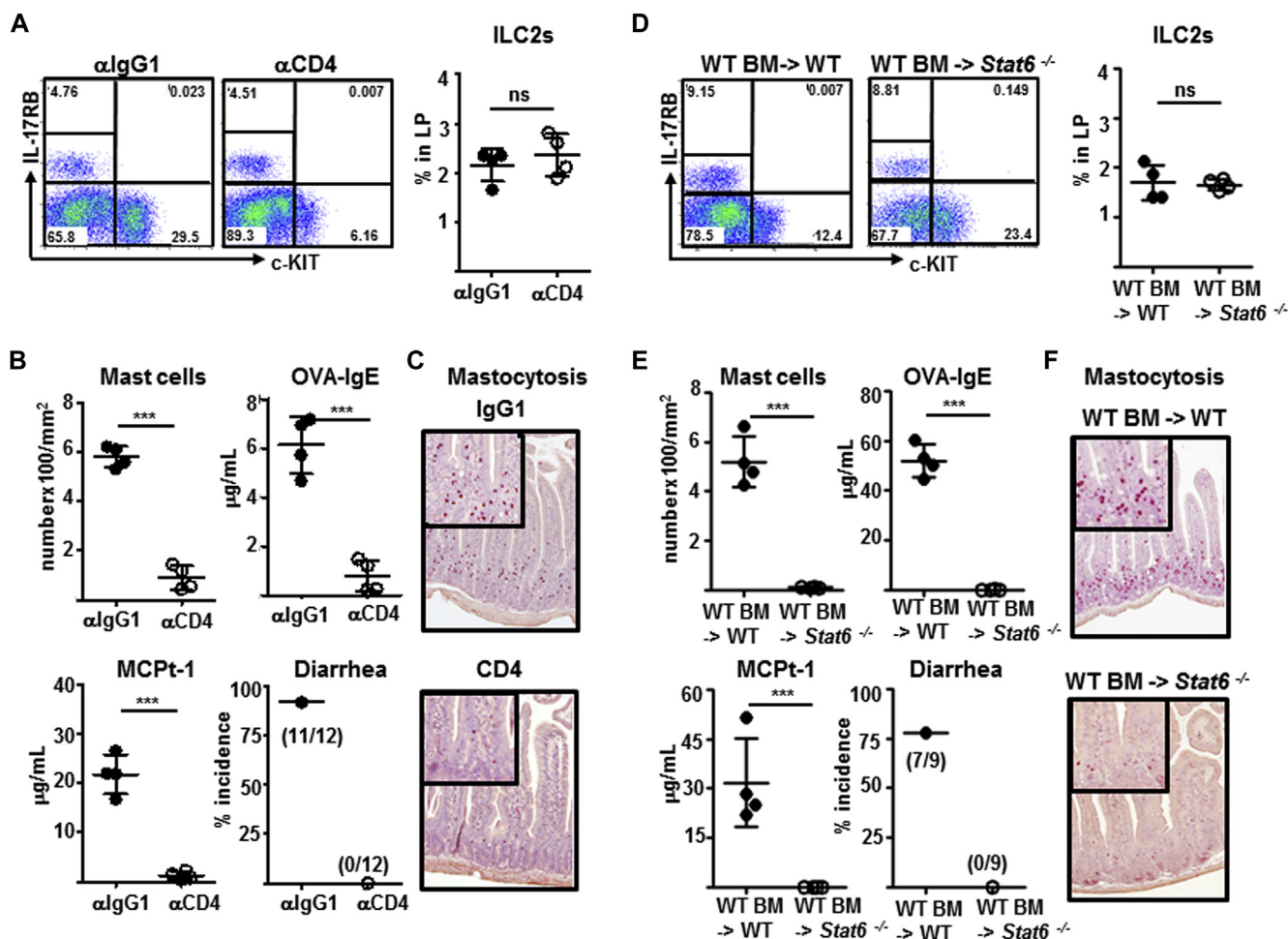


FIG 5. Detection and frequency of ILC2s (**A** and **D**), measurement of indicated features of experimental food allergy (**B** and **E**), and staining for intestinal mastocytosis (**C** and **F**) in sensitized WT BALB/c mice treated with indicated antibodies 1 day before the first and fourth intragastric 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 ≤ .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

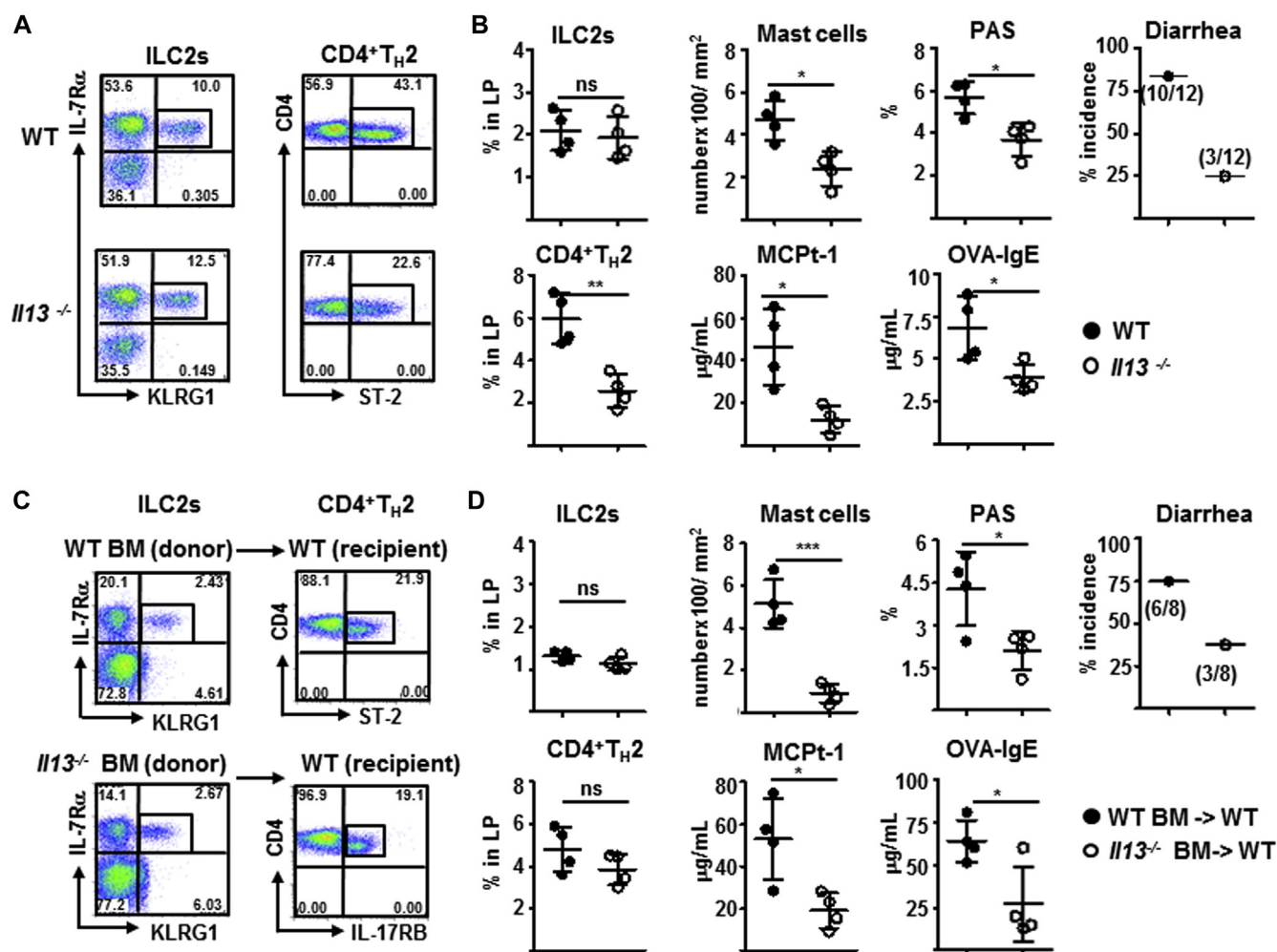


FIG 6. Detection (**A** and **C**) and frequency of Lin⁻IL-7Rα⁺KLRG1⁺ ILC2s and CD4⁺ST-2⁺ T_H2 cells in and measurements of indicated parameters of experimental food allergy of (**B** and **D**), sensitized WT or *Il13*^{-/-} mice (Fig 6, **A** and **B**) or irradiated WT BALB/c recipients reconstituted 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 ≤ .05, **P ≤ .01, and ***P ≤ .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 cells³⁵⁻³⁸ 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/FcεR1 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 *EcoRI* 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-1 [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-FcεR1α, 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'-TGACCACTCACTGCT-TAGC, reverse 5'-GGCAT GGACTGTGGTCATGAG), *Il13* (forward 5'-TGACCAACATCTCCAATTGCA, reverse 5'-TTGTTATAAAGTG GGCTA CTTGATTT), *Il25* (forward 5'-TGTACCAGGCTGTTGCATTC, reverse 5'-CTCCACTTCAGCCACTCCTC), *Tarc* (forward 5'-CAGGAAGTTGGT-GAGCTGGT, 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'-CTCCTTCTCTGCTGAGCCTGCG, reverse 5'-GTGATGAAGAT-GACCCCTGCCTT). 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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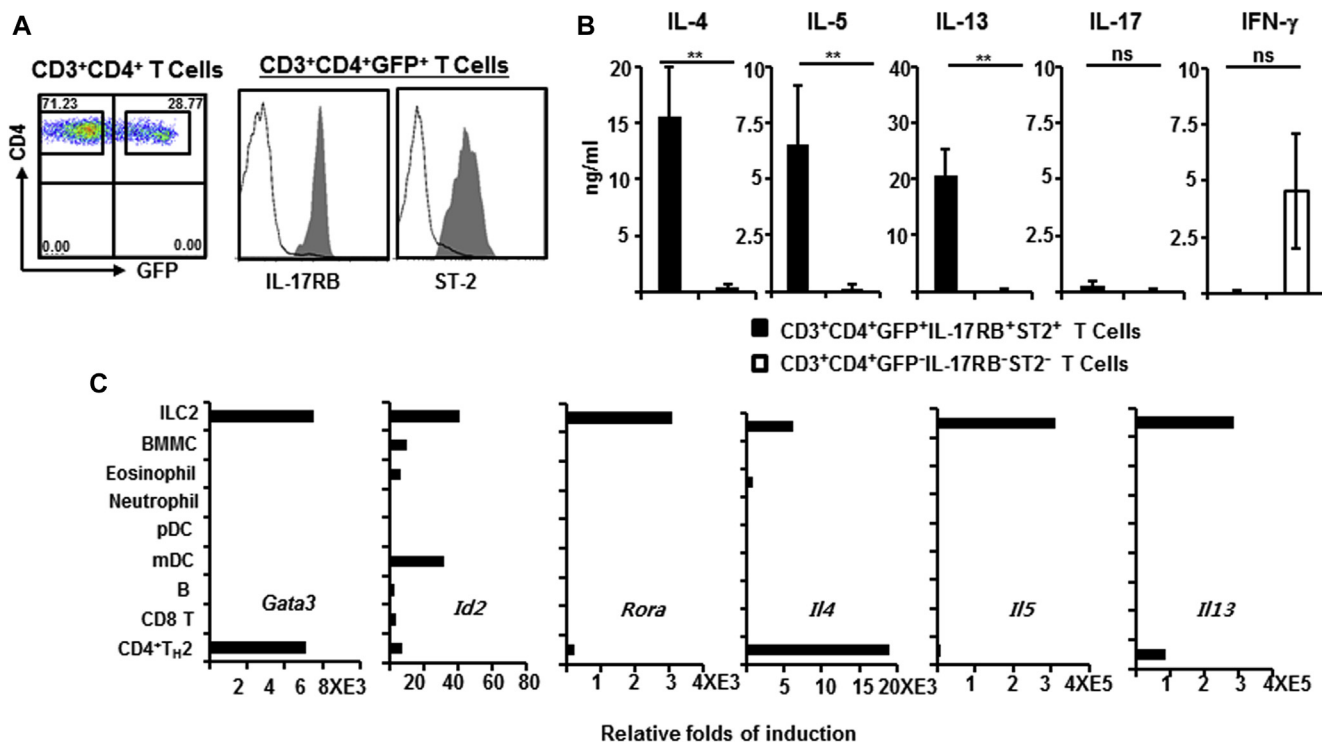


FIG E1. **A**, Detection of and phenotypic analysis of CD3⁺CD4⁺ T cells in LP of the small intestine of 4GET mice after 6 intragastric OVA challenges. **B**, Measurements by using ELISA of indicated cytokines secreted by sorted indicated T-cell subsets (1×10^5 cells) after