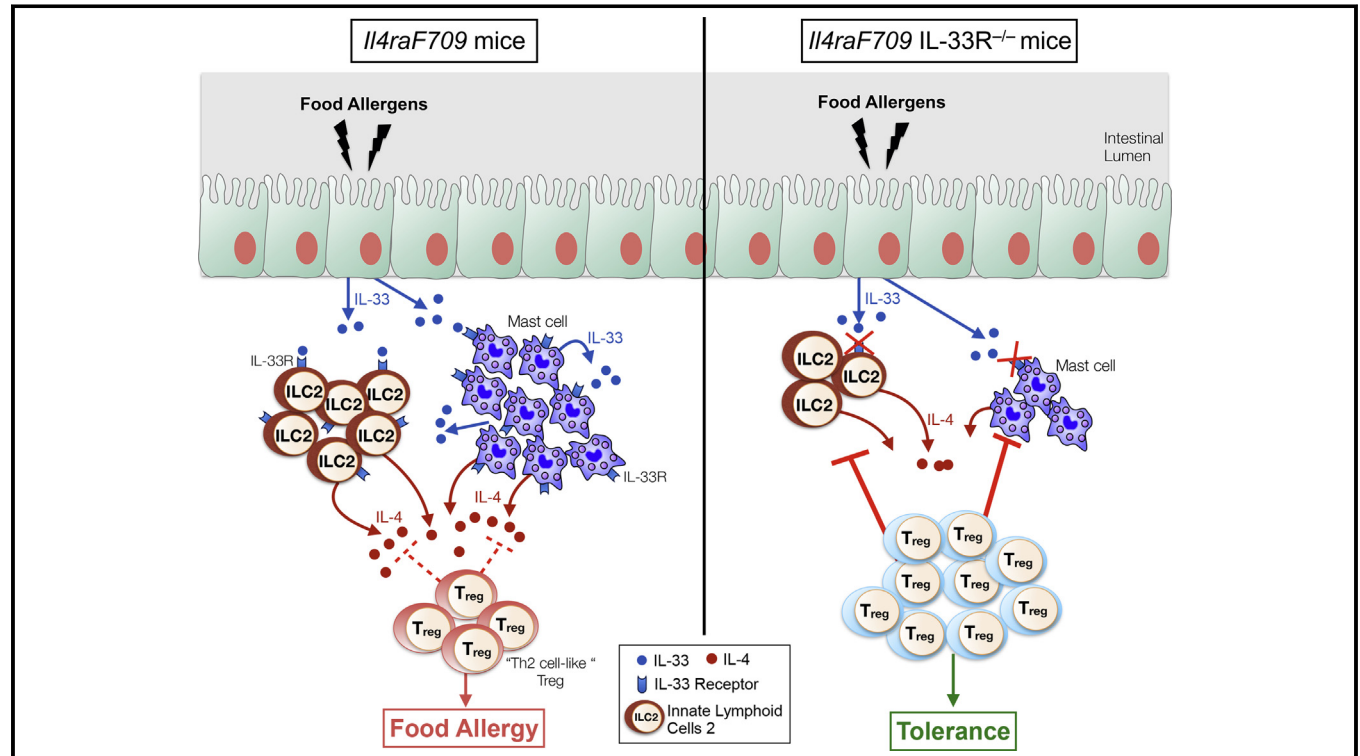


IL-4 production by group 2 innate lymphoid cells promotes food allergy by blocking regulatory T-cell function

Magali Noval Rivas, PhD,^{a,b,*§} Oliver T. Burton, PhD,^{a,b,*} Hans C. Oettgen, MD, PhD,^{a,b,‡} and Talal Chatila, MD, MSc^{a,b,‡}
Boston, Mass

GRAPHICAL ABSTRACT



From ^athe Division of Immunology, Boston Children's Hospital, and ^bthe Department of Pediatrics, Harvard Medical School.

*These authors contributed equally to this work.

‡These authors contributed equally to this work.

§Dr Noval Rivas is currently affiliated with the Division of Pediatric Infectious Diseases and Immunology, Department of Pediatric, Infectious and Immunologic Diseases Research Center, Cedars-Sinai Medical Center, Los Angeles, Calif.

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Corresponding author: Talal Chatila, MD, MSc, Division of Immunology, Boston Children's Hospital and the Department of Pediatrics, Harvard Medical School, Karp Family Building, Rm 10-214, 1 Blackfan St, Boston, MA 02115. E-mail: talal.chatila@childrens.harvard.edu.

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Background: Food allergy is a major health issue, but its pathogenesis remains obscure. Group 2 innate lymphoid cells (ILC2s) promote allergic inflammation. However their role in food allergy is largely unknown.

Objective: We sought to investigate the role of ILC2s in food allergy.

Methods: Food allergy-prone mice with a gain-of-function mutation in the IL-4 receptor α chain (*Il4raF709*) were orally sensitized with food allergens, and the ILC2 compartment was analyzed. The requirement for ILC2s in food allergy was investigated by using *Il4raF709*, IL-33 receptor-deficient (*Il1rl1^{-/-}*), IL-13-deficient (*Il13^{-/-}*), and IL-4-deficient (*Il4^{-/-}*) mice and by adoptive transfer of *in vitro*-expanded ILC2s. Direct effects of ILC2s on regulatory T (Treg) cells and mast cells were analyzed in coculture experiments. Treg cell control of ILC2s was assessed *in vitro* and *in vivo*.

Results: *Il4raF709* mice with food allergy exhibit increased numbers of ILC2s. IL-4 secretion by ILC2s contributes to the allergic response by reducing allergen-specific Treg cell and activating mast cell counts. IL-33 receptor deficiency in

Il4raF709 Il1rl1^{-/-} mice protects against allergen sensitization and anaphylaxis while reducing ILC2 induction. Adoptive transfer of wild-type and *Il13^{-/-}* but not *Il4^{-/-}* ILC2s restored sensitization in *Il4raF709 Il1rl1^{-/-}* mice. Treg cells suppress ILC2s *in vitro* and *in vivo*.

Conclusion: IL-4 production by IL-33-stimulated ILC2s blocks the generation of allergen-specific Treg cells and favors food allergy. Strategies to block ILC2 activation or the IL-33/IL-33 receptor pathway can lead to innovative therapies in the treatment of food allergy. (J Allergy Clin Immunol 2016;■■■:■■■-■■■.)

Key words: Anaphylaxis, food allergy, IL-4, IL-13, IL-33, group 2 innate lymphoid cells, innate lymphoid cells, nuocytes, mast cells, regulatory T cells, oral tolerance

Food allergy is a major health issue in westernized countries, with increasing prevalence over the last decades and a lack of curative treatment.¹ Several major food staples, including milk, soy, and wheat, can induce food-induced allergic reactions.² Food allergies reflect a failure of oral tolerance of innocuous food allergens, resulting in development of a dysregulated T_H2 immune response, secretion of allergen-specific IgE, and recruitment of effector cells to the gastrointestinal tract. Through their production of T_H2 cytokines, mostly IL-4 and IL-13, mast cells, basophils, and type 2 helper CD4⁺ T (T_H2) cells are key effector cells of the allergic reaction. Their T_H2 cytokine production amplifies the allergic immune response by blocking induction of allergen-specific regulatory T (Treg) cells, directing further T_H2 cell differentiation and IgE-driven mast cell activation.^{3,4} The sequence of early events in food allergen sensitization and the involvement of innate immune cells in triggering disease in susceptible subjects remain to be fully elucidated.

A population of innate mucosal T_H2 cytokine-secreting cells, called group 2 innate lymphoid cells (ILC2s), has emerged as central to a broad collection of allergic disorders, such as asthma, chronic rhinosinusitis, and atopic dermatitis.⁵⁻¹¹ ILC2s are characterized by the expression of CD90 (Thy1), CD25 (IL-2 receptor α), IL-25R (IL-17RB), IL-33 receptor (IL-33R; ST2), and CD127 (IL-7 receptor α) in the absence of specific markers that define other leukocytes lineages (Lin⁻; non-T/non-B cells).¹² They share morphologic characteristics with lymphocytes but do not express the rearranged antigen receptors characteristic of adaptive immune cells.^{13,14} ILC2s emerge from a common lymphoid progenitor present in bone marrow, and their development is critically dependent on the transcription factors Id2^{12,15} and retinoic acid-related orphan receptor (ROR) α ^{16,17} and the T_H2-specific transcription factor GATA-3.^{8,18} ILC2s are mostly found at barrier surfaces, including the skin, lung, and gastrointestinal tract.¹³ In response to cytokines produced by innate immune sources, such as thymic stromal lymphopoietin, IL-25, and IL-33, ILC2s rapidly expand at the mucosal surfaces in an innate, antigen-independent manner and produce large quantities of T_H2 cytokines, especially IL-5 and IL-13 but also IL-4 and IL-9.^{8,19-22}

One of the key issues in food allergy is the impaired induction of allergen-specific Treg cells and their subversion into nonfunctional T_H2 cell-like Treg cells that further promote disease.³ The cellular and molecular processes linking T_H2 effector cells to the defective regulatory immune response during food allergy are still not well understood. The role of ILC2s in

Abbreviations used

DT:	Diphtheria toxin
DTR:	Diphtheria toxin receptor
eGFP:	Enhanced green fluorescent protein
Foxp3:	Forkhead box P3
ILC2:	Group 2 innate lymphoid cell
ILC3:	Group 3 innate lymphoid cell
IL-4R:	IL-4 receptor
IL-33R:	IL-33 receptor
iTreg:	Induced regulatory T
MLN:	Mesenteric lymph node
MMCP-1:	Mouse mast cell protease 1
OVA:	Ovalbumin
ROR:	Retinoic acid-related orphan receptor
SI:	Small intestine
TCR:	T-cell receptor
Treg:	Regulatory T
WT:	Wild-type

disrupting tolerance in the setting of food allergy remains similarly obscure, with conflicting reports on the requirement for ILC2s in food allergen sensitization.^{23,24}

In this study we have made use of a well-characterized murine transgenic model of food allergy involving gain-of-function mutation in the IL-4 receptor (IL-4R) α chain to investigate the role of ILC2s during food allergy.^{3,4,25,26} We found that ILC2s play a critical role in the induction of food allergy by virtue of their secretion of IL-4, which acts to enhance mucosal mast cell activation and disrupt allergen-specific Treg cell induction and suppressive functions.

METHODS

Sensitization and challenge protocol

Mice were treated intragastrically once a week over 4 weeks with either PBS or 23 mg of peanut butter (Skippy; Hormel Foods, Austin, Minn), corresponding to 5 mg of peanut protein suspended in 250 μ L of 0.1 mol/L sodium bicarbonate (pH 8.0), as previously described.⁴ For ovalbumin (OVA) sensitization, OVA (250 μ g) together with 10 μ g of staphylococcal enterotoxin B (Toxin Technology, Sarasota, Fla) was gavaged once weekly for 8 weeks. On the ninth week, mice were challenged intragastrically with either 450 mg of peanut butter (approximately 100 mg of protein) or 150 mg of OVA, as previously described.^{3,26} Wild-type (WT) and *DO11.10⁺ Rag2^{-/-} Il4raF709* forkhead box P3 (*Foxp3*)^{eGFP} mice were fed with 1% OVA in drinking water for 7 days, followed by oral gavage every 2 days with 250 μ g of OVA the following week. Anaphylaxis was assessed by measuring changes in total body core temperature with transponders placed subcutaneously 2 days before challenge (IPTT-300; Bio Medic Data Systems, Seaford, Del) and a DAS-6001 console (Bio Medic Data Systems).

In vitro ILC2 and group 3 innate lymphoid cell culture and purification

Lamina propria lymphocytes from *IL-13YFP^{Cre}* mice were isolated, as previously described, and incubated at 37°C in complete RPMI medium supplemented with IL-33 (10 ng/mL; BioLegend, San Diego, Calif), IL-25 (10 ng/mL; Shenandoah Biotechnology, Warwick, Pa), and IL-2 (5 ng/mL, Shenandoah Biotechnology) for a week.²⁷ The ILCs were then sorted on a BD FACSaria II based on IL-13YFP expression and Lin⁻Thy1⁺Sca-1⁺c-Kit^{low} for ILC2s and the lack of IL-13YFP expression and Lin⁻Thy1⁺Sca-1⁻c-Kit⁺ for group 3 innate lymphoid cells (ILC3s). Cells were subsequently maintained in culture with IL-2, IL-25, and IL-33 for 3 to 4 weeks.

ILC adoptive transfer

ILCs were expanded *in vitro* from the lamina propria of WT, *Il13*^{-/-}, or *Il4*^{-/-} mice and cell sorted, as mentioned above. *Il4raF709 Il1rl1*^{-/-} mice were injected intravenously through the retro-orbital sinus with 2×10^5 ILCs each week the day before sensitization.

