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Intestinal Dysbiosis Featuring Abundance of *Ruminococcus gnavus* Associates With Allergic Diseases in Infants

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Abbreviations used in this paper: AHR, airway hyperresponsiveness; DCs, dendritic cells; IFA, immunofluorescence assay; IL, interleukin; ILC2s, type-2 innate lymphoid cells; LPS, lipopolysaccharide; NGS, next generation sequencing; OVA, ovalbumin; PCA, principle component analysis; PCoA, principle coordinates analysis; TSLP, thymic stromal lymphopoietin; TTGE, temporal temperature gradient gel electrophoresis.

Reprint requests

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Conflict of interest

All the authors disclose no conflicts of interest.

Author contributions

This study was designed by Y.H.N. & H.C.C. Patient recruitment was performed by Y.H.N. & H.C.C. Data acquisition was performed by Y.L.T., H.H.C. & C.C.L. Data were analyzed and reviewed by H.H.C. and Y.H.N. Besides, B.L.C. provided technical & material support. Statistical analysis was performed by H.H.L. Manuscript was drafted by H.H.C. and was critically revised by Y.H.N and H.H.L.

Abstract

Background & Aims: Dysbiosis of the intestinal microbiota has been associated with development of allergies in infants. However, it is not clear what microbes might contribute to this process. We investigated what microbe(s) might be involved in analyses of infant twins and mice.

Methods: We studied fecal specimens prospectively in a twin cohort (n=30) and age-matched singletons (n=14) born at National Taiwan University Children's Hospital, Taipei, Taiwan, from April 2011 to March 2013. Clinical parameters (gestational age, birth body weight, mode of delivery and feeding, immunizations and medical events) were recorded. Fecal samples were collected beginning immediately after birth and for 1 year; the children were followed until 3 years of age and allergic symptoms (repetitive and continuous for at least 6 months) were noted. A skin prick test was used to ascertain atopy. Bacterial communities in fecal samples were profiled by 16S rRNA-based PCR-temporal temperature gradient gel electrophoresis and next generation sequencing. BALB/c mice without and with ovalbumin sensitization/challenge were infected with candidate bacteria by oral gavage intra-gastric intubation. Fecal, serum, lung, and colon tissue samples were collected from mice and analyzed for mechanisms of allergy development.

Results: During the investigation period, 20 children (45.5%) developed allergic diseases, including respiratory (allergic rhinitis and asthma) and skin (atopic dermatitis and eczema) allergies. Lachnospiraceae were detected at significantly higher frequency in allergic infants than non-allergic infants ($P<.004$); the high fecal count of Lachnospiraceae in allergic subjects appeared at 2 months of age and persisted until 12 months of age. The enrichment of Lachnospiraceae in allergic infants was attributed to the overgrowth of *Ruminococcus gnavus*, which tended to have a low frequency in non-allergic subjects ($P=.0004$). Increased *R gnavus* was observed before the onset of allergic manifestations, and associated with respiratory allergies ($P<.002$) or respiratory allergies coexistent with atopic eczema ($P<.001$). In mice, endogenous *R gnavus* grew rapidly following sensitization and challenge with ovalbumin. Mice gavaged with purified *R gnavus* developed airway hyper-responsiveness and had histologic evidence of airway inflammation (asthma). Expansion of *R gnavus* in mice stimulated secretion of cytokines (interleukin 25 (IL25), IL33, and thymic stromal lymphopoietin) by colon tissues, which activated type-2 innate lymphoid cells and dendritic cells to promote differentiation of T-helper (Th)2 cells and production of their cytokines (IL4, IL5, and IL13). This led to infiltration of the colon and lung parenchyma by eosinophils and mast cells.

Conclusions: In a study of a twin cohort (some infants with, some without allergies), we associated development of allergies, particularly respiratory allergies, with increased fecal abundance of *R gnavus*. Mice fed *R gnavus* developed airway inflammation, characterized by expansion of Th2 cells in the colon and lung, and infiltration of colon and lung parenchyma by eosinophils and mast cells.

KEY WORDS: TSLP, ILC2, epithelium-derived cytokines, immune regulation

Introduction

Establishing intestinal microbiota in infancy is critical for building up immune tolerance against environmental antigens.¹ Young children gradually establish a stable gut microbial community and reach a dynamic balance at ≥ 3 years of age.¹ Early life gut microbial dysbiosis is currently recognized as a key determinant of immune dysregulation, which is associated with a broad spectrum of allergic disorders.²

Although a striking increase in the incidence of allergies has been reported worldwide during past decades, the World Health Organization has predicted further increases in the prevalence of bronchial asthma globally in forthcoming years.³ To date, the exact microbe(s) responsible for allergy establishment remains elusive. Some researchers observed high colonization of *Clostridium*/*Bacteroides*/*Escherichia coli* and a shortage of *Lactobacilli*/*Bifidobacterium* in allergic infants, while other groups demonstrated the completely opposite result.⁴⁻⁹ This discrepancy was most likely due to differences in the assay systems adopted in these studies, as well as the fact that traditional bacterial cultivation frequently caused lower than expected numbers of detectable species.^{7, 8, 10} Moreover, many of these studies were cross-sectional and lacked serial sampling.^{4, 7-9} Some studies enrolled subjects with ongoing atopic diseases and lacked sufficient data on the microbiota configurations before disease onset.^{5, 6} The control groups of these studies deserve the most critical appraisal, as they generally enrolled control subjects from different families without relationships to the allergic patients;⁴⁻¹¹ therefore, the genetic and environmental factors affecting microbial colonization could not be well controlled. All these drawbacks limited the chances of identifying the specific bacteria that predisposed individuals to the development of allergies and perturbed the development of an effective therapeutic strategy for treating the disease.

We hypothesized that dysbiosis of gut microbiota in infancy may predispose to later allergy development, and we intended to identify the causative bacteria. The aforementioned design drawbacks were ameliorated herein by enrolling a twin cohort to minimize the biases caused by familial and environmental determinants. Fecal collection was started on the first day of life to allow an ample time window for early detection of related microbial configurations before disease onset. Moreover, culture-free PCR-temporal temperature gradient gel electrophoresis (TTGE) and 16S rRNA-based next-generation sequencing (NGS) were applied to determine the composition of the entire microbiome down to the species level. Transitioning from the bedside to the bench, we established an asthmatic mouse model and *in vitro* mouse-derived cells to verify the role of the candidate bacteria in allergic manifestations. The immunoregulatory mechanisms underlying the ability of the candidate bacteria to control allergy development were studied not only in lung tissues but also in colons. This novel approach was expected to reveal the involvement of an as-yet-unknown gut-pulmonary axis in the pathogenesis of asthma that is linked to gut microbiota.

Materials and Methods

Patient enrollment

The subject group (n=44) of this study comprised 14 pairs of dizygotic twins, one pair of monozygotic twins and 14 age-matched unrelated singletons who were born at National Taiwan University Children's Hospital, Taipei, Taiwan, from April 2011 to March 2013 and were regularly followed from birth until 3 years of age. Newborns with a family history of allergies were encouraged to participate. Neonates receiving antibiotic treatment or with multiple congenital anomalies, for instance, meningoencephalocele, chromosomal anomalies and congenital intestinal atresia, were excluded from the study. All the subjects were enrolled on the first day after birth, and their clinical parameters, such as gestational age, birth body weight, mode of delivery and feeding, immunizations and medical events, were recorded. Allergic diseases were diagnosed according to case histories and to clinical manifestations defined by the criteria proposed by the Taiwan Academy of Pediatric Allergy Asthma and Immunology (<http://www.air.org.tw/air/publication.aspx>). Briefly, patients were diagnosed as having allergic rhinitis when they experienced disturbing nasal symptoms (sneezing, itching, runny and stuffy nose) that interfered with their sleep and daily activities. Children who had episodic wheezing, dyspnea and night cough were diagnosed with asthma. A diagnosis of atopic dermatitis (or atopic eczema) was made when pruritus was present along with an inflammatory skin condition featuring erythema/edema/vesicles in the acute stage and scaling/xerosis/lichenification (skin thickening) in the chronic stage. Allergic symptom occurrence must have been repetitive and continuous for at least 6 months. A skin prick test was used to ascertain the presence of atopy. The goals and procedures of this study were explained to the subjects' parents, who signed informed consent forms, and the study protocol was approved by the Institutional Review Board of National Taiwan University Hospital.

