

Consecutive Food and Respiratory Allergies Amplify Systemic and Gut but Not Lung Outcomes in Mice

Gregory Bouchaud,^{†,||} Paxcal Gourbeyre,^{†,||} Tiphaine Bihouée,^{‡,§,⊥,Δ,Π} Philippe Aubert,^{⊥,Π,⊗}
David Lair,^{‡,§,⊥,Δ,Π} Marie-Aude Cheminant,^{‡,§,⊥,Δ,Π} Sandra Denery-Papini,[†] Michel Neunlist,^{⊥,Π,⊗,Γ}
Antoine Magnan,^{‡,§,⊥,Δ,Π} and Marie Bodinier^{*,†}

[†]INRA, UR1268 BIA, rue de la géraudière, B.P. 71627, F-44316 Nantes, France

[‡]INSERM, UMR1087, l'institut du thorax, F-44000 Nantes, France

[#]CNRS, UMR 6291, F-44000 Nantes, France

[⊥]Université de Nantes, F-44000 Nantes, France

^ΔCHU Nantes, l'institut du thorax, Service de pneumologie, F-44000 Nantes, France

^ΠDHU2020 médecine personnalisée des maladies chroniques, F-44100 Nantes, France

[⊗]INSERM UMR S 913, Institut des Maladies de l'Appareil Digestif (IMAD), Faculté de Médecine, F-44000 Nantes, France

^ΓCHU Nantes, Institut des Maladies de l'Appareil Digestif (IMAD), F-44000 Nantes, France

ABSTRACT: Epidemiological data suggest a link between food allergies and the subsequent development of asthma. Although this progression may result from the additional effects of exposure to multiple allergens, whether both allergies amplify each other's effects remains unknown. This study investigated whether oral exposure to food allergens influences the outcomes of subsequent respiratory exposure to an asthma-inducing allergen. Mice were sensitized and orally challenged with wheat (FA) and then exposed to house dust mite (HDM) extract (RA). Immunoglobulin (Ig), histamine, and cytokine levels were assayed by ELISA. Intestinal and lung physiology was assessed. Ig levels, histamine release, and cytokine secretion were higher after exposure to both allergens than after separate exposure to each. Intestinal permeability was higher, although airway hyper-responsiveness and lung inflammation remained unchanged. Exposure to food and respiratory allergens amplifies systemic and gut allergy-related immune responses without any additional effect on lung function and inflammation.

KEYWORDS: asthma, allergy, cytokines, gut, house dust mite, immunity, mouse, T-cells, wheat

■ INTRODUCTION

Atopy is a predisposition to develop various forms of allergies that is influenced by genetic and environmental factors, with a rising prevalence in developed and developing countries.¹ Atopy is linked to immune system disorientation at birth marked by an uncontrolled Th2 response associated with delayed Th1 maturation.² Later, the allergic response is characterized by higher IgE production and an increased Th2 allergen-specific CD4⁺ T cell response with dampened regulatory T cells (Treg). Moreover, pro-inflammatory Th17 lymphocytes seem to play a role in the allergic response.^{3,4} Atopic dermatitis and respiratory allergies constitute the most common forms of atopy-related allergies observed in Western countries,^{5–7} affecting as much as 20–30% of the European population.⁸ Airway hyper-responsiveness and airflow obstruction in asthma are caused by a local influx of inflammatory cells, mainly eosinophils, under the action of cytokines and chemokines produced by Th2 cells. Atopic dermatitis develops as eczematous lesions and commonly occurs in children^{9,10} with a prevalence of 15–30% versus 2–10% in adults.¹¹ Food allergies affect approximately 5–8% of children, with a greatest prevalence in the first years of life.¹² Clinical symptoms are gastrointestinal, cutaneous, respiratory, or systemic.¹³ Food allergy is frequently involved in both atopic dermatitis and asthma. However, the typical progression of atopic diseases is

that change occurs with age from food allergen sensitization causing cutaneous symptoms in childhood¹⁴ to respiratory symptoms in adults.¹¹ This is mainly due to inhaled allergens such as house dust mites (HDM) in a progression called the “atopic march”.¹⁵ Thus, sensitization to food allergens seems to predispose patients to develop asthma induced by HDM.^{16–20} However, whether the atopic march results from two independent processes caused by various types of allergens in various organs or whether a primary sensitization to food primes the immune system for a subsequent respiratory allergy is unknown.

To address this question, we evaluated the impact of food allergy on the development of respiratory allergy to a new allergen in the context of the atopic march in a mouse model. In our model, exposure to food and respiratory allergens amplified systemic and gut allergy-related immune responses without any additional effect on lung function and inflammation.

Received: April 28, 2015

Revised: July 3, 2015

Accepted: July 6, 2015

Published: July 14, 2015

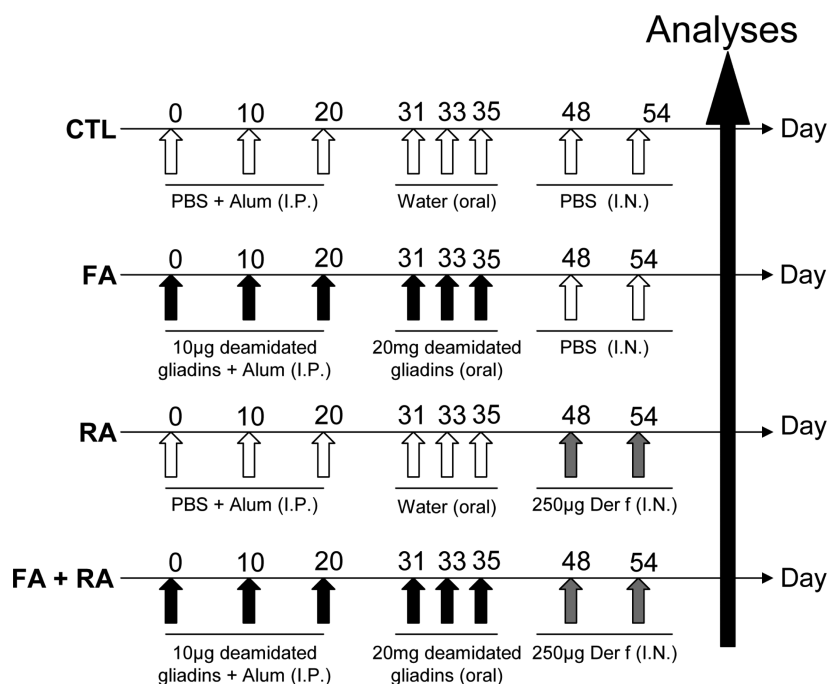


Figure 1. Study design. Mice were distributed into four groups: control (CTL), food allergy (FA), respiratory allergy (RA), and food and respiratory allergy (FA+RA).

MATERIALS AND METHODS

Animals. Balb/c mice (Charles River, France) were fed with an irradiated semisynthetic diet devoid of plant proteins (Safe, Augy, France). The Ethics Committee in Animal Experimentation of Pays de la Loire approved the protocol. (CEEA.2011.52; accreditation no. 4478), and all efforts were made to minimize suffering. All mice were maintained under specific pathogen-free conditions at the institutional animal facility. All animal procedures were conducted on mice between 8 and 12 weeks of age, in accordance with appropriate animal care. At the designated times, the animals were sacrificed according to institutional guidelines, and blood and tissues were collected for analyses.

