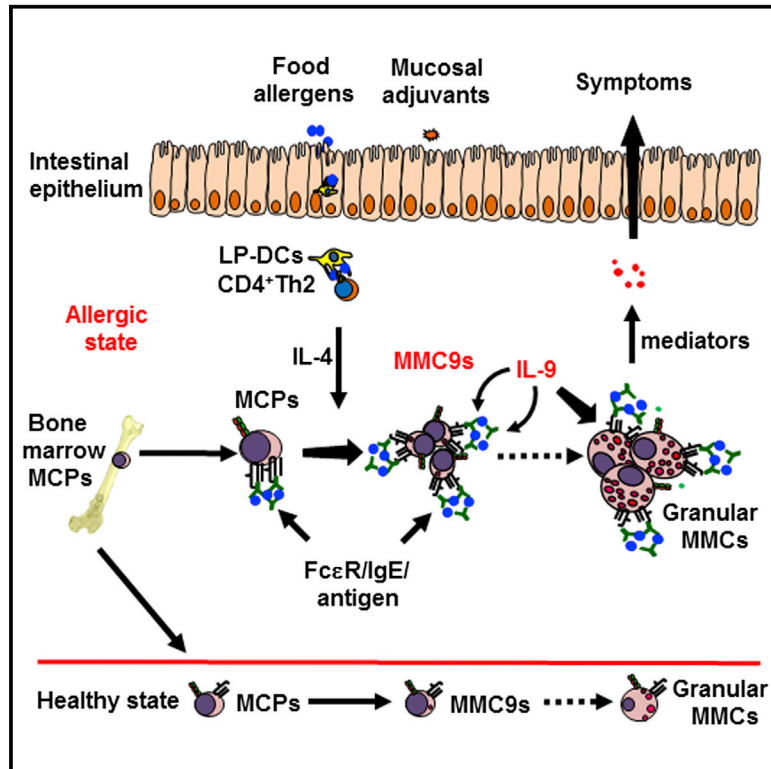


Immunity

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

Graphical Abstract



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In Brief

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

Highlights

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

Accession Numbers

GSE72921

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.

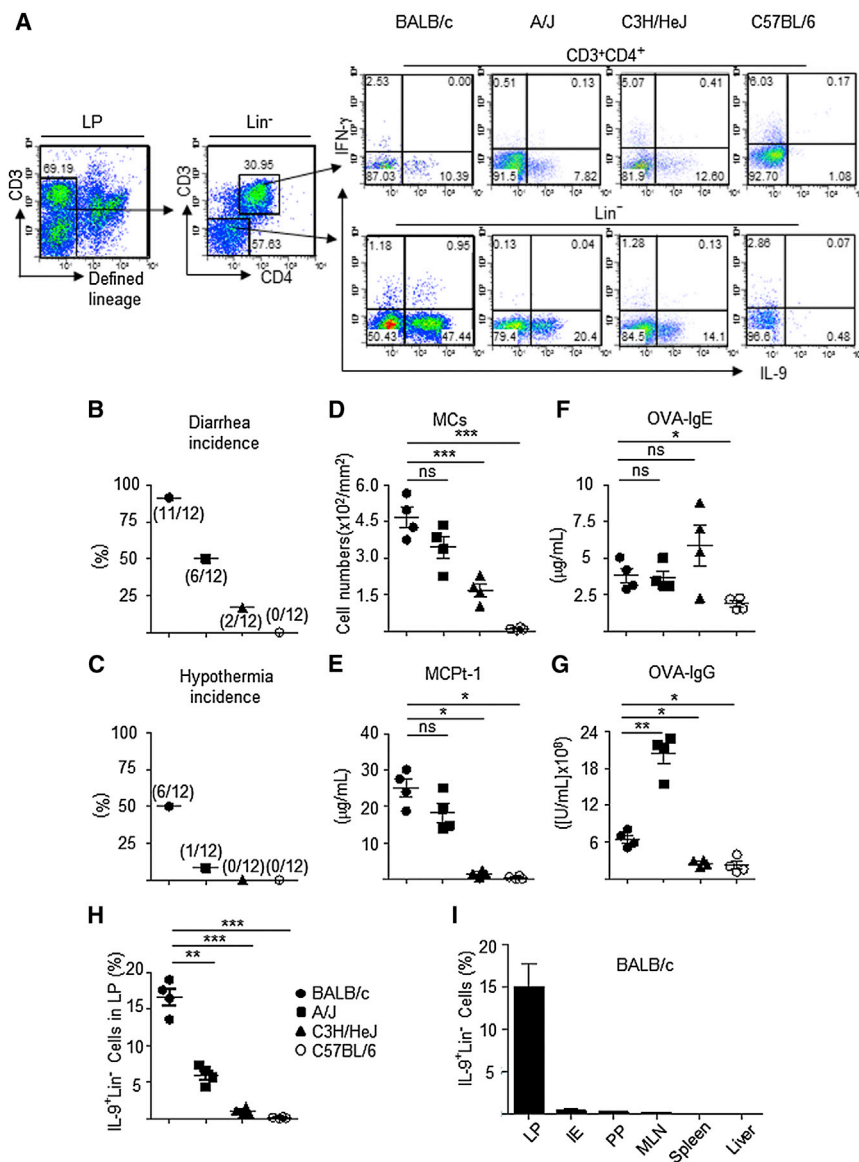


Figure 1. Lin⁻ IL-9-Producing Cell Frequency Correlates Positively with Susceptibility to Experimental Food Allergy

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

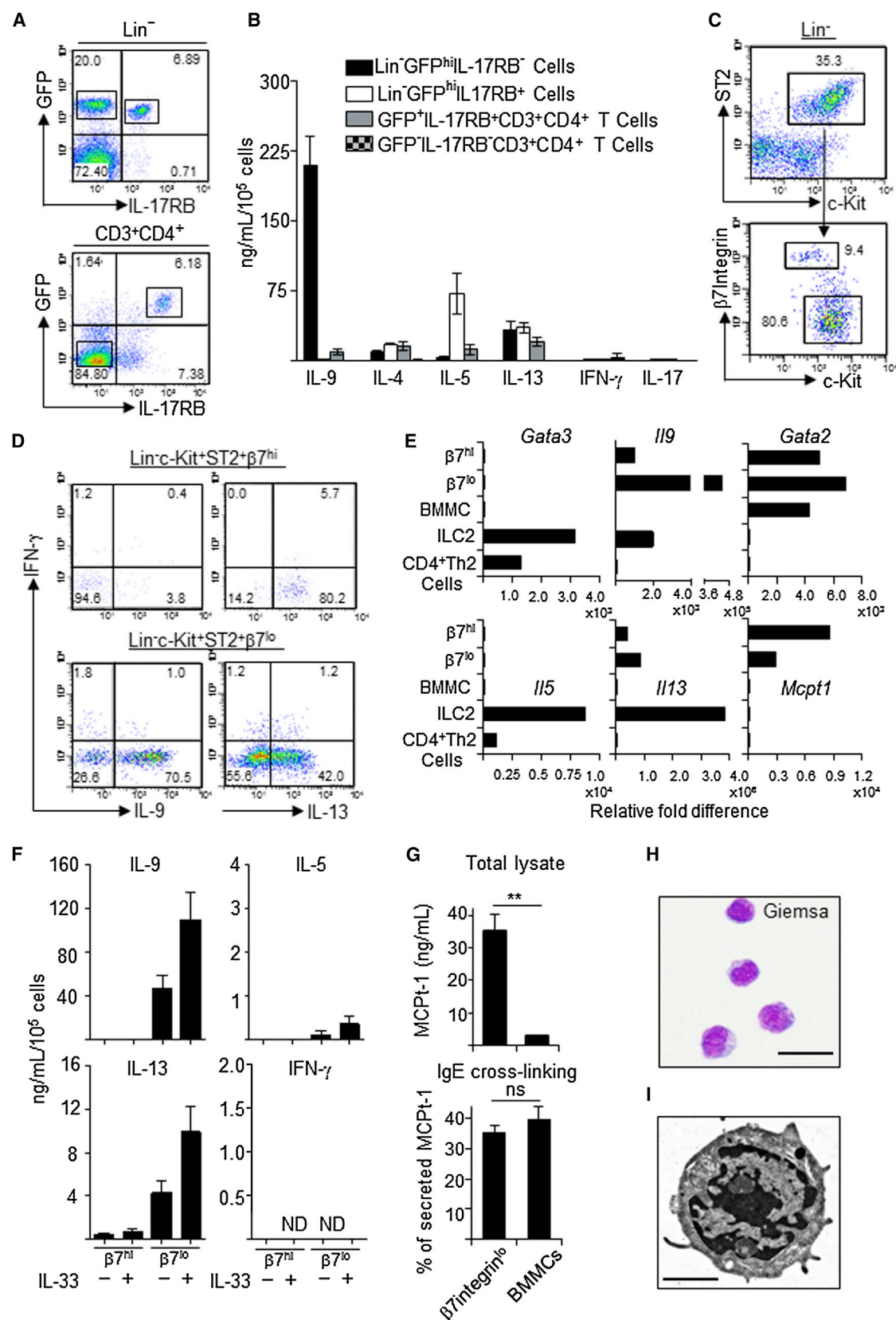
Innate MMC9s Are the Principal IL-9 Producers of Mast Cell Lineage

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

To better understand the lineage of these Lin[−]GFP^{hi} cells, we performed detailed cellular and molecular characterization. The purified IL-9-producing Lin[−]GFP^{hi}IL-17RB[−] cells were found to express c-Kit, Fc ϵ RI 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 RI 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 sublethal irradiation (protocol diagrammed 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 *Ilg13*, *Ilg13*, and *Mcp6*, 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⁺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^{hi}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.

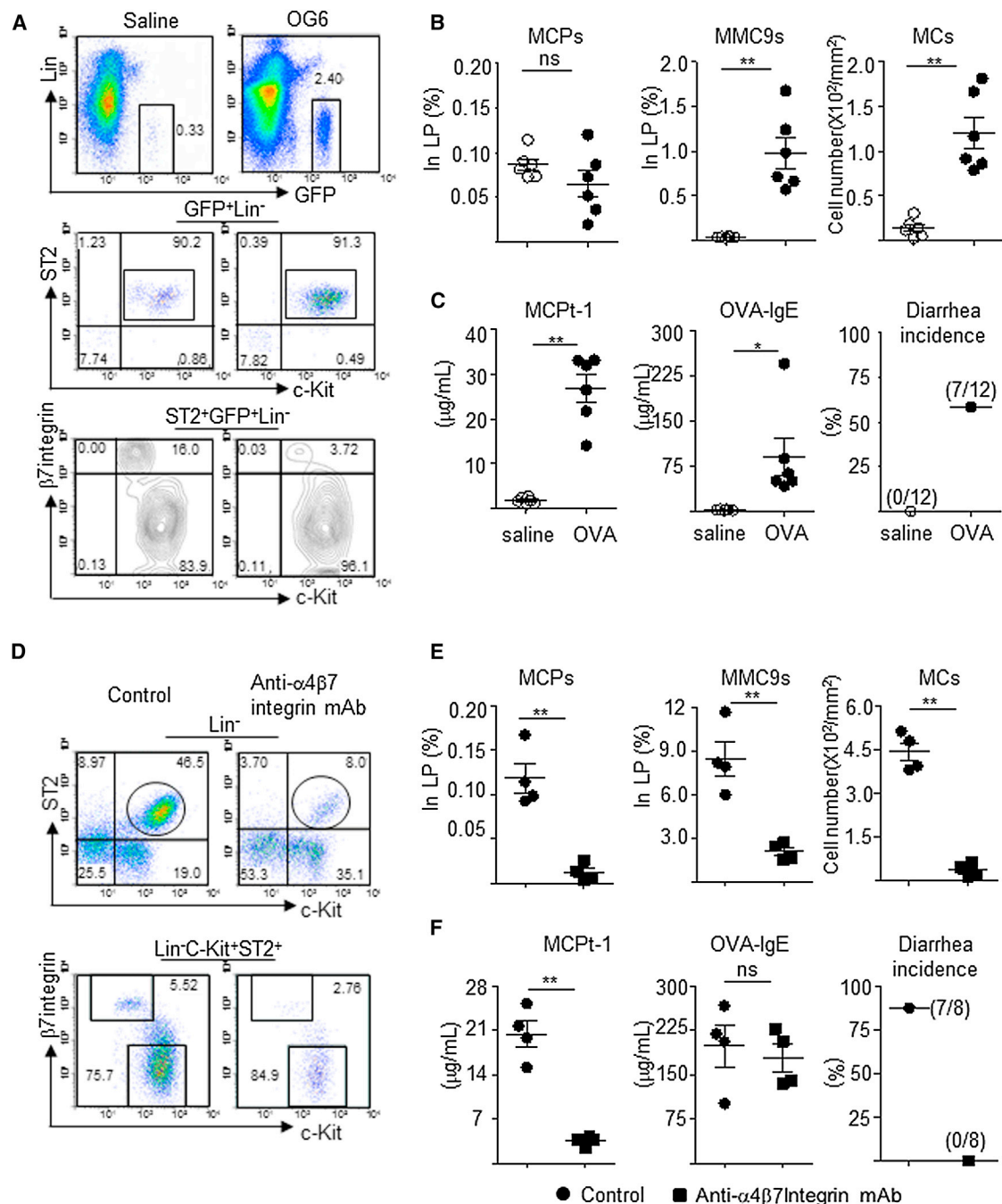


Figure 3. Blockade of Intestinal $\beta 7^{\text{hi}}$ MCP Recruitment from Bone Marrow Results in the Failure of MMC9 Induction

Detection and frequency of donor-derived MMC9s ($\text{Lin}^{-}\text{GFP}^{+}\text{ST2}^{+}\text{c-Kit}^{+}\beta 7^{\text{int}}^{\text{hi}}$) and MCPs ($\text{Lin}^{-}\text{GFP}^{+}\text{ST2}^{+}\text{c-Kit}^{+}\beta 7^{\text{int}}^{\text{hi}}$), 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 ($\text{Lin}^{-}\text{Ly-6c}^{-}\text{FcεR1α}^{-}\text{CD41}^{-}\text{CD71}^{-}\text{FLK2}^{-}\text{CD150}^{-}\text{c-Kit}^{+}\beta 7^{\text{int}}^{\text{hi}}$) 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,

