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Food allergen-sensitized CCR9$^+$ lymphocytes enhance airways allergic inflammation in mice

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Abstract

Background: The mechanisms of the atopic march, characterized by a natural progression from food and cutaneous allergies to rhinitis and asthma, are still unknown. However, as several organs can be involved, chemokines and their receptors might be implicated in this process and may be instrumental factors.

Objectives: We hypothesized that the T-cell gut-homing receptor CCR9 could be implicated in the evolution of allergic diseases.

Methods: We characterized the immune response and the role of CCR9 in a murine model combining food allergy to wheat gliadin and a model of acute airways inflammation in response to house dust mite.

Results: Compared with solely asthmatic-like mice, we demonstrated that the aggravation of pulmonary symptoms in consecutive food and respiratory allergies, characterized by an increase in pulmonary resistance and a higher Th17/Treg ratio, was abrogated in CCR9 knockout mice. Moreover, transfer of food-allergic CD4$^+$ T cells from wild-type but not from CCR9$^{-/-}$ aggravated airways inflammation demonstrating that CCR9 is involved in food allergy-enhanced allergic airway inflammation to unrelated allergens.

Conclusion: Taken together, our results demonstrated a crucial role of the T-cell homing receptor CCR9 in this model and validated its potential for use in the development of therapeutic strategies for allergic diseases.

KEYWORDS
allergy, atopic march, CCR9, homing, mouse model

1 | INTRODUCTION

The incidence of atopic disorders, including food allergies and allergic asthma, has increased tremendously in the last four decades. It is now well known that food allergy and/or atopic dermatitis (AD) during childhood increases the risk of developing asthma later in life.[1-3] The immunological mechanisms underlying atopy remain unclear. Several epidemiological reports suggest an association between food allergies and an increased risk of severity of asthma.[4-6] As a major food allergen, gluten induces atopic dermatitis,[7,8] and the history of wheat allergy in atopy and asthma is under investigation.[9,10] In this context, studies in mice have only evaluated the impact of food allergy on the development of respiratory allergy (RA) to new allergens, and few studies have attempted to mimic the influence of food allergy on allergic asthma[11-14] Moreover, although many studies

Abbreviations: AD, atopic dermatitis; AHR, airway hyperresponsiveness; BAL, bronchoalveolar lavage fluid; CCL, chemokine ligand; CCR, chemokine receptor; Der f, *Dermatophagoides farinae*; DMSO, dimethyl sulfoxide; ELISA, enzyme-linked immunosorbent assay; FRA, food and respiratory allergy; HDM, house dust mites; HRP, horseradish peroxidase; IBD, inflammatory bowel disease; IgE, immunoglobulin E; IM, intramuscular; IN, intranasal; IP, intraperitoneal; MCh, methacholine; PAS, periodic acid-Schiff; PC, percutaneous; PENH, enhanced pause; PMA, phorbol-12-myristate-13-acetate; RA, respiratory allergy.

have succeeded in inducing increased pulmonary symptoms of asthma after the induction of food allergy,[11,14] none have attempted to elucidate the underlying mechanisms. Cell trafficking is a key step in allergy,[15,16] and migration to the small intestine is largely regulated by chemokine (CC motif) receptor 9 (CCR9).[17,18] Recent research suggests that chemokine signaling directly modulates cellular and immune functions, and CCR9, in particular, is required for the development of normal gut immune function, including the development of gut-associated lymphoid tissues.[19] Mice lacking CCR9 exhibit impaired development of small intestine crypto patches, a reduced number of IELs and decreased function of regulatory T cells (Treg).[20] However, the role of gut-homing molecules in food allergy, and especially CCR9, is largely unknown. Therefore, the aim of this work was to develop a murine model of consecutive food and respiratory allergy (FRA), to analyze the possible interplay between non-cross-reactive food and respiratory allergies. To this end, mice were subjected to a wheat gliadin food allergy protocol and exposed to house dust mites (HDM) to induce subsequent airways allergic inflammation. This order was chosen because food allergy often precedes the development of asthma in humans. The impact of previous food allergy on asthma characteristics was investigated at the molecular, cellular, and anatomical levels. We demonstrated that food allergy later aggravated airway inflammation by modifying the immune reaction toward a more pronounced Th17 endotype. Additionally, CCR9-deficient mice (KO) did not display aggravation of asthma-like reaction observed after food allergy. Finally, adoptive transfer of food allergen-sensitized CCR9 lymphocytes enhanced allergic lung inflammation and asthma characteristics, demonstrating an unexpected role of CCR9 in the modulation of asthma.

2 | MATERIALS AND METHODS

2.1 | Animal model

BALB/c, C57BL/6, and Ly5.1 C57BL/6 mice were obtained from Charles River (France), and CCR9$^{-/-}$ C57 BL/6 mice were kindly provided by Dr. Malissen (CIML, Marseille, France). The mice were housed in a ventilated cage system. The protocol was approved by the Ethics Committee on Animal Experimentation of the Pays de Loire (Accreditation number: 3456). Gliadins were obtained as previously described,[21] and the mice were maintained on a special diet (SAFE, Augy, France) to avoid the development of tolerance. Wild-type 6-week-old BALB/c mice were sensitized for either food allergy[13] or RA,[14] or for both. Analysis was performed 24 hours after the last HDM challenge.

2.2 | Immunoglobulin assay

Blood was collected via cardiac puncture 24 hours after the last HDM challenge and then centrifuged at 3000 rpm for 15 minutes. The assay for the quantification of specific Der f IgE was performed in serum samples via indirect ELISA, as previously described.[14]

2.3 | AHR measurement

Airway hyperresponsiveness (AHR) was measured using the forced oscillation technique with a FlexiVent (SCIREQ) in response to nebulization of methacholine from 0 to 20 mg/mL, dose sufficient to induce a strong increase in pulmonary resistances in allergen-exposed mice but not in control.[14] FlexiWare software was used for data analysis. Enhanced pause (PENH) was measured through whole body plethysmography (EMKA Technologies) in response to nebulization of methacholine from 0 to 40 mg/mL, as previously described.[22]

2.4 | Histology

Lungs were fixed in 4% paraformaldehyde for at least 48 hours, then embedded in paraffin, cut, and stained with either periodic acid-Schiff (PAS) or hematoxylin-eosin for inflammatory scoring as previously described.[14] The histological score was calculated blindly on the basis of two parameters: bronchial morphology (4) and inflammation (8). The number of PAS-positive and PAS-negative cells was counted to evaluate the mucus production in the airways.