Allergy Models. Freeze-dried deamidated gliadins were solubilized in 70% ethanol at 5 mg/mL and then diluted to 0.1 mg/mL in sterile PBS. Mice were intraperitoneally sensitized with 10 µg of deamidated gliadins absorbed on aluminum hydroxide (Sigma-Aldrich, Saint Quentin Fallavier, France) on days 0, 10, and 20. Challenge was performed by intragastric administration of 20 mg of deamidated gliadins on days 31, 33, and 35. For this mouse model of atopic march, a delay of 2 weeks was set between the last oral challenge and the first intranasal sensitization. Respiratory allergy was induced by intranasal exposure to 40 µL, containing 250 µg of HDM extract (*Dermatophagoides farinae*, Stallergenes, Antony, France) and approximately 1200 UE/ml of endotoxin, as measured by LAL test (Thermo, Waltham, MA, USA) on mice anesthetized with a 200 µL of mixture of ketamine (80 mg/kg)/xylazine (10 mg/kg) intraperitoneally injected on days 48 and 54. Mice were sacrificed 1 day after the last HDM extract challenge on day 55. As described (Figure 1), mice were then exposed to different protocols: food allergen alone (wheat, FA), respiratory allergen alone (HDM extract, RA), and successive exposure to allergens (wheat and HDM extract, FA+RA). Unexposed mice were used as controls (CTL).

Immunoglobulin Assays. Blood was removed on day 55 by cardiac puncture and centrifuged to collect serum. Sera were stored at -20°C before immunoglobulin assays. Mouse IgE specific to deamidated gliadins was assayed by indirect F-ELISA as described by Gourbeyre et al.²¹ Plates were coated with 5 µg/mL deamidated gliadins in carbonate buffer (30 mM Na_2CO_3 and 70 mM NaHCO_3 , pH 9.6). Serum samples were diluted 1:50 in PBS with 0.1% Tween-20 and 0.5% gelatin. A detection rat antibody directed against mouse IgE

(diluted 1/250 in PBS with 0.1% Tween-20 and 0.5% gelatin) and conjugated to alkaline phosphatase (Southern Biotechnology Associates Birmingham, AL, USA) was used. Fluorescence intensity was measured (at 440 nm after excitation at 360 nm) after incubation with an alkaline phosphatase substrate (4-methylumbeliferin, Sigma-Aldrich). Specific anti-Der f 1 IgE was assessed by indirect ELISA. Briefly, 96-well plates (Nunc) were coated with 100 µL of a solution of 50 µM sodium bicarbonate and 0.25 µg of purified Der f 1 (Indoor Biotechnologies, Cardiff, UK) in each well and left overnight at 4°C . Then, wells were incubated with bovine serum albumin 1% in PBS for 12 h at 4°C . Sera were diluted in Can Get Signal immunoreaction enhancer solution 1 (CGS1, Cosmo Bio, Tokyo, Japan), and 100 µL of this solution was incubated overnight at 4°C . Specific immunoglobulins were detected after incubation at room temperature with an anti-mouse IgE (AbD serotec, Oxford, UK) antibody coupled to horseradish peroxidase and diluted in CGS 2 (Cosmo Bio). Substrate, 2,2'-azinobis[3-ethylbenzothiazoline-6-sulfonic acid] diammonium salt (ABTS, Roche, Basel, Switzerland), was added, and optical density was measured by spectrophotometry (PerkinElmer, Waltham, MA, USA).

Histamine Assay. Blood samples were collected on day 55, and histamine was measured by ELISA (Labor Diagnostika Nord, Nordhom, Germany), according to the manufacturer's recommendations.

Cell Proliferation. Organs were crushed using a tissue grinder (Wheaton, Rochdale, UK) after removal to obtain single-cell suspensions, and after red blood cell lysis, cells were suspended in supplemented RPMI. Cells from Peyer patches were transferred to HBSS/Hepes 1.5%. Fragments were incubated 1 h at 37°C in HBSS/Hepes 1.5%/FBS 2%/Dispase/DNase (Sigma-Aldrich) solution. Cells were plated at 10^6 cells/well for 96 h at 37°C in the presence of concanavalin A (1 µg/mL) (Sigma-Aldrich), and proliferation was measured by MTT assay.²¹

Flow Cytometry. Anti-mouse antibodies used were the following: CD3-APC, CD19 PE-Cy7, Ly6G-PerCP-Cy5.5, CD8 APC-H7, and CCR3-PE (BD Biosciences). Dead cells were excluded using DAPI. Stained cells resuspended in PBS-FCS 1% were acquired on a BD LSR II (BD Biosciences) and analyzed on BD FACSDiva software (BD Biosciences). The following cells were stained in the presence of Fc blocker (BD Biosciences): eosinophils (gated on SSC^{high} , Ly6G^{int}, and