Detailed protocols are provided in the Supplementary Materials and Methods.

ACCEPTED MANUSCRIPT

Results

A high abundance of Lachnospiraceae is prevalent among allergic twins

As we aimed to identify the gut microbes associated with the occurrence of allergic diseases during early childhood, we first enrolled 8 pairs of discordant twins. Among them, one featured a twin who suffered from allergic disorders and another twin who was free from disease. Besides, 7 pairs of concordant twins and 14 age-matched unrelated singletons were included in the study to allow substantial verification of our findings (Supplementary Table 1). During the investigation period, a total of 45.5% (20/44) subjects developed allergic diseases, including respiratory (allergic rhinitis and asthma) and skin (atopic dermatitis and eczema) allergies (Supplementary Table 2). Forty-five percent (9/20) of subjects manifested allergic symptoms before completing their first year of life, and the other patients had later allergy symptom onset (after 1 year, Supplementary Table 2). No significant relationships between human milk feeding, heredity, probiotic usage and allergy development were noted (Supplementary Table 2).

Stool specimens were collected serially at months 0 (meconium), 1, 2, 6 and 12, and fecal DNAs were subjected to 16S rRNA PCR-TTGE analysis for evaluating bacterial diversity. Each individual displayed a unique profile, while the members of each twin-pair were very similar to each other in terms of their band patterns (Supplementary Figure S1). All subjects displayed few TTGE bands at month 0. However, increases in the complexity of and dynamic changes in the bacterial populations were displayed by the subjects from months 1 to 12 (Supplementary Figure S1). Due to their promising characteristics, the TTGE profiles of the discordant twins were first analyzed for different taxa abundances, according to clinical phenotypes. Increased levels of the bacterial family Lachnospiraceae (formerly Clostridium cluster XIVa) were consistently

observed in all allergic discordant twins compared to their non-allergic siblings (Figure 1A). The data for the concordant twins and singletons were then analyzed. Similar to the above findings, Lachnospiraceae colonization showed a significantly higher frequency in allergic infants than in non-allergic subjects ($p < 0.004$, Supplementary Figure S2). Moreover, both the heat map and the line chart demonstrated that the high fecal count of Lachnospiraceae in allergic subjects manifested beginning at 2 months of age and persisted until 12 months of age (Figures 1B and 1C). A clear Lachnospiraceae amplicon was displayed in the month 12 fecal sample of the allergic discordant twin (Twin 1b), but not in the samples of the non-allergic Twin 1a and singletons (Inf 1 and 2), when the V3-V4 PCR products were separated in a TTGE gel (Figure 1D).

To further quantitate and evaluate the bacterial genomes, we subjected the month 12 fecal samples from 6 twin-pairs to 16S rRNA-based NGS analysis. Both Twins 4a and 4b had allergy symptoms although disease onset occurred much later in Twin 4b than in Twin 4a, whereas the remaining twin pairs are discordant (Supplementary Table 2). Principle component analysis (PCA) was employed to determine the frequencies and identities of the bacterial families in allergic and non-allergic twins, and the first (PC1, 27.8%) and second (PC2, 12.9%) principle components explained the total variation between the groups (Figure 2A). Moreover, principle coordinates analysis (PCoA) based on measures of dissimilarity and distance matrices between sample pairs indicated the existence of marked bacterial community structural differences between each allergic twin and non-allergy paired sibling (Figure 2B). To examine fecal 16S rRNA metagenomes in detail, we chose bacterial families with high scores on the NGS reads or families that had been implicated in allergic diseases for further comparisons. Only the family Lachnospiraceae exhibited a consistently higher abundance, an increment of at least 1.8 fold, in

allergic twins than in their non-allergic counterparts (Figures 2C and 2D), in line with the TTGE results. These data confirmed the reliable quantification of TTGE and suggested that Lachnospiraceae may be involved in the pathogenesis of allergies.

Early colonization with high counts of R. gnavus is predictive of allergy development

To identify the specific Lachnospiraceae bacteria that contribute to allergy manifestations, we further dissected all the TTGE-profiled bacteria from the family Lachnospiraceae down to species level. These bacteria are represented by a heat map displaying the total counts of each species from months 0 to 12 (Figure 3A). The enrichment of Lachnospiraceae in allergic infants was clearly attributed to the overgrowth of *Ruminococcus gnavus*, which tended to have a low frequency in non-allergic subjects ($p=0.0004$; Figure 3A). Likewise, our results regarding the NGS-surveyed microbiota composition of 6 twin-pairs showed that the fecal copy number of *R. gnavus* was profoundly and consistently augmented in allergic twins compared with other Lachnospiraceae species (Supplementary Figure S3A), as well as with a number of well-documented allergy-associated species, such as *Bifidobacterium longum*, *Lactobacillus rhamnosus*, *Clostridium difficile*, *Faecalibacterium prausnitzii*, and etc^{5, 7, 11-19} (Supplementary Figure S3B).

R. gnavus was originally categorized as a member of the family Ruminococcaceae but was recently reclassified into the genus *Blautia* of Lachnospiraceae based on the results of 16S rDNA gene sequencing studies.^{20, 21} For allergic infants, intestinal colonization of *R. gnavus* started at as early as 1-2 postnatal months and was followed by rapid growth exceeding the colonization rate of non-allergic controls (Figure 3B and Supplementary Figure S3C). In general, the fecal count of *R. gnavus* in allergic infants constituted an average of 2.9% of the total

commensal bacteria population at 6 month of age and rose to 3.8% at 1 year of age, as estimated by PCR-TTGE profiling, whereas it constituted averages of 0.4% and 0.8% at 6 months and 1 year of age, respectively, in non-allergic infants (Figure 3B). Regarding *R. gnavus*-positive subjects, their 3-year cumulative incidence of allergic diseases was significantly higher than that of subjects without *R. gnavus* colonization (Figure 3C), and the emergence of *R. gnavus* in the stool specimens of allergic subjects preceded the clinical manifestation of allergies (Supplementary Table 3). Furthermore, the rate of positive *R. gnavus* colonization in infants with a combination of respiratory and skin allergies or only respiratory allergies was 100%, and *R. gnavus* acquisition occurred at mean ages of 2-6 months, respectively (Figure 3D). In contrast, only 29.2% of non-allergic infants were colonized with *R. gnavus*, which happened at a mean age of 9.4 months, significantly later than the times at which infants with double-allergies were colonized ($p < 0.0001$, Figure 3D). Besides, the allergic subjects (50%, 10/20) were frequently colonized with *R. gnavus* before the initiation of solid-food intake, as compared with the non-allergic group (8.3%, 2/24, Supplementary Table S3), implying *R. gnavus* can be transmitted through caregiver's contact and/or environment exposure even before the introduction of solid-food. All these data supported the fact that infants colonized too early and too abundantly by *R. gnavus* are at risk for developing allergic disorders, especially respiratory allergic disorders.