stimulation with CD3 and CD28 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 (BMDCs), 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-1⁺B220⁺). Data represent one of 3 independent experiments. Error bars denote means $\pm$ SEMs. ** $P \leq .01$. ns, Not significant.

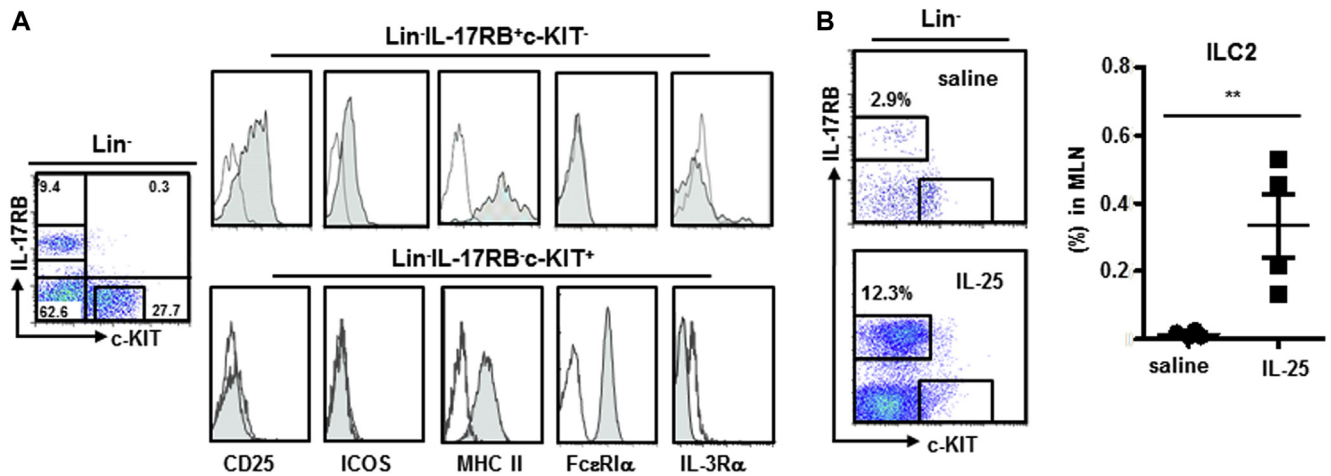


FIG E2. **A**, Phenotype of Lin⁻IL-17RB⁺c-KIT⁻ and Lin⁻IL-17RB⁻c-KIT⁺ cells in LP of the small intestine from mice with experimental food allergy was analyzed by using flow cytometry. **B**, Detection and frequency of ILC2s in mesenteric lymph nodes of BALB/c mice injected with recombinant IL-25 (0.5 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* ≤ .01.