2.5 | Flow cytometry

Bronchoalveolar lavage (BAL) was performed with 1 mL PBS administered intratracheally to mice through a flexible catheter. The collected fluid was centrifuged, and the total cell number was counted. The lungs and the gut mesenteric lymph nodes (mLn) were removed and crushed to obtain a single-cell suspension; the cells were suspended in PBS/5% FBS buffer. Cells were stained with the following surface markers: CD3-FITC, Ly6G-Percpcy5.5, CD4-BV421, CD11c-BV510 and CD25-BV510 (BD Biosciences, Le Pont-de-Claix, France), CCR3-APC (R & D system, Lille, France) and CCR9-PE (Ozyme, St Quentin en Yvelines, France) and in the presence of CD16/32 mAbs (BD Biosciences). Cells were stimulated for 5 hours with 50 ng/mL of PMA (phorbol-12-myristate-13-acetate) and 1 μg/mL of ionomycin with brefeldin A (Golgi plug, BD Biosciences, 1:1000). Cells were fixed and permeabilized using the Cytofix/Cytoperm Kit (BD Biosciences) and stained with anti-IL-17-APC and anti-Foxp3-Alexa-fluor 488 (BD Biosciences). The cells were analyzed on a Fortessa X20 cytometer (BD Biosciences). Data were acquired using DIVA software (BD Biosciences) and analyzed with FlowJo (TreeStar) as previously described[22,23] and using gating strategy describes in Figure S1.

2.6 | LEGENDplex assay

Cytokine levels were assessed via LEGENDplex bead-based immunoassays (Biolegend, London, UK) according to the manufacturer's instructions.

2.7 | Adoptive transfer

Spleen and mesenteric lymph nodes were sampled in food-allergic mice one hour after the last challenge and were then crushed and

suspended in PBS 5% FBS. $CD4^+$ T cells were sorted using the EasySep $CD4^+$ T Cell Enrichment Kit (Stemcell, Grenoble, France) according to the manufacturer's instructions. Then, 3×10^6 $CD4^+$ T cells were injected intravenously into 6-week-old Ly5.1 mice. On the next day, the mice began the acute asthma protocol, as described above.

2.8 | Human samples

Eleven atopic patients with a clinical history of house dust mite sensitization and allergic rhinitis were included in the analysis. In parallel, nine nonatopic healthy volunteers were included as controls. Blood was sampled on all subjects, and peripheral blood mononuclear cells (PBMC) were isolated using a Ficoll gradient from peripheral blood as described.[24] Samples were collected from Prevall DP cohort under recruitment (NCT02908360). Each subject provided a written informed consent.

2.9 | PCR

The total quantity of RNA extracted using Machery-Nagel DNA, RNA, and protein purification kit (Duren, Germany) was determined by Spectrophotometer Nanodrop ND-1000 (Thermo Fisher Scientific, Waltham, USA), and cDNA was synthesized from 500 ng of RNA using MMLV reverse transcriptase according to the manufacturer's instructions. Real-time quantitative PCR was performed as previously described[22] using the indicated primers: 5′-ATGTCAGGC AGTTTGCGAG-3′ and 3′-TGCAGTACCAGTAGACAAGGAT-5′ for CCR9 gene and 5′-GGGAAACTGTGGCGTGAT-3′ and 3′-TTCAGCTC AGGGATGACCTT-5′ for GAPDH as the housekeeping gene with the SYBR Green technology. IL-13, IL-10, IL-17, CCR4, and CCL25 genes were analyzed using the TaqMan technology (Applied Biosystems, Thermo Fisher) with the following assays: Hs00174379_m1, Hs00961622_m1, Hs00174383_m1, Hs00747615_m1, Hs00608-373_m1, respectively, and GAPDH (Hs03929097_g1) as the housekeeping gene.

3 | RESULTS

3.1 | Previous food allergy aggravates HDM-induced asthma

Thanks to our model, we compared the RA and FRA groups to determine the potential synergy between the two allergies (Figure 1A, Table 1). We assessed Der f1-specific IgE in serum as well as the pulmonary function and the histological parameters. Specific IgE was increased in the RA mice compared with the controls (CTL) (0.53 mA.U $\pm$ 0.02 vs 2.3 mA.U $\pm$ 0.8, $P < .05$). Compared with RA mice, the increase was more pronounced in FRA mice, as they displayed higher production of Der f1-specific IgE (2.3 mA.U $\pm$ 0.8 vs 5.56 mA.U $\pm$ 1.7, $P < .05$) (Figure 1B). At the physiological level, the Penh, measured via plethysmography, was significantly increased in the mice subjected to the HDM-induced airway inflammation protocol (RA and FRA groups) compared with the CTL mice, with the RA and FRA mice showing comparable values (Figure 1C). Using Flexivent®, as a more sensitive method for measuring airway responsiveness, we showed a more pronounced increase of airway resistance in HDM mice compared with control mice (from 0.53 to 2.7 cm H_2O.s/mL for control and 0.73 to 11.16 cm H_2O.s/mL for RA mice, $P < .001$). Moreover, we demonstrated that FRA mice displayed significantly greater AHR than RA mice, based on pulmonary resistance and elastance measurements (Figure 1D,E). Tissue inflammation was evaluated through histological examination of hematoxylin-eosin staining lung sections (Figure 1F). Compared with CTL, a significant increase in the histological score was observed in FRA and RA mice and there was a more pronounced inflammation in FRA than in RA mice, reflecting the aggravation of lung inflammation (Figure 1G). To assess the percentage of mucus-producing epithelial cells, PAS staining was performed in lung sections. Compared with RA mice, there were a significantly increased number of mucus-producing epithelial cells in the bronchioles of FRA mice, with both groups exhibiting an increase compared with CTL (Figure 1H). Taken together, these results show that previous food allergy aggravates HDM-induced AHR and the pathologic characteristics of asthma by priming the immune system to increase its response to HDM respiratory challenge.