Coculture experiments

Bone marrow–derived mast cells were prepared, as previously described, and cultured overnight at 10^5 cells/well with ILC2s at a 20:1 ratio in conical 96-well plates in the presence of IL-2 (500 pg/mL), IL-3 (2 ng/mL), and IgE anti-DNP (5 ng/mL SPE-7; Sigma, St Louis, Mo).⁴ IL-4 (10 ng/mL, Shenandoah Biotechnology) or anti-IL-4 (40 μg/mL, 11B11) was supplemented in some cases. Bone marrow–derived mast cells were stimulated for 10 minutes with 50 ng/mL DNP-albumin (Sigma-Aldrich) in the presence of anti-LAMP-1–eFluor 660 (eBio1D4B; eBioscience, San Diego, Calif), anti-c-Kit–phycoerythrin-Cy7, and fixable viability dye eFluor 780 to detect degranulation. CD4⁺ T cells (10^5 cells) from WT *DO11.10*⁺ *Rag2*^{-/-} *Foxp3*^{EGFP} mice were sorted to greater than 98% purity by using Miltenyi CD4 microbeads (Miltenyi Biotech, Bergisch Gladbach, Germany) and labeled with CellTrace Violet dye (Life Technologies, Grand Island, NY). Naive T cells were then cultured with ILC2s at a 20:1 ratio in round-bottom 96-well plates coated with 1 μg/mL anti-CD3 and 5 μg/mL anti-CD28. Recombinant human TGF-β1 (2 ng/mL) and IL-2 (1 ng/mL) were added to promote Foxp3 induction.

Statistical analysis

Anaphylaxis temperature curves were analyzed by using 2-way ANOVA. Student unpaired 2-tailed *t* tests were used for 2-group comparisons. For more than 2 groups, 1-way ANOVA with Bonferroni posttest analysis was used. Results are presented as means and SEMs, where each point represents 1 sample. In cases in which values were spread across multiple orders of magnitude, data were log-transformed for analysis with parametric tests.

Study approval

All animal studies were approved by the Boston Children's Hospital Institutional Animal Care and Use Committee.

Other methods

Information on mouse strains used, flow cytometry and related antibodies, reagents and methods, real-time PCR analysis, IgE and cytokine ELISAs, tissue histology, and TGF-β–mediated *in vitro* induced regulatory T (iTreg) cell induction is provided in the [Methods](#) section in this article's Online Repository at www.jacionline.org.

RESULTS

ILC2 enrichment in orally sensitized *Il4raF709* mice

Il4raF709 mice carry an IL-4Rα chain mutation that inactivates the receptor's immunotyrosine inhibitory motif. This mutation results in augmented signal transducer and activator of transcription 6 activation by IL-4 and IL-13 and renders the mice prone to oral allergic sensitization.^{25,26} *Il4raF709*, but not WT, mice are particularly susceptible to oral sensitization with food allergens, such as peanut or chicken egg OVA, and respond to oral challenge with a mast cell- and IgE-dependent anaphylactic response.^{3,4,28} Peanut or OVA-sensitized *Il4raF709* mice, but not WT control mice, had an anaphylactic reaction after oral challenge, as evidenced by a significant decrease in core body temperature (Fig 1, A, and see Fig E1, A, in this article's Online Repository at www.jacionline.org). *Il4raF709* mice with food allergy

produced peanut- and OVA-specific IgE antibodies, and they released mouse mast cell protease 1 (MMCP-1), a marker of mast cell degranulation, on allergen sensitization and challenge (Fig 1, B, and see Fig E1, B and C).

mRNA transcripts of *Il33*, production of which by epithelial cells promotes ILC2 differentiation, were significantly higher in the small intestine (SI) of *Il4raF709* mice after allergen sensitization and challenge (Fig 1, C, and see Fig E1, D). Accordingly, we analyzed the presence of ILC2s and ILC3s using flow cytometry in allergen-sensitized WT and *Il4raF709* mice. ILC2 and ILC3 identification in the draining mesenteric lymph nodes (MLNs) of orally sensitized mice was performed by analyzing their specific patterns of cell-surface markers: Lin⁻CD45⁺CD25⁺CD127⁺ and either Sca-1^{high}c-Kit^{low} (ILC2s) or Sca-1⁻c-Kit⁺ (ILC3s), as previously described (Fig 1, D, and see Fig E1, E).^{16,29} Compared with PBS-treated mice, the frequencies and absolute numbers of ILC2 and ILC3 populations were higher in the MLNs of allergen-sensitized *Il4raF709* mice (Fig 1, E, and see Fig E1, F). Compared with OVA-sensitized WT mice, increased ILC2 frequencies and absolute numbers were also observed in the SI lamina propria of OVA-sensitized *Il4raF709* mice (see Fig E1, G and H). Similar data were obtained by using intracellular cytokine staining to define ILC2s as CD45⁺Lin⁻IL-13⁺ and ILC3s as CD45⁺Lin⁻IL-17⁺ (Fig 1, E).

IL-33 receptor signaling regulates ILC2 expansion and allergic sensitization

IL-33 is a key cytokine that promotes the growth and maturation of ILC2s and their consequent T_H2 cytokine production.²⁹ IL-33 signaling through IL-33R, which is encoded by *Il1rl1*, and its associated IL-1 receptor accessory protein is implicated in airway inflammation after influenza virus infection and in several murine models of asthma.^{7,30,31} Because *Il33* mRNA levels are increased in the SI of allergen-sensitized *Il4raF709* mice (Fig 1, C, and see Fig E1, D), we investigated the dependence of allergic sensitization driven by ILC2 expansion on IL-33R signaling. Surprisingly, *Il4raF709 Il1rl1*^{-/-} mice were not sensitized to peanut, exhibiting no decrease in core body temperature, a lack of peanut IgE production, and minimal release of MMCP-1 after oral challenge (Fig 2, A and B). Flow cytometric analysis of ILC populations by means of the cellular markers Sca-1 and c-Kit revealed reduced ILC2 frequencies and absolute numbers in the MLNs and SI lamina propria of allergen-sensitized *Il4raF709 Il1rl1*^{-/-} mice (Fig 2, C and D).

GATA-3 is an essential transcription factor for ILC2s, and its expression can be used to identify those cells by using flow cytometry, as previously published.^{8,32} Using the markers Lin⁻CD45⁺Thy1.2⁺GATA-3⁺ and Lin⁻CD45⁺Thy1.2⁺RORγt⁺ to identify ILC2s and ILC3s, respectively, we confirmed that ILC2 frequencies and absolute numbers are increased in the MLNs and SI lamina propria of allergen-sensitized *Il4raF709* mice and that *Il1rl1* deficiency significantly reduced those numbers (see Fig E2 in this article's Online Repository at www.jacionline.org).

ILC2-derived IL-4 is indispensable for allergic sensitization

After activation with IL-2, IL-25, or IL-33, ILC2s secrete large amounts of T_H2 cytokines, such as IL-13 and IL-5, and it has become dogma that ILC2s produce minimal quantities of

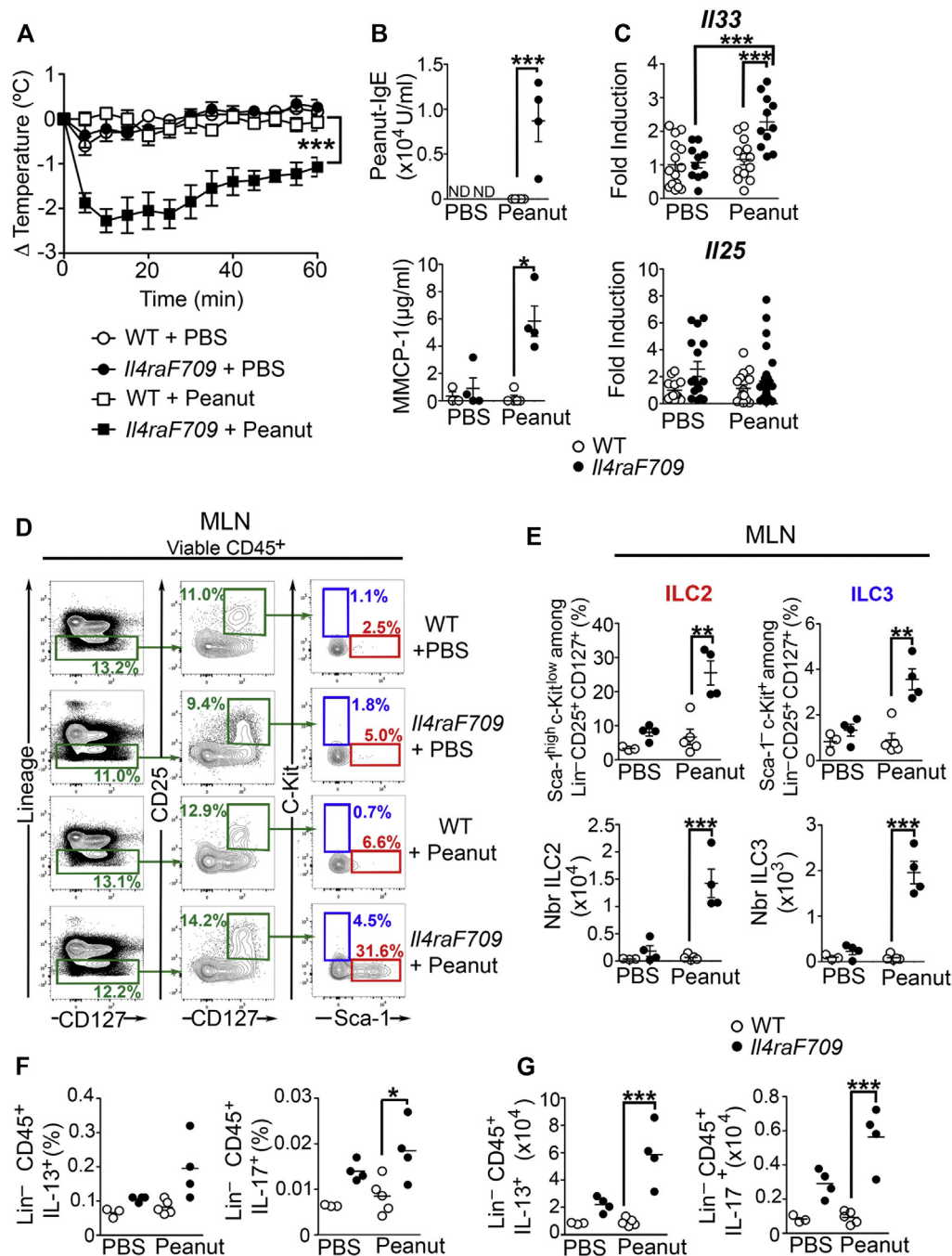


FIG 1. ILC2s are enriched in peanut-sensitized *Il4raF709* mice. **A**, Core body temperature changes in PBS- or peanut-sensitized WT and *Il4raF709* mice after oral peanut challenge. **B**, Serum concentrations of peanut-specific IgE and MMCP-1 after anaphylaxis. **C**, *Il33* and *Il25* mRNA levels in the SI of PBS- and peanut-sensitized WT and *Il4raF709* mice. **D** and **E**, Flow cytometric analysis (Fig 1, D), frequencies, and absolute numbers (Fig 1, E) of ILC2s ($\text{Lin}^- \text{CD25}^+ \text{CD127}^+ \text{Sca-1}^{\text{high}} \text{c-Kit}^{\text{low}}$) and ILC3s ($\text{Lin}^- \text{CD25}^+ \text{CD127}^+ \text{Sca-1}^{\text{low}} \text{c-Kit}^{\text{low}}$) in the MLNs. **F** and **G**, Frequencies and absolute numbers of IL-13^+ ILC2s and IL-17^+ ILC3s. Results represent data on 3 to 15 mice per group derived from 2 independent experiments. * $P < .05$, ** $P < .01$, and *** $P < .001$ by using 2-way ANOVA and 1-way ANOVA with posttest analysis.

