Commensal bacteria protect against food allergen sensitization

Andrew T. Stefka,1, Taylor Feehley,1, Prabhanshu Tripathi,1, Ju Qiu, Kathy McCoy, Sarkis K. Mazmanian, Melissa Y. Tjota, Goo-Young Seo, Severine Cao, Betty R. Theriault, Dionyssios A. Antonopoulos, Liang Zhou, Eugene B. Chang, Yang-Xin Fu, and Cathryn R. Nagler

Departments of Pathology, Medicine, and Surgery, The University of Chicago, Chicago, IL 60637; Department of Pathology and Microbiology-Immunology, Feinberg School of Medicine, Northwestern University, Chicago, IL 60611; Department of Clinical Research, University of Bern, 3010 Bern, Switzerland; Department of Biology, California Institute of Technology, Pasadena, CA 91125; and Argonne National Laboratory, Argonne, IL 60439

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Switzerland; dDepartment of Biology, California Institute of Technology, Pasadena, CA 91125; and gArgonne National Laboratory, Argonne, IL 60439

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Life-threatening anaphylactic responses to food are an increasingly important public health problem (1). Rising disease prevalence over a short period cannot be explained by genetic variation alone, renewing interest in the role of the environment in shaping allergic sensitization to food (2, 3). First proposed more than 20 years ago, the hygiene hypothesis suggested that societal efforts to reduce exposure to infectious microbes early in life have deprived the immune system of immunoregulatory stimulation necessary for protection against allergic disease (4). As our understanding of the profound influence of commensal microbes on the maturation of the immune system has grown, more recent iterations of this hypothesis have supported the idea that alterations in the composition of the intestinal microbiota induced by environmental factors (e.g., antibiotics, diet, vaccination, sanitation) play a central role in the regulation of allergic sensitization (5–7). In particular, antibiotic use during infancy potently perturbs intestinal bacterial populations and has often been cited as a contributing factor to the rising prevalence of allergic disease (8). However, the mechanisms by which changes in the composition of the intestinal microbiota regulate allergic responses to food remain poorly understood.

The gastrointestinal tract must maintain nonresponsiveness to both an enormous variety of food antigens and the trillions of bacteria that comprise the commensal microbiota (9). Mucosal IgA and regulatory T-cell (Treg) responses induced by commensal bacteria are critical for sustaining the homeostatic host–microbe relationship and preventing intestinal inflammation (10). In addition, recent work has revealed that a heterogeneous population of innate immune cells, known collectively as innate lymphoid cells (ILCs), plays a critical role in integrating signals from the commensal microbiota to maintain homeostasis at epithelial barriers and guide adaptive immunity (11). In this report we show that sensitization to a food allergen is enhanced in mice that have been treated with antibiotics (Abx) or are devoid of commensal microbes (germ free, GF). Selective colonization of gnotobiotic mice demonstrated that the allergy-protective capacity is contained within the Clostridia, a class of anaerobic spore-forming Firmicutes that reside in close proximity to the intestinal epithelium. Reintroduction of a Clostridia-containing microbiota to Abx-treated mice blocks sensitization to a food allergen. Using microarray analysis of intestinal epithelial cells from gnotobiotic mice revealed a previously unidentified mechanism by which Clostridia regulate innate lymphoid cell function and intestinal epithelial permeability to protect against allergen sensitization. Our findings will inform the development of novel approaches to prevent or treat food allergy based on modulating the composition of the intestinal microbiota.

Results

Neonatal Abx Exposure Alters the Commensal Microbiota and Enhances Food Allergen Sensitization. We evaluated the susceptibility of mice to food allergen sensitization by intragastric co-administration of peanut (PN) allergens and the mucosal adjuvant cholera toxin (CT), which induces PN-specific IgE, IgG1, and symptoms typical of systemic allergic hyperreactivity (12). Mice treated with Abx showed marked elevation in PN-specific IgE and...
Ileal Contents. Bacterial diversity, as shown by operational taxonomic unit (97% identity) portion of total reads (compared with no treatment [NT] controls. In the absence of a colonizing microbiota, GF mice displayed grossly enlarged cecal size (Fig. 2E) and spontaneously higher levels of circulating IgE with increasing age (Fig. S1A). Total IgE levels in GF mice were elevated by treatment with CT or PN/CT (Fig. 2C). GF mice colonized with an SPF microbiota (conventionalized) did not show elevated levels of PN-specific IgE (Fig. 2A) or IgG1 (Fig. 2B) or a reduced core body temperature (Fig. 2D) in response to sensitization with PN/CT. The concentration of total IgE detectable in the serum of conventionalized mice was also reduced to levels similar to those seen in SPF mice (Fig. 2C). In addition, conventionalized mice displayed the normal cecal size (Fig. 2E) and bacterial load characteristic of SPF mice (Fig. 2F).