3.2 | Previous food allergy redirects HDM-induced asthma toward a Th17 response

Having demonstrated the influence of double exposure to allergens on the canonical parameters of asthma-like reaction in mice, we next aimed to explore immune cell changes based on the total cell, lymphocyte, neutrophil, eosinophil, and dendritic cells counts in the BAL fluid (Figure 2A) and lungs (Figure 2B) via flow cytometry. Compared with RA, the numbers of BAL and lung inflammatory cells in FRA mice were increased twofold to threefold. Notably, the number of lymphocytes was increased by around 55% in BAL and 75% in lung in FRA mice compared with RA mice. In contrast, the number of eosinophils remained unaffected by previous food allergy in FRA mice compared with RA mice (Figure 2A,B). In addition, number of dendritic cells in lung is not modified between RA and FRA mice (Figure 2B). To further detail the mechanisms linking the two types of allergies, we analyzed cytokine secretion in BALs from immune cells via ELISA (Figure 2C-F). According to the increase in neutrophilic inflammation observed in FRA mice, the level of IL-17 was increased in FRA mice compared with RA mice (Figure 2C). In contrast, the levels of IL-4 and IL-5 were comparable between FRA and RA mice (Figure 2E,F). Finally, the level of IL-13, a cytokine involved in both Th2 inflammation and AHR, was elevated in FRA compared with RA mice (Figure 2D). Regarding the Treg response, the level of IL-10 and TGF-β was found to be decreased in FRA mice compared with RA mice (Figure 2G). These data demonstrate that FRA mice display mixed Th2/Th17 inflammation with an immune response oriented toward a Th17 phenotype in a more pronounced manner than RA mice, which results in more neutrophilic allergic airway inflammation. Moreover, we suggest that the Th17/Treg balance is altered in asthmatic-like mice with previous food allergy.

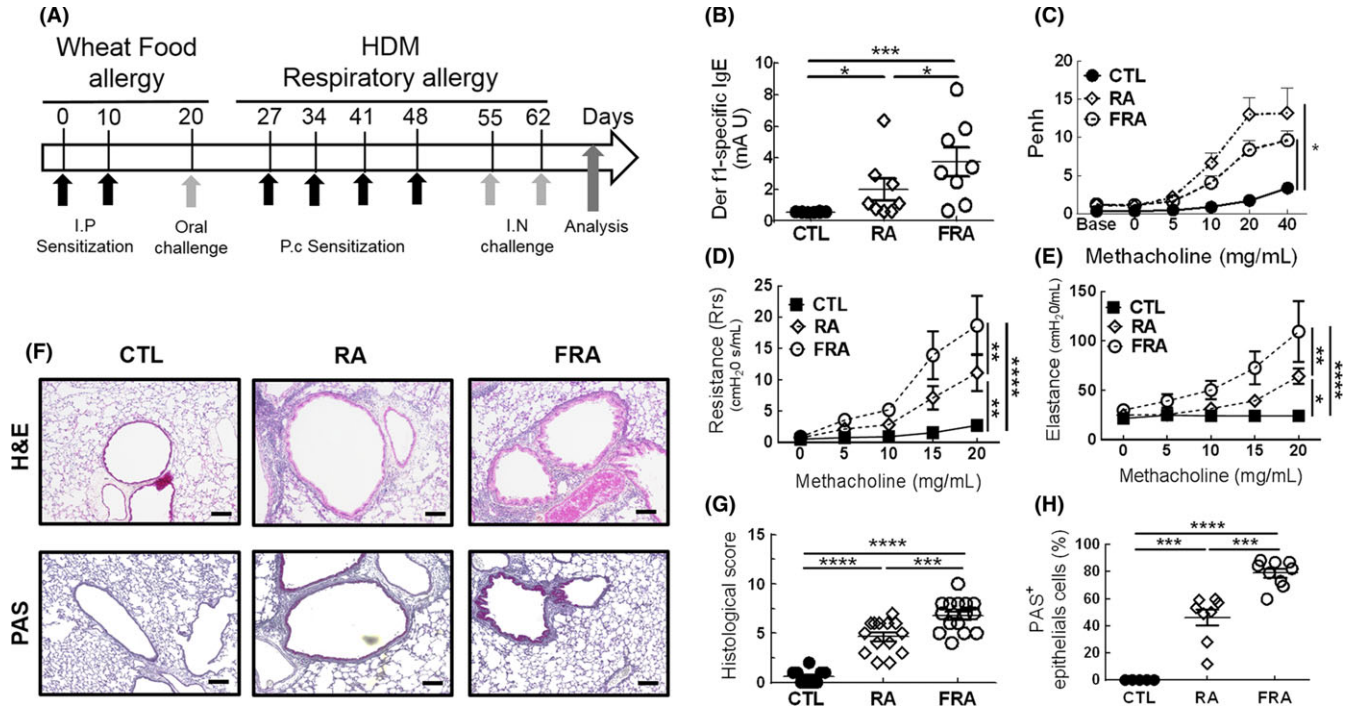

FIGURE 1 Previous food allergy aggravates house dust mites (HDM)-induced asthma. After wheat gliadin sensitization, mice were challenged via oral gavage; one week later, they were sensitized to HDM and then challenged intranasally (A); on the day after the last challenge, blood was collected, and Der f-specific IgE was measured (B); pulmonary functions were assessed based on Penh (C), resistance (D), and elastance (E). Lung sections were subjected to either hematoxylin-eosin or periodic acid-Schiff (PAS) staining (F) to allow the calculation of the histological score (G) and the percentage of mucus-producing epithelial cells (H). The results are expressed as the percentage of PAS-positive cells per bronchiole (PAS-positive cells per bronchiole divided by the total number of epithelial cells in each bronchiole). Data are represented as the mean ± SEM (n = at least 8 mice per group); *$P < .05$, **$P < .01$, ***$P < .001$, ****$P < .0001$. Scale bar = 250 μm [Colour figure can be viewed at wileyonlinelibrary.com]