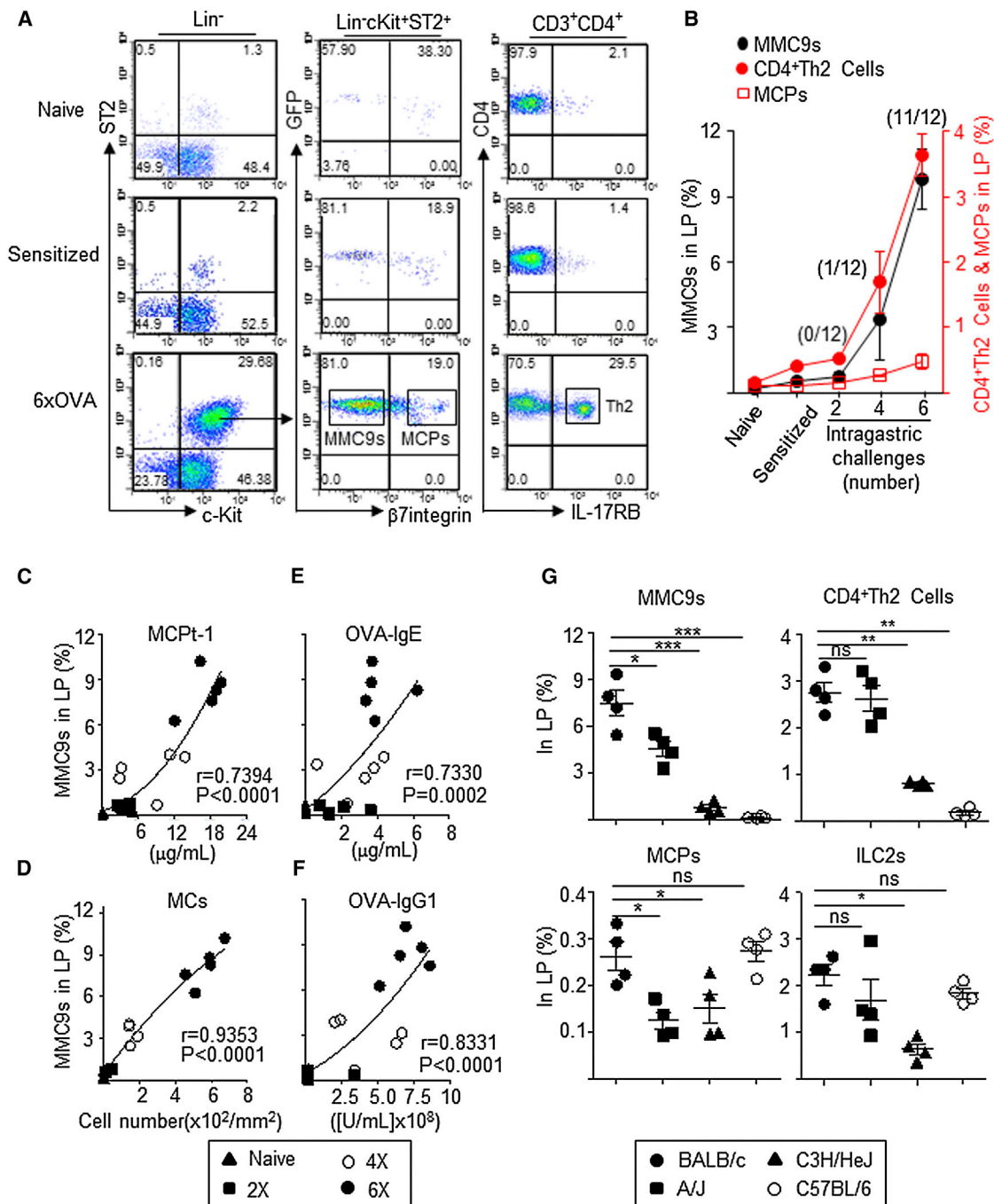


Figure 4. MMC9s and CD4⁺ Th2 Cell Frequencies Correlate Positively with Susceptibility to Experimental Food Allergy

(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

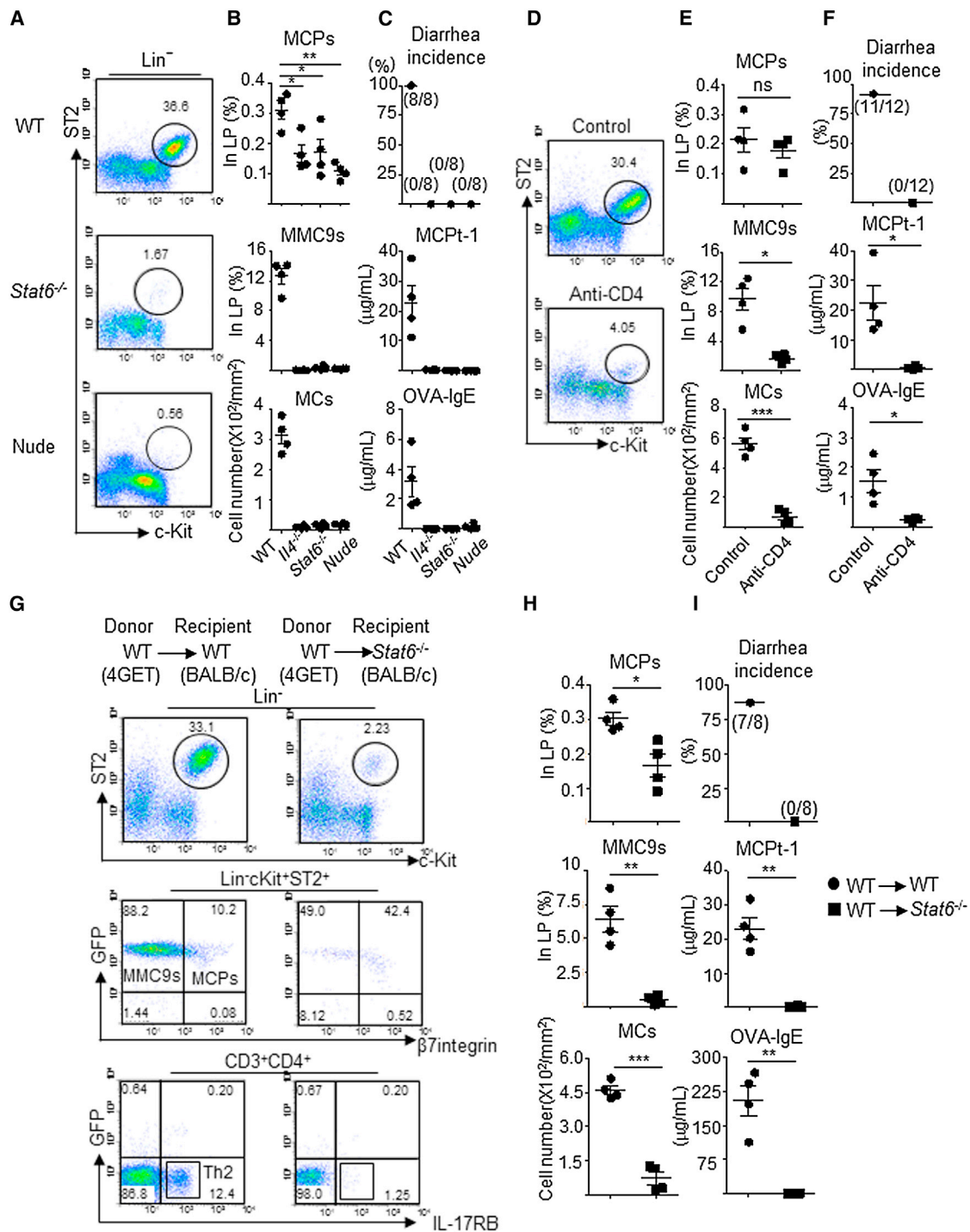


Figure 5. STAT6 Signaling and T Cells Are Required for MMC9 Induction that Promotes Experimental Food Allergy

(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.

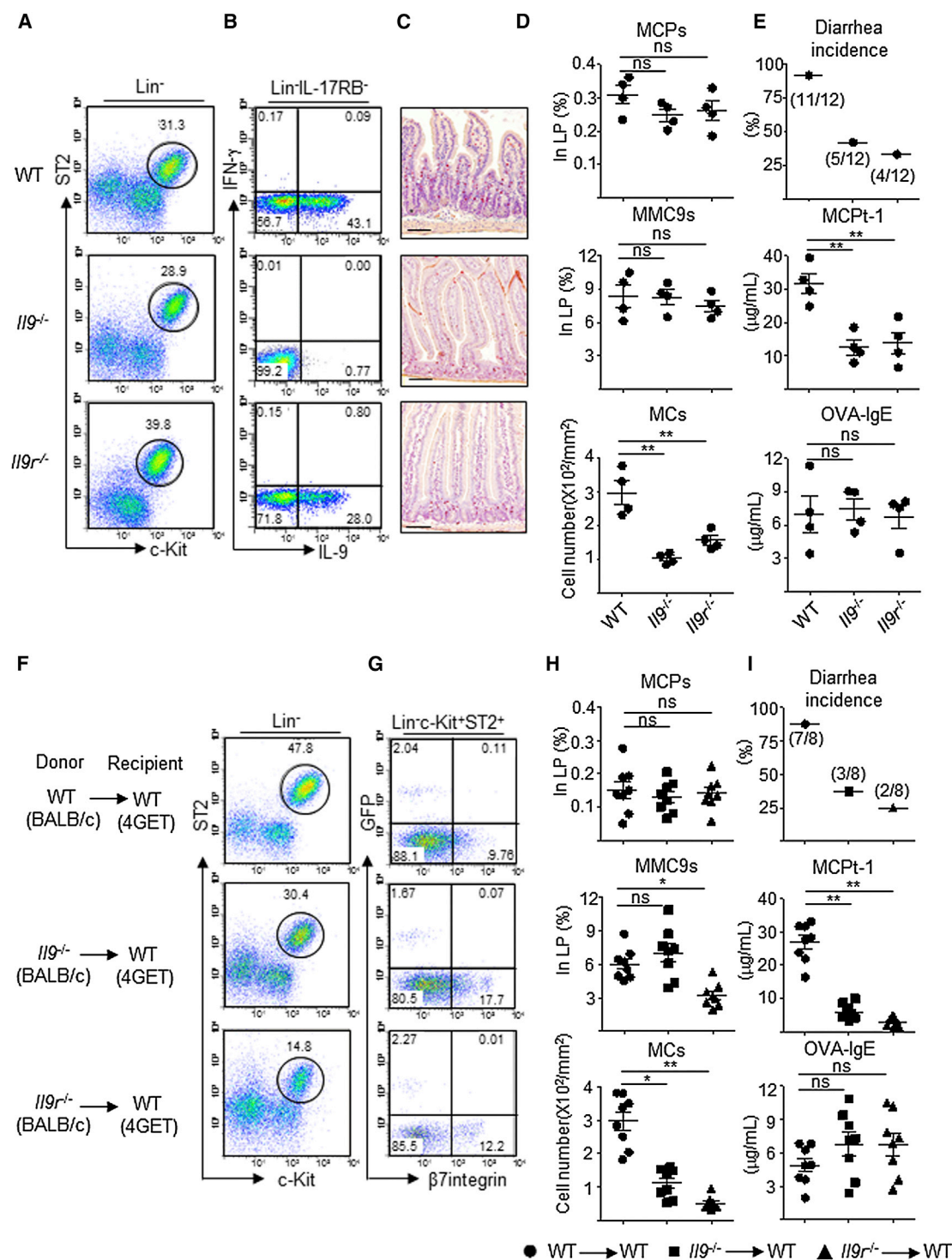


Figure 6. MMC9s Can Be Induced in IL-9- or IL-9R-Deficient Mice but Fail to Promote Experimental Food Allergy

(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⁺) and MCPs (Lin⁻ c-Kit⁺ ST2⁺ β 7 integrin⁺) (D) and measurement of indicated features of experimental food allergy (D, E) in wild-type BALB/c or indicated gene-deficient murine strains after six intragastric OVA challenges.

(F–I) Detection (F, G) and frequency (H) of donor-derived MMC9s (Lin⁻ c-Kit⁺ ST2⁺ β 7 integrin⁺ GFP⁺) and MCPs (Lin⁻ c-Kit⁺ ST2⁺ β 7 integrin⁺ 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.

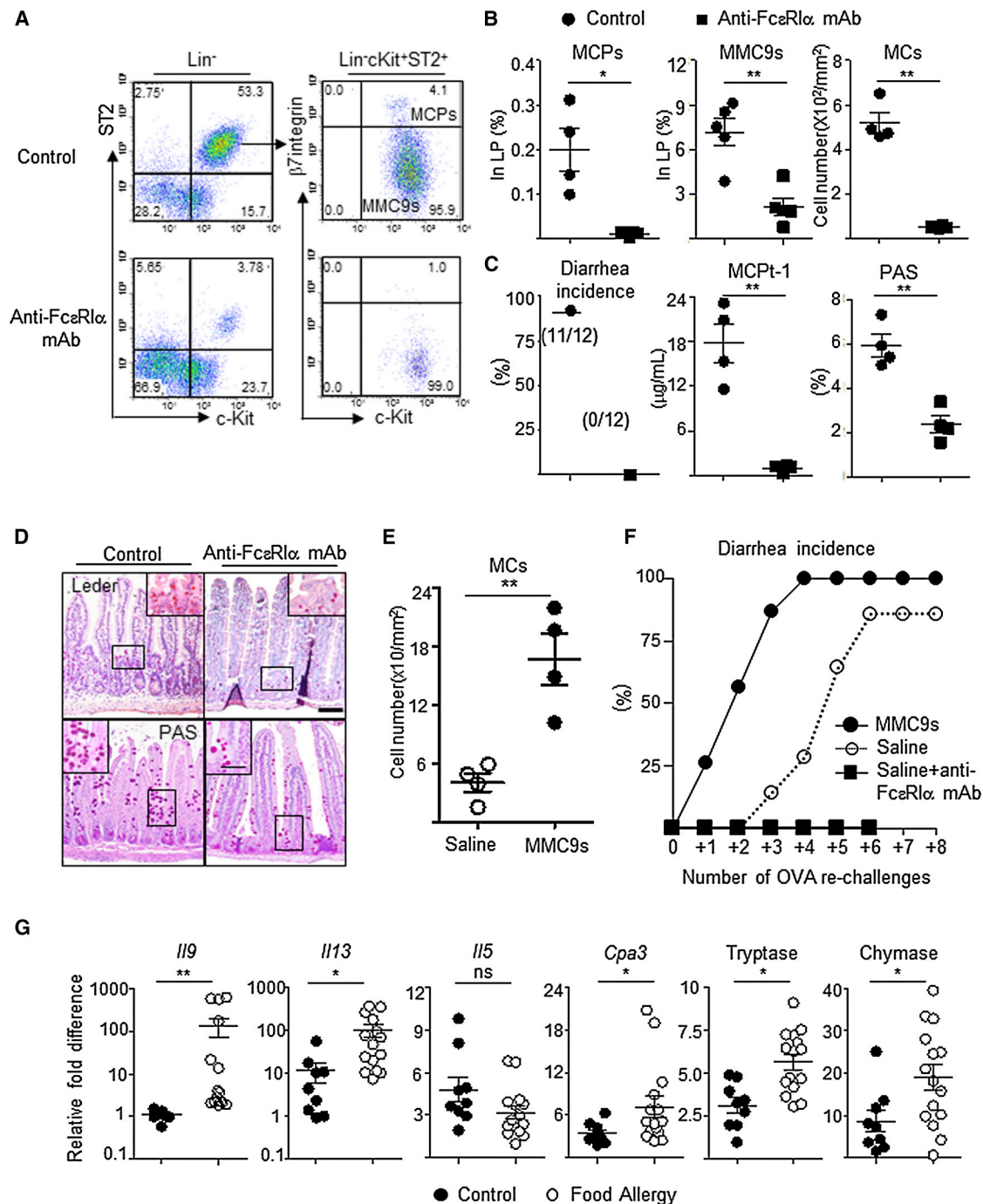


Figure 7. MMC9s Drive Intestinal Mastocytosis and IL9 Transcript Expression Is Increased in Food Allergy Patients

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

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

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

Data in (A)–(D) represent one of three independent experiments (n = 4 mice per group). Gene expression data are expressed as relative fold difference as described in (G). Error bars denote mean ± SEM. Scale bars represent 20 μm (D) and 100 μm (D, the corner insets). *p ≤ 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; Renaud 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 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; Ruitenberg and Elgersma, 1976; Urban et al., 2000).

Several murine models have been developed in an attempt to understand the immunological mechanisms underlying the

susceptibility of food allergy. In addition to adjuvant alum that induces a strong allergic sensitization, administration of CT intragastrically could also break oral tolerance and induce elevated histamine, IgE, and IgG1 titers, resulting in systemic anaphylactic responses to ingested allergen, which occurred selectively in C3H/HeJ strain (Li et al., 1999; Morafo et al., 2003). In contrast to sensitization via alum adjuvant, repeated intragastric OVA challenge failed to induce strong MMC9 (<1%) and CD4⁺ Th2 (<1%) immune responses in CT-sensitized C3H/HeJ or BALB/c mice, despite inducing a strong humoral immune response. Consequently, much fewer intestinal MC numbers and serum MCPT-1 titers (~15 cells/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-β7integrin (M293), allophycocyanin (APC)-conjugated anti-IL-17RB, APC-Cy7-conjugated anti-c-Kit (2B8), PE-Cy7-conjugated anti-CD3e (145-2C11), Horizon V500-conjugated CD4 (RPA-T4), and biotinylated anti-T1/ST2 (DJ8) antibodies. Subsequently, cells were counterstained with PerCP-Cy5.5-conjugated monoclonal antibodies against lineage (Lin) markers (CD11b [M1/70], CD11c [HL3], CD8 [53-6.7], B220 [RA3-6B2], Gr-1 [RB6-8C5], and CD335 [NKP46, 29A.4]) and with Brilliant Violet 421-labeled Streptavidin (Biolegend) before analysis with a 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-β7integrin, APC-Cy7-conjugated anti-c-Kit, and biotinylated anti-T1/ST2 antibodies. Subsequently, cells were counterstained with PerCP-Cy5.5-conjugated monoclonal antibodies against Lin markers (including CD3 and CD4) and with Brilliant Violet 421-labeled streptavidin. Stained cells were fixed and permeabilized for intracellular cytokine staining using FITC-anti-IFN-γ (XMG 1.2) and APC-anti-IL-9 (RM9A4) or APC-anti-IL-13 (eBio13A).