R. gnavus induces airway hyperresponsiveness (AHR) and Th2-allergic responses

To validate the potential role of *R. gnavus* in the pathogenesis of respiratory allergies, we established an asthmatic mouse model. BALB/c mice without (naïve) and with ovalbumin (OVA) sensitization/challenge were infected with *R. gnavus* intragastrically (Figure 4A) and were bred with free access to water and a standard laboratory diet. Repeated OVA

sensitization/challenges effectively triggered the production of mouse anti-OVA IgE (Supplementary Figure S4). Fecal samples from each mouse harvested at the indicated times (Figure 4A) were subjected to DNA isolation and 16S NGS analysis. A PCA-based similarity measure of species composition demonstrated that *R. gnavus* administration reduced microbial diversity and shared relevant structural commonalities with those of OVA-sensitized and OVA+*R. gnavus* co-stimulated mice (Figure 4B). In response to OVA sensitization/challenge, *R. gnavus* grew enormously in OVA mice to reach a mean level (0.43% of total commensal bacteria) higher than that of *R. gnavus*-infected mice (0.34% of total commensal bacteria), and administration of *R. gnavus* to OVA mice yielded a trend towards further increase in the mean fecal count of *R. gnavus* (0.53% of total commensal bacteria; Figure 4C, upper), implying that the allergic microenvironment supports the growth of *R. gnavus*. Although the total serum IgE level of *R. gnavus*-infected mice was less than that of OVA mice (Figure 4C, lower), these mice developed significant AHR in response to methacholine-induced bronchoconstriction and was comparable to that of OVA mice (Figure 4D). Moreover, OVA+*R. gnavus*-co-treated mice displayed a more prominent increase in AHR under methacholine challenge (Figure 4D). Histologic examination of the lung sections of *R. gnavus*-infected mice, as those of OVA mice with/without *R. gnavus* infection, revealed profound thickening of the bronchial walls and heavily lymphocytic infiltration in the peri-bronchial regions, which are the typical features of asthmatic inflammation (Figure 4E).

Th2 (represented by IL-4, IL-5 and IL-13), Th1 (IFN γ) and Th17 (IL-17) immune cells are necessary for mediating allergic airway responses.²² Accordingly, immunofluorescence assay (IFA) was performed to allow the identification of which cell-types are present in pulmonary lymphocytic infiltrates. An intensive accumulation of Th2 cells, characterized by the expression

of IL-4, IL-5 and IL-13, was observed in the parenchymal lung tissues of *R. gnavus*-infected mice, and the accumulation was as severe as that observed in OVA-co-stimulated mice (Figure 5A). Naïve mice that were naturally colonized with an increased abundance of *R. gnavus* ($\geq 0.3\%$ of total commensal bacteria) that was similar to that of the mean level of *R. gnavus*-infected mice also experienced allergic airway responses by recruiting Th2 cells to the pulmonary parenchyma and augmenting AHR (Supplementary Figure S5). In contrast to OVA-stimulation, *R. gnavus* infection did not induce an elevation in IFN γ and IL-17 levels (Supplementary Figure S6), reflecting that *R. gnavus* mounts a Th2 response to develop AHR.

Eosinophils and mast cells function as effectors in Th2 cytokine responses;²² therefore, immunohistochemistry was performed to validate the presence of these effectors in mouse lung tissues. Many eosinophils and mast cells, whose presences are specified by eosinophil peroxidase (EPX) and mast cell tryptase, respectively, accumulated in the pulmonary peri-vascular regions of mice with *R. gnavus* and/or OVA administration (Figure 5B). Additional mast cells could be found inside the airway lumens of OVA mice with/without *R. gnavus* co-stimulation (Figure 5B). Of the naïve mice, none displayed effector cell infiltration (Figure 5B and Supplementary Figure S7A). Collectively, our data indicated that a high abundance of *R. gnavus* is sufficient for the establishment of allergic pulmonary inflammation.

R. gnavus-induced pulmonary Th2 immunity arise from the colon

In view of the colonic colonization of *R. gnavus* and a broad migration of gut-derived immune cells to inflammatory sites has been hinted,²³ we hypothesized that *R. gnavus* may modulate the total pool of colonic immune cells, which would then migrate to lung to develop asthma. Thus, we examined the colonic microenvironments of these mice. The histologic

presence of lymphocytic infiltration was demonstrated in the colonic lamina propria of *R. gnavus*-infected mice, similar to OVA mice, and OVA+*R. gnavus* mice had the heaviest lymphocytic infiltrates; however, these infiltrates were absent in naïve mice (Figure 6A). Like the pulmonary lymphocytic infiltration, these colonic infiltrates comprised mainly Th2 cells, which express intracellular IL-13, as evidenced by the immunohistochemistry results (Figure 6B and Supplementary Figure S7B). Exacerbation of the histological score in the colon tissues was significantly enhanced upon *R. gnavus* infection (Figure 6C). When assessing colonic cytokine levels, we noted a significant increment in IL-4 and IL-13 levels and a less intense of IL-5 in *R. gnavus*-infected mice with/without OVA co-stimulation (Figure 6D). These Th2 cytokines increased the production of total IgE, which reached a peak level upon OVA co-stimulation, in the colons of *R. gnavus*-treated mice (Figure 6E). Compared to OVA mice, *R. gnavus*-infected mice displayed a slight up-regulation in colonic mast cell tryptase expression that paralleled their lower total IgE levels (Figures 6E and 6F). However, great increases in colonic eosinophils in response to *R. gnavus* administration were consistently revealed by western blotting and immunohistochemistry (Figures 6F and 6G). These eosinophils were found mainly in the colonic crypts, lamina propria and submucosa of *R. gnavus*-infected mice, and was absent in the naïve control (Figure 6G and Supplementary Figure S7C).

For human allergic cases, increasing serum level of eosinophil cationic protein (ECP), a granular protein secreted by activated eosinophils during allergic inflammation, was found to associate with high fecal count of *R. gnavus* ($p=0.0091$, $r^2=0.7054$; Supplementary Figure S8). This implied an increased entry of eosinophils into blood circulation system of *R. gnavus*-colonized infants, and proved that *R. gnavus* drives eosinophilic immune response not only in experimental mice but also human subjects.

R. gnavus primes ILC2s and DCs for greater Th2 differentiation by raising the production of epithelium-derived cytokines.

Th2 cell-mediated inflammation is initiated by activation of type-2 innate lymphoid cells (ILC2s) and dendritic cells (DCs) in response to the stimulation of epithelium-derived cytokines including IL-25, IL-33 and TSLP, which can be evoked by bacteria.²⁴ Activated ILC2s and DCs then prime naive T cells to differentiate into Th2 cells and orchestrate a prominence of Th2 cytokine production.²⁴ To explore the detailed mechanism of how *R. gnavus* induces the development of Th2 response, we investigated the role of colonic epithelium-derived cytokines. Using ELISA, we noted increased secretion of IL-25, IL-33 and TSLP upon *R. gnavus* exposure, and additional OVA treatment triggered further intensification of their production in adjacent colon cells (Figure 7A). However, only the levels of colonic IL-25 and IL-33, but not TSLP, increased when the mice received only OVA sensitization/challenge (Figure 7A). A concurrent expansion of both 33D1⁺ DCs and CD25⁺IL-9⁺ ILC2s upon the augmentation of epithelium-derived cytokines was generally found in the colons of *R. gnavus*-inoculated mice (Figures 7B and 7C). Greater ILC2 and DC populations were observed in mice co-treated with OVA and *R. gnavus* than in other mice (Figure 7D).