TABLE 1 Clinical parameters at inclusion for the atopic patients

Characteristics	Atopic patients
Age (mean ± SD)	26 ± 5.6
Female sex (%)	S3
Hight cm (mean ± SD)	163.7 ± 3.96
Weight kg (mean ± SD)	63.4 ±13.68
ARCT {mean ± SD)	16.1 ± 2.8
SCORAD (mean±SD)	2.84 ± 6.32
ACQ (mean± SD)	0.42± 0.5
Sensitization to Der f n (%)	11 (100)
Sensitization to cat n (%)	2 (18)
Sensitization to dog n (%)	1 (9)
Sensitization to grasses n {%)	3 (27)
Sensitization to birch n (%)	3 (27)

ARCT, allergic rhinitis control test (/25); SCORAD, score of atopic dermatitis; ACQ, asthma control questionnaire.
Sensitization is evaluated by skin prick test.

3.3 | Previous food allergy influences lung and gut-homing T cells

Accurate and efficient tissue-specific trafficking between blood, lymphoid organs, and peripheral tissues is a fundamental prerequisite for T cells to participate in the immune response. To determine whether the gut-homing chemokine receptor CCR9[25] is associated with particular T helper cell traffic and polarization during FRA, we assessed the nature of the T-cell response in CCR9-expressing cells (Figure 3) using flow cytometry to determine frequency of $CCR9^+$ Treg, Teff, and Th17 cells (Figure S1). After RA, frequency of $CCR9^+$ Treg cells and $CCR9^+$ Th17 cells was found more elevated in the lung compared with control mice. Surprisingly, frequency of $CCR9^+$ Treg cells was decreased and $CCR9^+$ Th17 was increased in FRA mice compared with RA mice, suggesting a possible role of CCR9 in the Treg/Th17 balance in food allergy exacerbation of airway inflammation (Figure 3A,B). To the opposite, frequency of $CCR9^+$ Treg cells remained unaffected in the mLN of control and RA mice but was decreased in FRA mice. Moreover, frequency of $CCR9^+$ Th17 cells was increased in FRA mice compared with RA mice (Figure 3C,D). Thus, the induction of allergic airway inflammation after food allergy increased the $CCR9^+$ Th17/Treg ratio suggesting a new role for CCR9 in asthma.

3.4 | CCR9 expressed by $CD4^+$ T cells is required for food allergy-aggravated asthma

To decipher the role of the gut-homing molecule CCR9 in the influence of food allergy on asthma, we analyzed $CCR9^{-/-}$ mice in our