We next examined the ability of selected members of the SPF microbiota to influence susceptibility to allergic sensitization to food. We focused on Bacteroides, Clostridium cluster XIVa, and Clostridium cluster IV, which constitute the numerically predominant taxa in the murine colon (14). Anaerobic cultures of fecal material from our SPF colony yielded Bacteroides uniformis as a representative Bacteroides species. Monocolonization of GF mice with B. uniformis resulted in a bacterial load similar to that seen in SPF and conventionalized mice (Fig. 2F) but did not reduce cecal size (Fig. 2E), rescue the drop in core body temperature in all mice (Fig. 2D), or significantly reduce the PN-specific IgE or IgG1 response seen in GF mice (Fig. 2A and B). To colonize GF mice with Clostridia, we used chloroform-extracted spores isolated from a mixed cecal/fecal sample from a healthy SPF mouse. Sequence analysis showed that this extract was consistently and predominantly composed of members of Clostridium clusters XIVa, XIVb, and IV (Fig. S1B–D). Colonization with this Clostridia consortium protected against sensitization to PN/CT, because levels of PN-specific and total IgE were reduced compared with GF controls (Fig. 2B and C), and no temperature drop was seen at challenge (Fig. 2D). Cecal size in Clostridia-colonized mice was comparable to that seen in SPF mice (Fig. 2E), although the bacterial load measured in feces was significantly lower (Fig. 2F). Collectively, these data suggest that Clostridia play a role in protection against sensitization to a food allergen. We then examined whether the changes in food allergen sensitization induced by neonatal Abx administration (Fig. 1) could be reversed by selectively restoring the intestinal microbial community. In addition, we examined the response to sensitization after recovery from 1 wk of preweaning antibiotic treatment (Abx Recov.) (Fig. S2). PN-specific IgE and IgG1 and total IgE levels were reduced in serum collected at challenge from Abx-treated, PN/CT-sensitized mice that had been conventionalized (Abx conv.), Clostridia-colonized (Abx Clost.), or allowed to recover (Abx Recov.) (Fig. S2A–C), suggesting that restoring a Clostridia-containing microbiota by either fecal gavage or removal of Abx-mediated selection is sufficient to protect against food allergen sensitization. In support of these findings, at termination, the abundance of Clostridia in fecal samples was restored to untreated levels in mice that received fecal gavage (Abx conv. or Abx Clost.) or were allowed to recover (Abx Recov., Fig. S2D and E), although their community structures remained distinct (Fig. S2F).

Clostridia Colonization Activates Innate Immune Genes in Intestinal Epithelial Cells. Several reports suggest that mucosa-associated Clostridia populations have a unique role in the induction of Foxp3+ Tregs and IgA, the two major arms of adaptive mucosal immunity (15–17). The ability of selected indigenous commensal bacteria to activate innate immune signaling in intestinal epithelial cells (IECs) is less well understood. We first confirmed that both conventionalized and Clostridia-colonized mice have

Fig. 1. Neonatal Abx exposure alters the commensal microbiota and enhances food allergen sensitization. Abx treatment was initiated before weaning as described in Methods. (A and B) 3-wk-old mice were sensitized by intragastric administration of PN plus CT (PN/CT, open symbols) or CT only (closed symbols) and challenged on day 35; feces and serum were collected on day 36. Serum concentration of (A) PN-specific IgE and (B) PN-specific IgG1 was measured by ELISA (n = 4–9 mice per group from three independent experiments; each circle represents an individual mouse; bars depict mean and SEM). (C) Bacterial load in the feces or ileal contents of mice treated with Abx compared with no treatment (NT) controls. (n = 4–5 mice per group). (D) Bacterial diversity, as shown by operational taxonomic unit (97% identity) rarefaction curves in Abx-treated mice compared with NT controls: black lines, NT; gray lines, Abx feces; red lines, NT ileal contents; blue lines, Abx ileal contents. (E) Taxonomic classifications for the mice in C represented as proportion of total reads (Methods). *P < 0.05, **P < 0.01, ***P < 0.001 determined by Student t test (B) or one-way ANOVA with Tukey posttest (C).
Clostridia. Microarray analysis showed that 38 genes in IEC from GF controls (Fig. 3≥ exhibited IECs from GF mice and from mice colonized with of sensitization to food allergens, we examined gene expression in they also differentially activate innate immunity. To gain insight and to induce colonic Tregs and fecal IgA (Fig. 3), suggesting that ability both to protect against food allergen sensitization (Fig. 2) of fecal IgA (Fig. 3 of colonization of GF mice with SPF (white), GF (red), or gnotobiotic mice (Conventionalized, blue), B. uniformis (gray), or with a consortium of Clostridia (green) were sensitized (Fig. 4A) in Clostridia-colonized mice (Fig. 3B). IL-22 also protects the intestinal epithelial barrier by promoting mucus secretion by goblet cells (19); the numbers of mucus-producing goblet cells were significantly increased in mice colonized with Clostridia but not in those colonized with B. uniformis (Fig. 4D). Having identified its cellular sources and confirmed a known barrier protective functional activity in our model, we asked whether IL-22 also plays a role in regulating epithelial permeability to protein antigens. Because antigen uptake from the intestinal lumen is the first step in sensitization to a food allergen, we reasoned that Clostridia-induced IL-22 production reinforces the epithelial barrier to reduce intestinal permeability to dietary proteins. To explore this hypothesis, we developed an assay to measure the transient presence of allergen in the blood after intragastric gavage. Several Ara h proteins have been identified as the immunodominant allergens of PN (Anachis hypo-}}
IL-22