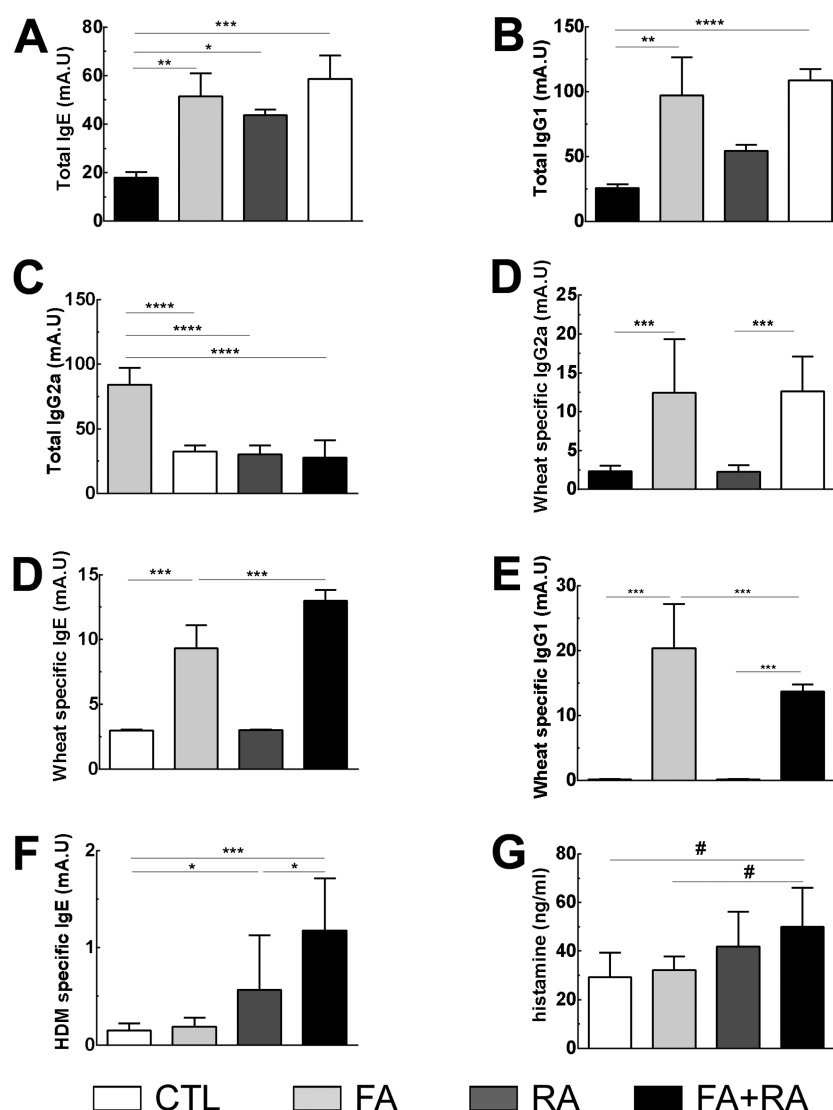


Figure 2. Ig and histamine levels are increased in food and respiratory allergy. Total and wheat-specific IgE (A, D), IgG1 (B, E), IgG2a (C, D), HDM-specific IgE (F), and histamine (G) were measured at day 54 in serum of mice after the last sensitization to HDM ($n = 8$ animals per group). Bonferroni multiple-comparison test: (*) $p < 0.05$, (**) $p < 0.01$, and (***) $p < 0.005$. Dunn's comparison test: (#) $p < 0.05$, (##) $p < 0.01$, and (###) $p < 0.005$.

CCR3⁺), neutrophils (gated on SSC^{high} and Ly6G^{high}), and lymphocytes (gated on CD3⁺).

Cytokine Measurements. In lymphoid organs and Peyer patches, cell culture supernatants IL-4 and IFN- γ were assayed using ELISA kits cytose (Invitrogen, Paisley, UK), and IL-10, IL-17A, and TGF- β were assayed using ELISA Ready-Set-Go! Kits (eBioscience, Paris, France) according to the manufacturer's recommendations. Broncho-alveolar lavages (BAL) were performed by intratracheally administering 1 mL of PBS and gentle aspiration to collect the epithelial lining fluid. IL-4, IL-10, IFN- γ , and IL-17 were quantified by Luminex technology (BioPlex 200 system, Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions (Bio-Rad).

Histopathological Analysis. The jejunum and proximal colon were collected, formalin fixed, and paraffin embedded. Sections (5 μ m thick) were stained with hematoxylin and eosin and inspected for the presence of mucosal inflammation. Histological analysis images were taken with an Axio Imager M1 microscope and an AxioCam HRC (Zeiss). Sections were analyzed using ImageScope for image acquisition (Aperio Technologies, Inc.), and a cell type count for goblet cells and leukocytes was performed on 10 villus/mouse. Grading of intestinal inflammation was determined in a blinded fashion, as previously described.²² Briefly, sections of mouse jejunum

were individually scored on the basis of three parameters. Inflammation was scored as follows: 0, rare inflammatory cells in the lamina propria; 1, increased numbers of cells in the lamina propria; 2, confluence of inflammatory cells extending into the submucosa; and 3, transmural extension of the inflammatory infiltrate. Crypt damage was scored as follows: 0, intact crypts; 1, loss of the basal one-third; 2, loss of the basal two-thirds; 3, entire crypt loss; 4, change of epithelial surface with erosion; and 5, confluent erosion. Ulceration was scored as follows: 0, absence of ulcer; 1, one or two foci of ulcerations; and 2, confluent or extensive ulceration. The total histological score represents the sum of all features evaluated and thus ranges from 0 to 10.

Evaluation of Gastrointestinal Functions. To assess permeability, the jejunum and proximal colon were removed and washed in cold Krebs solution, and segments were mounted in Ussing chambers (Physiological Instruments, San Diego, CA, USA). Paracellular and transcellular permeability were assayed by simultaneously measuring the flux of fluorescein-5.6 sulfonic acid (400 Da; 1 mg/mL, Invitrogen) and horseradish peroxidase (HRP) (44 kDa; 375 μ g/mL; Sigma, Saint Quentin Fallavier, France), respectively, over 3 h.

Airway Hyper-reactivity (AHR) Measurement. Enhanced pause (Penh) was measured by whole body plethysmography (Emka

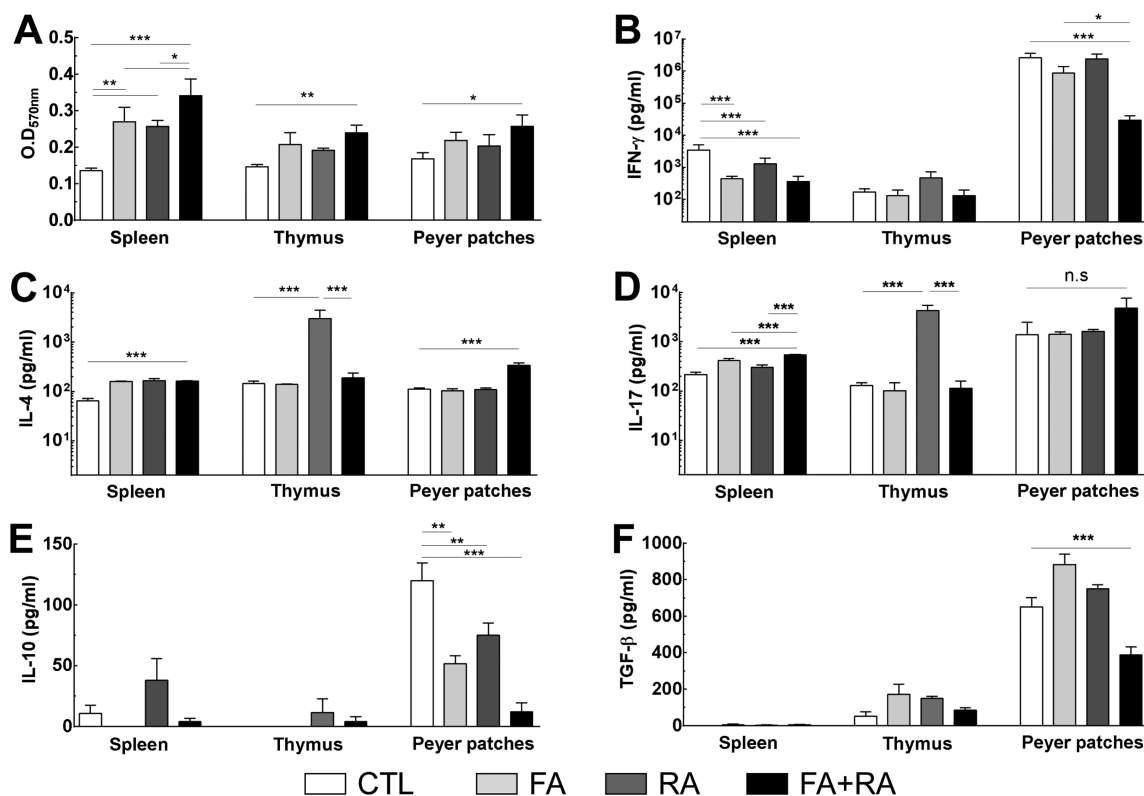


Figure 3. Allergens synergize to increase cell proliferation and cytokine secretion in lymphoid organs. Proliferation from spleen, thymus, and Peyer patch cells in response to ConA was measured (A) in control (white bar), food allergic (light gray bar), respiratory allergic (dark gray bar), and combined allergic (black bar) mice. IFN- γ (B), IL-4 (C), IL-17 (D), IL-10 (E), and TGF- β (F) from those cells were also measured by ELISA. The data represent the mean $\pm$ SEM (n = 8 animals per group). Bonferroni multiple comparison test: (*) p < 0.05, (**) p < 0.01, and (***) p < 0.005. Dunn's comparison test: (#) p < 0.05, (##) p < 0.01, and (###) p < 0.005.