IgE-Mediated Experimental Food Allergy

Mice were sensitized twice within a 2-week interval by intraperitoneal (i.p.) injection with 100 μg OVA and 1 mg alum and then orally gavaged with OVA (50 mg in 250 μl saline) six times within 2 weeks. In prophylactic experiments, sensitized BALB/c mice were injected intraperitoneally with 500 μg anti-FcεRIα (MAR-1) antibody (Biolegend) or hamster isotype control antibody (Bio X Cell) or with 500 μg of anti-CD4 (GK1.5) antibody (Bio X Cell) or rat isotype control antibody (Bio X Cell) twice 1 day before the first and fourth intragastric OVA challenges. Some antibody-treated mice were rested for 2 weeks after the sixth OVA challenge before reconstitution with 1 × 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). BMDCs 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 (A1090129-1 to Y.-H.W., A1073553 to S.P.H.), the Digestive Health Center (P30 DK078392, Pilot and Feasibility Award to Y.-H.W.), Campaign Urging Research For Eosinophilic Diseases (CURED) Foundation, the Buckeye Foundation, the Food Allergy Research Education Fund to M.E.R., and VA Merit Award to F.D.F.

Received: September 10, 2014

Revised: May 30, 2015

Accepted: August 20, 2015

Published: September 22, 2015

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Immunity

Supplemental Information

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

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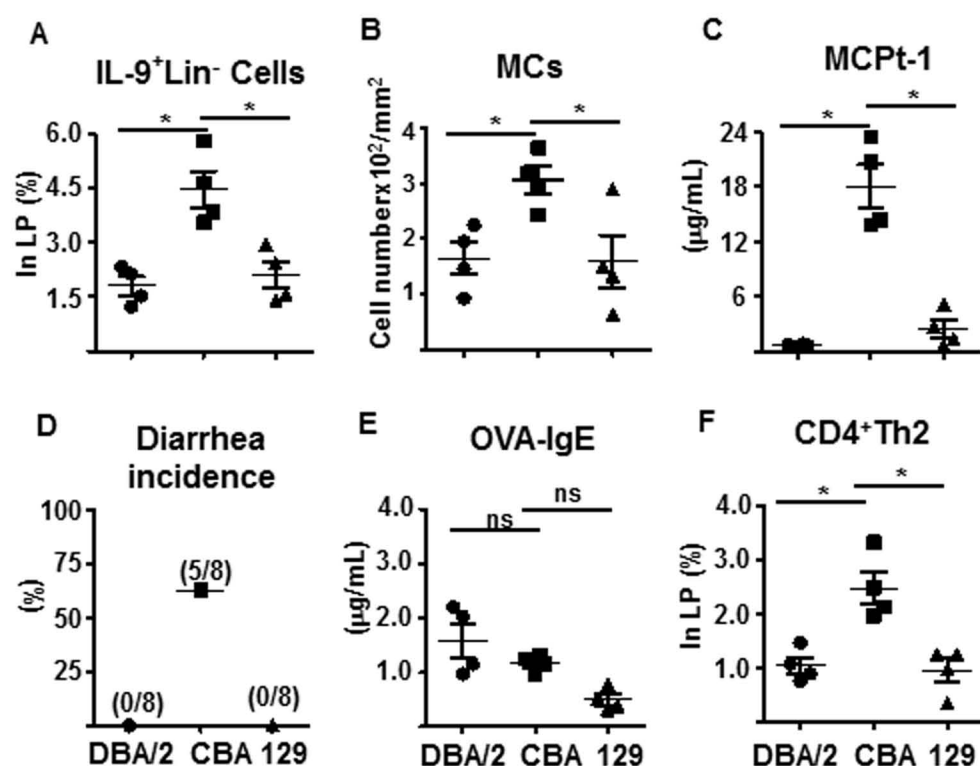


Figure S1. Frequencies of Intestinal IL-9-producing Lin⁻ and CD4⁺Th2 Cells Correlate Positively with the Susceptibility to Developing IgE-mediated Experimental Food Allergy. (related to Figure 1 and Figure 4) (A) Frequency of IL-9⁺Lin⁻ and (F) CD4⁺ST2⁺IL-17RB⁺ cells, (B) intestinal mast cell (MC) number,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

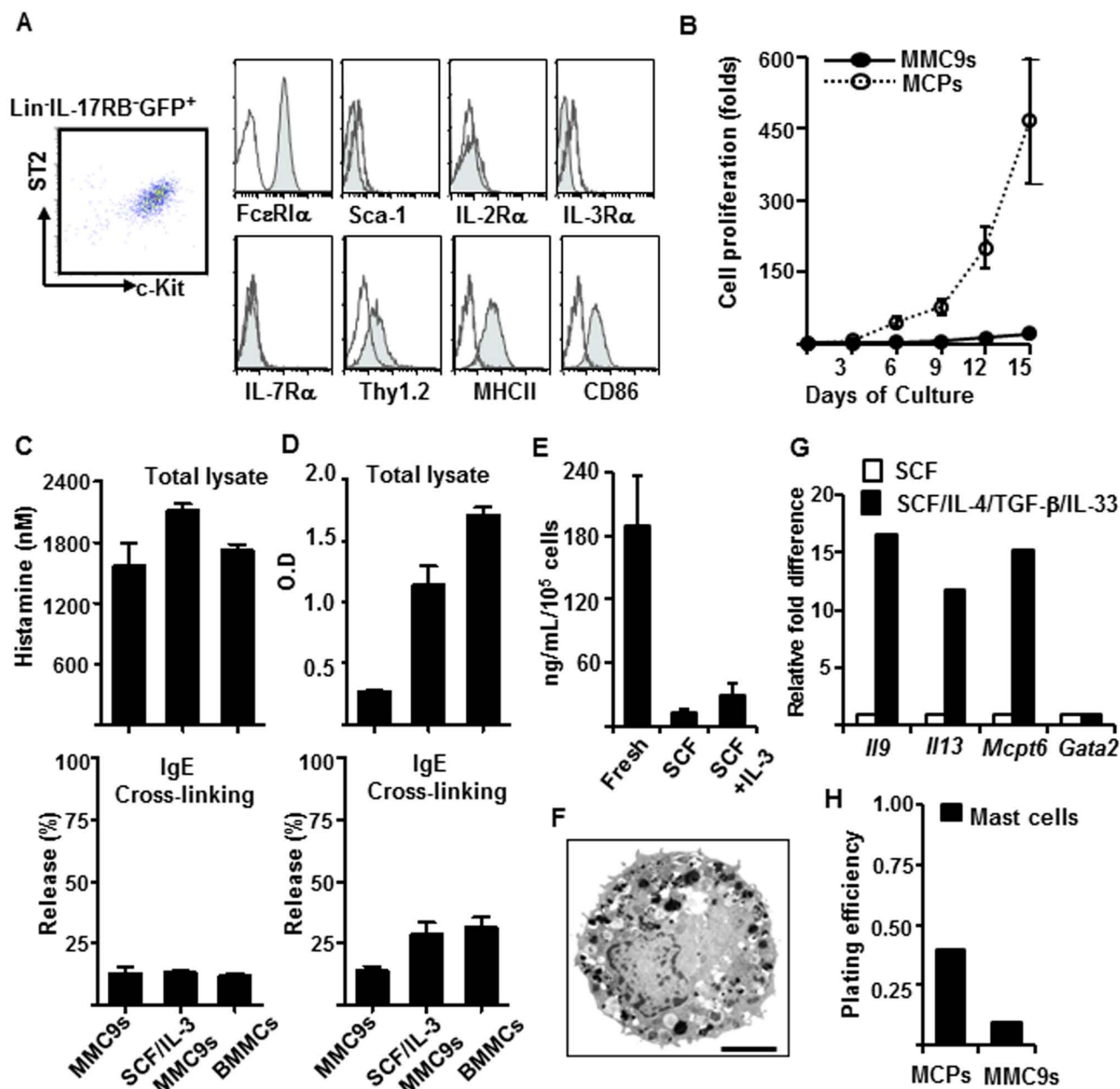


Figure S2. Characteristics of Intestinal MCPs and MMC9s. (related to Figure 2) (A) Phenotypic analysis of intestinal Lin⁺GFP^{hi}IL-17RB⁺c-Kit⁺ST2⁺ cells from BALB/c mice with active allergic diarrhea. (B) Comparison of proliferative response to SCF plus IL-3 between MCPs (Lin⁺IL-17RB⁺c-Kit⁺ST2⁺β7integrin^{hi}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

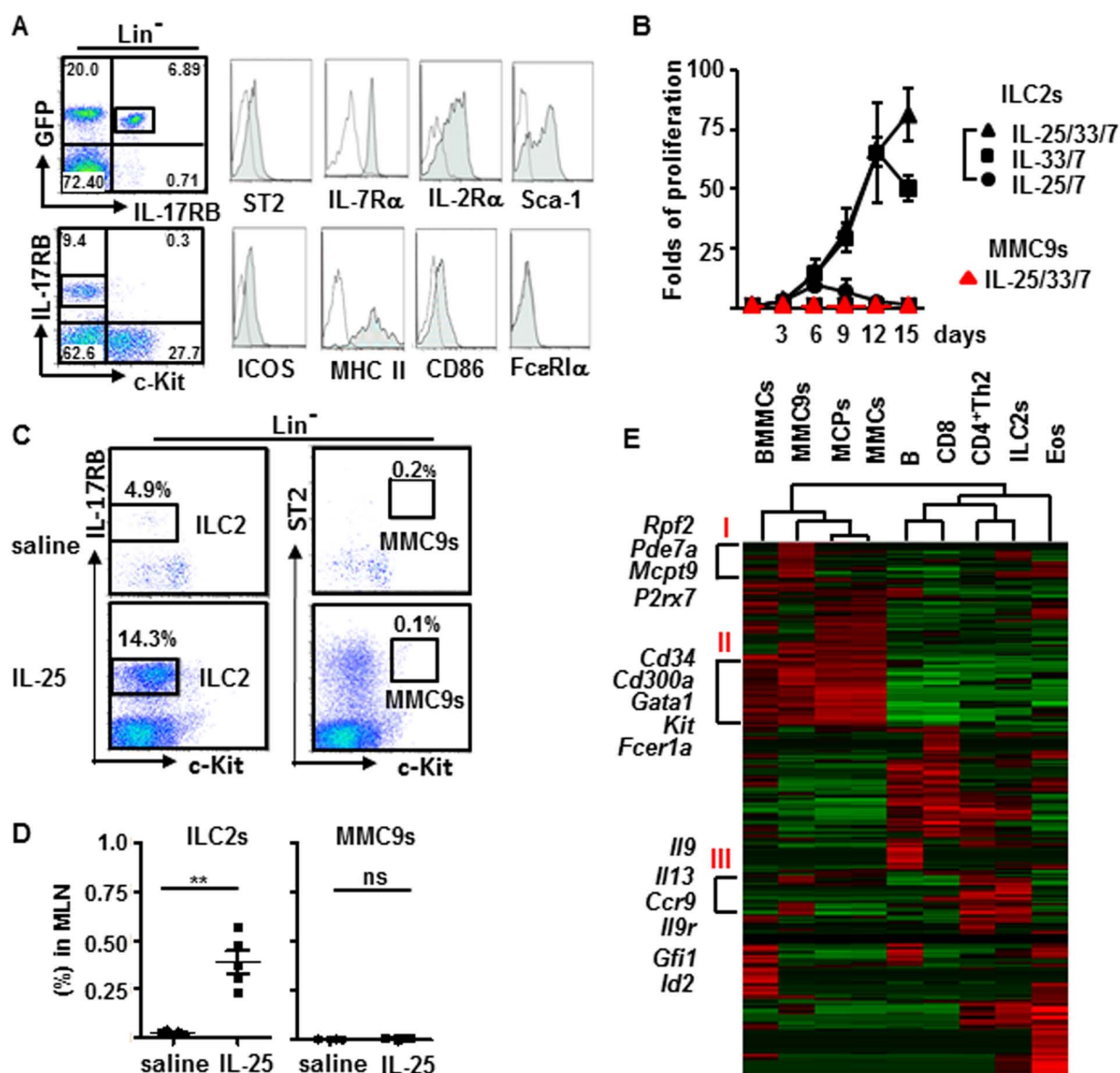


Figure S3. Characterizations of Intestinal ILC2s and MMC9s. (related to Figure 2)(A) Detection and phenotypic analysis of Lin⁻GFP^{hi}IL-17RB⁺c-Kit⁻ cells. **(B)** Proliferative response of purified MMC9s (Lin⁻GFP^{hi}IL-17RB⁺c-Kit⁺ β 7integrin^{lo} cells) and ILC2s (Lin⁻GFP^{hi}IL-17RB⁺c-Kit⁻IL-7R α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

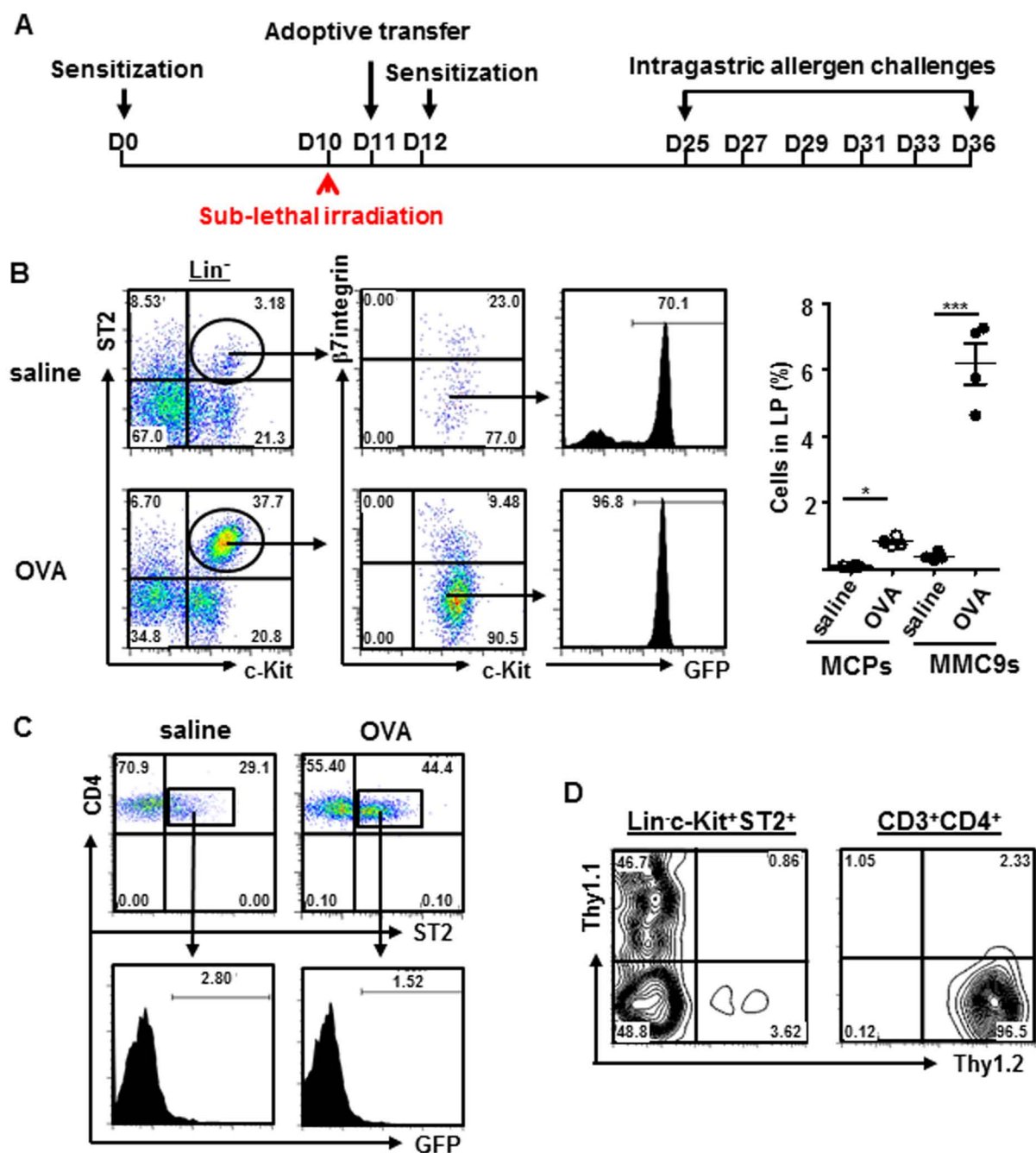


Figure S4. Origin of Intestinal MCPs, MMC9s, and CD4⁺Th2 Cells in a Reconstitution Model of Experimental Food Allergy. (related to Figure 3) (A) Diagram of adoptive transfer protocol in the mouse model of food allergy. (B-D) Detection and frequency of donor-derived MMC9s (Lin⁻IL-17RB⁺c-Kit⁺ β 7 integrin^{lo}) and MCPs (Lin⁻IL-17RB⁺c-Kit⁺ β 7 integrin^{hi}) (B), recipient-derived GFP-CD4⁺ST2⁺Th2 cells (C), or indicated cell subsets (D) in the small intestine of Thy1.2⁺ BALB/c recipient mice reconstituted with bone marrow progenitors from IL-4-eGFP (4GET) mice (A-C) or Thy1.1⁺ (D) BALB/c mice after six times of intragastric saline or OVA challenges. Data represent one of three independent experiments (n=4 mice per group). LP, lamina propria. * p<0.05, *** p<0.001.