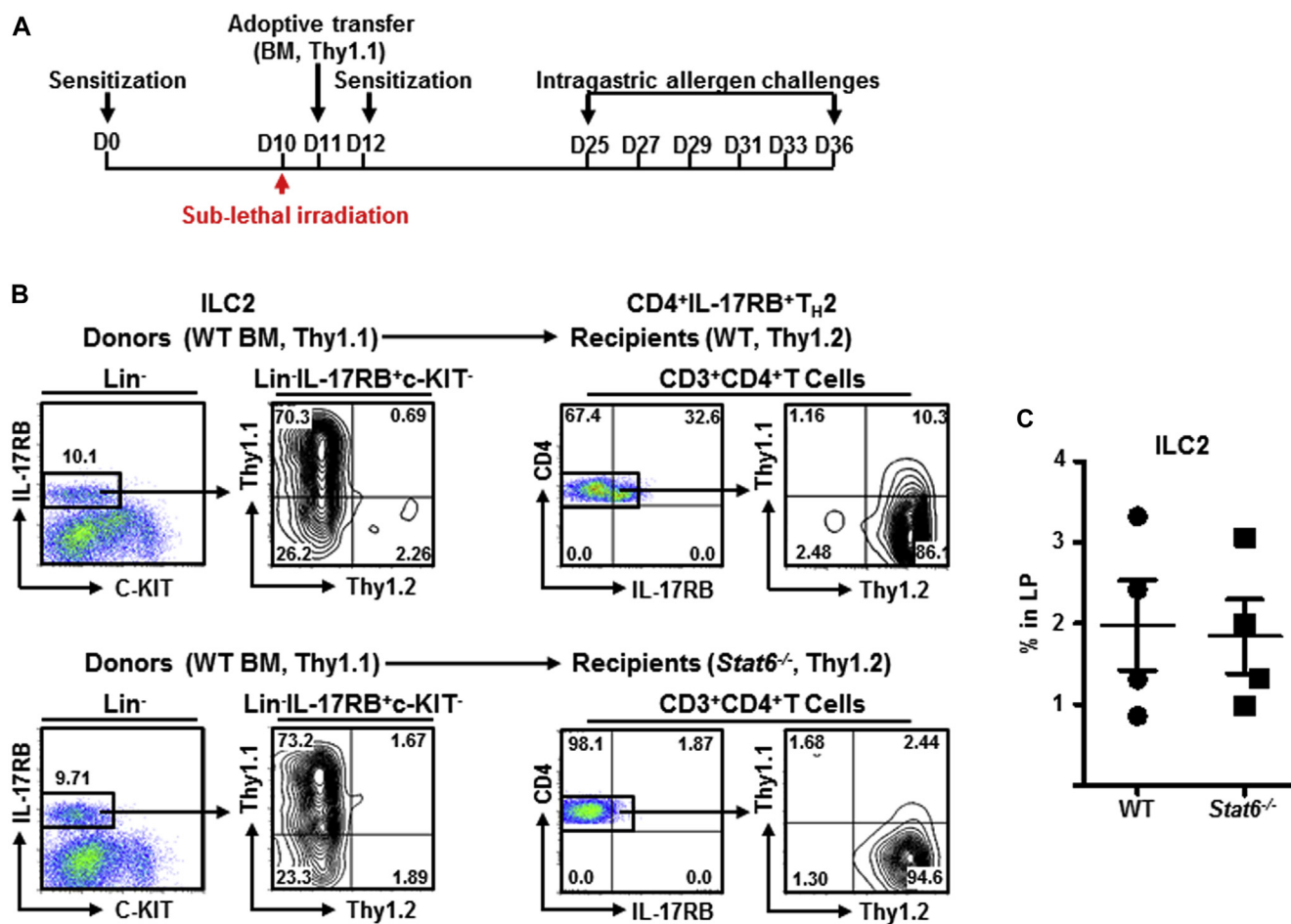


FIG E3. Origin of intestinal ILC2s and CD4⁺ T_H2 cells in irradiated recipient mice after reconstitution in a murine model of food allergy. **A**, Diagram of adoptive transfer protocol in the mouse model of food allergy. **B** and **C**, Detection (Fig E3, B) and frequency (Fig E3, C) of donor-derived 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 intra-gastric OVA challenges. Data represent one of 3 independent experiments (n = 4 mice per group).