B. uniformis, reduced the circulating concentrations of both proteins after gavage.

Clostridia-Induced IL-22 Regulates Allergen Access to the Bloodstream.

To determine whether IL-22 induced by Clostridia gavage is necessary and sufficient to reduce intestinal barrier permeability we used the Abx-depletion model. IL22 expression was significantly increased in the colon of Abx-treated Clostridia-colonized mice (Fig. 5A). Significantly higher concentrations of Ara h 6 were detected in the serum of Abx-treated mice compared with mice that received no treatment (NT; Fig. 5B); similar results were obtained for Ara h 2 (Fig. 5C). Serum Ara h 6 and Ara h 2 were reduced in Abx mice treated with an IL-22-Fc fusion protein (21) or colonized with Clostridia after 1 wk of Abx gavage (Fig. 5B and Fig. 5C), indicating that either Clostridia gavage or exogenous IL-22 is sufficient to reduce the concentration of serum allergen. To demonstrate that Clostridia-induced IL-22 regulates allergen access to the bloodstream, groups of Abx-treated Clostridia-colonized mice were given i.p. injections of a neutralizing antibody to IL-22 (22) or an isotype control before allergen challenge. Serum concentrations of Ara h 6 and Ara h 2 were significantly elevated in Clostridia-colonized mice treated with anti-IL-22 compared with mice treated with an isotype control (Fig. 5C and Fig. 5D), directly linking Clostridia-induced IL-22 production to the regulation of allergen uptake. Anti-IL-22 treatment did not affect Clostridia-mediated induction of Foxp3+ Tregs in the colonic LP (Fig. 5E). Together with the inability of IL-22-Fc to

Fig. 4. Clostridia colonization induces IL-22. (A) Reg3b and Reg3g expression from whole-tissue extracts isolated 4 d after colonization from the small intestine or colon of GF (red), B. uniformis-colonized (gray), or Clostridia-colonized (green) mice. Quantitative RT-PCR data are plotted relative to GF and normalized to Hprt (n = 8–9 mice per group from two independent experiments). (B) IL22 expression in LPL from mice in A. (C) IL-22 production by RORγt+ ILCs and T cells 6 d after colonization, determined by flow cytometric analysis of permeabilized cells (SI Methods; n = 3 mice per group representative of three independent experiments). (D) Representative images and quantification of goblet cells in distal colon of GF, B. uniformis-colonized, and Clostridia-colonized mice 6 d after colonization. n = 3–5 mice per group. (Scale bar, 100 μm.) (E) Serum Ara h 6 and Ara h 2 levels after PN gavage in GF, B. uniformis-colonized, or Clostridia-colonized mice 6 d after colonization (n = 5–12 mice per group from two independent experiments). *P < 0.05, **P < 0.01, ***P < 0.001 by two-way ANOVA with Bonferroni posttest (A and B) or one-way ANOVA with Tukey posttest (C).