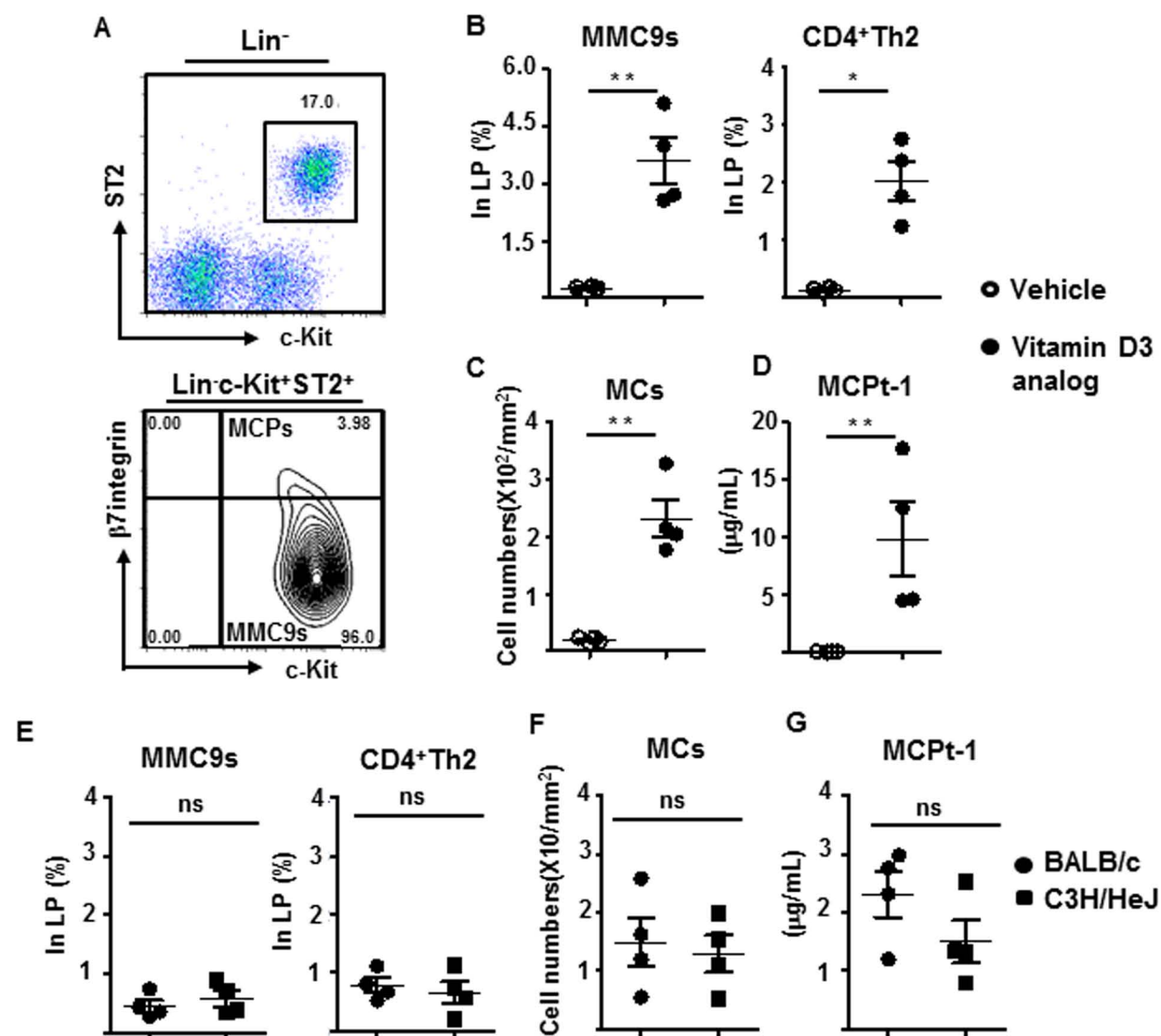
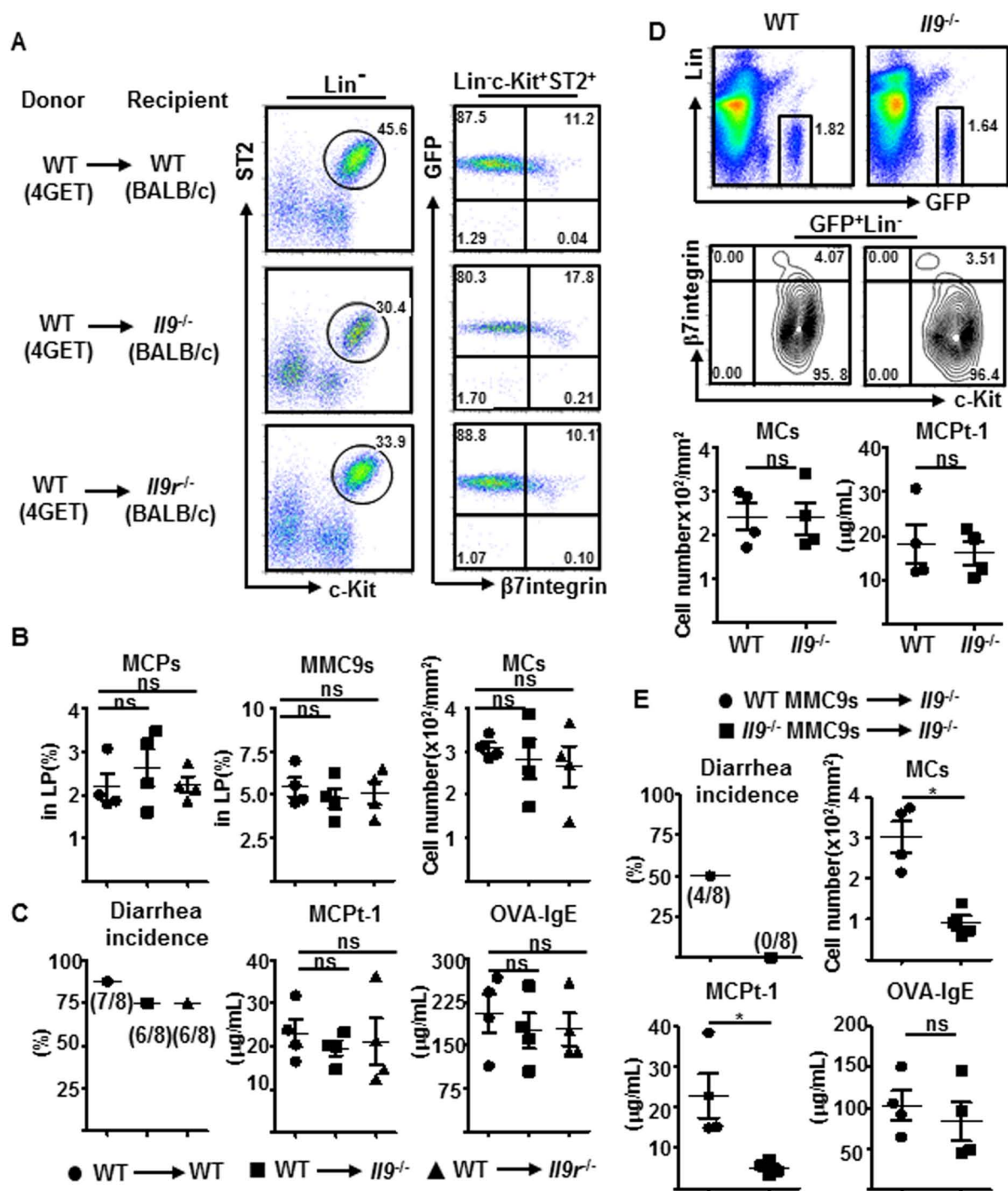


Figure S5. Induction of MMC9s and CD4⁺Th2 cells and Intestinal Mastocytosis in the Cholera Toxin- or Skin-sensitized Model of Experimental Food Allergy. (related to Figure 4) (A) Detection and (B, E) frequency of MMC9s (Lin⁻IL-17RB⁺c-Kit⁺ $\beta 7$ integrin^{lo}), and CD4⁺IL-17RB⁺Th2, (C, F) intestinal mast cell (MC) number, and (D, G) serum MCPt-1 titers of indicated murine strains after sensitization with vitamin D3 analog (Calcipotriol) (A-D) or cholera toxin (E-G) and six intragastric OVA challenges. Data represent one of two independent experiments (n=4 mice per group). LP, lamina propria. * p<0.05, ** p<0.01, NS, not significant.



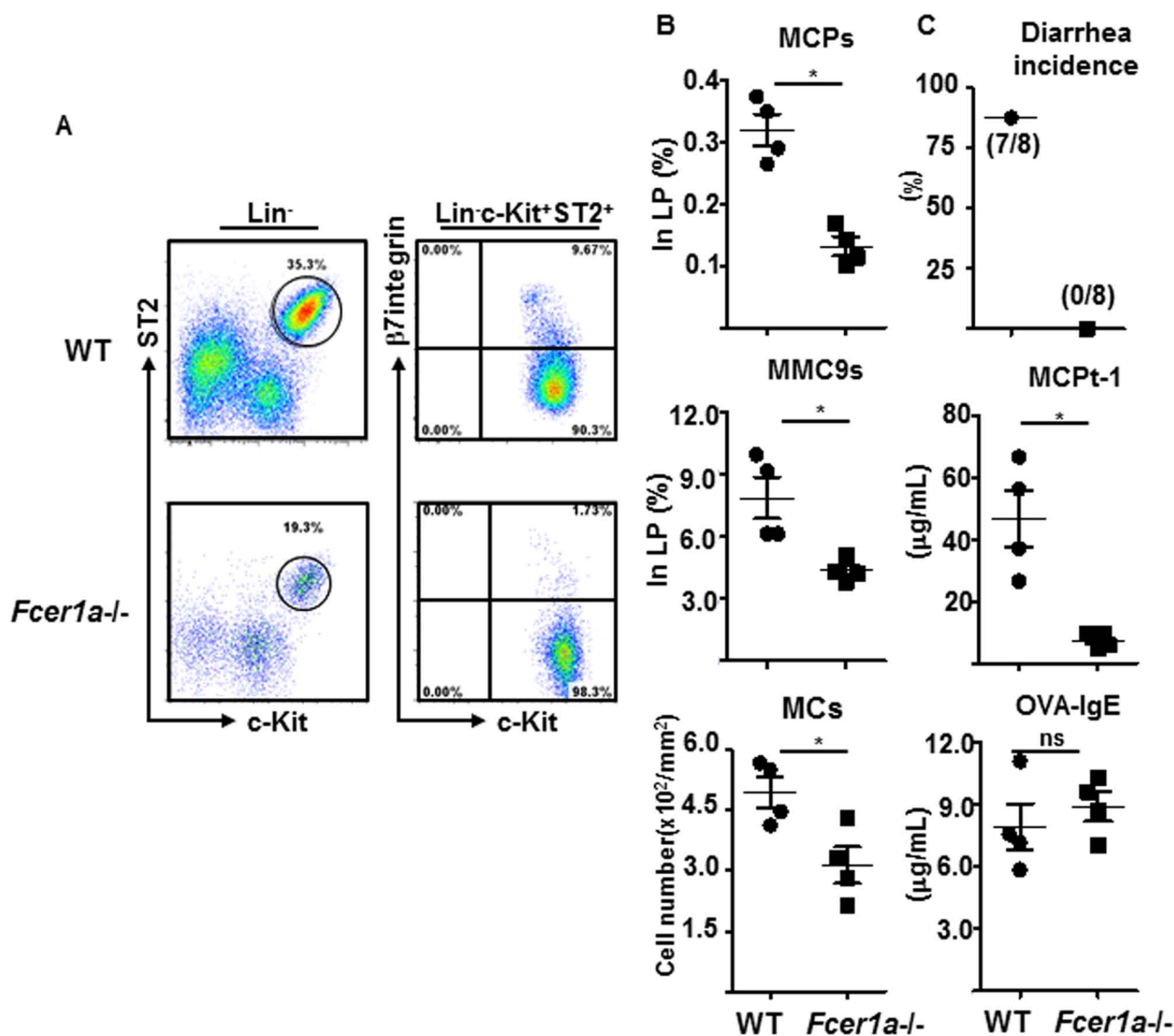


Figure S7. Diarrheal Response to Intragastric OVA Antigen Requires $Fc\epsilon R\alpha$ in a Murine Model of Food Allergy. (related to Figure 7) (A) Detection and (B) frequency of MCPs (Lin⁻IL-17RB⁻c-Kit⁺ $\beta 7$ integrin^{hi}) and MMC9s (Lin⁻IL-17RB⁻c-Kit⁺ $\beta 7$ integrin^{lo}), and intestinal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

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

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

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

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

^bAfter chart review, only one patient in the control group was examined for the serum IgE titer.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

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

Generation of rat anti-human/mouse IL-17RB monoclonal antibody (mAb) (clone SS13B)

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

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

consecutively and then orally gavaged with OVA (50 mg in 250 μ l saline) six times within two weeks. Following this application, solution was added directly over the site of the MC903 application for a total of 100 μ g of OVA. In reconstitution experiments, sensitized mice (first injection only) were intravenously injected with 5×10^6 BM cells one day after sub-lethal irradiation (9.5 Gy), then subjected to a second sensitization injection and intragastric challenges per the procedure above.

Measurement of parameters of food allergy Serum samples were analyzed using ELISA kits for OVA-specific IgE (MD Bioproducts), MCPt-1 (eBioscience), and OVA-specific IgG1 (alpha diagnostic international). For intestinal histological analyses, duodenal tissue was fixed in 10% formalin and processed by standard histological techniques. 5- μ m 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 $> 2^\circ\text{C}$) were performed as previously described (Osterfeld et al., 2010).