IL-4.^{15,33,34} Flow cytometric analysis of *in vitro*-expanded ILC2s derived from SI lamina propria of unsensitized WT, *Il13*^{-/-}, *Il4raF709*, *Il4raF709* *Il4*^{-/-}, and *Il4raF709* *Il1rl1*^{-/-} mice demonstrates that ILC2s identified based on expression of the

transcription factor GATA-3 ($\text{Lin}^- \text{Thy1.2}^+ \text{CD45}^+ \text{GATA-3}^+$) express IL-5, IL-13, and copious amounts of IL-4 (Fig 3). In contrast, IL-9 expression by ILC2s was minimal (data not shown). IL-4 expression by MLNs and SI ILC2s was further confirmed by

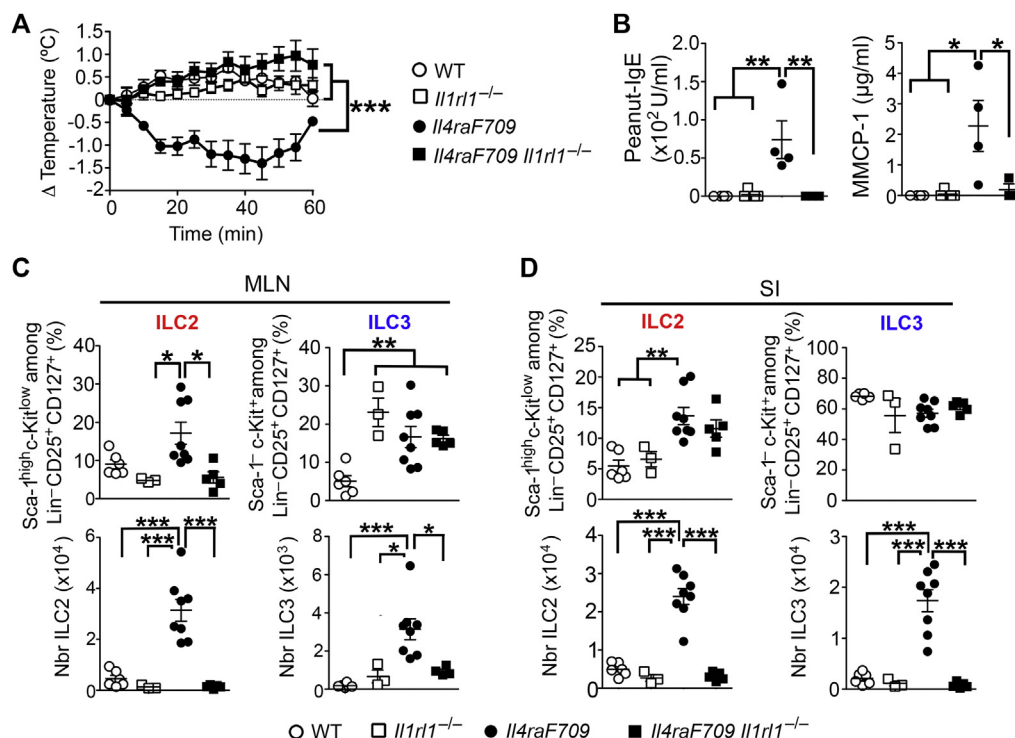


FIG 2. *IIR1* signaling drives ILC2 expansion and peanut allergy. **A**, Core body temperature change in peanut-sensitized WT, *IIR1*^{-/-}, *IIRaF709*, and *IIRaF709 IIR1*^{-/-} mice after oral challenge. **B**, Serum concentrations of peanut-specific IgE and MMCP-1 after anaphylaxis. **C** and **D**, Frequencies and absolute numbers of ILC2s and ILC3s in the MLNs (**C**) and SI (**D**) from the groups in **Fig 2, A**. Results represent 3 to 8 mice per group derived from 2 independent experiments. **P* < .05, ***P* < .01, and ****P* < .001 by using 2-way ANOVA and 1-way ANOVA with posttest analysis.

using reporter mice expressing the enhanced green fluorescent protein (eGFP) under control of the *IIR4* locus (4Get mice; see **Fig E3, A and B**, in this article's Online Repository at www.jacionline.org).³⁵ *IIR1* deficiency strongly reduced production of IL-4, IL-13, and IL-5 by intestinal ILC2s, even after peanut oral sensitization (see **Fig E4** in this article's Online Repository at www.jacionline.org). *IIR4* deletion in *IIRaF709* mice did not affect IL-13 production by intestinal ILC2s, whereas it completely abolished their IL-4 production (**Fig 3, B and C**). IL-13 receptor $\alpha 1$ was not expressed by CD4⁺ T cells, mast cells (Fc ϵ RI α ⁺c-Kit⁺), Treg cells (CD4⁺Foxp3⁺), or B cells (CD19⁺B220⁺), ruling out an effect by ILC2-derived IL-13 on these cell types (see **Fig E3, C**).

We reasoned that enforced restoration of the ILC2 compartment by means of adoptive transfer of *in vitro*-derived ILC2s might rescue allergic sensitization in *IIRaF709 IIR1*^{-/-} mice by acting as an early source of IL-4. *IIRaF709 IIR1*^{-/-} mice reconstituted with either *in vitro*-expanded WT or *IIR3*^{-/-} ILC2s and orally sensitized with peanut had food allergy, as evidenced by a decrease in their core body temperature, higher levels of peanut-specific IgE, and release of MMCP-1 in the serum after oral challenge with peanut (**Fig 4, A and B**, and see **Fig E5, A and B**, in this article's Online Repository at www.jacionline.org). Recovery from the decrease in core body temperature in mice reconstituted with *IIR3*^{-/-} ILC2s was faster, which is suggestive of a role for ILC2-derived IL-13 in potentiating the food-induced allergic response (see **Fig E5, A**). In contrast, adoptive transfer of *in vitro*-generated ILC3s into *IIRaF709 IIR1*^{-/-} mice subsequently orally sensitized and

challenged with peanut did not restore the allergic reaction, thereby demonstrating the specific requirement for ILC2s in food allergy (see **Fig E5**). Critically, adoptive transfer of IL-4-deficient ILC2s (*IIR4*^{-/-} ILC2s) did not restore oral allergic sensitization in *IIRaF709 IIR1*^{-/-} mice (**Fig 4, A and B**). ILC2 frequencies and absolute numbers were similarly restored in the MLNs and SI of mice receiving either WT, *IIR4*^{-/-} ILC2s, or *IIR3*^{-/-} ILC2, (**Fig 4, C and D**, and see **Fig E5**). These results revealed the importance of ILC2-secreted IL-4 in favoring allergic oral sensitization.

Treg cells suppress ILC2 expansion and activation

Establishment of oral tolerance and Treg cell suppressive functions are critical to prevent the development of oral sensitization and involved in the resolution process of food allergy.^{3,36,37} Failure of Treg cell-mediated oral tolerance or Treg cell absence exacerbates food allergy.^{3,38,39} To demonstrate that Treg cells can directly suppress ILC2 expansion during food allergy, we used transgenic *DO11.10⁺ Rag2^{-/-} Foxp3^{EGFP}* mice, which express the DO11.10⁺ T-cell receptor (TCR) specific to OVA₃₂₃₋₃₃₉ peptide on a RAG2-deficient genetic background. Those mice do not carry DO11.10⁺CD4⁺Foxp3⁺ T cells at steady state; however, supplying them with OVA in drinking water triggers the conversion of naive DO11.10⁺CD4⁺Foxp3⁻ T cells into induced DO11.10⁺CD4⁺Foxp3^{EGFP} iTreg cells.³ As previously established, the presence of the *IIRaF709* allele markedly impaired iTreg cell conversion in the MLNs and SI of DO11.10⁺ Rag2^{-/-} *IIRaF709* Foxp3^{EGFP}

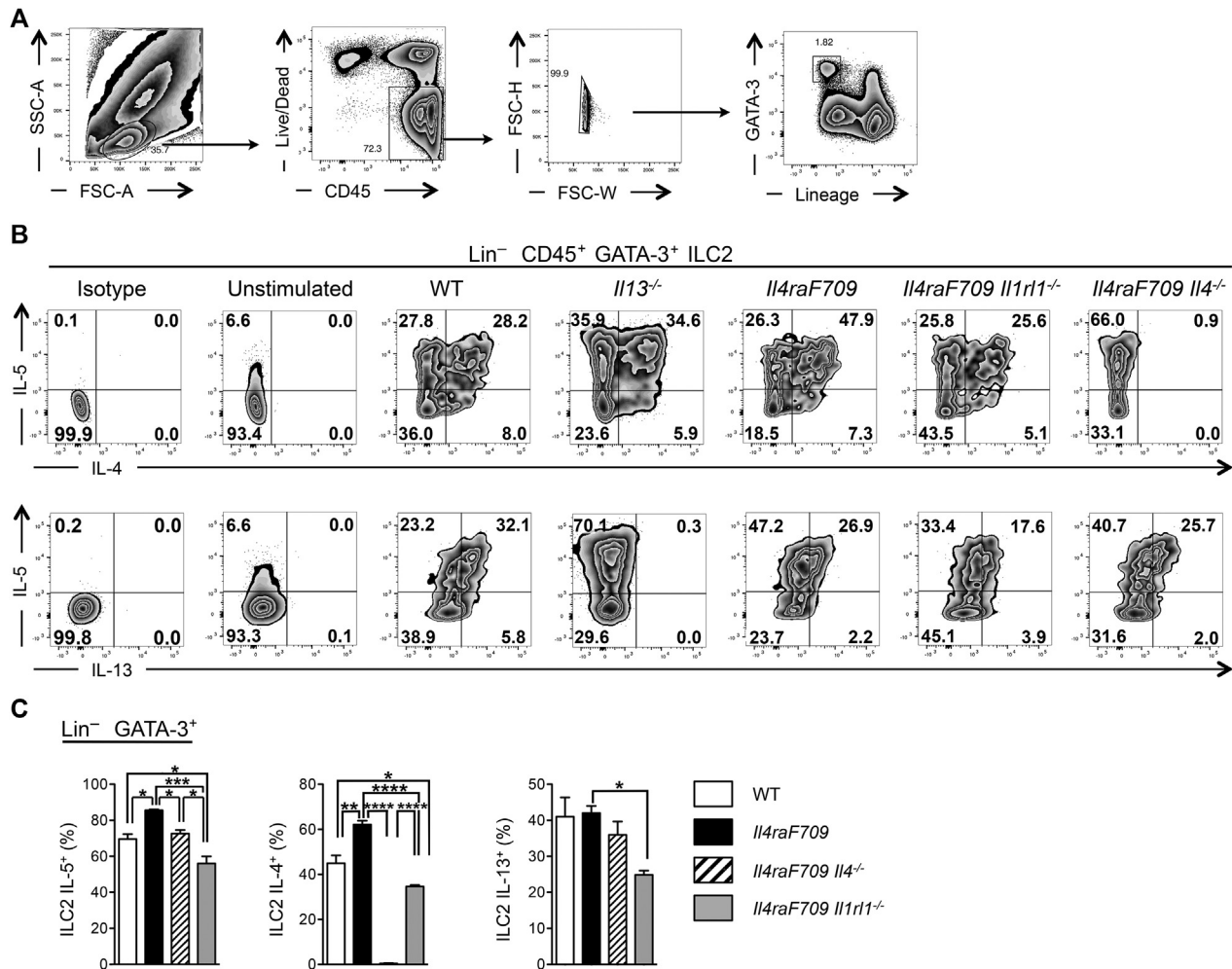


FIG 3. T_H2 cytokine production by *in vitro*-expanded SI ILC2s. **A**, Flow cytometric gating strategy used to identify *in vitro*-expanded ILC2s from the SI of naive mice. **B**, Flow cytometric analysis of IL-5, IL-4, and IL-13 expression by Lin⁻ CD45⁺ GATA-3⁺ ILC2s *in vitro* expanded from the SI of naive WT, *Il13*^{-/-}, *Il4raF709*, *Il4raF709 Il1r1*^{-/-}, and *Il4raF709 Il4*^{-/-} mice. **C**, Frequencies of IL-5⁺, IL-4⁺, and IL-13⁺ Lin⁻ CD45⁺ GATA-3⁺ ILC2s isolated from the SI of the groups from Fig 3, B. Results represent 3 to 5 mice per group derived from 2 independent experiments. **P* < .05, ***P* < .01, ****P* < .001, and *****P* < .0001 by using 1-way ANOVA with posttest analysis.

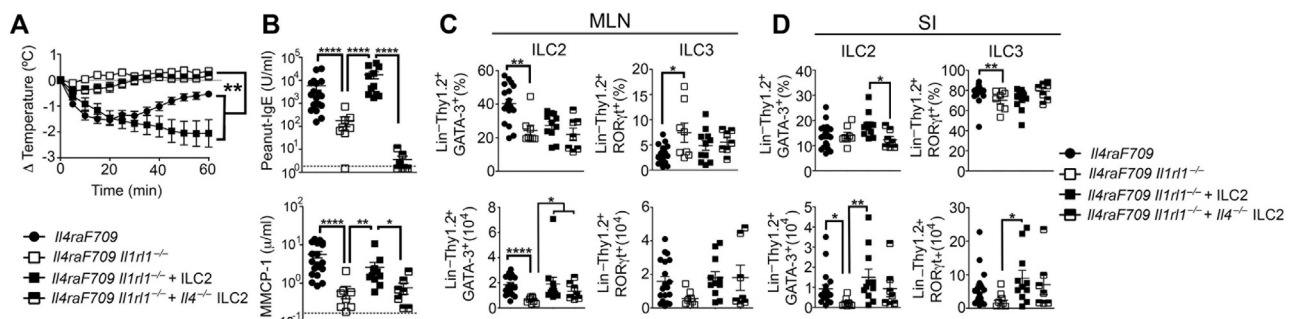


FIG 4. Oral allergic sensitization and anaphylaxis are obligately dependent on IL-4 production by ILC2s. **A**, Change in core body temperature after peanut oral challenge of peanut-sensitized *Il4raF709* and *Il4raF709 Il1r1*^{-/-} mice reconstituted with either WT or *Il4*^{-/-} ILC2s. **B**, Peanut-specific IgE concentration and MMCP-1 release after anaphylaxis. **C** and **D**, Frequencies and absolute numbers of ILC2s (Lin⁻Thy1.2⁺ GATA-3⁺) and ILC3s (Lin⁻Thy1.2⁺ RORγt⁺) in the MLNs (Fig 4, C) and SI (Fig 4, D) from the group in Fig 4, A. Results represent data of a minimum 7 to 18 mice per group derived from 2 independent experiments. **P* < .05, ***P* < .01, ****P* < .001, and *****P* < .0001 by using 2-way and 1-way ANOVA with posttest analysis.