Fig. 5. Clostridia-induced IL-22 regulates allergen access to the bloodstream. (A) Expression of IL22 in LPL from neonatal Abx-treated mice without Clostridia colonization, or at 6 d after weaning and colonization. (B) Serum Ara h 6 at indicated time points after PN gavage in NT or Abx mice treated with or without one i.p. injection of IL-22-Fc, or by Clostridia colonization. (C) Serum Ara h 6 at indicated time points after PN gavage in Abx-treated Clostridia-colonized mice injected i.p. with neutralizing antibody to IL-22 or an isotype control. All mice in B and C received PN at 6 d after weaning, and serum levels of Ara h 6 were measured by capture ELISA (n = 5–10 mice per group, pooled from at least two experiments). (D) Expression of Reg3b in whole-tissue extracts from Abx-treated Clostridia-colonized mice treated with neutralizing antibody to IL-22 or an isotype control and sensitized with PMNC (n = 11 mice per group, pooled from four experiments). (E) Concentration of IL-4 in culture supernatants from splenocytes of mice from D (n = 7 mice per group, representative of two experiments). (F) Concentration of PN-specific and total IgE in serum collected 24 h after challenge for mice in D (n = 11 mice per group, pooled from four experiments). (G) Concentration of IL-17 in culture supernatants from splenocytes from mice in D (n = 7 mice per group, representative of two experiments). (H) Concentration of PN-specific IgG in serum collected 24 h after challenge for mice in D (n = 11 mice per group, pooled from four experiments). (I) UniFrac analysis of fecal microbiota throughout the sensitization protocol (n = 4 mice per group). *P < 0.05, **P < 0.01 ***P < 0.001 by two-way ANOVA with Bonferroni posttest (A, B, D) or Student t test (C and G).
induce CD4⁺Foxp3⁺ Tregs in the colonic LP of Abx-treated mice (Fig. S4C), this result suggested that Clostridia-induced IL-22 does not expand the colonic Treg compartment. In addition, the concentration of Ara h 6 in the serum of Abx-treated mice 3 h after gavage with PN/CT was significantly higher than that detected in mice that received PN alone (Fig. S4D) compared with Fig. 5B; P < 0.05, in agreement with the role of adjuvants such as CT in increasing intestinal permeability to luminal antigens (23). Serum Ara h 6 and Ara h 2 were reduced in Abx-treated Clostridia-colonized mice even when PN was administered together with CT (Fig. S4 D and E). To examine whether Clostridia-induced IL-22 production by ILCs regulates allergen uptake, we repeated the Abx treatment/Clostridia colonization in Rag<sup>−/−</sup> mice depleted of ILCs with anti-CD90 antibody (as described in ref. 24). Elevated concentrations of Ara h 6 and Ara h 2 were detectable in the serum of Abx-treated Clostridia-colonized ILC-depleted Rag<sup>−/−</sup> mice compared with mice treated with an isotype control (Fig. S4 F and G). The efficacy of anti-CD90 treatment in depleting IL-22 transcripts in the intestinal LP was confirmed by quantitative PCR (Fig. S4H).

Finally, we examined whether Clostridia-induced IL-22 production in the intestinal LP regulates sensitization to food allergens. Abx-treated Clostridia-colonized mice sensitized with PN/CT as in Fig. 1 and Fig. S2 were treated with anti-IL-22 or isotype control beginning at the 35-d protocol. Examination at sacrifice showed that both intestinal Reg3β expression (Fig. S5D) and goblet cell numbers (Fig. S4I) were significantly reduced in mice treated with anti-IL-22 compared with isotype-treated controls, confirming that IL-22 was effectively neutralized by this treatment protocol. To assess sensitization to food, splenocytes harvested after allergen challenge were restimulated in vitro with anti-CD3 or PN as previously described (12). Oral administration of antigen with CT as a mucosal adjuvant typically induces a Th2 biased response to promote allergic sensitization (12). However, treatment of Abx-depleted Clostridia-colonized mice with anti-IL-22 throughout the course of the sensitization protocol did not result in elevated levels of IL-4 (Fig. S5E) or an increased PN-specific or total IgE response (Fig. S5F), in agreement with the absence of Th2 skewing (IL-13 and IFN-γ were also not significantly changed; Fig. S4 J and K).

Instead we detected significantly elevated production of IL-17 (Fig. S5G), consistent with other reports showing that depletion of innate IL-22 promotes an adaptive Th17 response (25). PN-specific IgG increased in anti-IL-22-treated mice compared with isotype controls (P = 0.09) (Fig. S5H). Interestingly, in keeping with the anti-microbial activity of REG3β, we found that anti-IL-22 treatment altered the composition of the fecal microbiota. UniFrac analysis showed that the microbiota of anti-IL-22-treated mice increasingly diverged from that of their isotype control treated littermates during the 5 wk of treatment (Fig. S5I). Neutralization of IL-22 increased the abundance of Clostridiales throughout most of the sensitization period, whereas the abundance of Bacteroidales remained unchanged (Fig. S4L). Taken together, these data support our hypothesis that mucosa-associated Clostridia play a critical role in regulating sensitization to food allergens.