Technologies, Paris, France) in response to nebulization of methacholine (Sigma-Aldrich) from 0 to 40 mg/mL, as described.²³ Because we previously ascertained in our mouse model using female Balb/c mice that Penh was similar to resistance or other measures determined by FLEXIVENT, intubation (not shown) and whole body plethysmography were determined to be sufficient to explore respiratory function.

Statistics. Values were expressed as the mean $\pm$ standard error (SEM). Statistical analysis was performed using GraphPad Prism (GraphPad Software, San Diego, CA, USA). Means were compared among groups using analysis of variance (ANOVA). In the case of positive ANOVA, means were compared by Mann–Whitney test. The results were corrected by a Bonferroni multiple-comparisons correction test. Statistically significant was marked as follow: (*) p < 0.05, (**) p < 0.01, and (***) p < 0.001. For data not following a normal distribution, a nonparametric Kruskal–Wallis test corrected using Dunn's test. Statistically significant was marked as follows: (#) p < 0.05, (##) p < 0.01, and (###) p < 0.001.

RESULTS

Peripheral Effects of Wheat and HDM Sensitization.

After the induction of a wheat-induced food allergy and/or the HDM-induced asthma-like reaction protocol (Figure 1), total and specific Ig and histamine levels were assessed in control (CTL), food allergy (FA), respiratory allergy (RA), and combined allergy (FA+RA) mice. Total IgE was increased in allergic mice groups compared to CTL but remained unchanged when compared to the FA, RA, and FA+RA groups (Figure 2A). The level of total IgG1 was also increased in all groups of allergic mice compared to CTL but with no statistically significant difference between the single and double allergies (Figure 2B). Conversely, total IgG2a was decreased in

the serum of all groups of allergic mice compared to CTL (Figure 2C). Wheat-specific IgG2a, IgE, and IgG1 were increased in the serum of FA mice (Figure 2D–F) compared to CTL mice. Conversely, they were at the threshold of detection in CTL and RA animals. In mice sensitized to both allergens, wheat-specific IgE and IgG1 levels were significantly increased compared to CTL or RA as well as the FA mice. Symmetrically, HDM-specific IgE was increased in the serum of RA mice (Figure 2F). In animals sensitized to both allergens, HDM-specific IgE levels were twice as high in mice sensitized to HDM only. FA+RA and RA sera but not FA displayed higher levels of histamine than CTL (Figure 2G). Specific IgE was therefore increased for each allergen by the sensitization to its counterpart, whereas histamine release increased only in mice sensitized to HDM, whether or not they were pre-sensitized to wheat.

Cell Proliferation and Cytokine Secretion in Lymphoid Organs.

Splenocyte proliferation in response to concanavalin A was evaluated by MTT in the spleen, thymus, and Peyer patches (Figure 3A). In FA+RA mice, immune cells from the spleen, thymus, or Peyer patches displayed increased proliferation by 2.52-, 1.63-, and 1.54-fold, respectively, compared to CTL mice. In FA and RA groups, increases of splenocyte proliferation of 2- and 1.9-fold, respectively, were also found. The proliferation was significantly higher in FA+RA mice compared to both single-sensitization groups only in the spleen.

To further detail the mechanisms linking both types of allergy, cytokine secretion by immune cells was measured in supernatants of spleen, thymus, and Peyer patches cells. After

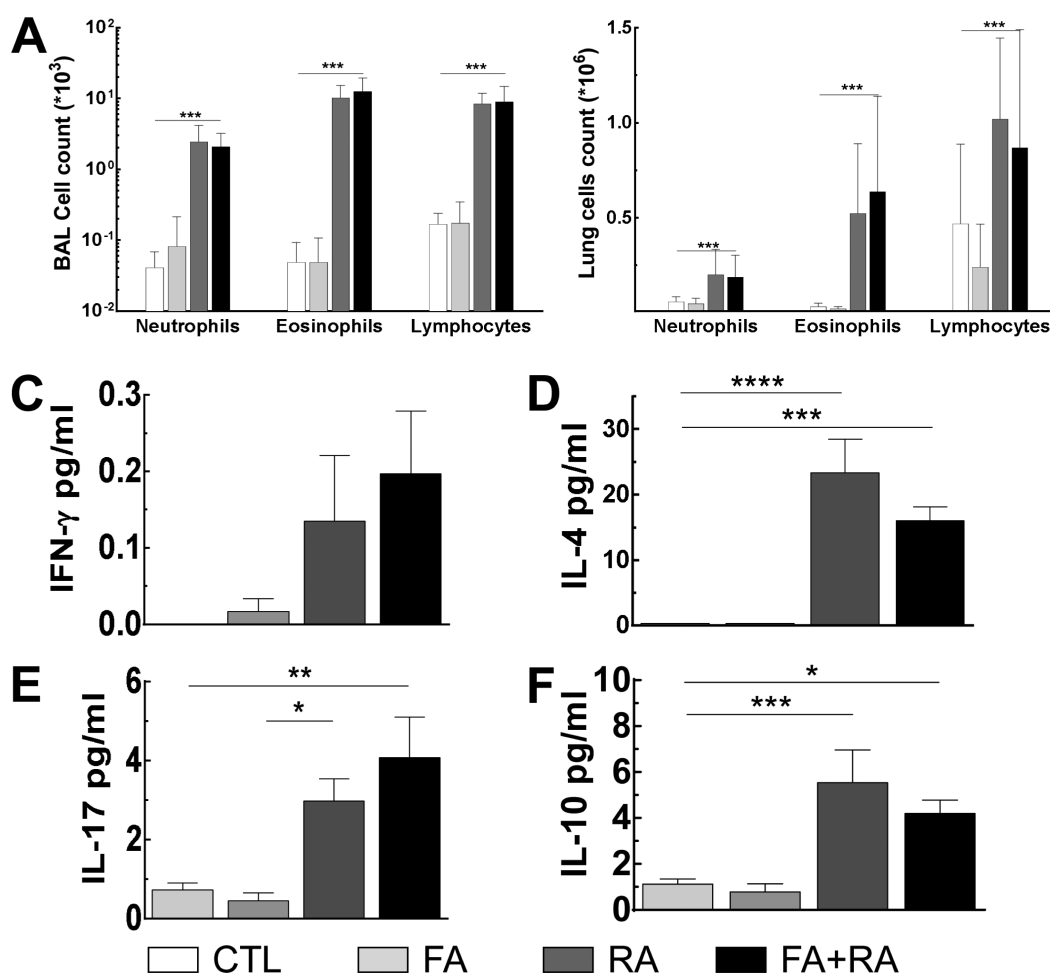


Figure 4. Successive allergies increase neither cell population nor cytokine secretion in the respiratory tract. Different cell populations from each group of mice were analyzed by flow cytometry in the broncho-alveolar lavage (BAL) (A, left panel) and lungs (A, right panel). Cell populations were polymorphonuclear neutrophil cells (neutrophils), polymorphonuclear eosinophil cells (eosinophils), and T lymphocytes (lymphocytes). IFN- γ (C), IL-4 (D), IL-17 (E), and IL-10 (F) were measured in parallel in BAL fluids after the last HDM intranasal challenge (day 54). The data represent the mean $\pm$ SEM ($n = 8$ animals per group). Bonferroni multiple-comparison test: (*) $p < 0.05$, (** $p < 0.01$, and (***) $p < 0.005$.