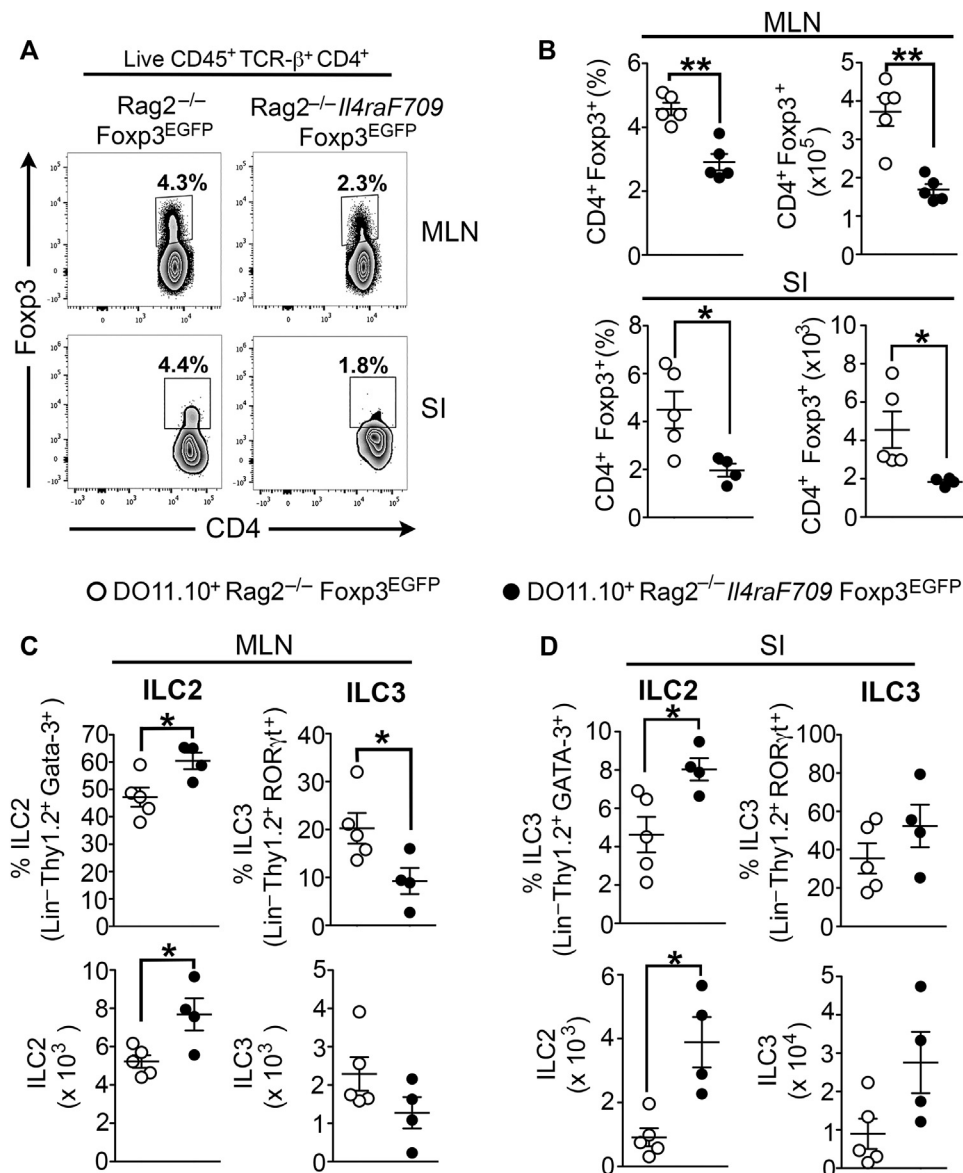


FIG 5. Defective allergen-specific Treg cell induction is associated with ILC2 expansion. **A**, Flow cytometric analysis of iTreg cells (CD4⁺Foxp3^{EGFP}) in the MLNs and SI of OVA-fed WT DO11.10⁺ Rag2^{-/-} Foxp3^{EGFP} and DO11.10⁺ Rag2^{-/-} Il4raF709 Foxp3^{EGFP} mice. **B**, Frequencies and absolute numbers of CD4⁺Foxp3⁺ iTreg cells in the MLNs and SI of the mouse groups from Fig 5, A. **C** and **D**, Frequencies and absolute numbers of ILC2s (Lin⁻Thy1.2⁺GATA-3⁺) and ILC3s (Lin⁻Thy1.2⁺RORγt⁺) in the MLNs (Fig 5, C) and SI (Fig 5, D) from the groups in Fig 5, A. Results represent data from 4 to 5 mice per group derived from 2 independent experiments. **P* < .05 and ***P* < .01, by using the unpaired 2-tailed Student *t* test.

mice (Fig 5, A and B).³ Impaired allergen-specific iTreg cell formation was associated with a marked increase in numbers of Lin⁻CD45⁺CD4⁺TCR-β⁺Thy1.2⁺GATA-3⁺ ILC2s but not RORγt⁺ ILC3s in the MLNs and SI of DO11.10⁺ Rag2^{-/-} Il4raF709 Foxp3^{EGFP} mice (Fig 5, C and D, and see Fig E6). These results established that defective iTreg cell induction and impaired oral tolerance are correlated with a significant expansion of the intestinal ILC2 population.

To further demonstrate regulation of the ILC2 compartment by Treg cells, we used Foxp3-deficient weanling mice (Foxp3^{K276X}), which lack functional Treg cells because of a nonsense mutation in Foxp3.^{40,41} Compared with age-matched WT mice, 20-day-old Foxp3^{K276X} exhibited a marked increase in the frequency and

absolute numbers of ILC2s in the SI and MLNs (see Fig E7 in this article's Online Repository at www.jacionline.org). ILC3s were also increased, although to a lesser extent. Because Foxp3^{K276X} mice succumb to early death because of an uncontrolled lymphoproliferative disease, we examined the effect of acute transient ablation of Treg cells on ILC2 expansion by using adult BALB/c Foxp3^{EGFP/DTR} mice.⁴¹ Those mice express the diphtheria toxin receptor (DTR) under the control of the Foxp3 promoter. Compared with age-matched WT Foxp3^{EGFP} mice, 2 weeks of diphtheria toxin (DT) treatment resulted in significant weight loss and a 2-fold decrease of MLN and SI Treg cell population in Foxp3^{EGFP/DTR} (see Fig E8 in this article's Online Repository at www.jacionline.org). Treg cell depletion with DT

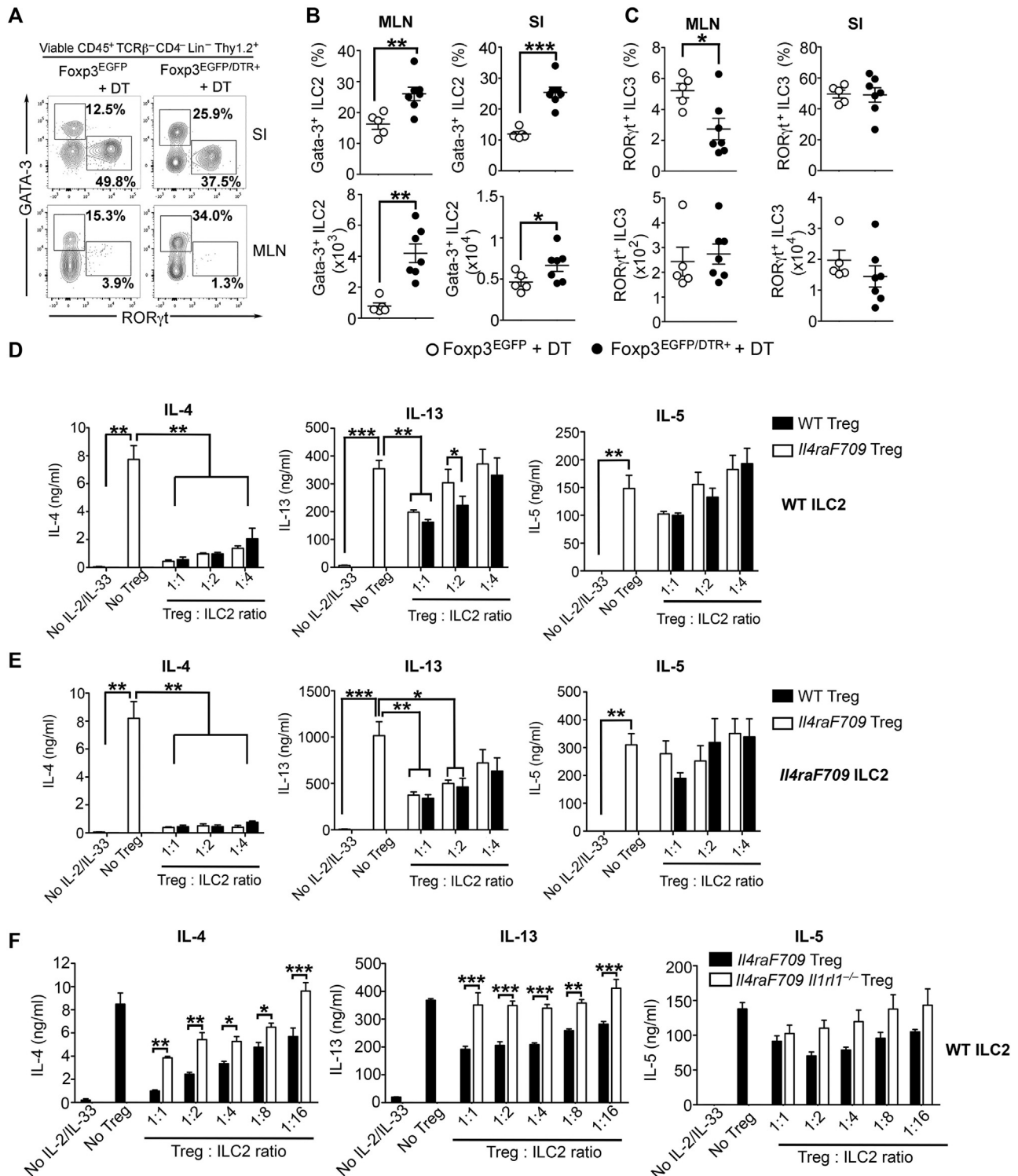


FIG 6. Treg cells inhibit ILC2 expansion and their Th2 cytokine production. **A**, Flow cytometric analysis of ILC2s (Lin⁻Thy1.2⁺GATA-3⁺) and ILC3s (Lin⁻Thy1.2⁺RORγt⁺) in the SI and MLNs of *Foxp3*^{EGFP} and *Foxp3*^{EGFP/DTR+} mice after DT administration. **B** and **C**, Frequencies and absolute numbers of Lin⁻Thy1.2⁺GATA-3⁺ ILC2s (Fig 6, **B**) and Lin⁻Thy1.2⁺RORγt⁺ ILC3s (Fig 6, **C**) in the MLNs and SI from the mouse groups in Fig 6, **A**. **D** and **E**, IL-4, IL-13, and IL-5 production by an increased gradient of cell-sorted WT ILC2s (Fig 6, **D**) or *I4raF709* ILC2s (Fig 6, **E**) cultured in the presence of either WT or *I4raF709* Treg cells. **F**, IL-4, IL-13, and IL-5 production by WT ILC2s cells cocultured in the presence of *I4raF709* or *I4raF709* *Il1r1*^{-/-} Treg cells. Results represent data from 5 to 7 mice per group. **P* < .05, ***P* < .01, and ****P* < .001, by using 1-way ANOVA with posttest analysis or the unpaired 2-tailed Student *t* test.