Discussion

Dietary antigens are absorbed in the small intestine and carried to the mesenteric lymph node by CD103<sup>+</sup> dendritic cells, ultimately generating food antigen-specific Tregs that then migrate to the small intestinal LP and expand to maintain tolerance to dietary antigen (26). Our data suggest a new paradigm in which both antigen-specific tolerance and a bacteria-induced barrier protective response are required to prevent sensitization to food antigens. We identify an innate mechanism through which a predominant component of the normal mucosa-associated commensal microbiota regulates sensitization to food. Using a sensitive capture ELISA to measure the concentration of two immunodominant PN allergens in serum within hours after gavage, we show that Clostridia-induced early innate IL-22 production by RORγ<sup>+</sup> ILCs and T cells reduces access of allergen to the bloodstream. Treatment of Abx-depleted Clostridia-colonized mice with neutralizing anti-IL-22 throughout the course of the PN/CT sensitization protocol induces enhanced production of IL-17 upon restimulation in vitro, in agreement with a role for innate IL-22 in regulating the adaptive Th17 response (25). PN-specific IgG responses increase in anti-IL-22-treated mice but, without Th2 skewing, the IgE response is unaltered. The composition of the microbiota was also transformed by treatment with anti-IL-22. The antimicrobial activity of REG3βγ is directed against Gram-positive bacteria (18). Clostridia induce both A22 and Reg3βγ expression and stably colonize gnotobiotic mice. In anti-IL-22-treated mice, however, increased abundance of Clostridiales correlates with reduced expression of Reg3β, suggesting that this antimicrobial peptide titrates Clostridia abundance in its colonic niche.

We also confirmed that the presence of a Clostridia-containing microbiota is associated with the adaptive expansion of the intestinal Treg compartment and class switching to IgA (16, 17), further reinforcing the immunoregulatory environment required to maintain tolerance to dietary antigen. Indeed, IgA likely contributes to immune exclusion to reduce allergen uptake; note the accelerated kinetics with which Ara h 6 and Ara h 2 reach the blood in Rag<sup>−/−</sup> mice in comparison with WT mice. Increased bacteria-induced luminal IgA and decreased systemic allergen-specific Ig in Clostridia-colonized mice may both be related to reduced systemic allergen uptake. However, Clostridia’s early induction of IL-22 may not be directly involved in the adaptive Treg and IgA phase of the Clostridia-induced protective response, because treatment with an IL-22Fc fusion protein does not result in an expansion of Tregs in the colonic LP. Instead, recent work suggests that microbial metabolites such as short chain fatty acids can regulate the proportions and functional capabilities of Foxp3<sup>+</sup> Tregs in the colonic LP (27–29).

Direct evidence for environment-induced dysbiosis in the increasing prevalence of food allergy among children is just beginning to emerge. Studies have tied urinary levels of the commonly used antibacterial agent triclosan to food and Aero-allergen sensitization (30) and prepartal or neonatal Abx use to cow’s milk allergy in infancy (31). Clostridia are enriched in the colon of both mice and humans (14). Recent work has shown that Clostridia strains isolated from healthy human feces potently induce Tregs in the colonic LP upon transfer to GF mice (17), suggesting our findings may be translatable to human disease. Oral and s.c. allergen-specific desensitization protocols are already showing promise for treating food allergy (32). Our data suggest that tolerance-inducing protocols could be effectively paired with Clostridia enrichment of gut microbiota to potentiate antigen-specific tolerance to prevent or treat food allergy.

Methods

Mice. C57BL/6, C57BL/6Foxp3<sup>gfp</sup> and Rag<sup>−/−</sup> mice on an inbred C57BL/6 background (33) were maintained in an SPF facility at The University of Chicago. Breeding pairs of GF C57BL/6 mice were initially provided by S. Mazmanian. C57BL/6Foxp3<sup>gfp</sup> mice were rederived GF by K. McCoy. All experiments were performed in accordance with the Institutional Biosafety and Animal Care and Use Committees.

Neonatal Abx Treatment. C57BL/6 or C57BL/6Foxp3<sup>gfp</sup> mice were treated with a mixture of Abx, beginning at 2 wk of age, as previously described (12). For the first week, mice were given a daily intragastric gavage with 100 μL of a mixture of kanamycin (4 mg/mL), gentamicin (0.35 mg/mL), colistin (8500 U/mL), metronidazole (2.15 mg/mL), and vancomycin (0.45 mg/mL) (Sigma-Aldrich; MP Biomedical). After weaning, the Abx were administered in the drinking water at 50-fold dilution except for vancomycin, which was maintained at 0.5 mg/mL.

Preparation of 16S rRNA-Based Amplicon Library and Data Analysis. PCR amplifications of the V4 region of the 16S rRNA gene were sequenced on the Illumina MiSeq platform and analyzed using QIIME as described in SI Methods.
Purified PN Extract and Intragastric Sensitization. Purified PN extract was prepared from roasted, unittest PN by a modification of van Wilijk et al., which omitted high-speed centrifugation at 10,000 × g (34). Purified sensitization was performed as in ref. 12 and is described in SI Methods.