nonspecific activation by concanavalin A, IFN- γ secretion was reduced in spleen and Peyer patches cell supernatants from FA+RA mice compared to CTL mice. In single-allergy groups, this difference was observed in the spleen only. IFN- γ reduction was not observed in the thymus (Figure 3B). Then, Th2 polarization was evaluated by IL-4 production, which was found to be elevated in FA+RA compared to CTL in the spleen and Peyer patches (Figure 3C). In these compartments, no additive effects were observed, as IL-4 levels remained unchanged between RA or FA and FA+RA in the spleen, even though IL-4 levels were increased compared to controls. In Peyer patches, an effect was observed, as IL-4 levels were higher in FA+RA mice compared to other groups. In the thymus of RA mice, a clear increase in IL-4 secretion was observed (Figure 3C). To obtain a broader view of T-helper polarization, IL-17 production was also measured and shown to be significantly enhanced in the spleen but not in Peyer patches or in the thymus of FA+RA mice compared to CTL, FA, or RA mice (Figure 3D). In the thymus, only cells exposed to respiratory allergens produced elevated levels of IL-17 compared to controls. As a read-out of Treg cell activation, IL-10 and TGF- β levels were measured in the spleen, Peyer patches, and thymus cell supernatants. Low levels of TGF- β and IL-10 were detected in splenocyte and thymocyte

supernatants from all groups, whereas in Peyer patches, as high levels of both cytokines were detected in CTL, a drastic reduction was observed in FA+RA mice, suggesting a defect in intestinal T-cell regulation (Figure 3E,F). Furthermore, IL-10 but not TGF- β cell secretion was also attenuated in the Peyer patches of FA or RA mice compared to controls but to a lesser extent than FA+RA.

Our data therefore revealed that in this mouse model of atopic march, the Th1/Th2-related cytokines leaned toward a Th2 response after unspecific activation. Then, inflammation was boosted by the development of Th17 and down-modulation of Treg differentiation and/or activation in lymphoid organs. However, we observed no major change in the thymus except in RA mice.

Cell Population and Cytokine Secretion in the Respiratory Tract. Because our results demonstrated an effect of a food allergy on lymphoid cell proliferation and cytokine secretion, we assayed cell populations and cytokine secretion in the respiratory tract of the mice, including broncho-alveolar lavage (BAL) fluid and lungs (Figure 4). Additionally, we measured cell numbers of different subsets in the BAL and the lungs from each group by flow cytometry. Total cell numbers, polymorphonuclear neutrophils, polymorphonuclear eosinophils, and T lymphocytes (either CD8⁺ or

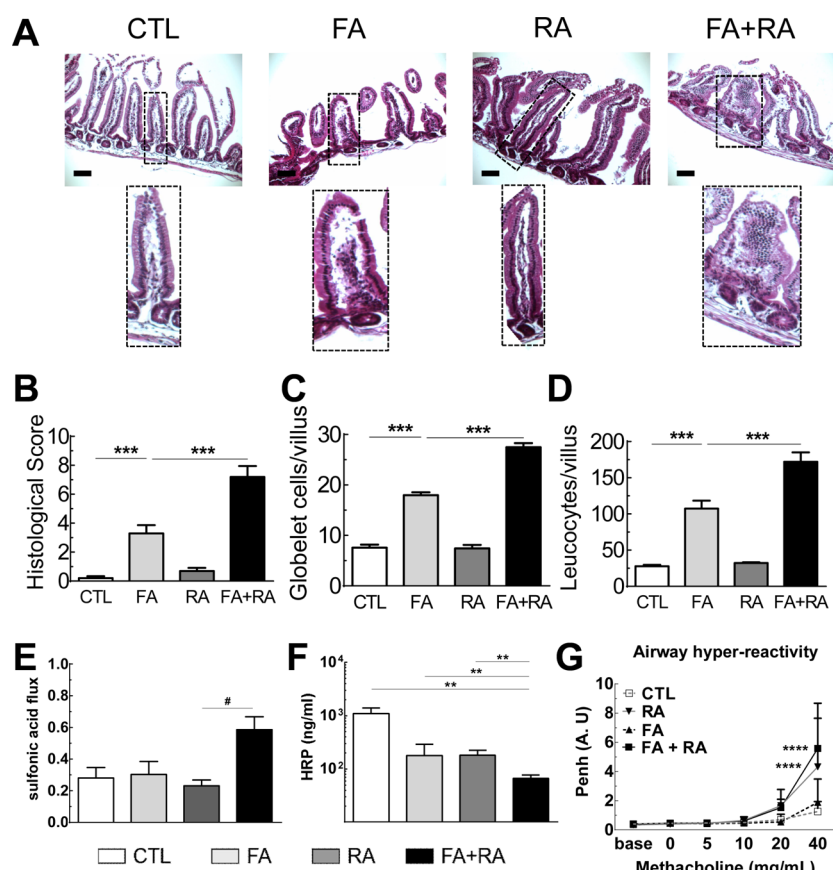


Figure 5. Successive exposure to allergens alters intestinal morphology and exacerbates symptoms without affecting respiratory parameters. At the end of the allergy-inducing protocol, gut segments were collected and processed for histopathology in jejunum (A), scored for inflammation (B), and counted for goblet cells (C) and leukocytes (D). As a critical feature of the gastrointestinal epithelium and function ex vivo paracellular permeability in proximal colon (E) and ex vivo transcellular permeability in jejunum (F) were evaluated in Ussing's chamber using intestine fragments. Respiratory function was determined by airway resistance measurements after methacholine nebulization (G). $n = 8$ animals per group. Bonferroni multiple-comparison test: (*) $p < 0.05$, (**) $p < 0.01$, and (***) $p < 0.005$. Dunn's comparison test: (#) $p < 0.05$, (##) $p < 0.01$, and (###) $p < 0.005$. Scale bar = 250 μm .

CD4⁺) were increased in mice with respiratory or mixed allergy compared to FA or CTL in both the BAL and lungs (Figure 4A, left and right panels, respectively). However, the inflammatory cell counts were equivalent in mice with the mixed or the single-respiratory allergy. Cytokine secretion was also assayed in BAL fluids. As expected, lower levels of cytokines were observed in the BAL compared to cell culture after nonspecific activation. The levels of all cytokines were increased in both groups of HDM-induced asthma-like reaction mice compared to the two other groups (Figure 3C–F). However, no further increase was observed in FA+RA mice. Similar results were observed in the lungs (data not shown). Taken together, our results establish that double allergy impacts the systemic and intestinal but not lung immune response.