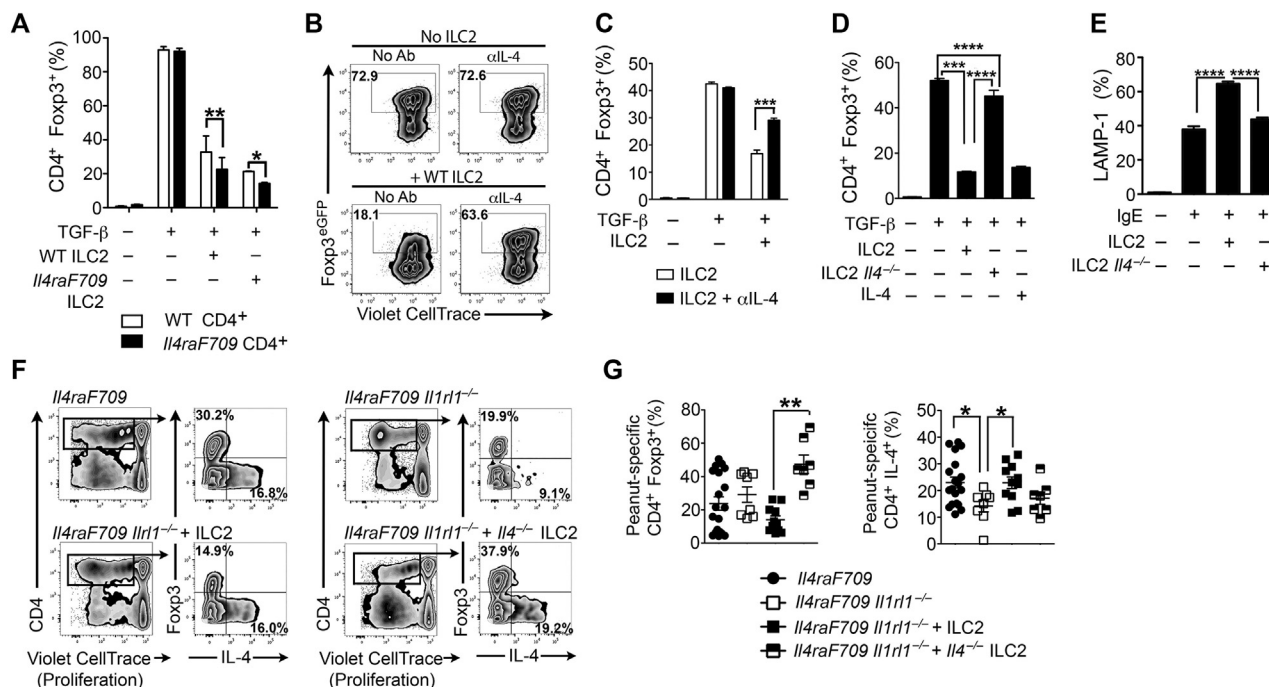


FIG 7. IL-4 secretion by ILC2s promotes food allergy by decreasing allergen-specific Treg cell induction. **A**, TGF- β induction of iTreg cells from naive CD4⁺ WT or *Il4raF709* T cells cocultured with cell-sorted WT or *Il4raF709* ILC2s. **B**, Flow cytometric analysis of the effect of ILC2s and anti-IL-4 (α IL-4) treatment on TGF- β -driven *in vitro* iTreg cell induction. **C**, Frequency of TGF- β -induced iTreg cells cocultured in the presence of WT ILC2s without or with α IL-4. **D**, Frequency of TGF- β -induced iTreg cells cocultured with either WT ILC2s, *Il4*^{-/-} ILC2s, or recombinant IL-4. **E**, IgE-mediated mast cell degranulation after coculture with ILC2s, ILC2 supernatants, or IL-4 in the presence or absence of α IL-4. **F**, IgE-mediated degranulation of mast cells cocultured with WT ILC2 or *Il4*^{-/-} ILC2s. **G**, Flow cytometric analysis of peanut extract-induced *in vitro* proliferation of CD4⁺Foxp3⁺ Treg cells and CD4⁺Foxp3⁻ conventional T cells isolated from the MLNs of peanut-sensitized *Il4raF709* and *Il4raF709 Il1r1*^{-/-} mice reconstituted with either WT or *Il4*^{-/-} ILC2. **H**, Frequency of peanut-specific CD4⁺Foxp3⁺ Treg cells and CD4⁺ T cells producing IL-4. Results represent data of 5 mice per group (Fig 7, A-F) and 7 to 18 mice (Fig 7, G and H) derived from 2 independent experiments. **P* < .05, ***P* < .01, ****P* < .001, and *****P* < .0001 by using 2-way and 1-way ANOVA with posttest analysis.

treatment resulted in increased ILC2 frequencies and absolute numbers in the SI and draining MLNs of *Foxp3*^{EGFP/DTR+} (Fig 6, A-C). These results indicate that Treg cell deficiency, either congenital or inducible, results in dysregulated ILC2 expansion.

We further analyzed the capacity of Treg cells to regulate ILC2 TH2 cytokines production using an *in vitro* coculture system. Cell-sorted iTreg cells *in vitro* derived from either WT or *Il4raF709* naive CD4⁺ T cells were used in a suppressive assay and cultured in the presence of an increased gradient of cell-sorted WT or *Il4raF709* ILC2s (Fig 6, D and E). In the absence of iTreg cells and under IL-2 and IL-33 stimulation, purified ILC2s derived from either WT or *Il4raF709* mice secrete substantial quantities of IL-4, IL-13, and IL-5 (Fig 6, D and E). Addition of iTreg cells resulted in a dose-dependent reduction in IL-4 and IL-13 production, with IL-4 being more amenable to suppression (Fig 6, D and E). IL-33R expression on Treg cells plays a critical role in suppression of intestinal inflammation.⁴² When cocultured with *in vitro*-derived ILC2s, *Il4raF709 Il1r1*^{-/-} iTreg cells displayed significantly less efficient suppressive function than their *Il4raF709* counterparts, being only half as potent in reducing IL-4 levels and completely failing to reduce IL-13 secretion by ILC2s (Fig 6, F). In summary, Treg cells restrain ILC2 expansion in the gastrointestinal mucosa and

directly suppress ILC2 activation in an IL-33R-dependent manner.

IL-4 secretion by ILC2s antagonizes allergen-specific Treg cell induction and impairs their suppressive function

In vitro differentiation of naive CD4⁺ T cells into CD4⁺Foxp3⁺ iTreg cells is disrupted by the presence of IL-4.^{3,43} Accordingly, we examined the capacity of ILC2-derived IL-4 to disrupt oral tolerance in the setting of food allergy by impairing allergen-specific Treg cell formation and function. When naive CD4⁺Foxp3^{EGFP} T cells are cultured in Treg cell-inducing conditions, addition of ILC2s to the culture resulted in a significant reduction of CD4⁺Foxp3^{EGFP} iTreg cell conversion in an IL-4-dependent fashion (Fig 7, A-D). Proliferation was not markedly altered, indicating a specific effect on Treg cell lineage commitment rather than outgrowth of the Foxp3⁻ compartment. IL-4 is a potent modulator of mast cell activation.⁴⁴ Coculture of IgE-sensitized mast cells in the presence of ILC2s resulted by upward of a 50% increase of degranulating lysosomal-associated membrane protein 1 (LAMP-1)⁺ mast cells, which was largely abrogated when ILC2s were derived from IL-4-deficient mice (Fig 7, E).

We further examined the effect of ILC2s on allergen-specific Treg cells *in vivo* by using the ILC2 reconstitution approach described in Fig 4. MLNs isolated from peanut-sensitized *Il4raF709 Il1r1^{-/-}* mice that received either WT or *Il4^{-/-}* ILC2s were labeled with a proliferative cell dye and cultured in the presence of peanut. Analysis revealed significantly higher frequencies of peanut-specific Treg cells in recipients treated with *Il4^{-/-}* ILC2s (Fig 7, F and G). Together, these results established that ILC2-derived IL-4 might act to promote food allergy by antagonizing the induction of allergen-specific Treg cells and inhibiting their regulatory function, which feeds into dysregulated mast cell activation.

DISCUSSION

In this report we demonstrate that ILC2s play a requisite role in mediating oral sensitization to food allergens and delineate the mechanisms involved in such action. *Il4raF709* mice, which are genetically prone to food allergy, do not have allergy when their ILC2 differentiation is compromised by concurrent IL-33R deficiency. Furthermore, for the first time, we have established, using several genetic approaches, that Treg cells regulate ILC2 expansion and suppress their T_H2 cell cytokine secretion. In turn, ILC2 activation and expansion promote food allergy by secreting IL-4, which compromises Treg cell function and augments mast cell activation. Our results define a cellular network involving ILC2s, Treg cells, and mast cells that is normally poised toward tolerance to food allergens but the perturbation of which on ILC2 activation might tip the balance toward food allergy.

A key finding in our studies relevant to food allergy is that Treg cells and ILC2s have been shown to engage in reciprocal regulation centered on ILC2-derived IL-4. Although some previous reports have documented IL-4 secretion by ILC2s, most have focused on the production of IL-5 and IL-13 by ILC2s.^{8,15,33,45} Our present investigations reveal that ILC2s produce copious amounts of IL-4 that are sufficient to suppress allergen-specific iTreg cell differentiation and promote food allergy. Dysregulated ILC2 expansion and activation might well be an initiating step in the pathogenesis of food allergy. The ensuing impairment in the mucosal regulatory compartment contributes to a T_H2 -skewed, food allergen-specific adaptive immune response and disease persistence.

The function of ILC2s in the mucosal innate immune response and their localization at the environmental interface indicate that they might play a critical role in fueling food allergy in response to alarmin signals emanating from the intestinal epithelium, such as IL-33.⁴⁶ Of note, production of the alarmin IL-33 is augmented in the intestinal tract of mice with food allergy. Increased expression of alarmins by intestinal epithelial cells could be induced by an altered intestinal microbial flora.^{47,48} We have demonstrated that food allergy is associated with intestinal microbial dysbiosis, which in turn promotes oral allergic sensitization.²⁶ A dysbiotic flora might upregulate the expression of alarmins in the intestinal epithelium, creating an environment conducive to ILC2 activation and expansion after oral exposure to allergen. Some allergens, including peanut, have been shown to activate alarmin secretion by epithelial cells and provide one trigger leading to T_H2 cytokine production and food allergy induction.⁴⁹

In addition to disabling Treg cells, IL-4 production by ILC2s amplified mast cell activation. We have previously shown that Treg cell inhibition of mast cell degranulation is critical to forestalling food allergy.^{3,4} In turn, dysregulated IgE–mast cell interaction profoundly inhibits iTreg cell induction and mediates the latter's T_H2 cell–like reprogramming by an IL-4–dependent mechanism, providing a requisite step in food allergy pathogenesis.³ Allergen re-exposure of intestinal IgE-stimulated mast cells could also lead to robust IL-33 production and consequently create an ILC2 activation and expansion loop that positively feeds back on mast cell activation and negatively regulates allergen-specific iTreg cell induction.^{50,51} Thus ILC2s can also sustain immune dysregulation during the adaptive phase of the allergic immune response to food allergens.

Although oral immunotherapy has been effective in inducing a desensitized state with short-lived unresponsiveness to food allergens, it has had more limited success in conferring true long-term tolerance after cessation of therapy.⁵² Our previous results and those reported herein emphasize the importance of interrupting the ILC2–Treg cell–mast cell circuit of immune dysregulation that acts to suppress oral tolerance by blocking effective allergen-specific iTreg cell responses in the setting of food allergy. Resetting this circuit either pharmacologically and/or with an immunomodulator, such as anti-IL-4R antibody therapy, might be essential in conferring long-term tolerance to food allergens.

We thank Drs Calvin B Williams and Dipica Haribhai for provision of *Foxp3^{EGFP/DTR+}* mice on a BALB/c background and Dale Umetsu and Andrew McKenzie for the gift of *Il1r1^{-/-}* mice.

Key messages

- Food allergy is accompanied by ILC2 expansion and activation in the gastrointestinal tract.
- Treg cells control ILC2 expansion and activation.
- IL-4 secretion by ILC2 blocks allergen-specific Treg cell induction.

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METHODS

Animals

C.129X1-*Il4ra*^{tm3.1Tch} (*Il4ra*F709), C.Cg-*Foxp3*^{tm2Tch}/J (*Foxp3*^{EGFP}), *DO11.10*⁺ *Foxp3*^{EGFP}, *Foxp3*^{EGFP/DTR}+, and *Foxp3*^{K276x} mice (all BALB/c congenics), were previously described.^{E1-E7} C.129S6(B6)-*Rag2*^{tm1Fwa} (*Rag2*^{-/-}) mice were obtained from Taconic (Hudson, NY) and crossed with *DO11.10*⁺ *Foxp3*^{EGFP} and *DO11.10*⁺ *Il4ra*F709 *Foxp3*^{EGFP} mice.^{E8} C.129S4(Cg)-*Il13*^{tm2.1Lky}/J (*IL-13*YFP^{-Cre}), C.129-*Il4*^{tm1Lky}/J, C.129Ola-*Il13*^{tm1.1Anjm}, and BALB/c-*Il4*^{tm2Nnt}/J (*Il4*^{-/-}) mice were obtained from the Jackson Laboratories and maintained in our animal facility. BALB/c congenic *Il1rl1*^{-/-} mice, a gift from Dr Andrew McKenzie, were backcrossed with *Il4ra*F709 mice to generate *Il4ra*F709 *Il1rl1*^{-/-} mice.^{E9} Mice were bred and housed under specific pathogen-free conditions and used according to the guidelines of the Institutional Animal Research Committee at the Boston Children's Hospital of Animal Care Resources.