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

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

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

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

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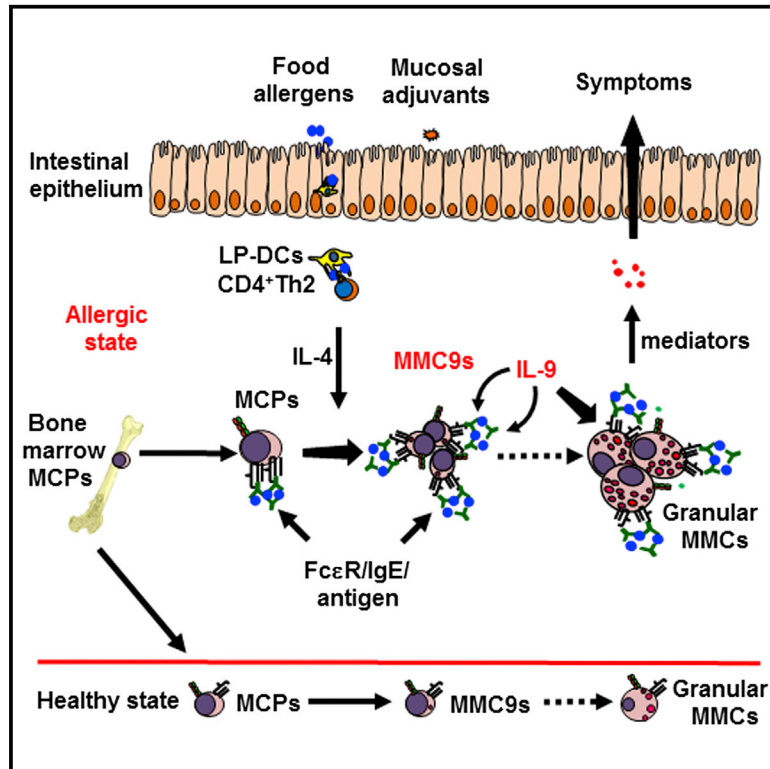
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Immunity

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

Graphical Abstract



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In Brief

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

Highlights

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

Accession Numbers

GSE72921

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.

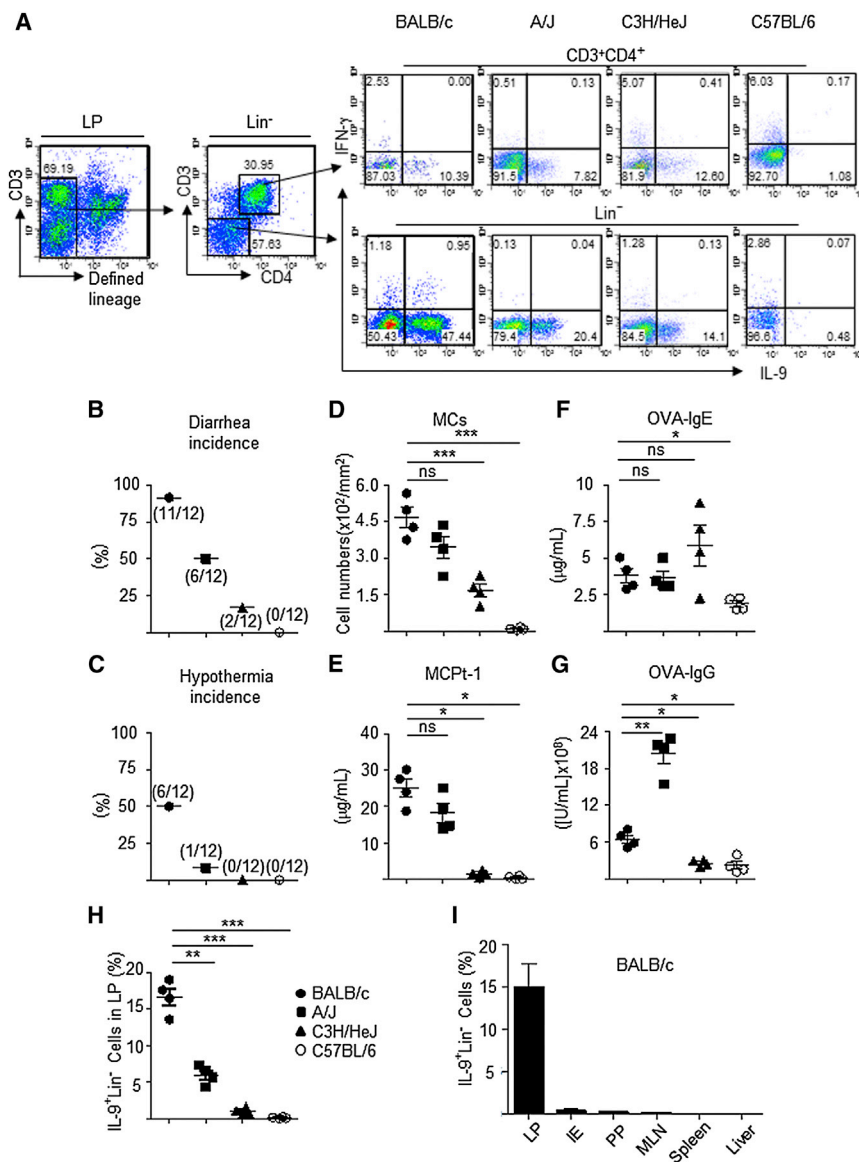


Figure 1. Lin⁻ IL-9-Producing Cell Frequency Correlates Positively with Susceptibility to Experimental Food Allergy

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

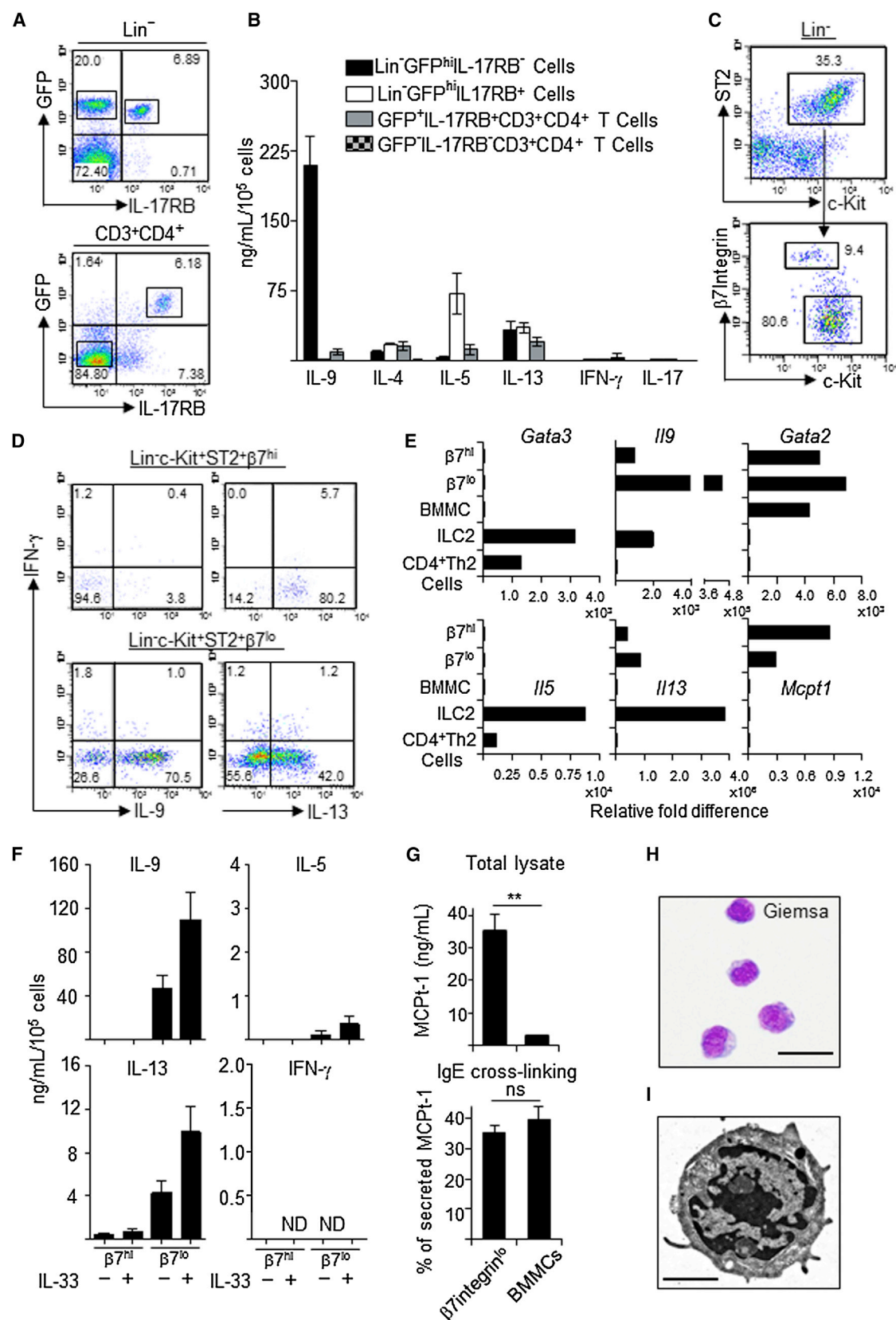
Innate MMC9s Are the Principal IL-9 Producers of Mast Cell Lineage

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

To better understand the lineage of these Lin[−]GFP^{hi} cells, we performed detailed cellular and molecular characterization. The purified IL-9-producing Lin[−]GFP^{hi}IL-17RB[−] cells were found to express c-Kit, Fc ϵ RI 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 RI 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 sublethal irradiation (protocol diagrammed 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 *Ilg1*, *Ilg13*, and *Mcp16*, 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⁺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^{hi}IL-17RB⁺ and GFP^{hi}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^{hi}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.

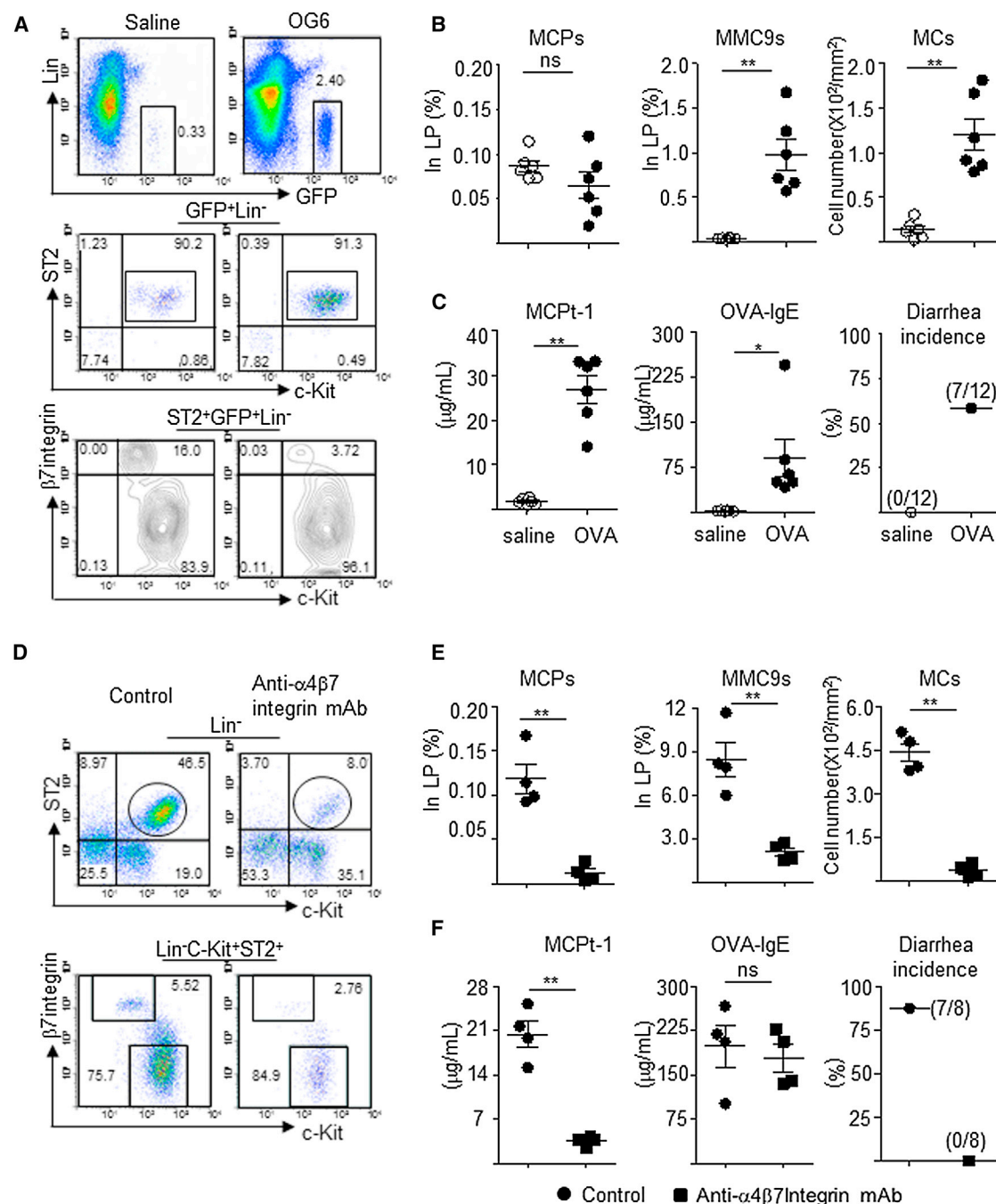


Figure 3. Blockade of Intestinal $\beta 7^{\text{hi}}$ MCP Recruitment from Bone Marrow Results in the Failure of MMC9 Induction

Detection and frequency of donor-derived MMC9s ($\text{Lin}^{-}\text{GFP}^{+}\text{ST2}^{+}\text{c-Kit}^{+}\beta 7^{\text{int}}^{\text{hi}}$) and MCPs ($\text{Lin}^{-}\text{GFP}^{+}\text{ST2}^{+}\text{c-Kit}^{+}\beta 7^{\text{int}}^{\text{lo}}$), 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 ($\text{Lin}^{-}\text{Ly-6c}^{-}\text{FcεRIα}^{-}\text{CD41}^{-}\text{CD71}^{-}\text{FLK2}^{-}\text{CD150}^{-}\text{c-Kit}^{+}\beta 7^{\text{int}}^{\text{hi}}$) 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,

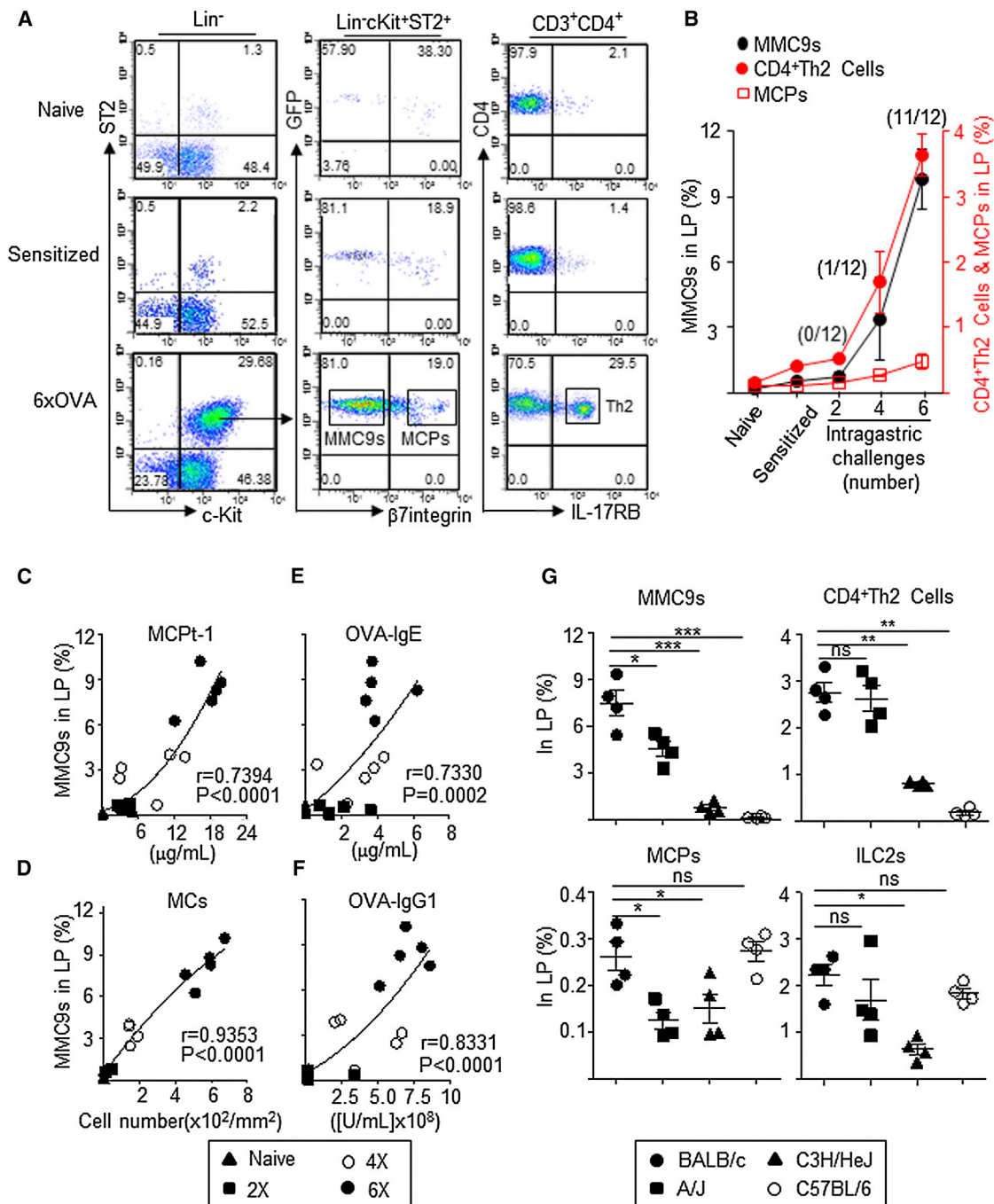


Figure 4. MMC9s and CD4⁺ Th2 Cell Frequencies Correlate Positively with Susceptibility to Experimental Food Allergy