Ig Detection, Isolation of Lymphocytes, and Flow Cytometry. Methods were modified from refs. 12 and 33 and are described in SI Methods.

Microbial Isolation and Colonization of GF or Abx-Treated Mice. B. uniformis was isolated from GFP mice by chloroform treatment. Some experimental mice were colonized from live gnotobiotic repository mice; one fecal pellet was homogenized in 1 mL sterile PBS, PBS were allowed to settle, and 100 μL of the liquid phase was administered by gavage. A detailed description of bacterial colonization is given in SI Methods.

Quantitative Real-Time PCR. RNA was prepared from freshly homogenized intestinal tissue or isolated LP cells from the small intestine and colon of GF, B. uniformis, or Clostridia-colonized mice at 4 d after colonization, 6 d after colonization, or Clostridia-colonized mice at 6 d after colonization by shaking (35). Primer sequences for B. uniformis (EUB338) and B. uniformis (Bio-Rad) on the StepOnePlus system (Applied Biosystems). Primer sequences for SJ2, Regβ2, Reg3γ, and Hprt are described in ref. 35. Expression of target genes was normalized to Hprt.

Microarray Analysis. IECs were isolated from colons of GF, B. uniformis-colonized, or Clostridia-colonized mice at 6 d after colonization by shaking tissue fragments at 100 rpm for 20 min at 37 °C in 5 mM EDTA followed by vigorous vortexing and Percoll gradient centrifugation. IECs from three mice were used for each sample in two independent experiments per condition. RNA was isolated as above. Samples were run on a single Illumina MouseRef-8 array at The University of Chicago Functional Genomics Facility. SI Methods provides analysis detail.

In Vivo Antibody Treatment. SPF mice were treated with Abx by gavage for 1 wk before weaning. At weaning, mice were either placed on Abx-containing diet or were colonized with or without anti-CD90.2 treatment at 13150 μg of neutralizing antibody to IL-22 (clone BE11, Genentech) (22) or an isotype control (GP120 106T1D2, Genentech) (36) administered throughout the sensitization protocol by i.p. injection three times per week as previously described (37, 38). To deplete ILCs, 250 μg of anti-CD90.2 (clone 30H12, BioCell) or isotype control (LT-F-2, Bio-XCell) was administered i.p. every 3 d beginning 3 d before weaning, modified from ref. 24. The requirement for Clostridia-induced IL-22 production for the expression of colonic Foxp3+ Tregs was examined by i.p. injection of 500 μg of clone IL-22-OP (eBioScience), as previously described (39).

Assessment of Allergen Uptake. To assess allergen uptake into serum, mice were bled before receiving 20 mg PN by gavage (±5 μg CT). Mice were bled at 4 indicated time points, and PN allergen concentration in serum was measured with capture ELISAs for Ara h 2 or Ara h 6 (Indoor Biotechnologies).

Statistical Analysis. Statistical analysis was performed using GraphPad Prism 5. Normally distributed data were analyzed by one-way ANOVA with Tukey posttest, two-way ANOVA with Bonferroni correction, or Student t test as appropriate to the number of comparisons to be made. Data that did not exhibit a normal distribution were analyzed using the nonparametric Kruskal-Wallis test with Dunn’s posttest.

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Bacterial Colonization. For Bacteroides isolation, full-length 16S rDNA was PCR amplified and sequenced from individual colonies from SPF feces after growth on Anaerobic Neomycin Blood Agar plates (Remel). A strain with >99% sequence identity to Bacteroides uniformis (NR_040866) was further propagated in Schaedler's media. For Clostridia isolation, a chloroform-treated fecal/cecal suspension was prepared with slight modifications from ref. 1. A single fecal pellet plus an equivalent volume of cecal material from the same donor was suspended in 18 mL of prereduced PBS in an anaerobic chamber. Chloroform was added to 3% (vol/vol) concentration; the sample was shaken vigorously before weaning and then incubated at 37 °C for 1 h. Chloroform was removed by percolation with CO2 from a compressed cylinder. This suspension was then administered to an adult germ-free (GF) mouse as a repository for all subsequent colonizations, which were performed as described in the main text. Conventionalization of GF or antibiotic (Abx)-treated mice was performed by suspending a fecal pellet and an equivalent volume of cecal material from the same donor in 18 mL of prereduced PBS in an anaerobic chamber. Chloroform was added to 3% (vol/vol) concentration; the sample was shaken vigorously and then incubated at 37 °C for 1 h. Chloroform was removed by percolation with CO2 from a compressed cylinder. This suspension was then administered to an adult germ-free (GF) mouse as a repository for all subsequent colonizations, which were performed as described in the main text. Conventionalization of GF or antibiotic (Abx)-treated mice was performed by suspending a fecal pellet and an equivalent volume of cecal material from the same donor SPF mouse in 1.5 mL sterile PBS and administering 100 µL of the liquid phase to weanling C57BL/6 or C57BL/6 Foxp3<sup>−/−</sup> recipients by gavage. A second gavage was given 2 wk after weaning in Abx conventionalized and Abx Clostridia sensitization experiments. For Abx recovery experiments, mice received 1 wk of Abx by gavage before weaning and then were left unmanipulated; no additional bacteria were administered. Whenever possible, litters were used to minimize variation in the composition of the microbiota pretreatment. If litters were not used, all pups were related by maternal lineage.