To strengthen this observation, an *in vitro* model was set up by incubating the *R. gnavus* culture with mouse colon tissues, which were cut open longitudinally after slicing into 2-cm segments with carefully protecting the integrity of the mucus layer. A striking release of IL-33 and, to a lesser extent, IL-25 from the colon tissues was found in the presence of *R. gnavus* (Supplementary Figure S9), suggesting *R. gnavus* potentially penetrates the mucus layer and stimulates the underlying colonic epithelium to secrete epithelium-derived cytokines. Subsequent

in vitro assay further demonstrated *R. gnavus* primed the bone marrow-derived DCs to induce naive T cell differentiation into Th2 subset (Supplementary Figure S10). In that *R. gnavus*-pulsed DCs were competent to trigger the release of IL-5 from Th2 cells regardless of the presence or absence of anti-CD3/28, which cross-linked the T cell-associated CD3 and CD28 molecules to provide second signal for T cell activation (Supplementary Figure S10C). Further enhancement of IL-5 production was dependent upon co-stimuli from IL-33 (Supplementary Figure S10C). In contrast, the crosslinking signal of anti-CD3/28 was necessary for *R. gnavus*-pulsed DCs to prompt the secretion of IL-13 (Supplementary Figure S10D). IL-33 acted synergistically with *R. gnavus* in Th2 differentiation to allow for additional production of IL-13 (Supplementary Figure S10D). It has been documented that IL-33 amplifies both Th1 and Th2 immune responses by augmenting the secretion of IFN γ , IL-5 and IL-13,²⁵ which were in line with our finding (Supplementary Figure S10C to S10E). However, the effect of *R. gnavus* was clearly restricted to the production of Th2-skewed cytokines. *R. gnavus* by itself did not influence the release of IFN γ unless IL-33 was concurrently provided (Supplementary Figure S10E).

Taken together, these findings are illustrative of a scenario in which *R. gnavus* primarily triggers a Th2-dominated allergic response by stimulating the colonic epithelium to secrete epithelium-derived cytokines, which elicit an expansion of ILC2s and DCs to prime the differentiation of Th2 cells and the production of Th2 cytokines, thereby causing tissue eosinophilic infiltration and exerting a minor effect on IgE-mast cell axis induction. Consequently, Th2 lymphocytes and the effector cells circulate to the lung to direct the asthmatic inflammatory cascade (Figure 7E).

Discussion

The present study, which is based upon a prospective discordant twin cohort design, disclosed that dysbiosis with *R. gnavus* overgrowth in early infancy contributes significantly to the development of respiratory allergies. *R. gnavus* is affiliated with Lachnospiraceae, a family of gram-positive obligate anaerobes within the order Clostridiales.²⁶ Bacteria from Lachnospiraceae rarely inhabit the GI tract of human newborns, but the populations of these bacteria increase moderately in infancy and reach their peak in adulthood.²⁷ This family comprises not only potent probiotics that counteract allergic inflammation, such as *Lachnospiraceae* bacterium 7_1_58FAA and *Lachnospira* spp.,^{28, 29} but also includes *R. gnavus*, which, in contrast to the above bacteria, acts to promote allergic development. Our data highlighted that early bacterial colonization accompanied by a high abundance of *R. gnavus* may predispose individuals to the development of allergic rhinitis and asthma.

In the mouse asthmatic model, the results of gastric administration of *R. gnavus* were in good agreement with the clinical investigation involving human infants. Parallel increases in the tissue concentrations of Th2-specific cytokines, the infiltration of Th2 effectors, and the signs of AHR all confirm an immunopathological role for *R. gnavus* in mediating the Th2 inflammatory processes of asthma. These results contrast with those regarding the effect of lipopolysaccharide (LPS) produced by gram-negative bacteria. LPS increases AHR by augmenting the pulmonary levels of Th17 (IL-17 and IL-1) and Th1 (IFN γ and TNF α) cytokines, and subsequently increasing the proliferation of neutrophils and CD8⁺ T cells.³⁰ *R. gnavus* has no effect on the pulmonary concentration of IFN γ and IL-17 in our present study; in addition, *R. gnavus* has been reported to have an inhibitory effect on TNF α secretion.³¹

Overgrowth of *R. gnavus* is associated with colonic infiltration of Th2 lymphocytes and eosinophils. This may have an adverse effect on intestinal health, as seen that high abundance of *R. gnavus* is prevalent specifically in patients with intestinal inflammatory disorders, for instance, Crohn's disease,³² inflammatory bowel disease,^{33, 34} ileal pouchitis,³⁵ ulcerative colitis³⁴ and diverticulitis.³⁶ Additionally, a previous report described poor disease-free survival in pouchitis patients with *R. gnavus* colonization after ileal pouch-anal anastomosis.³⁵ Besides, *Sel1L* (a protein involved in endoplasmic reticulum-associated degradation)-deficiency mice with an abundance of *R. gnavus* in their gut ecosystem developed spontaneous enteritis by causing extensive eosinophilic infiltration.³⁷ Our data also indicated that increases in the numbers of colonic ILC2s, DCs and Th2 cells are one of the causal mechanisms underlying *R. gnavus*-induced eosinophilia and that colonic eosinophils may be recruited to inflammatory organs systematically through the bloodstream and the lymphatic route, thereby developing immune disorders manifesting as allergies.

R. gnavus is a mucolytic bacterium capable of degrading the human colonic mucin MUC2, which is a major form of secretory glycosylated mucin coating the epithelia of the intestine, particularly the colon, so that commensal flora can inhabit colonic mucus without triggering inflammatory response.^{34, 38} The *nan* genes of the *R. gnavus* strain ATCC 29149 have been noted to be responsible for degrading the colonic mucus layer and utilizing mucin-derived glycans.³⁹ This may allow *R. gnavus* to penetrate the mucus layer, be recognized by DCs, and succeed in activating the inflammatory cascades that lead to immune disorders. A future metatranscriptomic study may fulfill the need to identify the possible bacterial gene product(s) responsible for microbial and host immune crosstalk.

We conclude that an abundance and premature colonization of *R. gnavus* have shaped the aberrant structure of the intestinal microbial consortium of respiratory allergic infants. Our *in vivo* and *in vitro* mouse models revealed the ability of *R. gnavus* to drive Th2-dominated inflammation and uncovered a translation-related mechanism explaining the role of *R. gnavus* in pulmonary allergy. This bacterium and its gene products may be the targets of therapies for allergic diseases in the future.

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Figure legends

Figure 1. Lachnospiraceae are predominantly overgrown in allergic infants. (A) Heat map on a black (high)-to-yellow (low) scale showing the relative percent abundance of the bacterial families that constituted the microbial consortium of the discordant twin pairs. Fecal DNA of months 0, 1, 2, 6 and 12 was subjected to 16S rRNA-based PCR-TTGE analysis. Each color-coded square represents the sum of the TTGE bands of each bacterial family revealed from month 0 to 12 relative to the total band numbers of all bacterial families detected in each patient. Arrowhead indicates the bacterial family consistently showing a significant difference between non-allergic and allergic subject. (B) Heat map overview of the time-course change of Lachnospiraceae. The amount of Lachnospiraceae of each indicated month was estimated by calculating the percentage of TTGE band numbers of Lachnospiraceae relative to the total band numbers of all bacterial families in each subject, and the results were symbolized by the color-coded squares. (C) The relative abundance of Lachnospiraceae between allergic and non-allergic subjects was plotted as the mean percentage $\pm$ SEM. *p* values of the Mann-Whitney U test comparing the differences between groups are depicted. (D) TTGE gel images of singleton control infants (Inf 1 and 2) and the discordant twin pair [Twin 1a (non-allergic) and 1b (allergic)]. Fecal DNA harvested at the indicated postnatal months was analyzed by 16S rRNA-primed PCR-TTGE. Each band represents microbe(s) residing in an individual GI tract, and the band corresponding to Lachnospiraceae is indicated.