(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

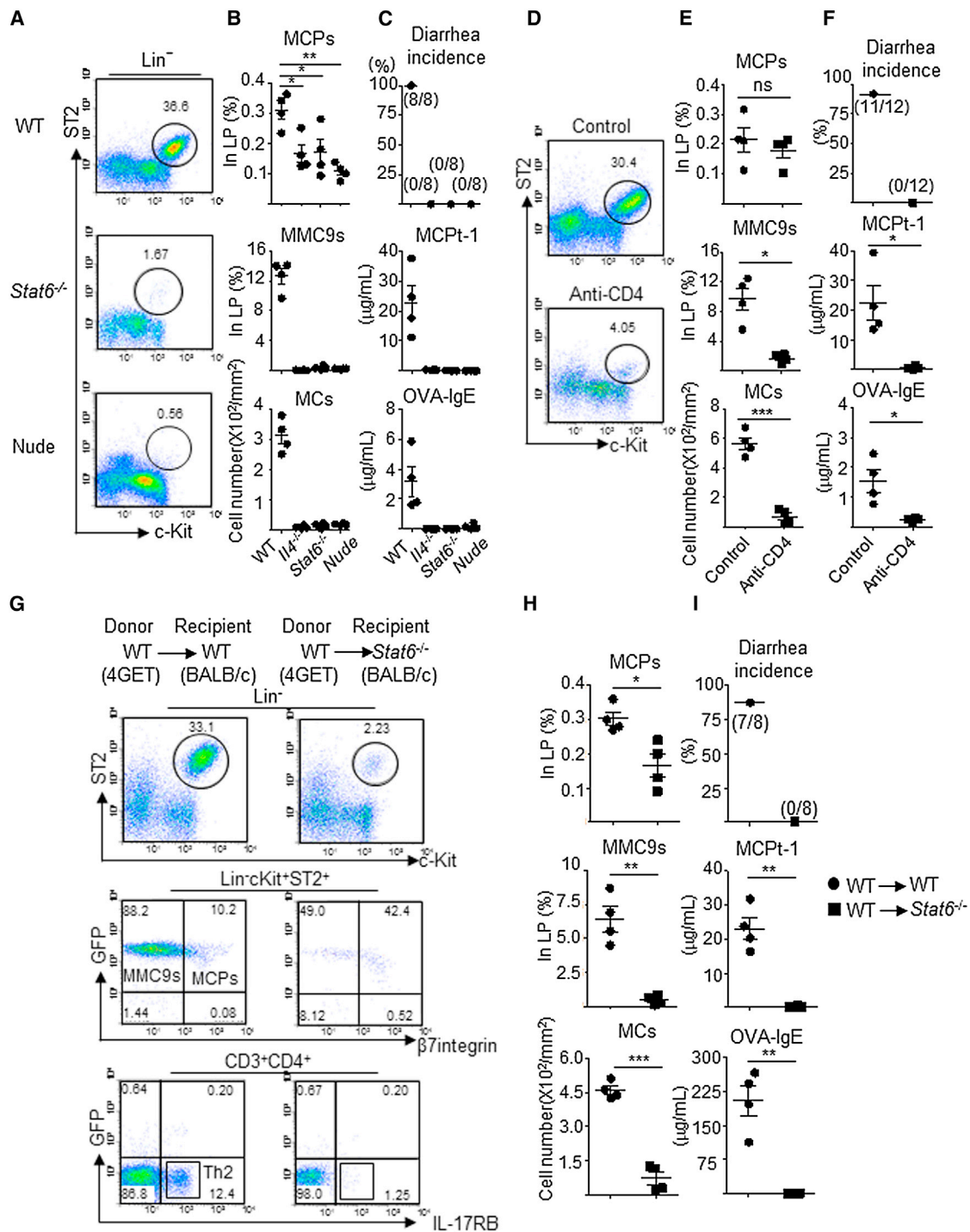


Figure 5. STAT6 Signaling and T Cells Are Required for MMC9 Induction that Promotes Experimental Food Allergy

(A–F) Detection (A, D) and frequency (B, E) of MMC9s (Lin⁻ c-Kit⁺ ST2⁺ β 7 integrin^{hi}) and MCPs (Lin⁻ c-Kit⁺ ST2⁺ β 7 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 $\pm$ SEM. *p < 0.05; **p < 0.01; ***p < 0.001. NS, not significant.

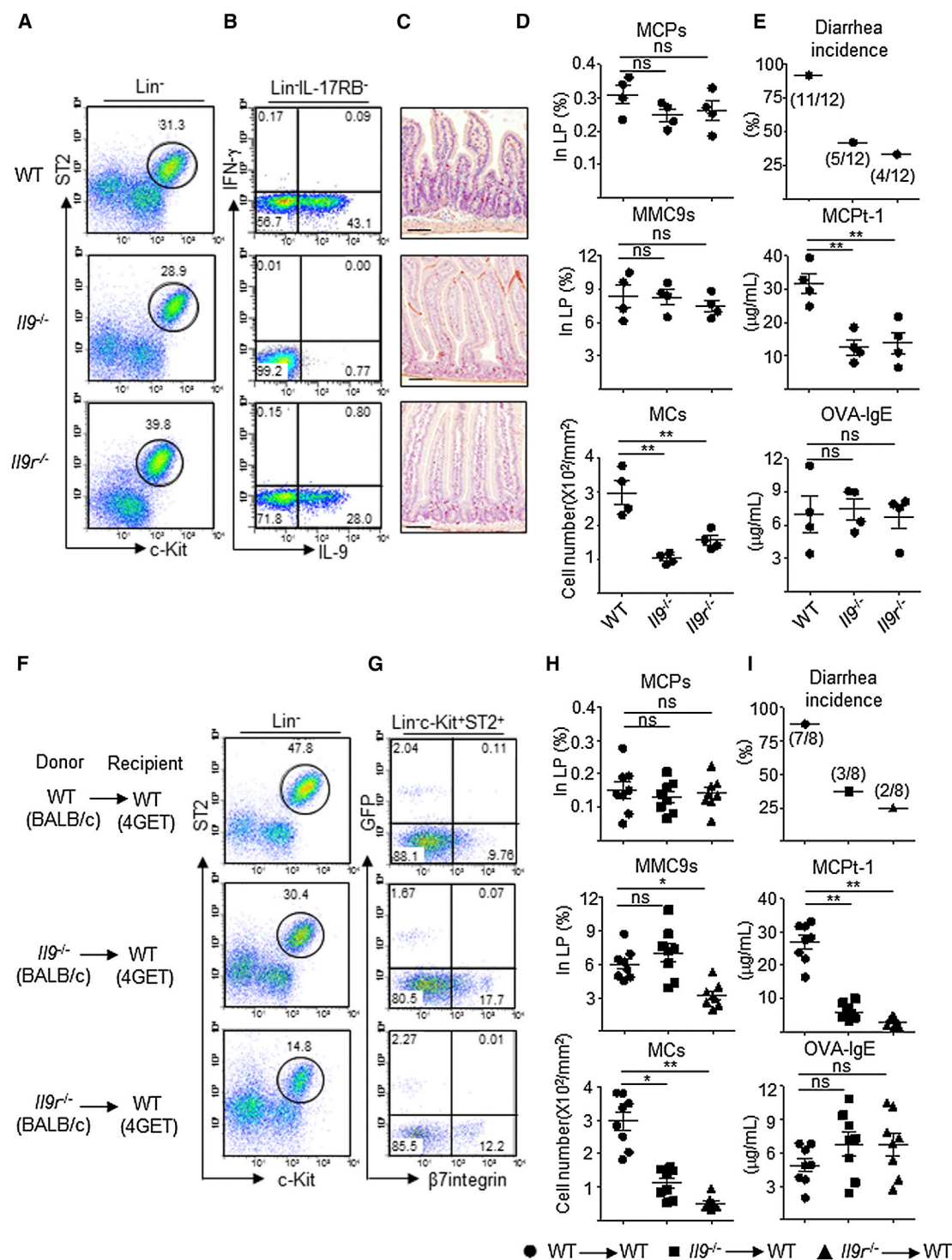


Figure 6. MMC9s Can Be Induced in IL-9- or IL-9R-Deficient Mice but Fail to Promote Experimental Food Allergy

(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 7integrin⁺) and MCPs (Lin⁻ c-Kit⁺ ST2⁺ β 7integrin⁺) (D) and measurement of indicated features of experimental food allergy (D, E) in wild-type BALB/c or indicated gene-deficient murine strains after six intragastric OVA challenges.

(F–I) Detection (F, G) and frequency (H) of donor-derived MMC9s (Lin⁻ c-Kit⁺ ST2⁺ β 7integrin⁺ GFP⁺) and MCPs (Lin⁻ c-Kit⁺ ST2⁺ β 7integrin⁺ 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.

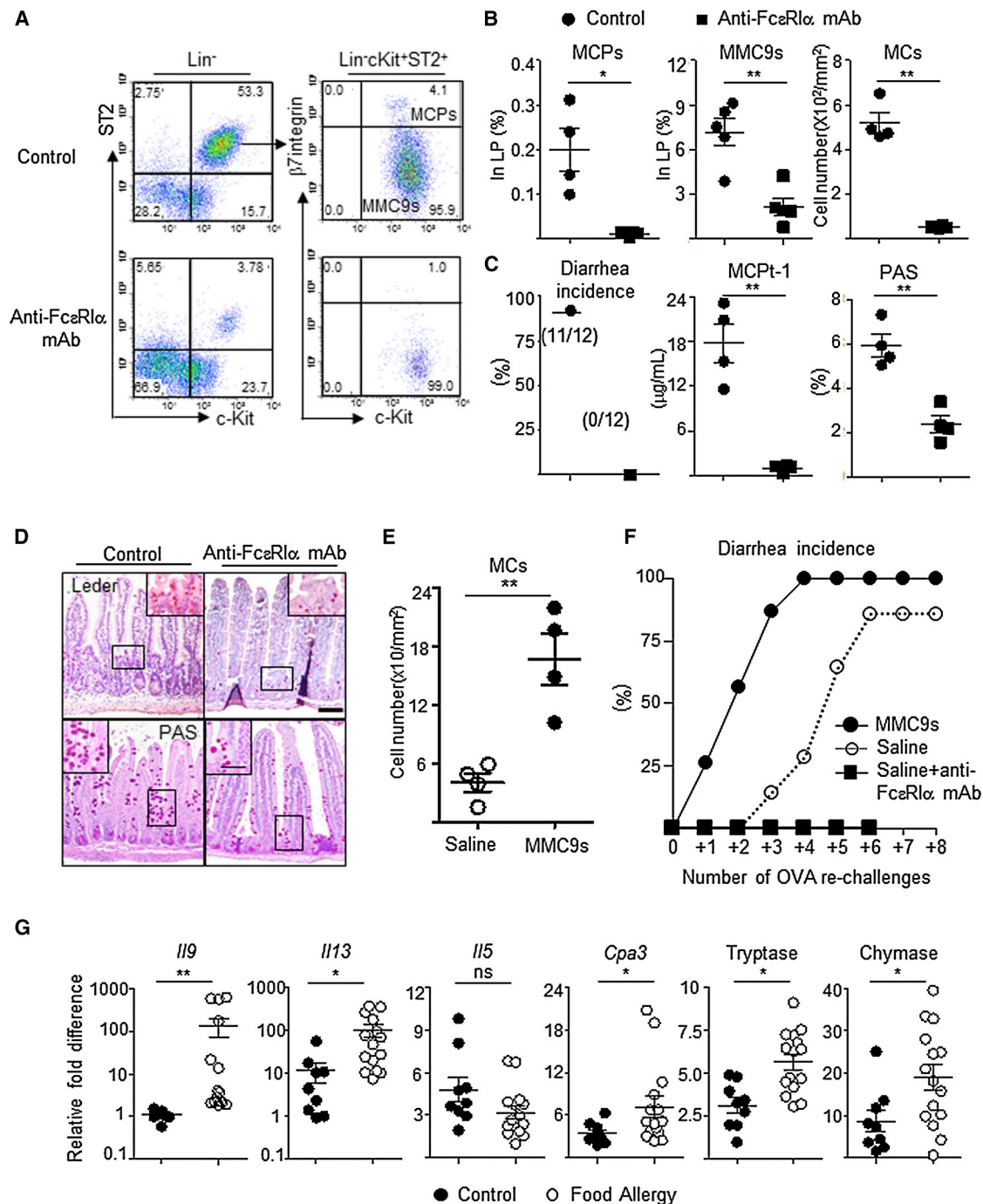


Figure 7. MMC9s Drive Intestinal Mastocytosis and IL9 Transcript Expression Is Increased in Food Allergy Patients

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

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

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

Data in (A)–(D) represent one of three independent experiments (n = 4 mice per group). Gene expression data are expressed as relative fold difference as described in (G). Error bars denote mean ± SEM. Scale bars represent 20 μm (D) and 100 μm (D, the corner insets). *p ≤ 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; Renaud 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 or deficient in T cells or IL-4 signaling had reduced capacity to accumulate intestinal MCPs and failed to develop MMC9s. Thus, the ability of murine strains to mount the intestinal CD4⁺ Th2-cell-mediated immune response to ingested dietary antigens, not their MHC H2 haplotype, correlated positively with MMC9 frequency, and consequently, susceptibility to food allergy. Whether our findings of MMC9s and its dependence on CD4⁺ Th2 cells pertain to helminth-induced T-cell-dependent mucosal MCs and IL-9 dependence of the protective response to *Trichinella spiralis* infection remains to be investigated (Angkasekwinai et al., 2013; Dillon and MacDonald, 1986; Liu et al., 2013; Ruitenberg and Elgersma, 1976; Urban et al., 2000).

Several murine models have been developed in an attempt to understand the immunological mechanisms underlying the

susceptibility of food allergy. In addition to adjuvant alum that induces a strong allergic sensitization, administration of CT intragastrically could also break oral tolerance and induce elevated histamine, IgE, and IgG1 titers, resulting in systemic anaphylactic responses to ingested allergen, which occurred selectively in C3H/HeJ strain (Li et al., 1999; Morafo et al., 2003). In contrast to sensitization via alum adjuvant, repeated intragastric OVA challenge failed to induce strong MMC9 (<1%) and CD4⁺ Th2 (<1%) immune responses in CT-sensitized C3H/HeJ or BALB/c mice, despite inducing a strong humoral immune response. Consequently, much fewer intestinal MC numbers and serum MCPT-1 titers (~15 cells/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-β7integrin (M293), allophycocyanin (APC)-conjugated anti-IL-17RB, APC-Cy7-conjugated anti-c-Kit (2B8), PE-Cy7-conjugated anti-CD3e (145-2C11), Horizon V500-conjugated CD4 (RPA-T4), and biotinylated anti-T1/ST2 (DJ8) antibodies. Subsequently, cells were counterstained with PerCP-Cy5.5-conjugated monoclonal antibodies against lineage (Lin) markers (CD11b [M1/70], CD11c [HL3], CD8 [53-6.7], B220 [RA3-6B2], Gr-1 [RB6-8C5], and CD335 [NKP46, 29A.4]) and with Brilliant Violet 421-labeled Streptavidin (Biolegend) before analysis with a 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-β7integrin, APC-Cy7-conjugated anti-c-Kit, and biotinylated anti-T1/ST2 antibodies. Subsequently, cells were counterstained with PerCP-Cy5.5-conjugated monoclonal antibodies against Lin markers (including CD3 and CD4) and with Brilliant Violet 421-labeled streptavidin. Stained cells were fixed and permeabilized for intracellular cytokine staining using FITC-anti-IFN-γ (XMG 1.2) and APC-anti-IL-9 (RM9A4) or APC-anti-IL-13 (eBio13A).