Effects of Wheat and HDM Sensitization on Intestinal and Respiratory Functions. Having shown the influence of a double exposure to allergens on immune cell homeostasis and cytokine production, we aimed to explore the morphologic and phenotypic impact linking observations on cell and cytokine levels to the development of allergic symptoms. Therefore, we first determined whether combined allergy was associated with intestinal histological alterations by evaluating the development of inflammation in the jejunum of mice. FA+RA mice developed intestinal inflammation characterized by epithelial ulceration, edema, and leukocyte infiltration in the lamina

propria (Figure 5A). These lesions were, to a lesser extent, also present in FA mice. In contrast, in control or RA mice, no mucosal inflammation or edema was observed in the jejunum or proximal colon (Figure 5A and data not shown). Using histological inflammation semiquantitative scoring, we showed an absence of intestinal alterations in CTL and RA mice. In contrast, the inflammatory score was significantly larger in RA mice compared to CTL and even larger in FA+RA compared to FA mice (7.2 versus 3.3, respectively, $P < 0.05$). (Figure 5B). This inflammation is linked to an increase of goblet cells and leukocytes within the villus of the intestine in FA mice compared to CTL mice (Figure 5C,D). Moreover, in line with the histological score, the number of cells was increased in FA+RA mice compared to single-FA mice (Figure 5C,D).

Then, we measured jejunal and proximal colon paracellular and transcellular permeability ex vivo. Paracellular permeability was increased in the proximal colon of FA+RA mice compared to the other groups (Figure 5E). In contrast, HRP flux in the jejunum was significantly reduced in FA+RA compared to the other groups (70.3 versus 1078 and 179 ng/mL for FA+RA, CTL, and FA, respectively) (Figure 5F). No change in HRP flux was observed in the proximal colon (data not shown).

The effect of each allergy protocol on lung function was also evaluated by measuring mouse airway reactivity in response to methacholine. As expected, a gain in methacholine sensitivity

was noted in the RA and FA+RA groups compared to the CTL and FA groups (Figure 5G). However, airway hyper-responsiveness was not higher in RA allergic mice in cases of pre-existing food sensitization.

DISCUSSION

In this study, we aimed to establish that food sensitization primes the immune system to enhance the effects of a subsequent respiratory allergy. Contrasting results were obtained, with a clear effect observed on the systemic and gut hallmarks of allergy but without modification of respiratory inflammation and function.

Dual exposure to food and respiratory allergens exerted effects that can be considered at various levels: (1) the systemic compartment composed of blood, thymus, and spleen; (2) the intestinal mucosa; and (3) the lung mucosa. Exposure to FA and RA led to a higher production of specific IgE directed against each allergen that may cause blood basophils to degranulate and release higher levels of histamine, as observed. The marked secretion of IgE specific to each allergen might be enhanced by a dysregulation of the T-cell population balance, as suggested by the higher levels of IL-4 observed in spleen, whereas IFN- γ levels were moderated in FA+RA mice compared with mice allergic to a single substance. IL-17 was also elevated, suggesting that Th17 could act in synergy with Th2 cells to exacerbate the immune response. Interestingly, a role for Th17 cells in food allergy and asthma has been suggested.^{4,5} In contrast, thymocyte cytokine production was much more related to respiratory allergy. In fact, cytokine secretion in the thymus was increased in respiratory allergic mice but not in food allergic mice. Because thymocyte analysis was carried out shortly after the intranasal challenge and at a distance from the gut sensitization, it cannot be excluded that the thymocytes lost their food allergy-related reactivity at the time of analysis. However, in combined allergic mice, the effect of respiratory allergy on thymocytes was no longer observed. Although a negative feedback of food allergy on thymocyte reactivity can be evoked as a potential explanation, it is more likely that the thymocytes may have become unresponsive after the first food allergen stimulation.^{24,25}

At the level of intestinal Peyer patches, a clear Th2 inflammatory profile was demonstrated in FA+RA mice, with higher IL-4 and lower IFN- γ , IL-10, and TGF- β production. IL-10 secretion by Peyer patch cells was already dampened in mice exposed to a single allergen. It is noteworthy that intranasal exposure to RA induced a decrease in the secretion of IL-10 in the small intestine. Thus, the intestinal mucosal immune system of allergic mice may be more vulnerable to novel allergenic sensitizations by locally dampening tolerance-related cytokines (IL-10 and TGF- β) and increasing Th2 commitment (IL-4). Numerous studies have addressed the role of gut-associated lymphoid tissues (GALT) for oral tolerance induction.^{26–29} Notably, Peyer patch dendritic cells (DCs) were shown to produce much more IL-10 than spleen DCs.²⁶ In addition, gut DCs efficiently promote the differentiation of naïve Th0 cells into inducible Treg cells.³⁰ Furthermore, GALT were demonstrated to be a preferential site for the induction of the forkhead box P3⁺ Treg.²⁶ In healthy animals, CD4⁺ T cells activated by Peyer patch DCs produce much more IL-4 and IL-10 than CD4⁺ T cells activated by spleen DCs and promote the differentiation of Th2 cells.²⁷ Thus, GALT appear to be highly involved in allergy induction through their capacity to induce Th2 cells.

The observed perturbations in T-cell homeostasis induced by FA and RA affected intestinal functions such as a reduced HRP flux observed in FA+RA mice. This reduced flux could reflect either a drop in lysosomal transcellular transport or an increase in the lysosomal activity of epithelial cells. Indeed, an increase in the lysosomal degradation of HRP was observed in cecum treated with toxin A from *Clostridium difficile*.³¹ Under our experimental conditions, we could not determine the total amount of HRP that was transported across the epithelium, which could have been accomplished using [³H]HRP. Reduced HRP flux could also be a consequence of villus atrophy (as less surface exchange), as was reported in FA and RA+FA. This discrepancy could be due to the measurement of intact protein but not total HRP in the latter. Proximal colon, but not small intestine, paracellular permeability was increased in FA+RA mice.

Hence, these mice displayed signs of gut permeability perturbation as well as altered intestine morphology linked with allergy pathophysiology.¹³ Moreover, the stronger gut alteration in FA+RA mice may be due to a boost in local leucocytes after airway challenge. The mechanisms of intestinal modification under the action of RA are questionable. The ingestion of RA extract during intranasal challenge cannot be excluded. However, the huge pulmonary inflammation observed in lungs after intranasal challenge shows that most if not all of the extract is inhaled. Moreover, the absence of an effect in non-wheat-sensitized mice renders a direct local effect of HDM in the gut most unlikely. An effect of RA-induced systemic effect in wheat-sensitized mice is likely and is in accordance with the Ig and histamine results as well as the spleen data. A local priming by wheat sensitization of intestinal cells is also likely, as demonstrated by results in Peyer patches, and could account for the effects of HDM in wheat-sensitized mice. Interestingly, HDM exposure was necessary to obtain an effect on intestinal functional parameters that were not perturbed by the food allergy alone. However, the latter had a clear effect on intestinal architecture that was largely enhanced by subsequent HDM respiratory exposure.