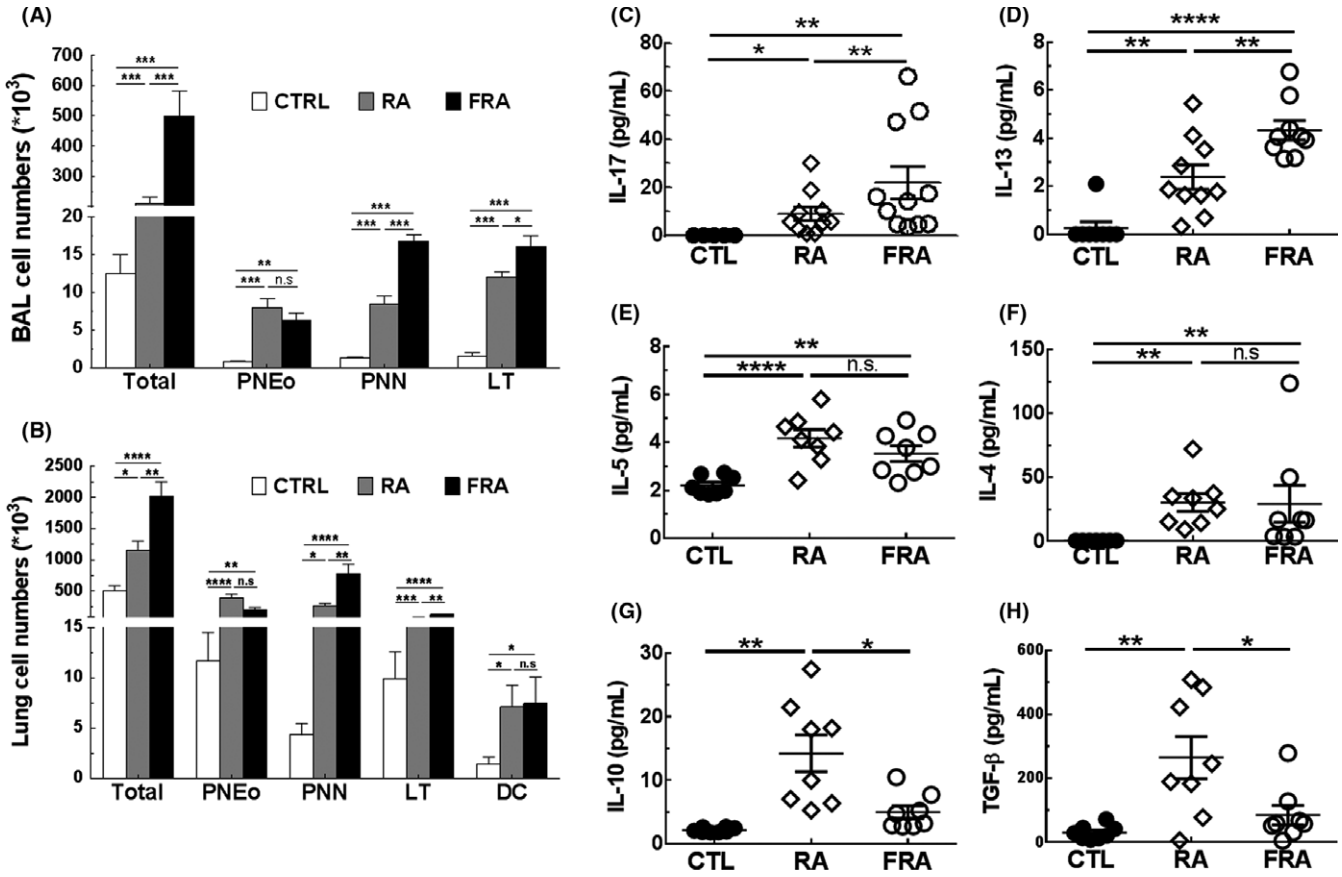

FIGURE 2 Previous food allergy redirects inflammation in house dust mites-induced asthma toward a Th17 phenotype and modulates the Th17/Treg cytokine balance. Total, polynuclear eosinophil, polynuclear neutrophil, and lymphocyte cell numbers in bronchoalveolar lavage (BAL) fluid (A) and in lung together with dendritic cells (B) were measured via cytometry. IL-17 (C), IL-13 (D), IL-5 (E), IL-4 (F), IL-10 (G), and TGF-β (H) levels were assessed in supernatant of BAL fluid using LEGENDplex. Data are represented as the mean ± SEM (n = at least 8 mice per group); *$P < .05$, **$P < .01$, ***$P < .001$, ****$P < .0001$. Detection limit was 0.95 pg/mL for IL-17, 1.14 pg/mL for IL-13, 0.97 pg/mL for IL-5, 0.53 pg/mL for IL-4, 1.25 pg/mL for IL-10 and 2 pg/mL for TGF-β

model (Figure 4). As our model was developed in BALB/c genetic background and CCR9$^{-/-}$ was on a C57BL/6 background, we first confirmed our results in C57BL/6 mice (Figure S2). Interestingly, CCR9$^{-/-}$ mice showed a decrease in lung resistance after a dual consecutive allergic protocol (FRA CCR9$^{-/-}$), suggesting a crucial role for CCR9 in the aggravation of airway inflammation after food allergy (Figure 4A). In addition, FRA CCR9$^{-/-}$ mice exhibited a lower number of total cells in BAL especially T lymphocytes compared with FRA WT mice (Figure 4B). Finally, staining of lung sections revealed decreases in epithelial cell hyperplasia and mucus production and extensive perivascular and peri-bronchial cell infiltration in FRA CCR9$^{-/-}$ mice compared with FRA WT mice (Figure 4C). Then, as FRA mice display higher Th17 and lower Treg response, we aimed to determine the role of CCR9 in this balance. To do so, we measured Th17 and Treg response in the lung and the mLN of dual WT and CCR9$^{-/-}$-allergic mice (Figure 4D,E). First, we confirmed that naïve C57BL/6 CCR9$^{-/-}$ mice do not show differences in the Th17/Treg responses compared with naive C57BL/6 WT (Figure S3A-C). Unexpectedly, FRA CCR9$^{-/-}$ mice did not show decrease in CD4^{+} IL-17-producing Th17 cells compared with FRA WT mice neither in the lung nor in mLN (Figure 4D), suggesting a minor role of CCR9 in the number of Th17 cells during allergies. To the opposite, we observed an increase in Tregs cell number in the lung and in the mLN of FRA CCR9$^{-/-}$ compared with FRA WT mice (Figure 4E), reinforcing the crucial role of CCR9 in the food allergy-induced aggravation of airway inflammation in mice. Taken together, our results demonstrated a major role of CCR9 in regulating the Th17/Treg balance during allergy by acting mainly on Treg cells number within the organs. We next sought to confirm the role of CCR9 expressed by CD4^{+} T cells using adoptive transfer of CD4^{+} T lymphocytes from food-allergic WT or CCR9$^{-/-}$ into congenic Ly5.1^{+} mice before the induction of RA (Figure 5A). Then, on the day after the last intranasal HDM challenge cell numbers in BAL fluid were assessed. Our results demonstrated a decrease of cell infiltrates in mice receiving food allergen-sensitized lymphocytes from CCR9$^{-/-}$ mice (Figure 5B), but no effect was observed in mice receiving WT or CCR9$^{-/-}$ cells from nonallergic mice (Figure 5C). Then, we measured decreases in IL-17 and IL-13 levels in the BAL of mice receiving food-allergic cells from CCR9$^{-/-}$ compared with mice receiving WT cells (Figure 5D). Finally, we found similar levels of the Th2