IgE-Mediated Experimental Food Allergy

Mice were sensitized twice within a 2-week interval by intraperitoneal (i.p.) injection with 100 μg OVA and 1 mg alum and then orally gavaged with OVA (50 mg in 250 μl saline) six times within 2 weeks. In prophylactic experiments, sensitized BALB/c mice were injected intraperitoneally with 500 μg anti-FcεRIα (MAR-1) antibody (Biolegend) or hamster isotype control antibody (Bio X Cell) or with 500 μg of anti-CD4 (GK1.5) antibody (Bio X Cell) or rat isotype control antibody (Bio X Cell) twice 1 day before the first and fourth intragastric OVA challenges. Some antibody-treated mice were rested for 2 weeks after the sixth OVA challenge before reconstitution with 1 × 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). BMDCs 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 (A1090129-1 to Y.-H.W., A1073553 to S.P.H.), the Digestive Health Center (P30 DK078392, Pilot and Feasibility Award to Y.-H.W.), Campaign Urging Research For Eosinophilic Diseases (CURED) Foundation, the Buckeye Foundation, the Food Allergy Research Education Fund to M.E.R., and VA Merit Award to F.D.F.

Received: September 10, 2014

Revised: May 30, 2015

Accepted: August 20, 2015

Published: September 22, 2015

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Immunity

Supplemental Information

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

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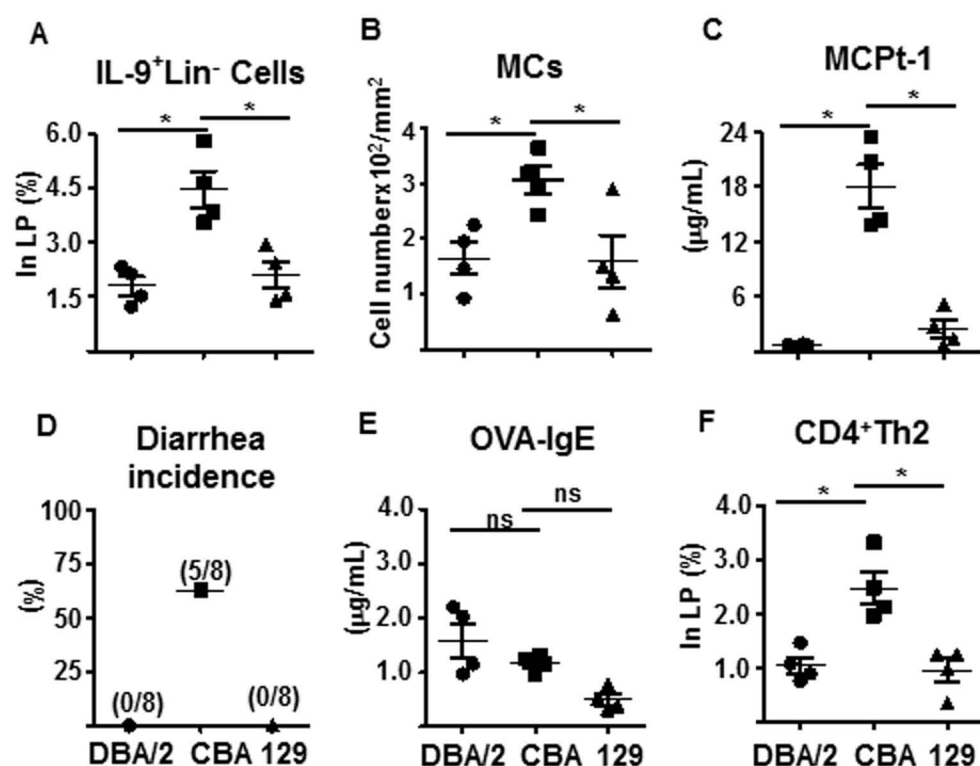


Figure S1. Frequencies of Intestinal IL-9-producing Lin⁻ and CD4⁺Th2 Cells Correlate Positively with the Susceptibility to Developing IgE-mediated Experimental Food Allergy. (related to Figure 1 and Figure 4) (A) Frequency of IL-9⁺Lin⁻ and (F) CD4⁺ST2⁺IL-17RB⁺ cells, (B) intestinal mast cell (MC) number,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

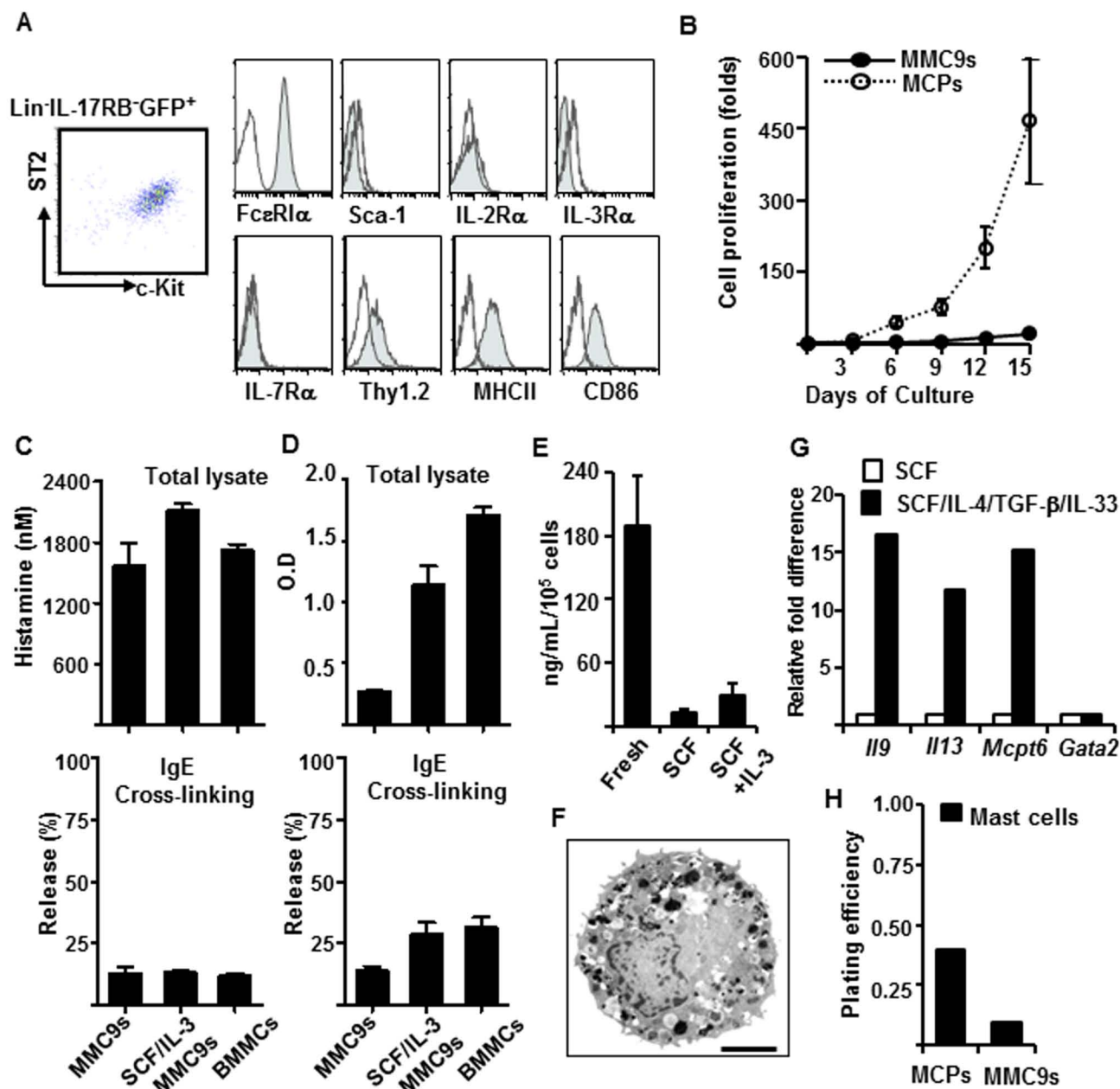


Figure S2. Characteristics of Intestinal MCPs and MMC9s. (related to Figure 2) (A) Phenotypic analysis of intestinal Lin⁻GFP^{hi}IL-17RB⁻c-Kit⁺ST2⁺ cells from BALB/c mice with active allergic diarrhea. (B) Comparison of proliferative response to SCF plus IL-3 between MCPs (Lin⁻IL-17RB⁻c-Kit⁺ST2⁺β7integrin^{hi}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

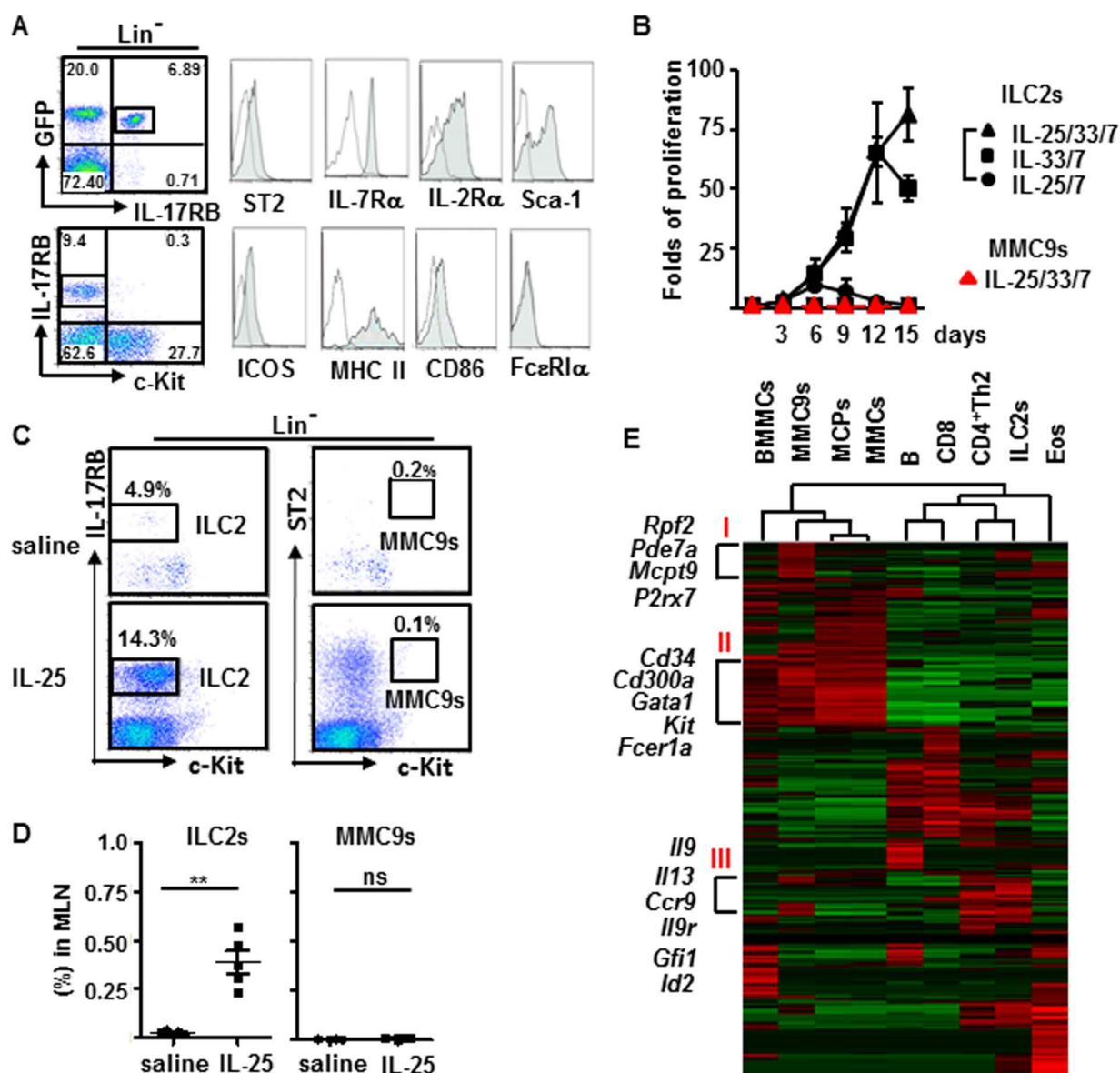


Figure S3. Characterizations of Intestinal ILC2s and MMC9s. (related to Figure 2)(A) Detection and phenotypic analysis of Lin-GFP^{hi}IL-17RB⁺c-Kit⁻ cells. **(B)** Proliferative response of purified MMC9s (Lin-GFP^{hi}IL-17RB⁺c-Kit⁺ β 7integrin^{lo} cells) and ILC2s (Lin-GFP^{hi}IL-17RB⁺c-Kit⁻IL-7R α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

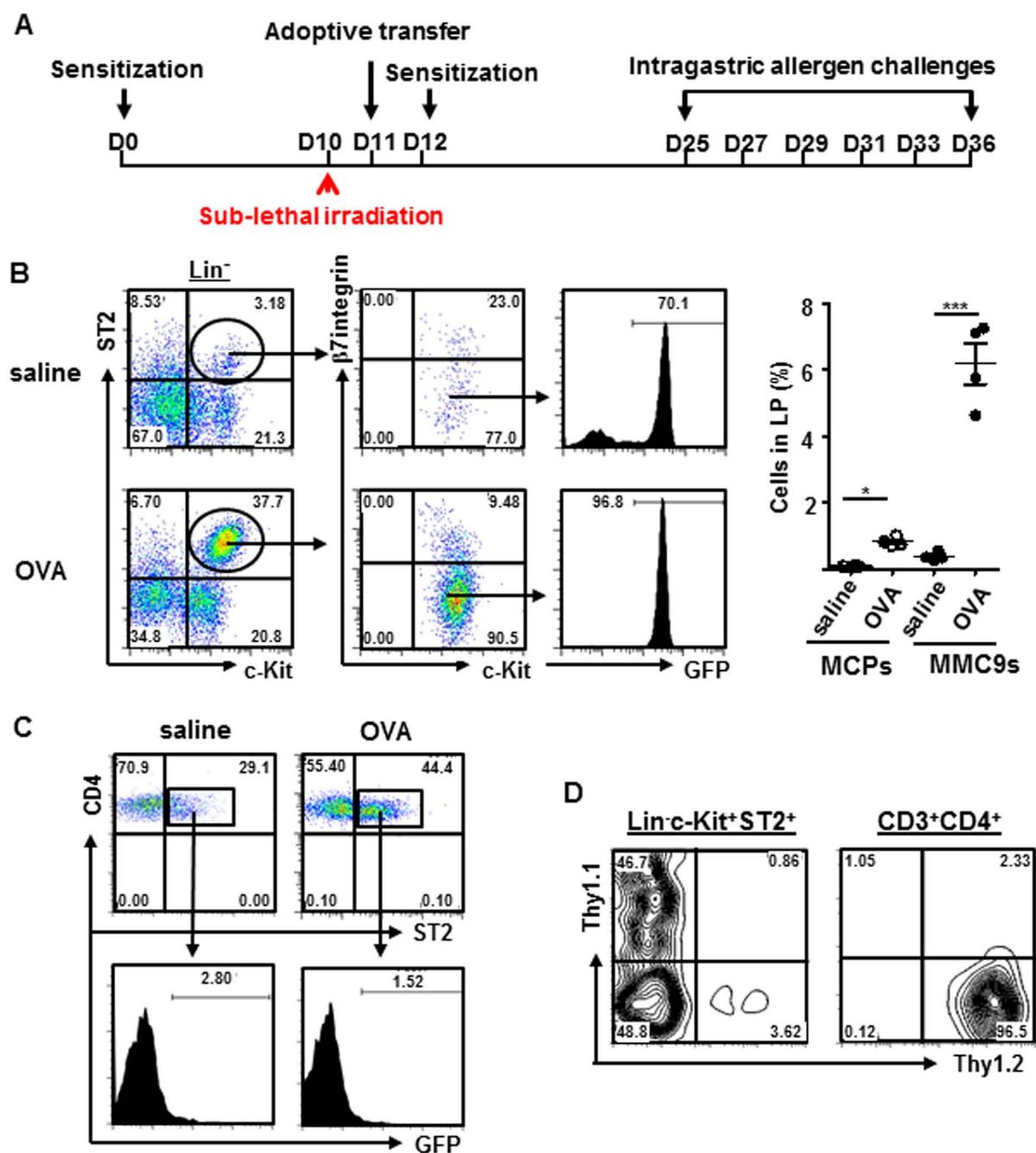


Figure S4. Origin of Intestinal MCPs, MMC9s, and CD4⁺Th2 Cells in a Reconstitution Model of Experimental Food Allergy. (related to Figure 3) (A) Diagram of adoptive transfer protocol in the mouse model of food allergy. (B-D) Detection and frequency of donor-derived MMC9s (Lin⁻IL-17RB⁺c-Kit⁺ $\beta 7$ integrin^{lo}) and MCPs (Lin⁻IL-17RB⁺c-Kit⁺ $\beta 7$ integrin^{hi}) (B), recipient-derived GFP-CD4⁺ST2⁺Th2 cells (C), or indicated cell subsets (D) in the small intestine of Thy1.2⁺ BALB/c recipient mice reconstituted with bone marrow progenitors from IL-4-eGFP (4GET) mice (A-C) or Thy1.1⁺ (D) BALB/c mice after six times of intragastric saline or OVA challenges. Data represent one of three independent experiments (n=4 mice per group). LP, lamina propria. * p<0.05, *** p<0.001.