By contrast, dual exposure did not exert any additive effects on the lung compartment, as all inflammatory parameters (cells and cytokines) were similarly increased in mice sensitized to HDM, regardless of the previous sensitization to gliadins. Moreover, in the lung, contrary to the results in the intestine, dual exposure increased IL-10 production in BAL. In addition, in the lungs, both Th2 and Th17 activation was observed in RA and FA+RA mice. In BAL fluids of asthmatic patients, this pattern of cytokine responses is frequently observed³² and may be related to a particular property of the lung mucosa. Thus, it can be suggested that the allergy mechanism is much more related to the Th2 pathway in the intestine than in the lung. In accordance with cell and cytokine results, respiratory function was not modified by dual exposure to wheat and HDM allergens compared to HDM only. The absence of a supplementary effect of food allergy on lung inflammation of HDM-sensitized mice could be related to the insensitivity of the lung compartment to the previous wheat sensitization. Another hypothesis is that the second exposure influences only the compartment in which the first sensitization operated. To validate this hypothesis in a further study, it would be interesting to reverse the two sensitization phases with an RA +FA model. However, these views contradict the typical sequence observed in humans, in whom food allergy predisposes to subsequent asthma and in whom respiratory

allergy is not known to aggravate symptoms related to food allergy. It is therefore more likely that the huge inflammation due to the sole HDM sensitization cannot be further extended. Indeed, a synergy between the two processes is still very well documented in the periphery through the increase of specific IgE and histamine release when both allergies are present.

These results are complementary to those we obtained in a model of successive allergies using ovalbumin (OVA) as food allergen and HDM extract. In fact, Bihouée et al.³³ previously demonstrated that food allergy to OVA increases AHR, hypereosinophilia, and global hypercellularity in response to subsequent HDM challenges, although no further increase in OVA-specific IgE was observed. However, physiological intestinal parameters and intestinal architecture were not assessed in dual allergic mice, leaving a gap in the intestinal impact of prior food allergy after respiratory allergy. Moreover, the absence of AHR and lung immunology modification in our experiment could result from the respiratory allergic protocol, which consists of two intranasal doses of HDM. However, the results from Bihouée et al.³³ were obtained after four sensitizations and two intranasal challenges.

The possibility that cross-reaction between the food allergen and the airborne allergen generates IgE that recognizes both antigens has never previously been described. Although cross-reactivity between aero-allergens such as pollen or plant allergens and food allergens such as shrimp and apple has been described^{34,35} cross-reactivity with wheat proteins, especially gliadin cross-reactivity with HDM, has not yet been described. Gliadin is known to cross-react with other foods such as milk or rice.

Our study demonstrates that sensitization to a first allergen imprints the immune response and enhances the effects of a novel subsequent sensitization. It therefore shows that multiple allergies observed in atopic subjects in the process of the atopic march are not independent events that occur within a common genetic background, but they synergize to mutually increase the effects of each allergen involved. Further studies are needed to decipher the mechanisms involved in this reciprocal intestine and lung allergic cross talk.

AUTHOR INFORMATION

Corresponding Author

*(M.B.) E-mail: marie.bodinier@nantes.inra.fr.

Author Contributions

[†]G.B. and P.G. contributed equally to this work.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We thank Manon Pietri for her technical assistance with the mouse model. We also thank the Cytocell Cytometry Facility in Nantes for expert technical assistance and the "Therassay platform and the UTE animal facility for animal welfare.

ABBREVIATIONS USED

Alum, aluminum hydroxide; ANOVA, analysis of variance; BAL, broncho-alveolar lavage; CTL, control group; *Der f*, *Dermatophagoides farinae*; DMSO, dimethyl sulfoxide; DNase, deoxyribonuclease; FA, food allergy; FA+RA, combined allergy group; F-ELISA, fluorometric enzyme-linked immunosorbent assay; FBS, fetal bovine serum; HBSS, Hank's balanced salt solution; HDM, house dust mite; HRP, horseradish peroxidase;

IFN, interferon; Ig, immunoglobulin; IL, interleukin; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrasodium bromide; PBS, phosphate-buffered saline; RA, respiratory allergy; RPMI, Roswell Park Memorial Institute; SD, standard deviation; TGF, transforming growth factor; Treg, regulatory T cell; TSLP, thymic stromal lymphopoietin

REFERENCES

- (1) Pawankar, R.; Canonica, G. W.; Holgate, S. T.; Lockey, R. F. *WAO White Book on Allergy 2011–2012: Executive Summary*; World Allergy Organization: Milwaukee, WI, USA, 2011.
- (2) Prescott, S.; Nowak-Węgrzyn, A. Strategies to prevent or reduce allergic disease. *Ann. Nutr. Metab.* **2011**, *59* (Suppl. 1), 28–42.
- (3) Agache, I.; Ciobanu, C.; Agache, C.; Anghel, M. Increased serum IL-17 is an independent risk factor for severe asthma. *Respir. Med.* **2010**, *104*, 1131–1137.
- (4) Herberth, G.; Daegelmann, C.; Roder, S.; Behrendt, H.; Kramer, U.; Borte, M.; Heinrich, J.; Herbarth, O.; Lehmann, I. IL-17E but not IL-17A is associated with allergic sensitization: results from the LISA study. *Pediatr. Allergy Immunol.* **2010**, *21*, 1086–1090.
- (5) Asher, M. I.; Montefort, S.; Bjorksten, B.; Lai, C. K.; Strachan, D. P.; Weiland, S. K.; Williams, H. Worldwide time trends in the prevalence of symptoms of asthma, allergic rhinoconjunctivitis, and eczema in childhood: ISAAC Phases One and Three repeat multicountry cross-sectional surveys. *Lancet* **2006**, *368*, 733–743.
- (6) Bjorksten, B.; Clayton, T.; Ellwood, P.; Stewart, A.; Strachan, D. Worldwide time trends for symptoms of rhinitis and conjunctivitis: Phase III of the International Study of Asthma and Allergies in Childhood. *Pediatr. Allergy Immunol.* **2008**, *19*, 110–124.
- (7) Strachan, D.; Sibbald, B.; Weiland, S.; Ait-Khaled, N.; Anabwani, G.; Anderson, H. R.; Asher, M. I.; Beasley, R.; Bjorksten, B.; Burr, M.; Clayton, T.; Crane, J.; Ellwood, P.; Keil, U.; Lai, C.; Mallol, J.; Martinez, F.; Mitchell, E.; Montefort, S.; Pearce, N.; Robertson, C.; Shah, J.; Stewart, A.; von Mutius, E.; Williams, H. Worldwide variations in prevalence of symptoms of allergic rhinoconjunctivitis in children: the International Study of Asthma and Allergies in Childhood (ISAAC). *Pediatr. Allergy Immunol.* **1997**, *8*, 161–176.
- (8) Valovirta, E. *Respiratory Allergies: Raise Awareness, Relieve the Burden*; EFA: Brussels, Belgium, 2011.
- (9) Patrizi, A.; Pileri, A.; Bellini, F.; Raone, B.; Neri, I.; Ricci, G. Atopic dermatitis and the atopic march: what is new? *J. Allergy* **2011**, *2011*, 279425.
- (10) Zheng, T.; Yu, J.; Oh, M. H.; Zhu, Z. The atopic march: progression from atopic dermatitis to allergic rhinitis and asthma. *Allergy, Asthma Immunol. Res.* **2011**, *3*, 67–73.
- (11) Fuiano, N.; Incorvaia, C. Dissecting the causes of atopic dermatitis in children: less foods, more mites. *Allergol. Int.* **2012**, *61*, 231–243.
- (12) Sampson, H. A. Update on food allergy. *J. Allergy Clin. Immunol.* **2004**, *113*, 805–819 quiz 820, .
- (13) Hagel, A. F.; de Rossi, T. M.; Zopf, Y.; Lindner, A. S.; Dauth, W.; Neurath, M. F.; Raithel, M. Small-bowel capsule endoscopy in patients with gastrointestinal food allergy. *Allergy* **2012**, *67*, 286–292.
- (14) Cingi, C.; Demirbas, D.; Songu, M. Allergic rhinitis caused by food allergies. *Eur. Arch. Otorhinolaryngol.* **2010**, *267*, 1327–1335.
- (15) Malmberg, L. P.; Saarinen, K. M.; Pelkonen, A. S.; Savilahti, E.; Makela, M. J. Cow's milk allergy as a predictor of bronchial hyperresponsiveness and airway inflammation at school age. *Clin. Exp. Allergy* **2010**, *40*, 1491–1497.
- (16) Allen, K. J.; Dharmage, S. C. The role of food allergy in the atopic march. *Clin. Exp. Allergy* **2010**, *40*, 1439–1441.
- (17) Boralevi, F.; Hubiche, T.; Leaute-Labreze, C.; Saubusse, E.; Fayon, M.; Roul, S.; Maurice-Tison, S.; Taieb, A. Epicutaneous aeroallergen sensitization in atopic dermatitis infants – determining the role of epidermal barrier impairment. *Allergy* **2008**, *63*, 205–210.
- (18) Bergmann, R. L.; Edenharter, G.; Bergmann, K. E.; Forster, J.; Bauer, C. P.; Wahn, V.; Zepp, F.; Wahn, U. Atopic dermatitis in early