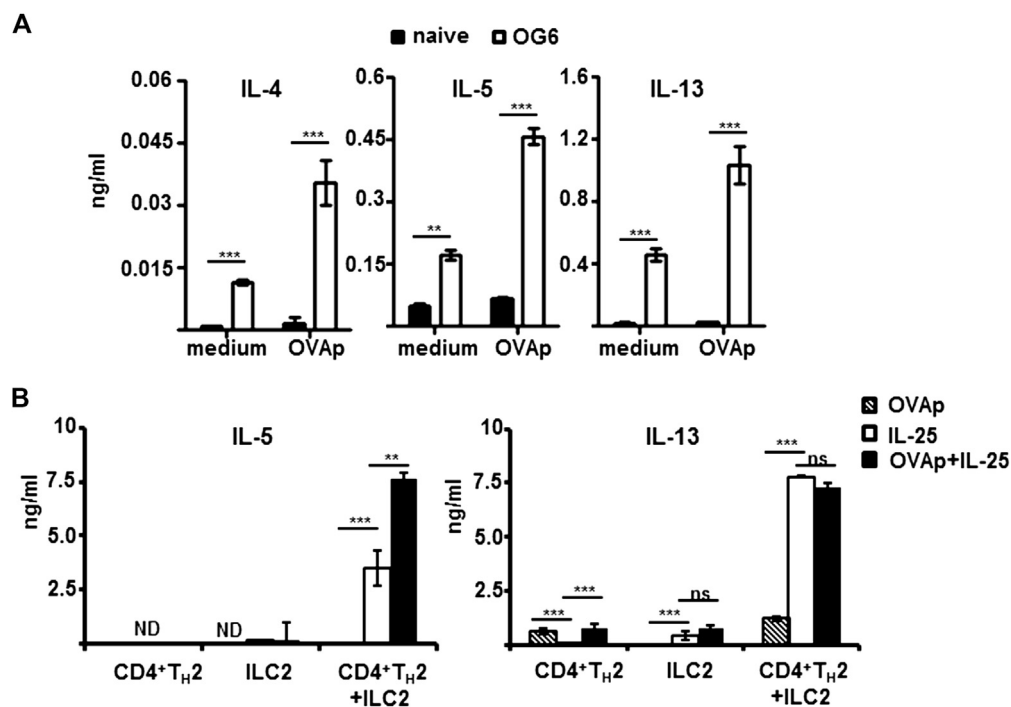


FIG E4. A, Measurement of indicated cytokines produced by LP cells of naive mice or mice with experimental food allergy after culture with medium only or OVA₃₂₃₋₃₃₉ peptides (10 μ mol/L) for 3 days by using ELISA. **B,** Measurement of IL-5 and IL-13 production by ILC2s, CD4⁺ TH₂ cells, or ILC2s cocultured with CD4⁺ TH₂ cells after stimulation with OVA₃₂₃₋₃₃₉ peptides, IL-25, or both for 3 days by using ELISA. Data are representative of 2 independent experiments. Error bars denote means $\pm$ SDs. ** $P \leq .01$ and *** $P \leq .001$. N. D., Not detected; ns, not significant.