Flow cytometry

The following murine antibodies were used: CD45 (30-F11), Thy1.1 (His51), Thy1.2 (30-H12), CD25 (PC61.5), CD4 (RM4-5), CD3 (145-2C11), IL-17 (TC11-18H10.1), c-Kit (2B8), Sca-1(D7), IL-5 (TRFK5), TCRβ (H57-597), F4/80 (BM2), CD124 (IL-4Rα; I015F8; BioLegend), *Foxp3* (FJK-16S), *DO11.10* (KJ126), IL-13 (eBio13a), GATA-3 (TWAJ), inducible costimulator (C398.4A), IFN-γ (XMG1.2), RORγt (B2), Rat IgG₁ Isotype control (eBRG1), CD213a1 (IL-13 receptor α; 13MOKA; eBioscience), ST2 (DJ8; MD Biosciences, Paul, Minn), IL-4 (11B11), and CD127 (SB/199; BD Biosciences, San Jose, Calif). Exclusion of lineage marker-expressing cells was accomplished by using a cocktail of fluorescein isothiocyanate- or phycoerythrin-conjugated antibodies comprised of B220 (RA3-6B2), CD3ε (145-2C11), CD4 (RM4-5), CD8α (53-6.7), CD49b (DX5), CD11b (M1/70), CD11c (N418), CD19 (6D5), Gr-1 (RB6-8C5), FcεRIα (MAR-1), NKp46 (29A1.4), and TCRγδ (UC7-13D5) (BioLegend). For cytokine and nuclear factor intracellular staining, cells were stimulated for 4 hours with phorbol 12-myristate 13-acetate (50 ng/mL, Sigma-Aldrich) and ionomycin (500 ng/mL, Sigma-Aldrich) in the presence of GolgiPlug (BD Biosciences) and stained with BD Cytotfix/Cytoperm buffers (BD Biosciences) or the *Foxp3* Transcription Factor buffer set (eBioscience). Cell proliferation assays were performed with the Violet CellTrace Dye (Invitrogen, Carlsbad, Calif), as previously described.^{E1} Dead cells were routinely excluded from analysis based on the staining of eFluor 780 fixable viability dye (eBioscience), and analyses were restricted to single cells by using forward scatter-H and forward scatter-W signals. Stained cells were analyzed on an LSRII Fortessa (BD Biosciences), and data were processed with FlowJo Software (Tree Star, Ashland, Ore).

TGF-β-mediated *in vitro* iTreg cell induction

Sorted naive CD4⁺ T cells (1×10^6 /mL) were cultured with plate-bound anti-CD28 (5 μg/mL, BioLegend), anti-CD3 (1 μg/mL, BioLegend), recombinant TGF-β (10 ng/mL; R&D Systems, Minneapolis, Minn), retinoic acid (100 nmol/L, Sigma-Aldrich), anti-IFN-γ (5 μg/mL, XMG1.2; BioLegend), IL-2 (2 ng/mL, Shenandoah Biotechnology), and anti-IL-4 (20 μg/mL, 11B11, prepared in house). After 7 days, the induced iTreg cells were analyzed by means of flow cytometry or resorted on eGFP fluorescence.

DT Treg cell depletion

For *in vivo* Treg cell depletion, *Foxp3*^{EGFP/DTR} and control *Foxp3*^{EGFP} mice were given 50 μg/kg DT (Sigma-Aldrich) administered intraperitoneally every other day during 2 weeks, as previously described.^{E6} Mice were analyzed the day after the last DT injection, and Treg cell depletion was assessed by flow cytometry analysis of MLN and SI lymphocytes of treated mice.

Quantitative real-time PCR analysis

Jejunal sections were snap-frozen in liquid nitrogen and homogenized in TRIzol (Life Technologies). RNA was extracted from the homogenized tissues by using the RNeasy kit (Qiagen, Hilden, Germany). Reverse transcription was performed with Superscript III and oligo dT (Invitrogen). TaqMan gene probes were used with TaqMan Universal Fast Master Mix (Applied Biosystems, Foster City, Calif) and run on a Step-One-Plus machine (Applied Biosystems). Glyceraldehyde-3-phosphate dehydrogenase was used as an endogenous control (Applied Biosystems), and WT mouse RNA was used as the exogenous control. Samples were run in triplicates, and relative expression was calculated by using the ΔΔ cycle threshold method.

ELISAs

MMCP-1 and total and OVA-specific IgE concentrations were measured in the sera of treated mice by using ELISAs, as described previously.^{E5} Peanut-specific IgE was captured onto ELISA plates coated overnight with 3 μg/mL anti-IgE (R35-92, BD) and detected by using 200 ng/mL biotinylated peanut extract, as previously described.^{E2}

Peanut-specific T-cell assays

CD4⁺ T cells from sensitized mice were assessed for peanut responsiveness, as previously described.^{E2} Briefly, MLN cells were labeled with CellTrace Violet and cultured with aqueous protein extract from peanut flour for 5 days before analysis by using flow cytometry. Cells undergoing proliferation (dye dilution) in response to stimulation were considered peanut specific.

Histologic analysis

Intestinal mast cells were enumerated by means of microscopic examination of jejunal sections fixed in 10% formaldehyde and stored in 70% ethanol before toluidine blue staining by the Harvard Rodent Histopathology Facility (Cambridge, Mass).

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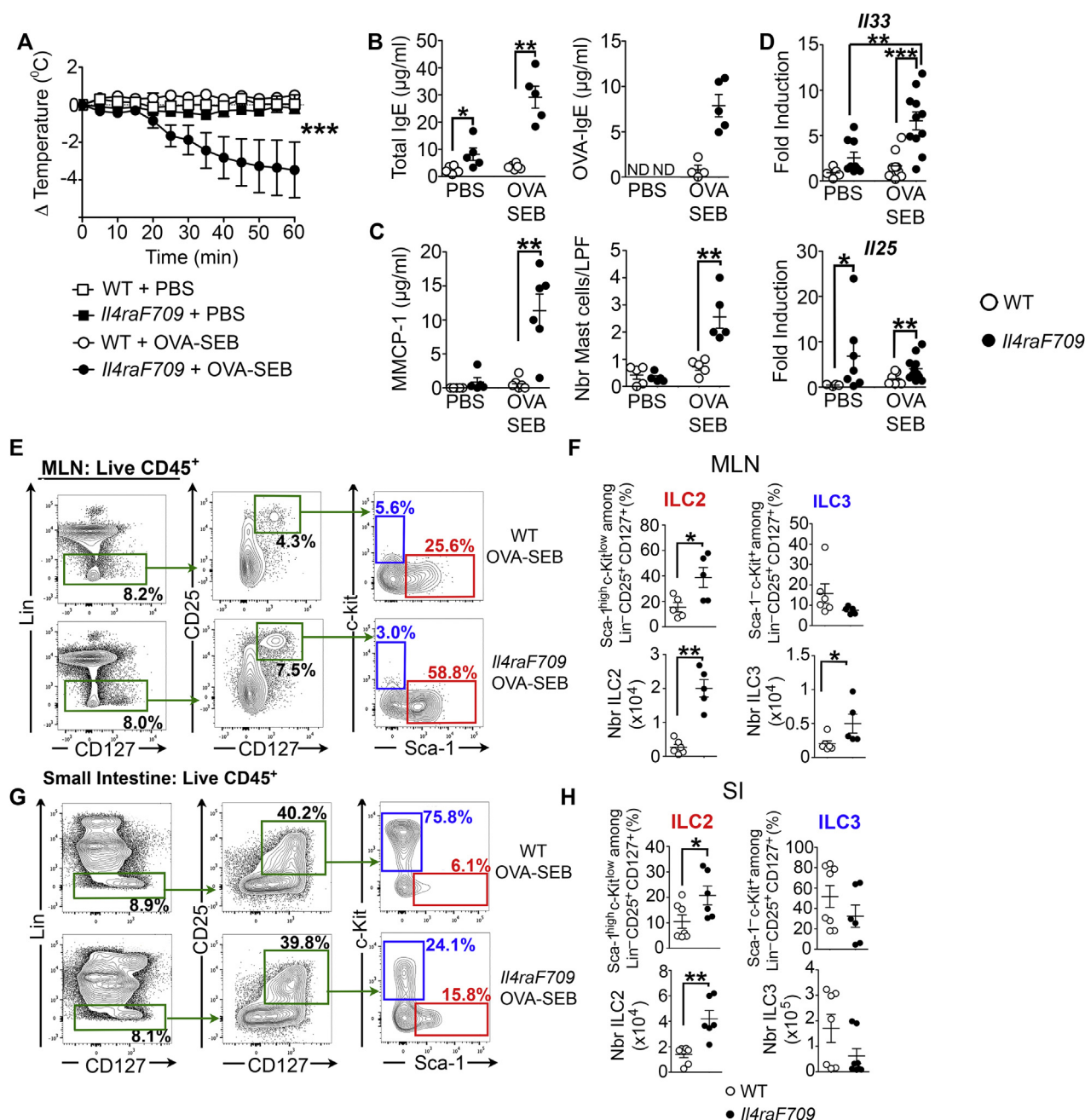


FIG E1. OVA sensitization expands ILC2s in *Il4raF709* mice. **A**, Core body temperature changes of PBS- or OVA/staphylococcal enterotoxin B (SEB)-sensitized WT and *Il4raF709* mice after oral OVA challenge. **B** and **C**, Serum levels of total and OVA-specific IgE (Fig E1, B); serum MMCP-1 release and intestinal mast cell numbers after anaphylaxis (Fig E1, C). **D**, *Il25* and *Il33* mRNA in the SI. **E** and **F**, Flow cytometric analysis of ILC2s (Lin[−]Thy1.2⁺CD25⁺CD127⁺Sca-1^{high}c-Kit^{low}) and ILC3s (Lin[−]Thy1.2⁺CD25⁺CD127⁺Sca-1[−]c-Kit⁺) in the MLNs (Fig E1, E) and SI of OVA/SEB-sensitized mice (Fig E1, F). **G** and **H**, Frequencies and absolute numbers of ILC2s and ILC3s in the SI (Fig E1, G) and MLNs (Fig E1, H) of OVA/SEB-sensitized WT and *Il4raF709* mice. Results are representative of 2 independent experiments with 5 mice per group. **P* < .05, ***P* < .01, and ****P* < .001 by using 2-way ANOVA and 1-way ANOVA with posttest analysis.