Ig Detection. For antigen-specific assays, plates were coated with PN, and antigen-specific serum antibodies were detected with goat anti-mouse IgG1-HRP (Southern Biotech), rat anti-mouse IgE-AP (23G3, Southern Biotech), or goat anti-mouse IgG1-HRP (Southern Biotech). PN-specific standards were prepared from the serum of sensitized mice by affinity purification on a Serotonin IgA-AP (Southern Biotech). OD was normalized to a commercial mouse IgA standard (BD Biosciences).

Preparation of 16S rRNA-Based Amplicon Library and Data Analysis. Fecal samples were mixed with 0.1 mm zirconia/silica beads in 1.4 mL ASL buffer (Qiagen) in a Mini-Beadbeater (Biospec); DNA was extracted with the QIAamp DNA Stool Mini Kit (Qiagen) using 1/2 inhibitEX tablet and 100 µL Buffer AE for elution. DNA template was amplified using the 515F/806R region of the 16S rRNA gene with primers and cycling conditions modified slightly from ref. 2, specifically adapted for the Illumina MiSeq by adding nine extra bases in the adapter region of the forward amplification primer to support paired-end sequencing. Briefly, the V4 region of the 16S rRNA gene was amplified with region-specific primers that included the Illumina flowcell adapter sequences and 12-base barcodes on the reverse primer. PCR reactions were completed in triplicate, and products were pooled. Each pool was then quantified using Invitrogen's PicoGreen, pooled with equal amounts of DNA per sample, and cleaned using the UltraClean PCR Clean-Up Kit (MoBio). Amplicons were then sequenced on an Illumina MiSeq at the Next-Generation Sequencing Core at Argonne National Laboratory using custom sequencing primers and procedures described in the supplementary methods of ref. 3. Paired-end Illumina reads were joined using fastq-join (E. Aronesty, code.google.com/p/ea-utils). All analysis of high-throughput sequencing data was performed using QIIME v.1.6.0 as previously described (4), except that uclust was used for operational taxonomic unit (OTU) selection (with 97% identity threshold). Where the UniFrac metric was used, principal coordinates analysis of the unweighted pairwise distance matrix is shown; even sampling was performed at a depth of 7,500 (Fig. S2F) or 13,500 (Fig. S5F) sequences per sample. Phylogenetic reconstruction of Clostridia OTUs (Fig. S1C) includes sequences described in ref. 5.

Calculation of Bacterial Load. Bacterial load was determined by quantitative real-time PCR using a protocol modified from ref. 6. Fecal DNA was extracted as for sequencing, and bacterial load was quantified against a standard curve derived from a pCR4TOPO-TA vector containing a nearly full-length copy of the 16S rRNA gene from a member of Porphyrromonadaceae. Bacterial DNA was amplified with universal primers 8F and 338R using the IQ SYBR green supermix (Bio-Rad Life Science) and the StepOnePlus system (Applied Biosystems). The results were normalized to fecal weight.

Isolation of Lymphocytes and Flow Cytometry. Single cell suspensions from spleen, mesenteric lymph node, and lamina propria (LP) were stained with anti-CD4-PE (RM 4.4, eBioscience). Intracellular staining with anti-Foxp3-FITC (FJK-16s, eBioscience) was performed after permeabilization with Foxp3 Fix/Perm buffer (eBioscience). In experiments with gnotobiotic mice, Tregs were analyzed 2 wk after colonization. For innate lymphoid cell (ILC) cytokine analysis, cells were isolated and stained as described in ref. 7. Briefly, mononuclear cells from the colon were isolated using mechanical/enzymatic digestion and Percoll density centrifugation. The isolated cells were incubated for 4 h with 50 ng/mL phorbol myristate acetate and 500 ng/mL ionomycin before fixation and permeabilization using Foxp3 Fix/Perm buffer (eBioscience) and staining for CD3, CD4, TCRβ, NKp46, RORγt, and IL-22. All samples were acquired on FACSCanto or LSRII, and data were analyzed with FlowJo software (TreeStar).
**Microarray Analysis.** Data were normalized using Illumina software and then analyzed with dChip. Genes were considered induced if the detection $P$ value for each sample was $>0.05$ and there was $\geq1.5$-fold increase in expression in either colonized group compared with GF. For verification of results, cDNA was produced as described in Methods followed by quantitative real-time PCR using Taqman primer/probe sets and Master Mix (Applied Biosystems). Results were normalized to Gapdh.