infancy predicts allergic airway disease at 5 years. *Clin. Exp. Allergy* **1998**, *28*, 965–970.

(19) Host, A.; Halken, S.; Jacobsen, H. P.; Christensen, A. E.; Herskind, A. M.; Plesner, K. Clinical course of cow's milk protein allergy/intolerance and atopic diseases in childhood. *Pediatr. Allergy Immunol.* **2002**, *13* (Suppl. 15), 23–28.

(20) Simpson, A. B.; Glutting, J.; Yousef, E. Food allergy and asthma morbidity in children. *Pediatr. Pulmonol.* **2007**, *42*, 489–495.

(21) Gourbeyre, P.; Denery-Papini, S.; Larre, C.; Gaudin, J. C.; Brossard, C.; Bodinier, M. Wheat gliadins modified by deamidation are more efficient than native gliadins in inducing a Th2 response in Balb/c mice experimentally sensitized to wheat allergens. *Mol. Nutr. Food Res.* **2012**, *56*, 336–344.

(22) Wan, H.; Winton, H. L.; Soeller, C.; Taylor, G. W.; Gruenert, D. C.; Thompson, P. J.; Cannell, M. B.; Stewart, G. A.; Garrod, D. R.; Robinson, C. The transmembrane protein occludin of epithelial tight junctions is a functional target for serine peptidases from faecal pellets of *Dermatophagoides pteronyssinus*. *Clin. Exp. Allergy* **2001**, *31*, 279–294.

(23) Beilvert, F.; Tissot, A.; Langelot, M.; Mevel, M.; Chatin, B.; Lair, D.; Magnan, A.; Pitard, B. DNA/amphiphilic block copolymer nanospheres reduce asthmatic response in a mouse model of allergic asthma. *Hum. Gene Ther.* **2012**, *23*, 597–608.

(24) Konkel, J. E.; Jin, W.; Abbatiello, B.; Grainger, J. R.; Chen, W. Thymocyte apoptosis drives the intrathymic generation of regulatory T cells. *Proc. Natl. Acad. Sci. U. S. A.* **2014**, *111*, E465–473.

(25) Tepper, R. I.; Levinson, D. A.; Stanger, B. Z.; Campos-Torres, J.; Abbas, A. K.; Leder, P. IL-4 induces allergic-like inflammatory disease and alters T cell development in transgenic mice. *Cell* **1990**, *62*, 457–467.

(26) Coombes, J. L.; Powrie, F. Dendritic cells in intestinal immune regulation. *Nat. Rev. Immunol.* **2008**, *8*, 435–446.

(27) Mowat, A. M. Anatomical basis of tolerance and immunity to intestinal antigens. *Nat. Rev. Immunol.* **2003**, *3*, 331–341.

(28) Pabst, O.; Bernhardt, G.; Forster, R. The impact of cell-bound antigen transport on mucosal tolerance induction. *J. Leukocyte Biol.* **2007**, *82*, 795–800.

(29) Varol, C.; Zigmond, E.; Jung, S. Securing the immune tightrope: mononuclear phagocytes in the intestinal lamina propria. *Nat. Rev. Immunol.* **2010**, *10*, 415–426.

(30) Coombes, J. L.; Siddiqui, K. R.; Arancibia-Carcamo, C. V.; Hall, J.; Sun, C. M.; Belkaid, Y.; Powrie, F. A functionally specialized population of mucosal CD103⁺ DCs induces Foxp3⁺ regulatory T cells via a TGF- β and retinoic acid-dependent mechanism. *J. Exp. Med.* **2007**, *204*, 1757–1764.

(31) Heyman, M.; Corthier, G.; Lucas, F.; Meslin, J. C.; Desjeux, J. F. Evolution of the caecal epithelial barrier during *Clostridium difficile* infection in the mouse. *Gut* **1989**, *30*, 1087–1093.

(32) Jang, M. H.; Sougawa, N.; Tanaka, T.; Hirata, T.; Hiroi, T.; Tohya, K.; Guo, Z.; Umemoto, E.; Ebisuno, Y.; Yang, B. G.; Seoh, J. Y.; Lipp, M.; Kiyono, H.; Miyasaka, M. CCR7 is critically important for migration of dendritic cells in intestinal lamina propria to mesenteric lymph nodes. *J. Immunol.* **2006**, *176*, 803–810.

(33) Bihouée, T.; Bouchaud, G.; Chesne, J.; Lair, D.; Rolland-Debord, C.; Braza, F.; Cheminant, M. A.; Aubert, P.; Mahay, G.; Sagan, C.; Neunlist, M.; Brouard, S.; Bodinier, M.; Magnan, A. Food allergy enhances allergic asthma in mice. *Respir Res.* **2014**, *15*, 142.

(34) Vieths, S.; Scheurer, S.; Ballmer-Weber, B. Current understanding of cross-reactivity of food allergens and pollen. *Ann. N. Y. Acad. Sci.* **2002**, *964*, 47–68.

(35) Klinglmayr, E.; Hauser, M.; Zimmermann, F.; Dissertori, O.; Lackner, P.; Wopfner, N.; Ferreira, F.; Wallner, M. Identification of B-cell epitopes of Bet v 1 involved in cross-reactivity with food allergens. *Allergy* **2009**, *64*, 647–651.