Figure 2. NGS reveals the specific outgrowth of Lachnospiraceae in allergic twins. Fecal samples collected at month 12 of 6 twin-pairs were subjected to 16S rRNA-based NGS analysis.

(A) Multivariate analysis by PCA based on bacterial family abundances. Scatterplot expressing the frequency and similarity matrices allows differentiation between allergic and non-allergic subjects. (B) PCoA using an individual-by-individual distance matrix distinguishes the diversity of bacterial families between allergic and non-allergic populations. (C) The relative abundance of bacterial families was calculated according to NGS read numbers and was expressed as a percentage. The colors of the rectangles correspond to the five most abundant families (Enterobacteriaceae, Lachnospiraceae, Ruminococcaceae, Streptococcaceae, Enterococcaceae) and five well-known allergy-associated families (Bifidobacteriaceae, Lactobacillaceae, Bacillaceae, Bacteroidaceae, Clostridiaceae). (D) The percent abundance of Lachnospiraceae in allergic twins relative to their non-allergic paired siblings is expressed as a fold-change. The green dots denote subjects with allergic diseases.

Figure 3. High fecal counts of *R. gnavus* are indicative of a risk of allergy development. (A) Heat map represents the fecal counts of bacterial species from Lachnospiraceae. Fecal samples collected at months 0, 1, 2, 6 and 12 were subjected to 16S rRNA-primed PCR-TTGE analysis. Saturated-colored squares signified the mean percentage of the bacterial species from Lachnospiraceae, including *Blautia luti*, *Clostridium lentocellum*, *Clostridium aminophilum*, *Clostridium clostridioforme*, *Clostridium polysaccharolyticum*, *Clostridium spenoides*, *Robinsoniella peoriensis*, *Roseburia hominis*, *Roseburia inulinivorans*, *Ruminococcus gnavus*, *Syntrophococcus sucromutan* and *Tyzzzeria nexilis*. (B) The relative abundance of *R. gnavus* revealed by 16S rRNA PCR-TTGE was compared between groups and the significance was analyzed by Mann-Whitney U. All cases from discordant twins, concordant twins and age-matched singletons were included in this statistical analysis. (C) Three-year cumulative

incidence of allergic disorders was determined including the twin and singleton cohorts with or without *R. gnavus* colonization throughout first year of life using Kaplan-Meier analysis. (D) Proportional analysis estimated the differences in the case positivity rate (*upper*), as well as mean ages of initial acquisition $\pm$ SEM (*lower*), of *R. gnavus* colonization among groups. The significance was calculated by Mann-Whitney U test.

Figure 4. *R. gnavus* triggers airway allergic responses. (A) Experimental design and time schedule depicting the daily progression of the establishment of an OVA-induced mouse asthmatic model (n=4-7/group). *R. gnavus* (10^6 CFU) was inoculated to BALB/c mice by oral gauge. OVA sensitization was performed by intraperitoneal (ip) injection, and OVA challenge was administered via the intranasal (in) route. (B) Taxonomic microbiota composition at the species level, which profiled by 16S rRNA NGS, was analyzed by PCA. (C) Histogram (*upper*) represents the fold changes (mean $\pm$ SEM) in the fecal counts of *R. gnavus* estimated by NGS. Serum levels of total IgE were measured by ELISA (*lower*; mean $\pm$ SEM). (D) AHR in each mouse was examined by whole-body plethysmography upon methacholine-induced bronchoconstriction. The result was expressed as enhanced pause (Penh), which is correlated with the measurement of airway resistance, and plotted as the mean percentage of baseline (PBS) values $\pm$ SEM. (E) Light microscopical examination of H&E-stained mouse lung sections, and a representative histological image of each group is shown. Arrowheads indicate bronchial wall thickening, and arrows specify lymphocytic infiltrates surrounding the peri-bronchial regions. A bar chart demonstrating histological score in the lung tissues of naive and treated groups is shown.

Figure 5. High frequency of *R. gnnavus* induces pulmonary Th2 responses. (A) IFA of mouse lung tissues was conducted using α -IL-4, -IL-5 and -IL-13 antibodies. Positive intracytoplasmic staining is indicative of the presence of Th2-type immune cells. DAPI staining identifies all cell nuclei. Cells expressing these cytokines were counted and plotted as the means of the percentages of IL-4/IL-5/IL-13-positive cells over a total of 100 cells $\pm$ SEM. (B) EPX and mast cell tryptase immunohistochemistry of mouse lung tissues were performed to specify the amounts of eosinophils and mast cells, respectively. The brownish DAB signals indicated the presence of eosinophils (*upper*) and mast cells (*lower*). BV: blood vessel; BE: bronchial epithelium. The numbers of eosinophils and mast cells were counted, and the values representative of mean cell numbers $\pm$ SEM from five fields per section are shown. * p <0.05, ** p <0.005 versus naïve group, Student's t test.

Figure 6. *R. gnnavus* prompts massive infiltration of Th2 cells and eosinophils in the mouse colon. (A) Microphotographs of H&E-stained colon sections are shown and the immune cell infiltrates are indicated. (B) IL-13-specific immunohistochemistry was accomplished in ascending colon tissue sections. (C) Histological score of H&E-stained colon sections was determined and shown as mean $\pm$ SEM. (D) A multiplex bead-based ELISA targeting IL-4, IL-5, IL-13 and IL-17A was conducted on the colon tissue lysate (mean levels $\pm$ SEM. * p <0.05, ** p <0.005 versus naïve, Mann-Whitney U test). (E) ELISA determining total IgE levels in colon tissues was performed. (F) Western blotting assessed the levels of EPX and mast cell tryptase in the colon tissues. Blotting of α -tubulin is provided as an internal control. (G) Quantification of the total numbers of colonic eosinophils and mast cells specified by immunohistochemistry of EPX and mast cell tryptase, respectively. For each section, the cell numbers from five fields were counted (mean $\pm$

SEM. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0007$ versus naïve, Student's t test). A representative EPX-stained colon section from an *R. gnavus*-infected mouse is shown.

Figure 7. *R. gnavus*-mediated the secretion of IL-25, IL-33 and TSLP for efficient activation of ILC2s and DCs. (A) ELISA determining the levels of IL-25, IL-33 and TSLP in the mouse ascending colon was performed and plotted (mean levels $\pm$ SD). (B) IFA of colon tissues was accomplished with antibodies recognizing 33D1, a mature DC marker. (C) Co-staining of CD25 and IL-9 revealed the presence of ILC2s (arrows) that co-expressed both antigens and yielded a yellow signal in the cytoplasm and plasma membrane. (D) Quantification of the total numbers of colonic DCs and ILC2s assessed by IFA. Cell numbers from 3 sections of each group were counted (mean $\pm$ SD). (E). Hypothetical mechanism of *R. gnavus* in the pathogenesis of asthma. *R. gnavus* induces Th2 dominance in colonic mucosal sites via DC recognition coincident with the secretion of IL-25, IL-33 and TSLP by colonic epithelium, resulting in the activation of DCs and ILC2s. Autocrine IL-9 is specifically produced by ILC2s as a survival and growth factor. ILC2-produced IL-13 promotes DC migration to mediate the differentiation of Th2 cells. In the presence of IL-4, possibly released by ILC2s and naïve T cells, facilitates DCs to polarize naïve T cells toward Th2 cells, which successively release additional amounts of IL-4, IL-5 and IL-13. Both ILC2- and Th2-secreted IL-5 plays a major role in eosinophil recruitment, whereas IL-4 and IL-13 produced by Th2 cells are responsible for IgE synthesis and subsequent IgE-dependent mast cell degranulation. *R. gnavus*-mediated Th2 immunity favorably causes colonic infiltration by eosinophils and, to lesser extent, mast cells. These effectors may then enter the circulatory/lymphatic systems along with Th2 cells, moving to the lung to initiate the manifestation of asthma.