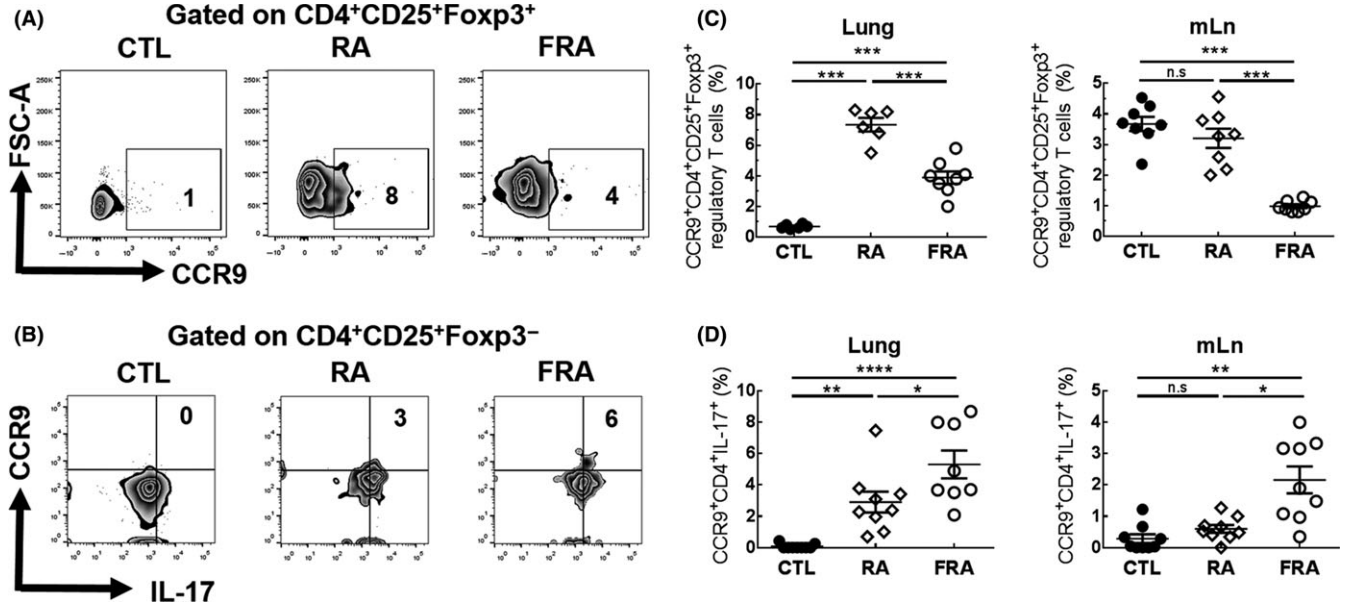

FIGURE 3 Previous food allergy induces an increase in effector Th17 cells and impairs regulatory T-cell development in CCR9$^+$ CD4$^+$ T cells. Dot plot (left panel) and frequency (right panel) of CCR9 expression on CD4$^+$Foxp3$^+$ regulatory T cells (A) and effector Th17 cells (B) in the lung of CTL, respiratory allergy (RA) and food and respiratory allergy (FRA) mice measured by flow cytometry. Frequency of CCR9 expression on Treg and Th17 cells in the gut mesenteric lymph nodes of CTL, RA, and FRA mice (C and D). Data are represented as the mean ± SEM (n = at least 8 mice per group); *P < .05, **P < .01, ***P < .001, ****P < .0001

cytokines IL-4 and IL-5, whereas the level of IL-10 and TGF-β was increased (Figure 5D). These results confirmed the pivotal role of the gut-homing receptor CCR9 expressed in CD4$^+$ T cells under the influence of previous food allergy on inflammation during subsequent asthma, especially on lung function and Th17 inflammation. Finally, to assess whether the increased expression of CCR9 by immune cells would be relevant for human atopy, CCR9 gene expression was assessed in PBMC from allergic rhinitis patients. CCR9 gene expression was markedly more elevated in PBMC of atopic patients compared with healthy volunteers (Figure 5E). Analysis of Th2 related gene was performed in atopic patients (Figure 5F), and we could observe an increase in the mRNA levels of IL-13 and CCR4 gene for the atopic patients compared to the healthy volunteers.

4 | DISCUSSION

In the present study, we first confirmed that food allergy aggravates airway inflammation induced by HDM, as previously suggested by our group and others.[11,13,14] The induction of airway inflammation after food allergy increases pulmonary resistance and histological scores. Moreover, the inflammatory cell count in BAL increases, showing potentiation of pulmonary inflammation by food allergy. Unexpectedly, we observed a significant increase of IL-17 secretion in BAL. These results are reminiscent of data obtained in severe asthmatic patients[26,27] and mouse models,[28,29] in which the secretion of IL-17 in airways was shown as related to the high proportion of neutrophils. Moreover, we observed an increase in IL-13 levels, indicating a persistence, of the Th2 phenotype, despite the lack of change in the levels of IL-4 and IL-5. Finally, those mice display a deficiency in the regulatory response, as suggested by the decrease in IL-10 and TGF-β levels. Thus, these results suggest that food allergy aggravates asthma through a mixed Th2/Th17 phenotype inflammation and a modulation of the Th17/Treg balance. We were then interested in analyzing the role of CCR9 in the regulation of the inflammatory process during allergic airway inflammation after food allergy. Indeed, CCR9 is the main gut-homing receptor and is implicated in gut diseases, such as Crohn's[30] or celiac disease,[31] and is suggested to play a role in food allergies.[24,25,32,33] Thus, we analyzed the role of CCR9 in our model. Interestingly, we observed an increase in IL-17-producing effector T cells, along with a decrease of Treg cells in FRA mice, among cells expressing CCR9. This decrease in CCR9 expressing Treg cells might be linked to a modification of the Teff/Treg balance toward the T effector response in food allergy which in turn impact the later response. Therefore, we were able to demonstrate that deficiency of CCR9 alleviates lung inflammation in mice. CCR9 is the main chemokine receptor involved in chemotaxis to the gut and is responsible for the migration of T cells toward the intestine.[20,34,35] Its deletion in knockout mouse models or the use of CCR9-specific antagonists leads to a decrease in gut inflammation in mouse models of Crohn's disease or ulcerative colitis.[36,37] Here, we show that CCR9 deficiency in a model of successive allergy reduces inflammation. A previous study by Lopez-Pacheco et al analyzing airway inflammation in CCR9$^{-/-}$ showed that CCR9 deficiency impairs lung inflammation through decreased recruitment of eosinophils.[38] In our hand, CCR9 deficiency has no effect on allergic airway inflammation. These different results might be due to the use of different