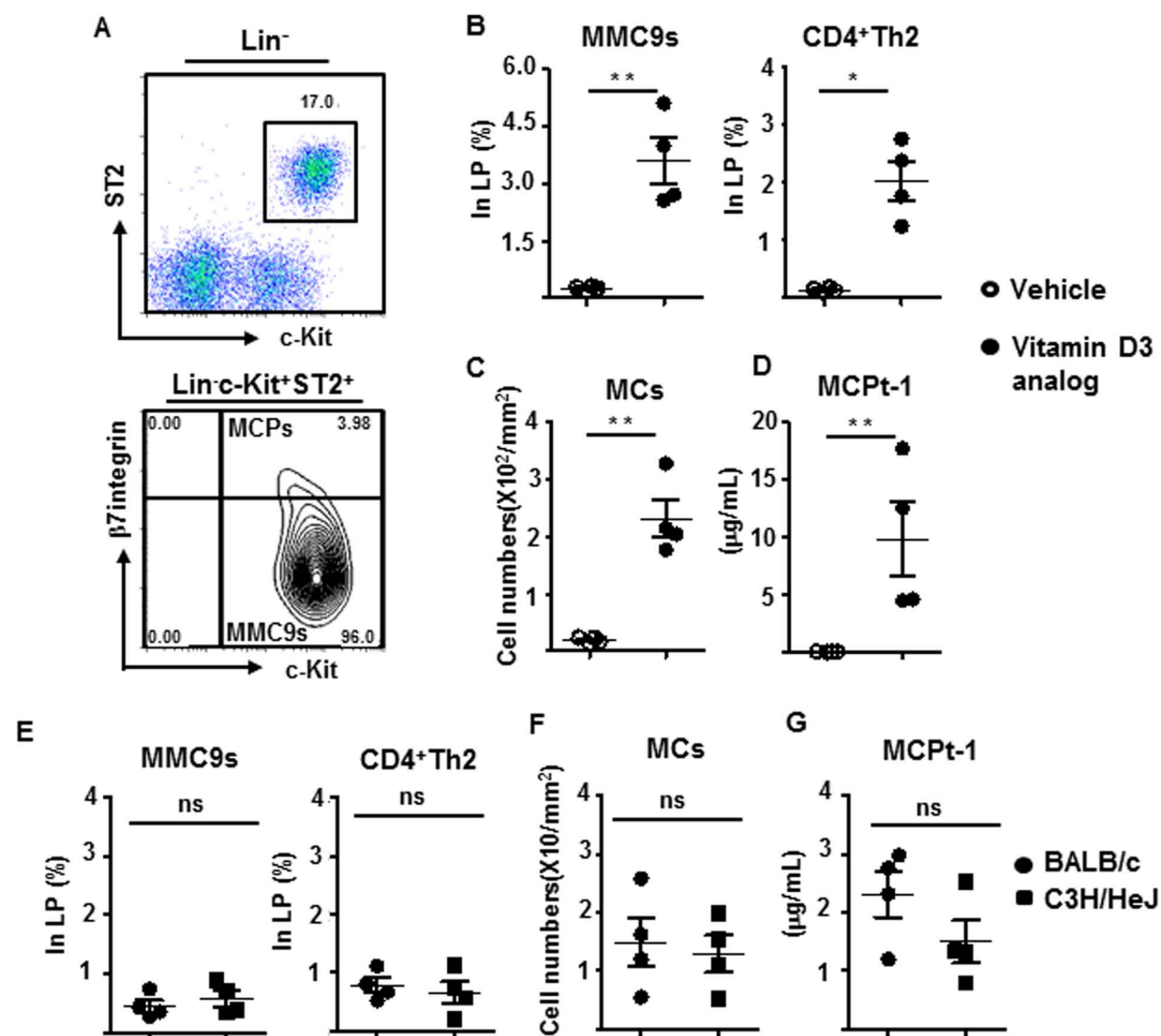
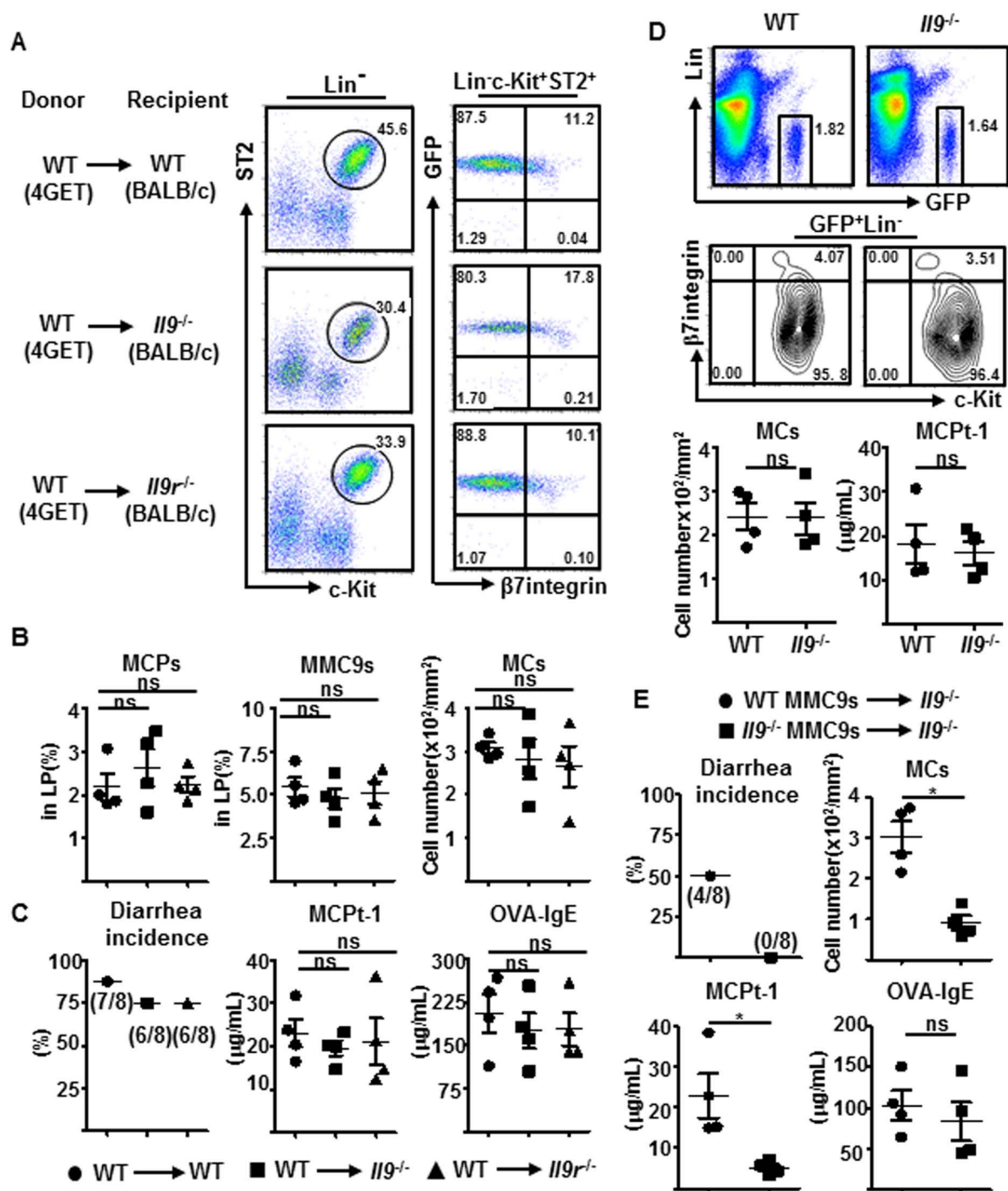


Figure S5. Induction of MMC9s and CD4⁺Th2 cells and Intestinal Mastocytosis in the Cholera Toxin- or Skin-sensitized Model of Experimental Food Allergy. (related to Figure 4) (A) Detection and (B, E) frequency of MMC9s (Lin⁻IL-17RB⁻c-Kit⁺ $\beta 7$ integrin^{lo}), and CD4⁺IL-17RB⁺Th2, (C, F) intestinal mast cell (MC) number, and (D, G) serum MCPt-1 titers of indicated murine strains after sensitization with vitamin D3 analog (Calcipotriol) (A-D) or cholera toxin (E-G) and six intragastric OVA challenges. Data represent one of two independent experiments (n=4 mice per group). LP, lamina propria. * p < 0.05, ** p < 0.01, NS, not significant.



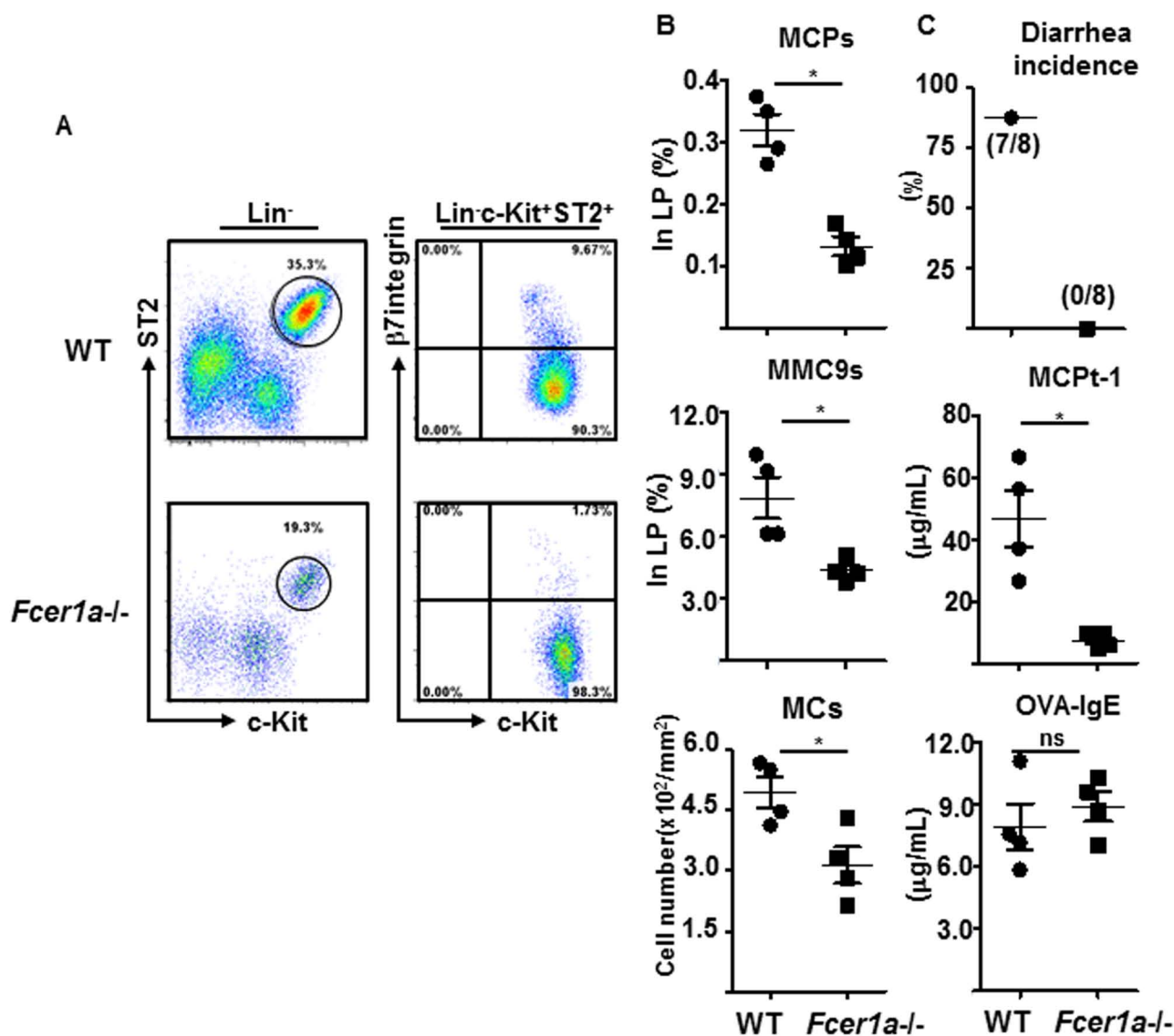


Figure S7. Diarrheal Response to Intragastric OVA Antigen Requires $Fc\epsilon R\alpha$ in a Murine Model of Food Allergy. (related to Figure 7) (A) Detection and (B) frequency of MCPs (Lin⁻IL-17RB⁺c-Kit⁺β7integrin^{hi}) and MMC9s (Lin⁻IL-17RB⁺c-Kit⁺β7integrin^{lo}), and intestinal mast cell (MC) numbers and (C) diarrhea incidence, and titers of serum MCPt-1 and OVA-IgE of indicated mouse strains after OVA/alum sensitization and six intragastric OVA challenges. Data represent one of two independent experiments (n=4 mice per group). LP, lamina propria. * p<0.05, NS, not significant.

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

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

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

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

^bAfter chart review, only one patient in the control group was examined for the serum IgE titer.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

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

Generation of rat anti-human/mouse IL-17RB monoclonal antibody (mAb) (clone SS13B)

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

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

consecutively and then orally gavaged with OVA (50 mg in 250 μ l saline) six times within two weeks. Following this application, solution was added directly over the site of the MC903 application for a total of 100 μ g of OVA. In reconstitution experiments, sensitized mice (first injection only) were intravenously injected with 5×10^6 BM cells one day after sub-lethal irradiation (9.5 Gy), then subjected to a second sensitization injection and intragastric challenges per the procedure above.

Measurement of parameters of food allergy Serum samples were analyzed using ELISA kits for OVA-specific IgE (MD Bioproducts), MCPt-1 (eBioscience), and OVA-specific IgG1 (alpha diagnostic international). For intestinal histological analyses, duodenal tissue was fixed in 10% formalin and processed by standard histological techniques. 5- μ m 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 $> 2^\circ\text{C}$) were performed as previously described (Osterfeld et al., 2010).

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

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

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

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

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