Figure 1

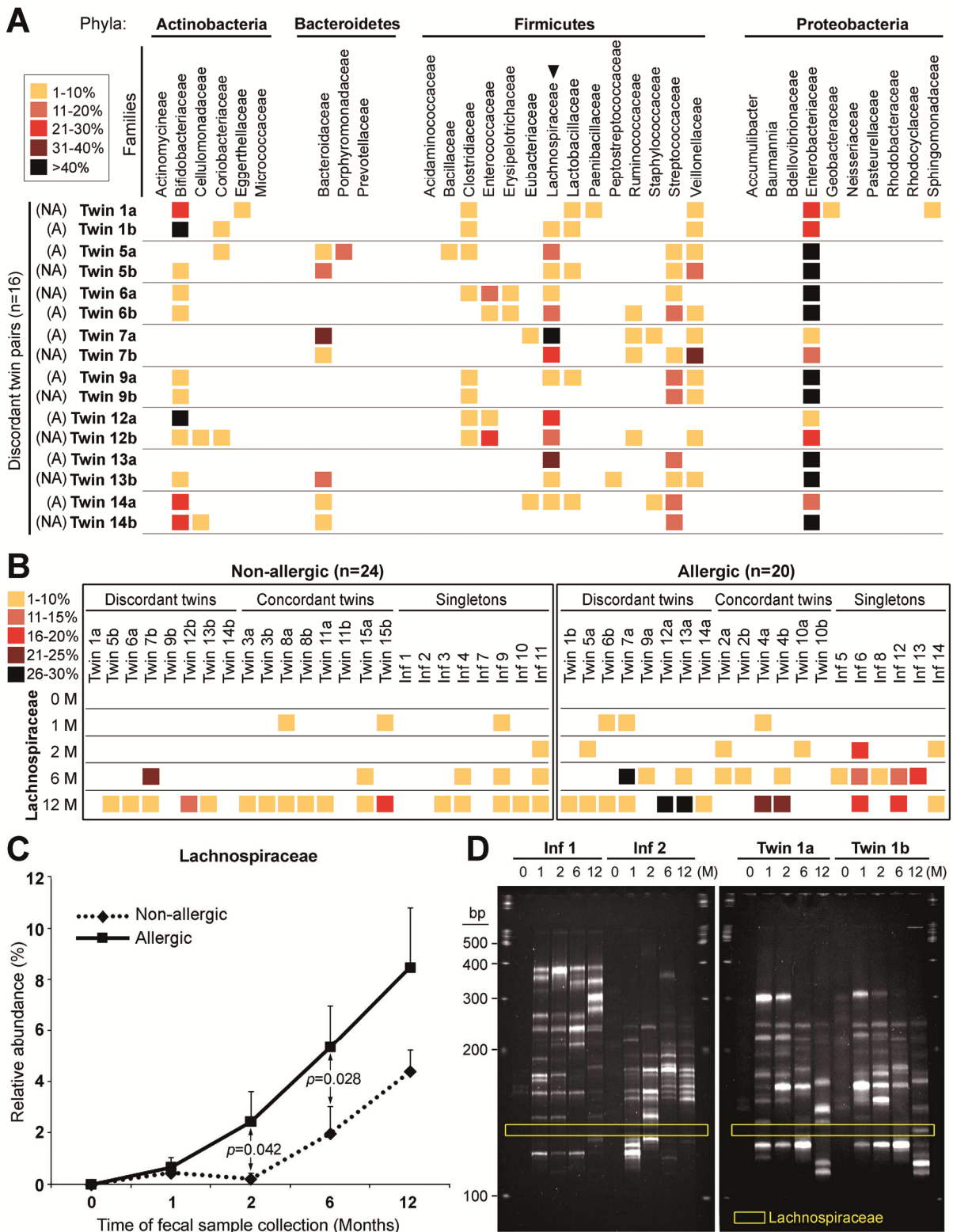


Figure 2

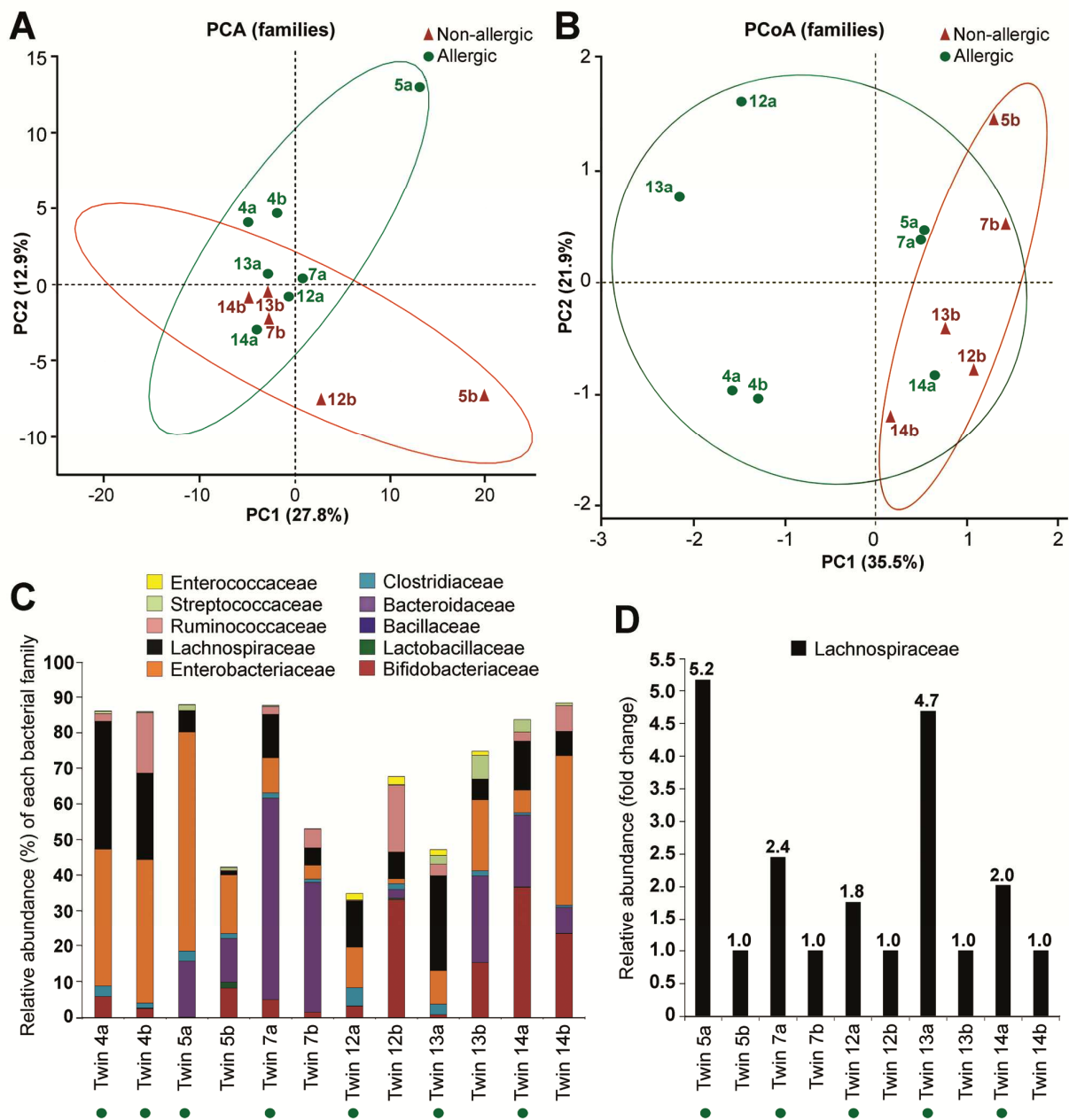