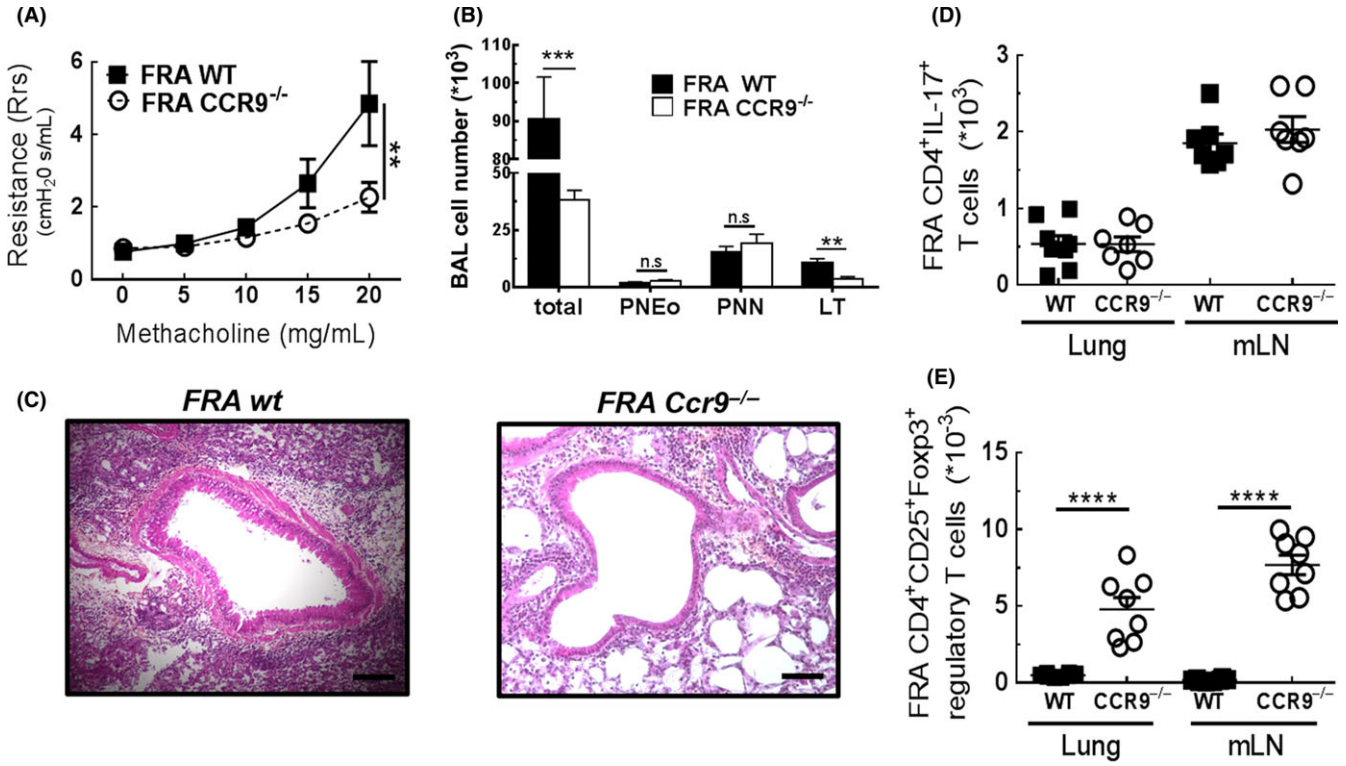

FIGURE 4 CCR9 deficiency abrogates the effects of previous food allergy on asthma. Pulmonary resistance (A) was measured in WT (black square) and CCR9$^{-/-}$ (white square) mice with consecutive food and respiratory allergies using a FlexiVent. Total, eosinophil, neutrophil, and lymphocyte cell numbers in bronchoalveolar lavage fluid (B) were measured via flow cytometry in food and respiratory allergy (FRA) WT (black bars) and FRA CCR9$^{-/-}$ (white bars) mice. Hematoxylin-eosin staining of lung sections (C) from WT (left panel) and CCR9$^{-/-}$ (right panel) mice was performed to measure lung lesions. Th17 cells (D) and Treg cells (E) numbers were measured in the lungs and in the gut mesenteric lymph nodes of FRA WT (black square) and FRA CCR9$^{-/-}$ (white circle) mice by flow cytometry. Data are represented as the mean ± SEM (n = at least 7 mice per group); *P < .05, **P < .01, ***P < .001, ****P < .0001. Scale bar = 250 μm [Colour figure can be viewed at wileyonlinelibrary.com]

animal model. In fact, Lopez-Pacheco et al use a model consisting of an intraperitoneal sensitization of ovalbumin with adjuvant which could induce a gut inflammation and a systemic response. Our model uses a percutaneous sensitization with a total extract of house dust mite without adjuvant to induce a local and mix inflammatory response. Therefore, both studies suggest that CCR9 and CCL25 expression may therefore be key drivers of the early stages of airway inflammation by regulating immune cell recruitment. To better understand the mechanism underlying the food allergy-induced aggravation of asthma and the role of CCR9, we performed adoptive transfer of CD4$^+$ T cells from food-allergic mice into naïve mice, which were subsequently sensitized to HDM. This allowed us to demonstrate that food-sensitized CD4$^+$ T cells were required to enhance asthma characteristics, as we observed more severe inflammation in asthmatic mice receiving food-sensitized CD4$^+$ T cells compared with asthmatic mice receiving nonsensitized CD4$^+$ T cells. Interestingly, the aggravation of asthma induced by food allergen CD4$^+$ lymphocytes was abolished when mice received food allergen-sensitized lymphocytes from mice deficient in CCR9. Thus, these results confirm that CCR9 is a driver of the exacerbation of lung inflammation in food-allergic mice. This discrepancy may not be linked to a difference in cell survival after adoptive transfer as we found the donor cells in similar proportions in each condition (data not shown). One explanation could be a dysregulation of the cell migration involving CCR9 or the expression of another chemokine receptor such as CCR4 or CCR8 which target T cells to the lung and/or to the skin.[39,40]

Our mouse model allowed us to determine the implications of homing receptors in the interconnection between food and respiratory allergies. The increase in CCR9$^+$ CD4$^+$ T cells in the gut might be linked to an accumulation during food allergy-related inflammation and could subsequently produce IL-17 in the lungs, in turn inducing neutrophil influx and aggravation of asthma. Another source of IL-17 might be ILC3. Indeed, it has been showed that IL-17-producing ILC3 are involved in asthma.[41] Furthermore, CCR6$^+$ IL-17$^+$ ILC3 cells are active in the gut in some forms of inflammatory bowel disease and in mice model of intestinal inflammation.[42,43]