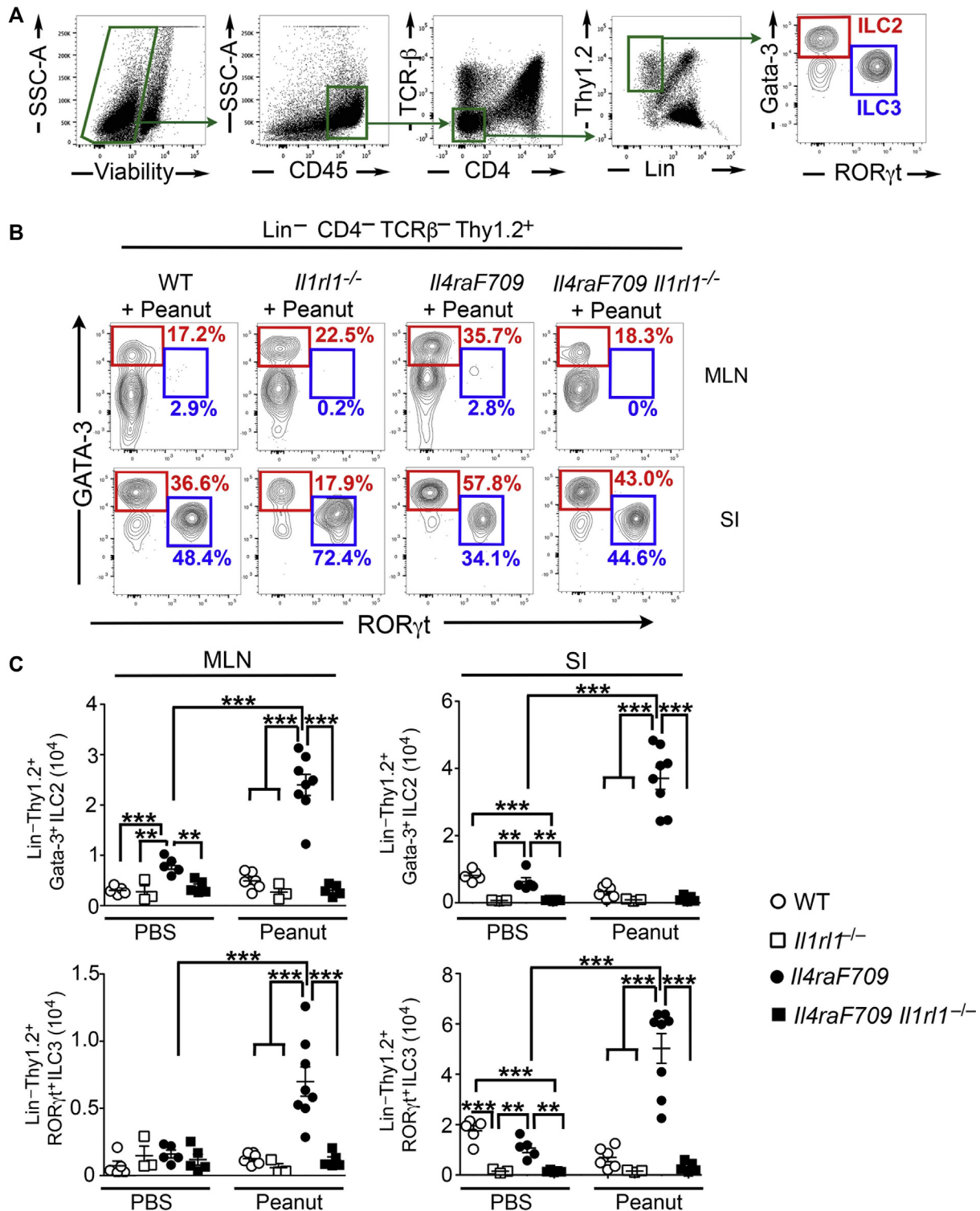


FIG E2. Lin⁻GATA-3⁺ ILC2s are enriched during food allergy. **A**, Flow cytometric gating strategy used to identify ILC2s and ILC3s based on their respective expression of the transcription factors GATA-3 and RORγt. SSC, Side scatter. **B**, Flow cytometric analysis of GATA-3⁺ ILC2s and RORγt⁺ ILC3s in the MLNs and SI of peanut-sensitized WT, *Il1rl1*^{-/-}, *Il4ra*F709, and *Il4ra*F709 *Il1rl1*^{-/-}. **C**, Frequencies and absolute numbers of GATA-3⁺ ILC2s and RORγt⁺ ILC3s in the MLNs and SI of peanut-sensitized WT, *Il1rl1*^{-/-}, *Il4ra*F709, and *Il4ra*F709 *Il1rl1*^{-/-} mice. Results are representative of 2 independent experiments, with 5 to 8 mice per group. **P* < .05, ***P* < .01, and ****P* < .001 by using 1-way ANOVA with posttest analysis.

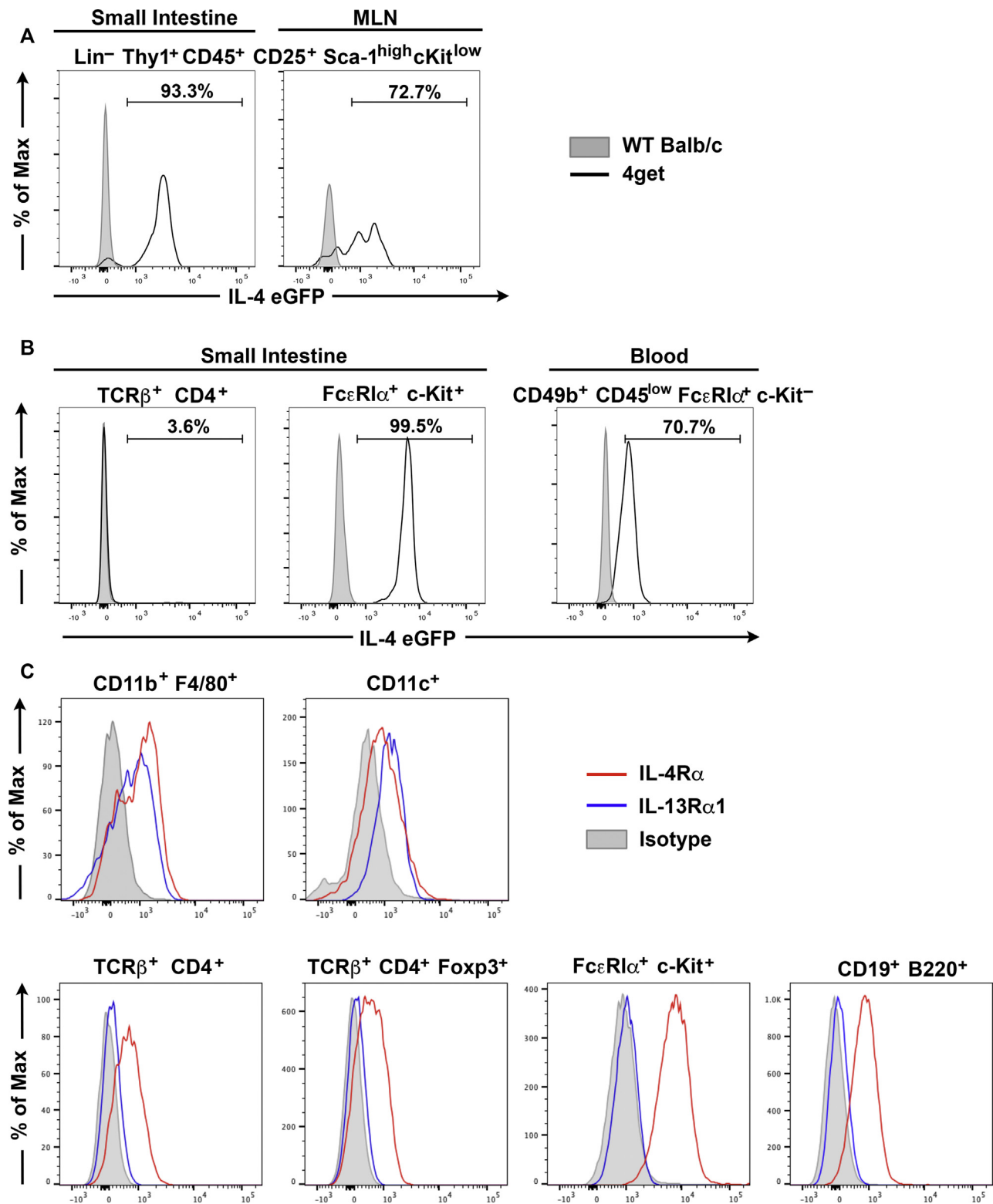


FIG E3. IL-4 expression by ILC2s. **A**, Flow cytometric analysis of IL-4 production by Lin⁻CD45⁺CD25⁺CD127⁺Sca-1^{high}c-Kit^{low} ILC2s isolated from the SI lamina propria and MLNs of IL-4 eGFP mice based on eGFP expression. **B**, Detection of IL-4 signal in TCR β ⁺CD4⁺ T cells, Fc ϵ R1 α ⁺c-Kit⁺ mast cells from the SI lamina propria, and DX5⁺Fc ϵ R1 α ⁺c-Kit⁻ basophils from peripheral blood of IL-4 eGFP mice. **C**, Flow cytometric analysis of IL-4R α and IL-13 receptor α 1 expression on F4/80⁺ CD11b⁺ macrophages, CD11c⁺ dendritic cells, TCR β ⁺ CD4⁺ T cells, Foxp3⁺ Treg cells, Fc ϵ R1 α ⁺c-Kit⁺ mast cells, and CD19⁺B220⁺ B cells.

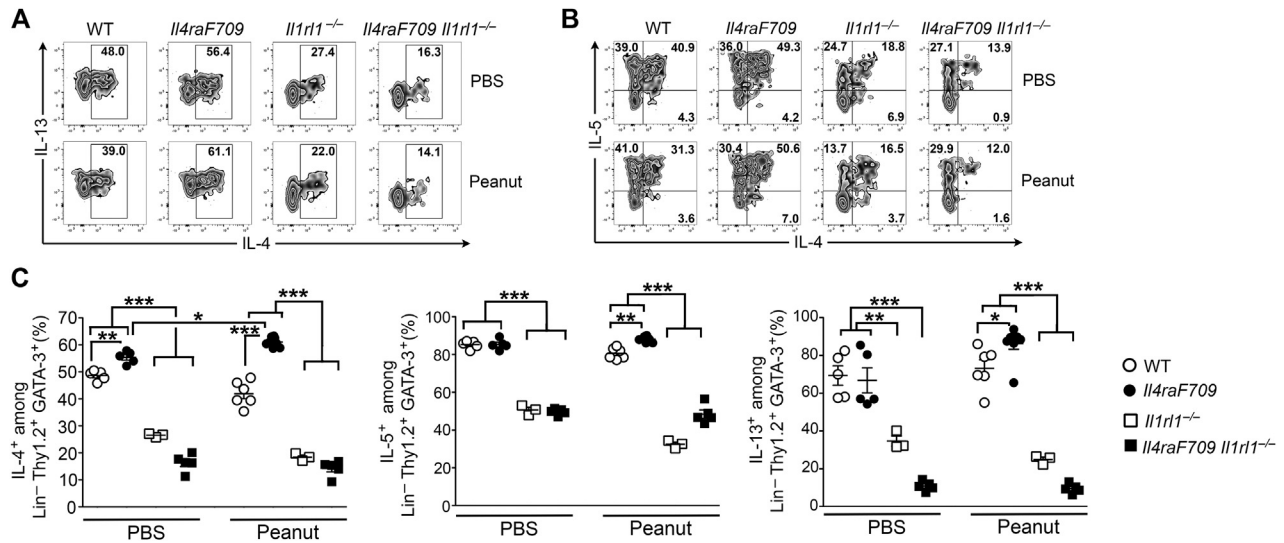


FIG E4. *Il1rl1* signaling regulates ILC2 expansion in peanut-sensitized mice. **A** and **B**, Flow cytometric analysis of IL-13 and IL-4 (Fig E4, **A**) and IL-5 and IL-4 (Fig E4, **B**) expression by intestinal GATA-3⁺ ILC2s isolated from the SI of PBS- or peanut-sensitized WT, *Il4raF709*, *Il1rl1*^{-/-}, and *Il4raF709 Il1rl1*^{-/-} mice. **C**, Frequencies of IL-4⁺, IL-5⁺, and IL-13⁺ cells among GATA-3⁺ ILC2s isolated from the SI of PBS- or peanut-sensitized WT, *Il4raF709*, *Il1rl1*^{-/-}, and *Il4raF709 Il1rl1*^{-/-} mice. Results represent data on 3 to 6 mice per group derived from 2 independent experiments. **P* < .05, ***P* < .01, and ****P* < .001 by using 1-way ANOVA with posttest analysis.

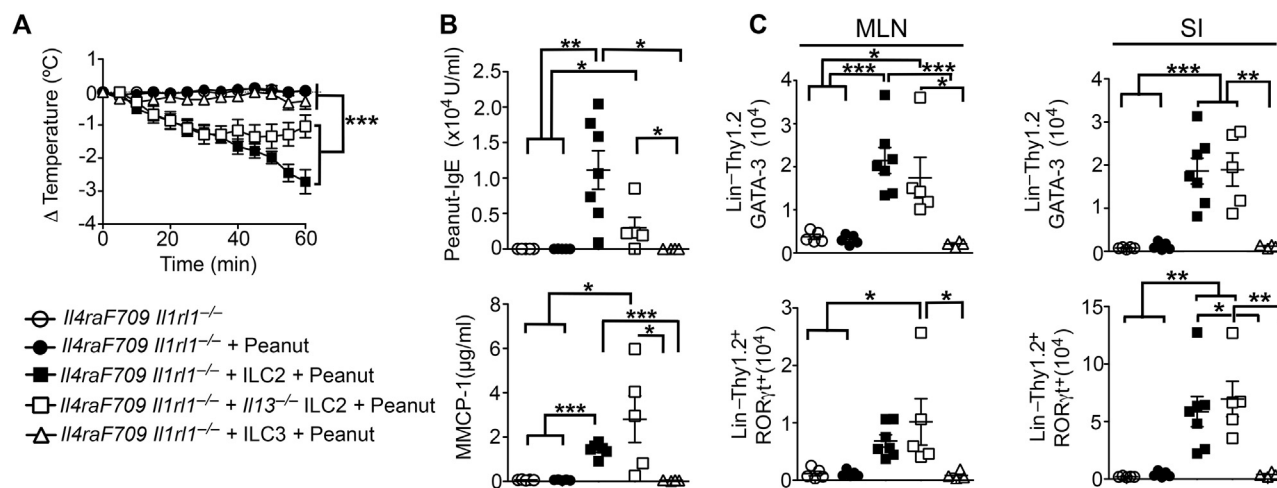


FIG E5. Adoptive transfer of *I13^{-/-}* ILC2s but not ILC3s restores oral sensitization in *I4raF709 I1rl1^{-/-}* mice. **A**, Core body temperature changes of PBS- or peanut-sensitized *I4raF709 I1rl1^{-/-}* mice in the absence or after adoptive transfer of *in vitro* WT ILC2s or *I13^{-/-}* ILC2s or ILC3s. **B**, Serum levels of peanut-specific IgE and MMCP-1 release after anaphylaxis. **C**, Absolute numbers of GATA-3⁺ ILC2s and ROR γ t⁺ ILC3s from the MLNs and SI of PBS or peanut-sensitized *I4raF709 I1rl1^{-/-}* mice without or after adoptive transfer of either *in vitro*-expanded and cell-sorted WT ILC2s, *I13^{-/-}* ILC2s, or ILC3s. Data are representative of 4 to 5 mice per group. * $P < .05$, ** $P < .01$, and *** $P < .001$ by using 2-way ANOVA and 1-way ANOVA with posttest analysis.