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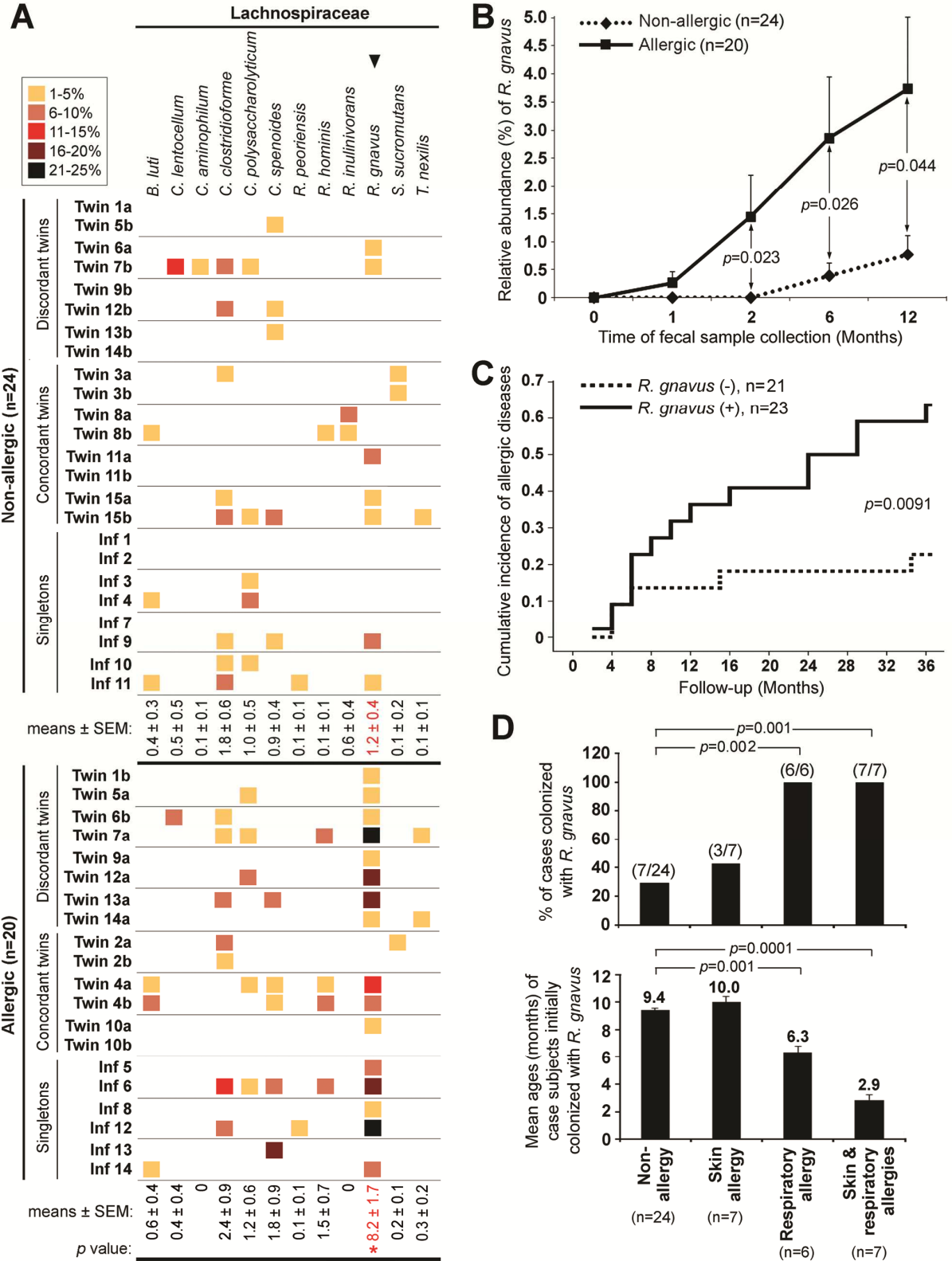