Additionally, a minor increase in CD4$^+$ T regulatory cells was observed in the lungs of successive allergic mice compared to only respiratory allergic mice, which is relevant to the increase in inflammation. It has been shown in vitro that CCR9 can inhibit the development of regulatory T cells through its ligation to CCL25.[44] This observation suggests that migration of CCR9$^+$ T cells into the lungs could inhibit the proliferation of Tregs, prevent their anti-inflammatory effect, and, thus, exacerbate pulmonary inflammation. However, the antigen specificity of such migrating CCR9$^+$ cells might be a

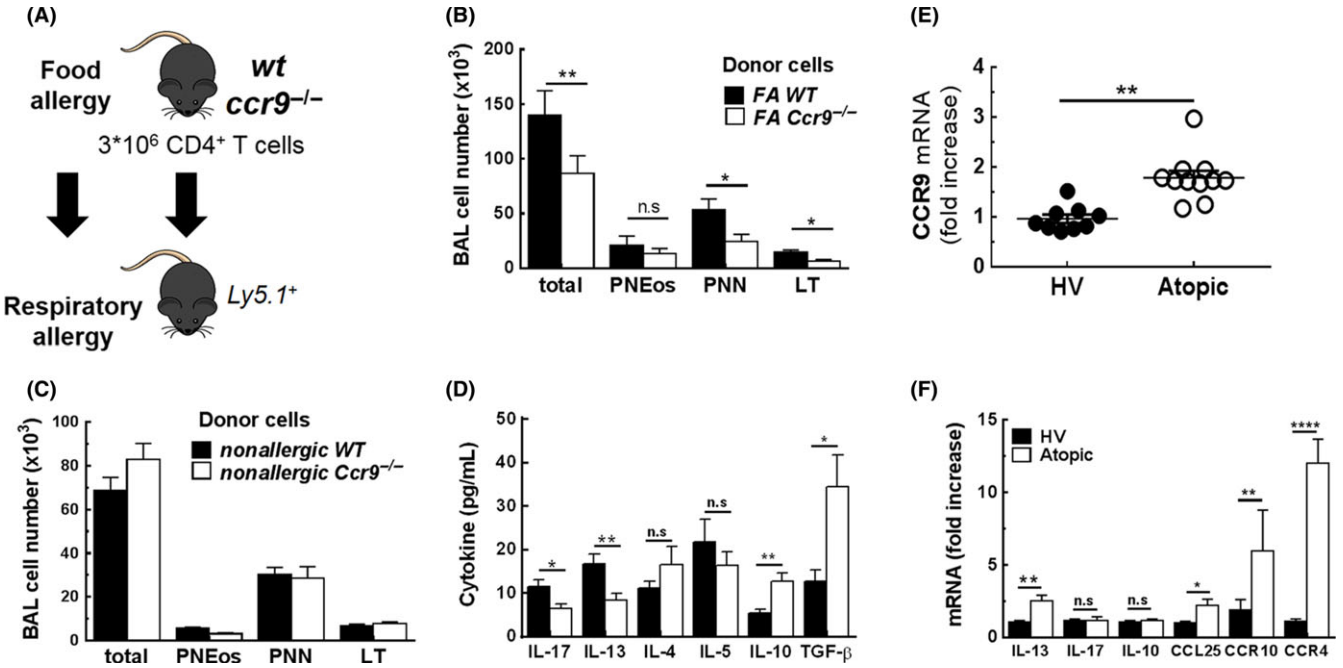

FIGURE 5 CCR9 expressed by $CD4^+$ T cells is indispensable for food allergy-aggravated asthma. First, 3.10^6 food allergen-sensitized $CD4^+$ lymphocytes from WT (black bars) or CCR9-deficient (white bars) mice were injected intravenously into naïve congenic Ly5.1 mice. The next day, Ly5.1-receiving mice were subjected to the respiratory protocol (A). Total, eosinophil, neutrophil, and lymphocyte cell numbers in bronchoalveolar lavage (BAL) fluid were measured in mice receiving WT or CCR9-deficient cells from food-allergic mice (B) and naïve mice as control (C). IL-17, IL-13, IL-4, IL-5, IL-10, and TGF-β levels in the BAL of mice receiving food allergens sensitized WT or $CCR9^{-/-}$ cells were measured using LEGENDplex (D). Data are represented as the mean ± SEM (n = at least 6 mice per group); $*P < .05$, $**P < .01$. CCR9 mRNA expression (E) measured by qPCR in healthy volunteers (black circle) and in atopic rhinitis patients (white circle). IL-10, IL-13, IL-17, CCR4 and CCL25 mRNA expression measured by qPCR in atopic patients and healthy volunteers (F). Data are represented as the mean ± SEM (n = 8) $**P < .01$. Allergic status of all the patients was determined by skin prick testing for common aeroallergen, and the main characteristics are described in Table 1. [Colour figure can be viewed at wileyonlinelibrary.com]

crucial point to understand the mechanism by which those cells could interplay between organs in allergy. According to our previous results, there is an increase of $CCR9^+$ T cells and a decrease of $CD25^+Foxp3^+$ regulatory T cells in the blood of patients suffering from allergic conjunctivitis,[45] in line with the increase in CCR9 mRNA levels we have observed in atopic rhinitis patients compared with healthy volunteers. Finally, the structure of CCR9 has been revealed and presents similarities with CCR5.[46] These findings suggest that a cross-reactivity between CCR5 ligand RANTES and CCR9 receptor cannot be excluded and needs to be taken into account to develop new CCR9 antagonist. Thus, CCR9 appears as a promising therapeutic target for both food and respiratory allergies.

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CONFLICT OF INTEREST

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AUTHOR CONTRIBUTIONS

LC performed the experiments, analyzed the data, and wrote the manuscript. GB participated in the analysis of the cell populations and wrote the manuscript. MAC provided support for the experiments in mice. LC recruited and characterized patients. SB gave scientific inputs and help in manuscript writing. AM designed and elaborated the study and supervised manuscript writing.

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SUPPORTING INFORMATION

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