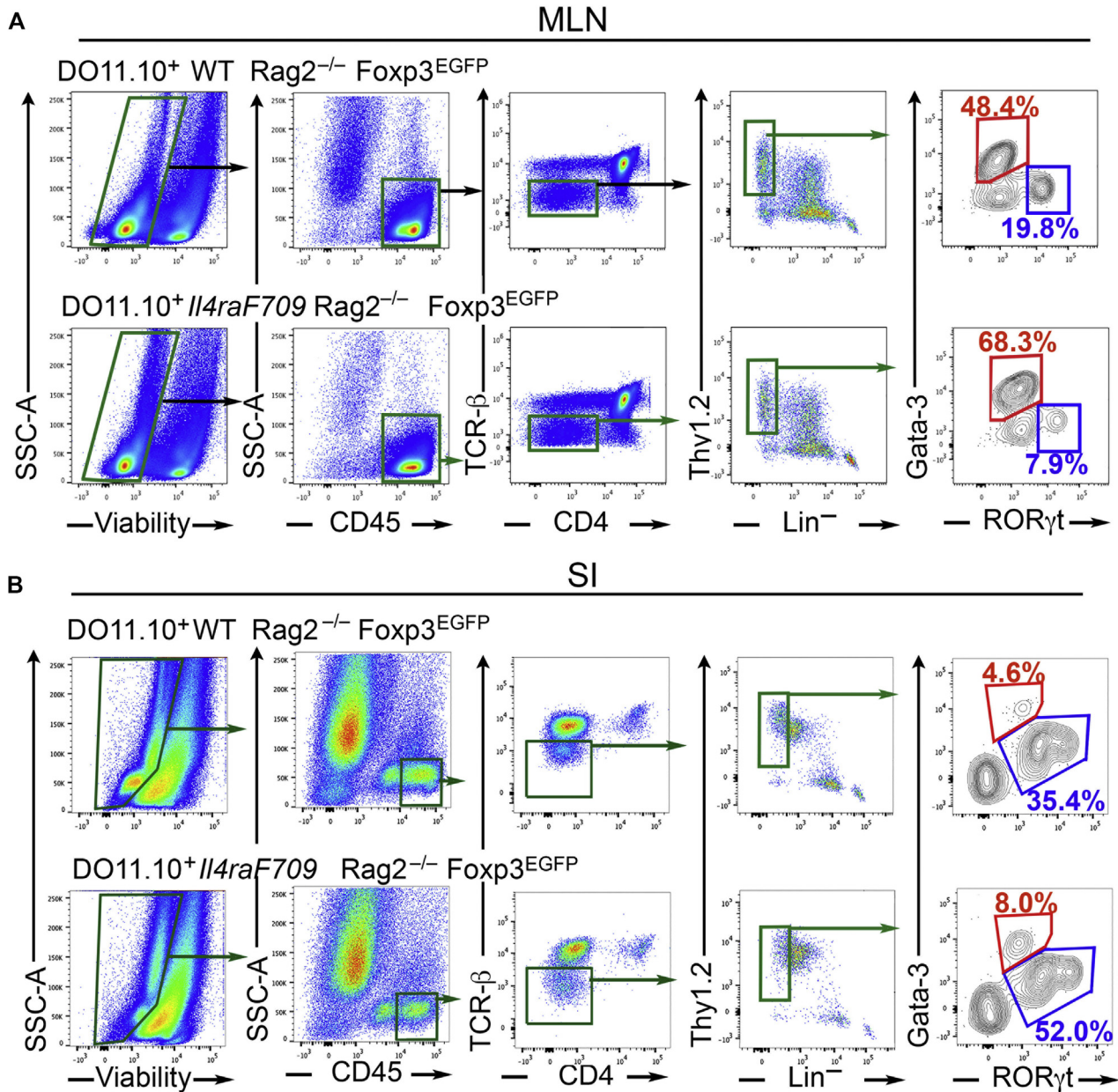


FIG E6. Impaired allergen-specific iTreg cell induction is permissive to ILC2 expansion. Flow cytometric gating strategy used to identify ILC2s (CD45⁺CD4⁻TCR-β⁻Lin⁻Thy1.2⁺GATA-3⁺) and ILC3s (CD45⁺CD4⁻TCR-β⁻Lin⁻Thy1.2⁺RORγt⁺) in the MLNs (**A**) and SI (**B**) of WT DO11.10⁺ Rag2^{-/-} Foxp3^{EGFP} and DO11.10⁺ Rag2^{-/-} *Il4ra*^{F709} Foxp3^{EGFP} mice fed with 1% OVA in drinking water is shown. SSC, Side scatter.

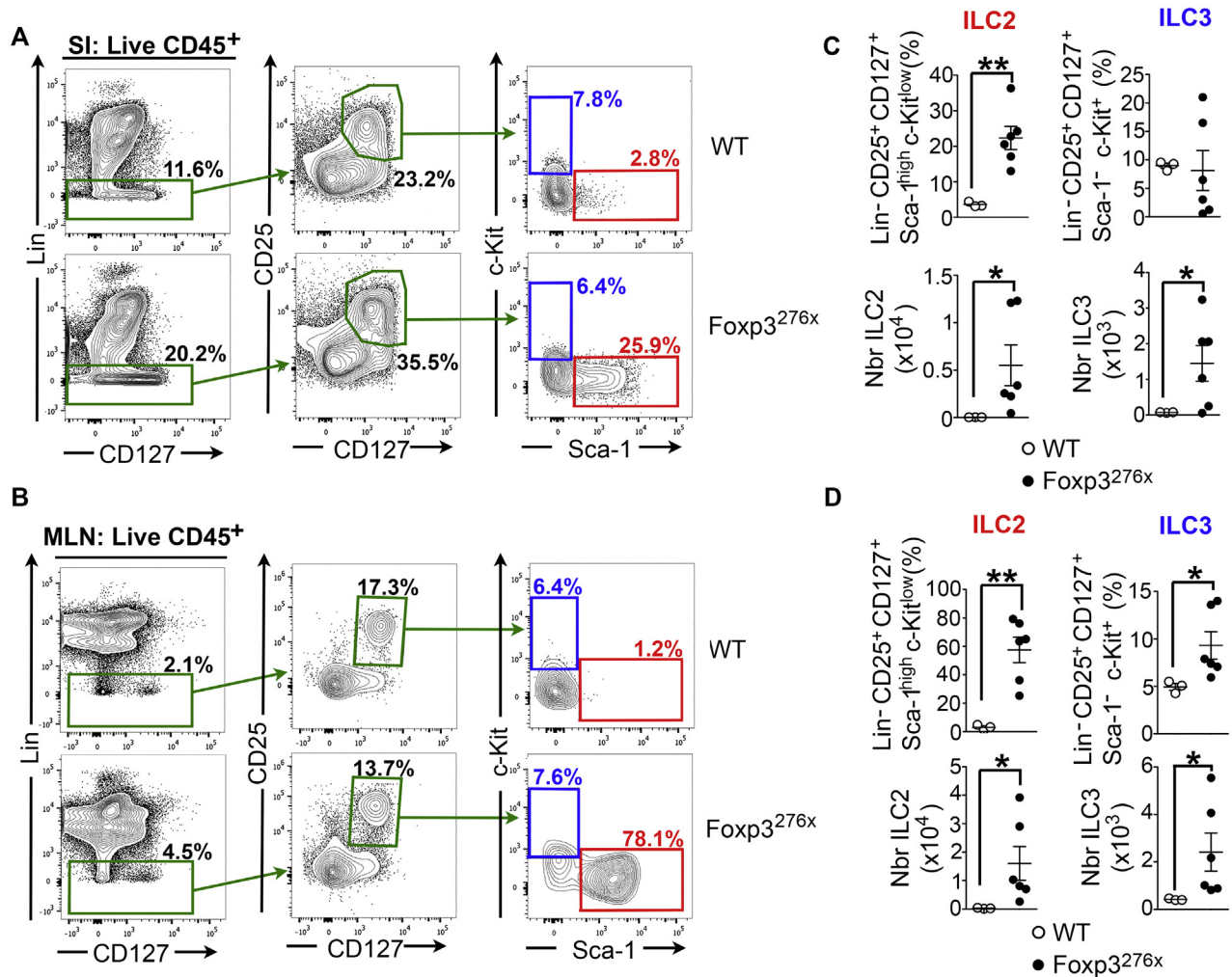


FIG E7. Treg cell deficiency in *Foxp3*^{276x} mice results in ILC2 expansion. **A** and **B**, Flow cytometric analysis of Sca-1^{high} c-Kit^{low} ILC2s and Sca-1⁻ c-Kit⁺ ILC3s in the SI (Fig E7, **A**) and MLNs (Fig E7, **B**) of 21-day-old WT and *Foxp3*^{276x} mice. **C** and **D**, Frequencies and absolute numbers of ILC2s and ILC3s in the SI (Fig E7, **C**) and MLNs (Fig E7, **D**) of 21-day-old WT and *Foxp3*^{276x} mice. Results are representative of 2 independent experiments, with 3 to 6 mice per group. **P* < .05 and ***P* < .01 by using the unpaired 2-tailed Student *t* test.

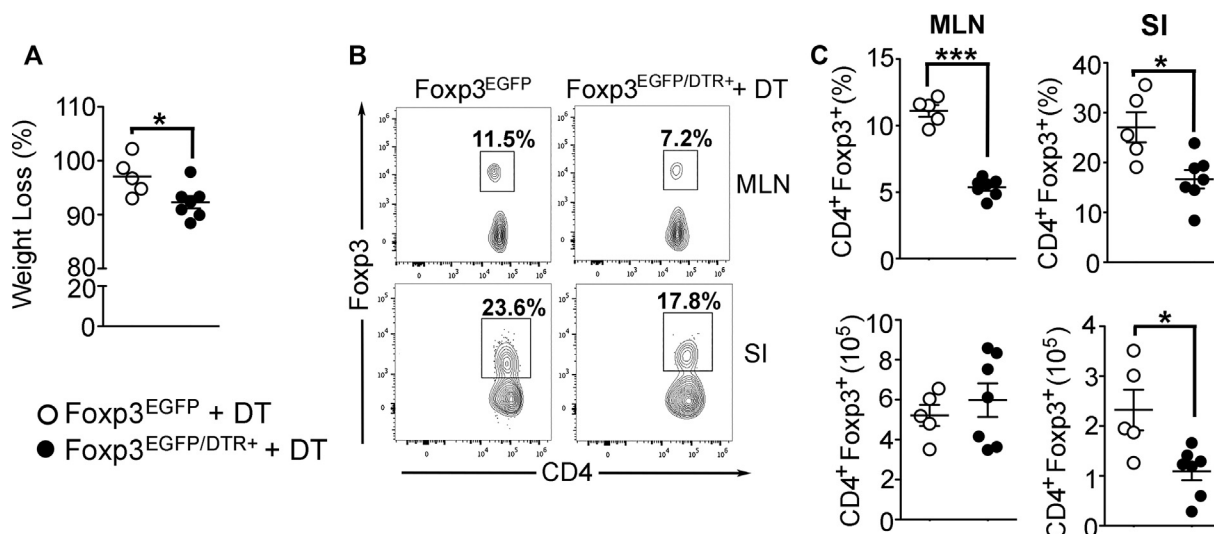


FIG E8. *Fcpx3*^{EGFP/DTR} Treg cell depletion after DT administration. **A**, Body weight of *Fcpx3*^{EGFP} and *Fcpx3*^{EGFP/DTR} mice after 2 weeks of DT treatment. **B**, Flow cytometric analysis of CD4⁺*Fcpx3*^{EGFP} Treg cells in the MLNs and SI of the mice from Fig E8, A. **C**, Frequency and absolute number of CD4⁺*Fcpx3*^{EGFP} Treg cells in the mice from Fig E8, A. Results are representative of 2 independent experiments, with 5 mice per group. **P* < .05, ***P* < .01, and ****P* < .001 by using the unpaired 2-tailed Student *t* test.