Figure 4

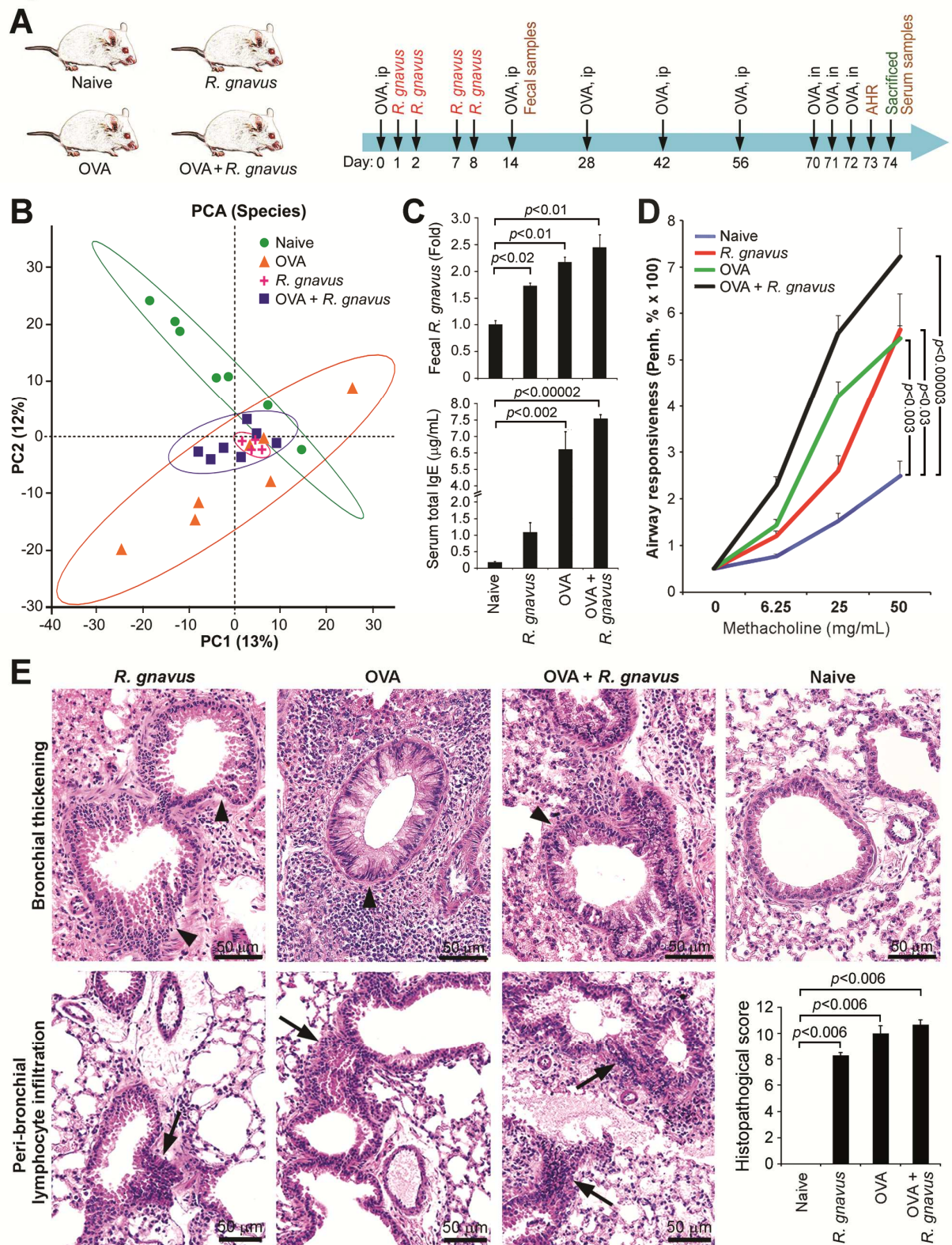


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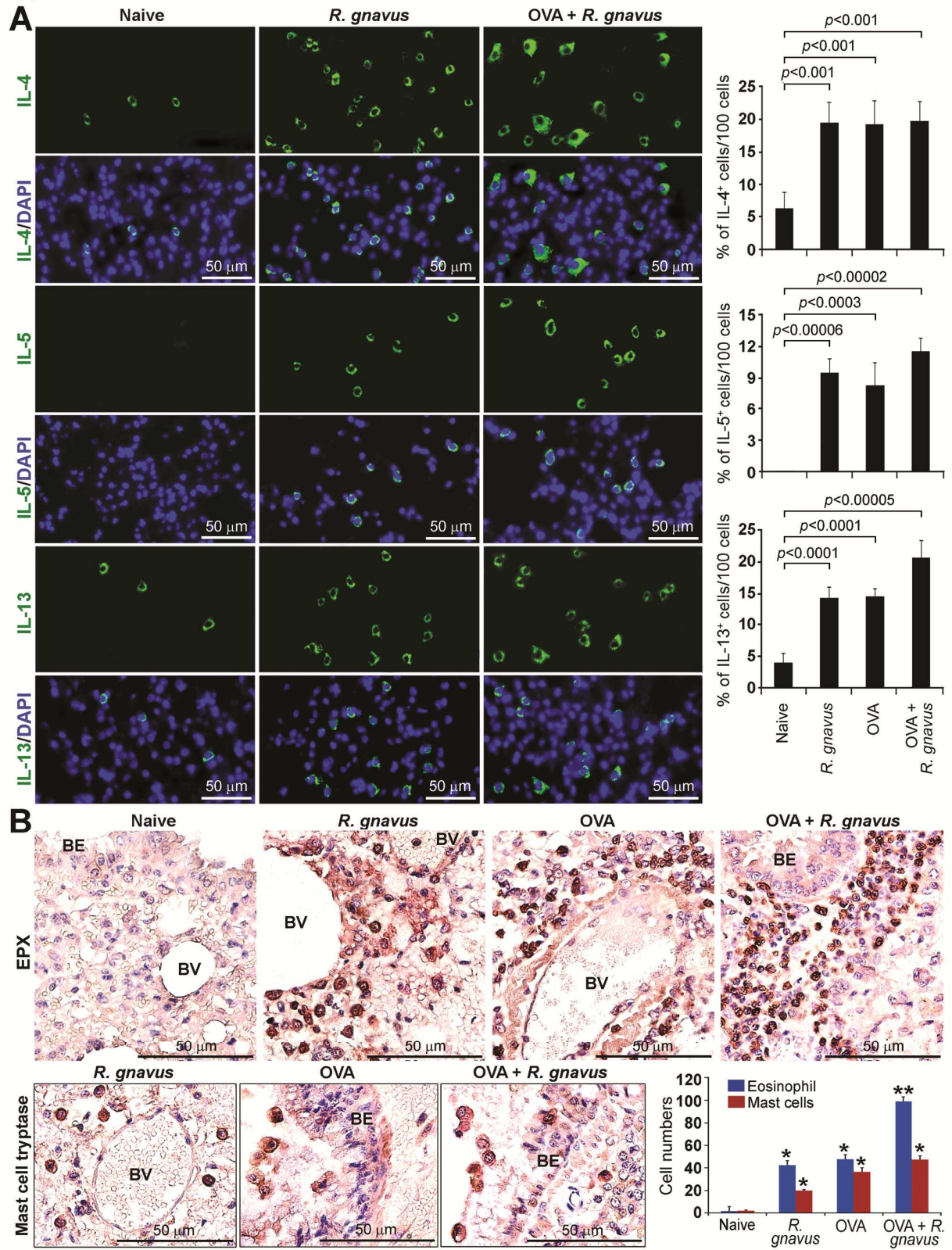


Figure 6

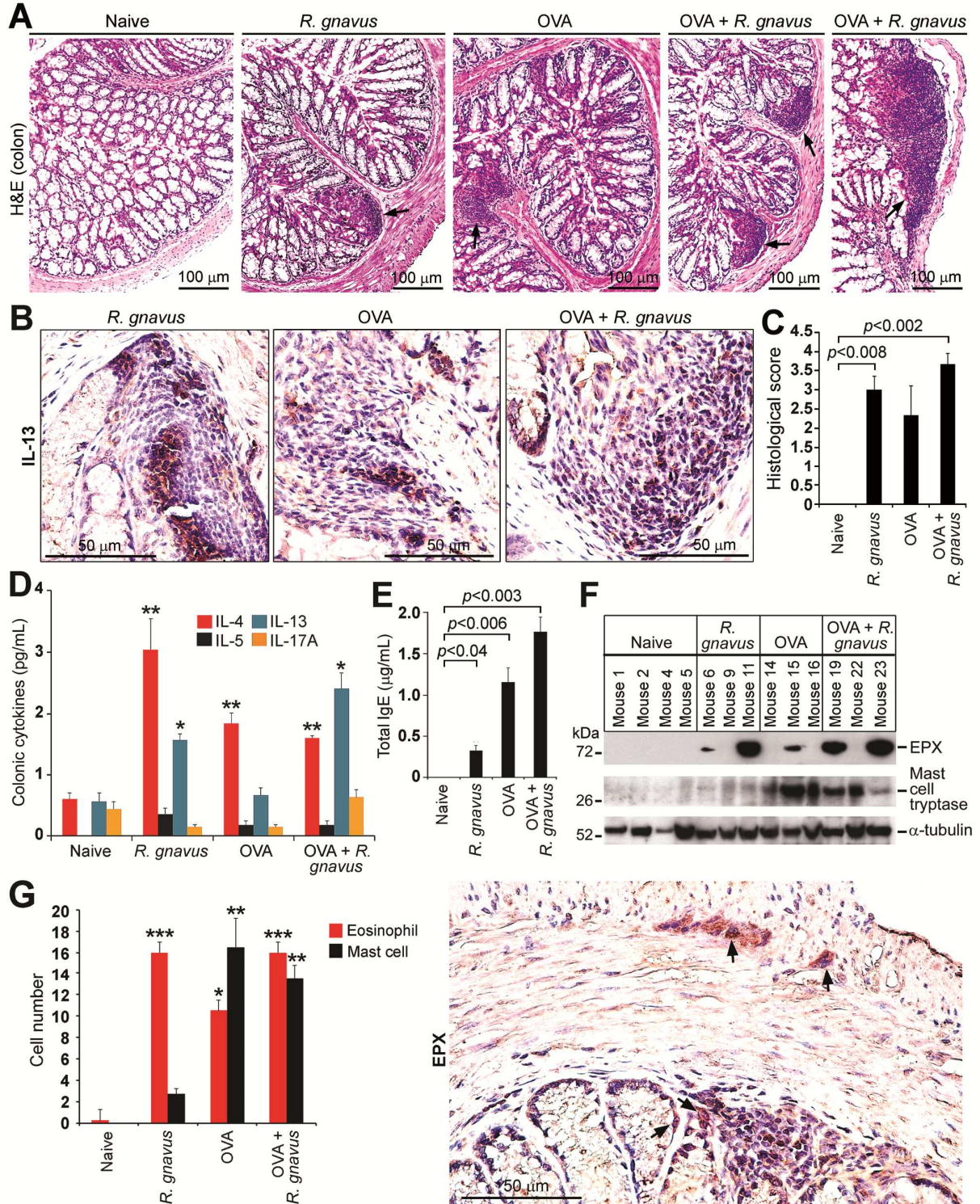


Figure 7