**Mucus Staining and Goblet Cell Quantification.** Tissue from the center of the distal colon was collected from GF, *B. uniformis*, and Clostridia-colonized mice 6 d after colonization or from sensitized Abx-treated, Clostridia-colonized mice with or without anti-IL-22 treatment 24 h after challenge. Tissue was fixed for 4 h at 4 °C in Carnoy’s fixative and then transferred to 70% EtOH for paraffin embedding. Cross-sections of 5-μm thickness were stained with periodic acid Schiff as previously described (8). Slides were imaged on a CRi Panoramic Scan Whole Slide Scanner and analyzed with Panoramic Viewer. Goblet cells were quantified in at least 7 crypts per mouse, average 11 crypts per mouse.

**Cell Culture and Cytokine Measurement.** Single-cell suspensions were prepared from spleens harvested 24 h after challenge from Abx-treated, Clostridia-colonized mice treated with anti-IL-22 or an isotype control and sensitized with PN and CT. Cells were plated at $2 \times 10^5$ cells per well with media alone, 1 μg/mL anti-CD3 (clone 2C11), or 200 μg/mL PN and incubated at 37 °C for 72 h as previously described (9). After 72 h, plates were frozen at −20 °C. Cytokine concentrations in supernatants were measured using Milliplex MAP Mouse Cytokine magnetic bead panel (Millipore) and read on a Bio-Plex machine (BioRad) as previously described (10).

Fig. S1.  (A) IgE concentrations determined by ELISA. n = 7–8 mice per group. (B) Average taxonomic classifications from paired-end Illumina sequencing of fecal 16S rDNA amplicons from 41 Clostridia-colonized mice over a 15-mo period. (C) Phylogenetic reconstruction of OTUs present in feces from the same mice as in B. Among reads classified as Clostridia, all OTUs (defined as 97% identical) with >0.1% abundance were considered for alignment with representative sequences (red text; pynast). OTUs are numbered 1–70, and their abundance is shown as a percentage. (D) Abundance of the 70 OTUs depicted in C in individual mice over time. **P < 0.01 by one-way ANOVA with Tukey posttest.
Fig. S2. (A–D) Groups of mice were not treated (NT, white) or treated with Abx by gavage before weaning and then in the drinking water afterward for 5 wk (as in Fig. 1; Abx, dark gray). Alternatively, groups of mice received Abx by gavage preweaning and then were colonized at weaning with conventional SPF feces and cecal contents (Abx conv., blue), or with a consortium of Clostridia (Abx Clost., green), or were allowed to recover without colonization (Abx Recov., black). Mice in each treatment group were sensitized with PN/CT and challenged on day 35. (A) Concentration of PN-specific IgE, (B) IgG1, and (C) total IgE in the serum of sensitized mice, collected 24 h after challenge, as determined by ELISA. n = 4–10 mice per group. (D) Bacterial load at killing. n = 3–5 mice per group. (E) Taxonomic classifications and (F) UniFrac analysis for Illumina-derived sequences of fecal 16S rDNA from mice in A–D. n = 4–5 mice per group. Each group was housed in two cages, separated by sex. Data shown are pooled from two independent experiments. Bar graphs depict mean and SEM; in A–D and F, each circle represents an individual mouse. *P < 0.05, **P < 0.01, ***P < 0.001 by one-way ANOVA with Tukey posttest (A–D).
Fig. S3. (A) Representative flow cytometry plots for data in Fig. 4B. (B) Percentage of Lti0 (CD3−CD4−RORγt−NKp46−), Lti4 (CD3−CD4+RORγt+NKp46−), and NK-22 (CD3−RORγt+NKp46+) ILC subsets among CD3−TCRβ− cells and the percentage of CD4+TCRβ+ cells in the colonic LP of GF (red) and Clostridia-colonized (green) mice. n = 3 mice per group. Data are representative of three independent experiments.
Fig. S4. (A) Serum Ara h 2 levels after PN gavage in NT or Abx-treated mice and Abx-treated mice colonized with Clostridia or given one i.p. injection of IL-22-Fc (mice from Fig. 5B; n = 6–9 mice per group) and (B) Abx-treated Clostridia colonized mice treated with neutralizing antibody to IL-22 or an isotype control (mice from Fig. 5C; n = 5–8 mice per group). (C) Flow cytometric analysis of Foxp3+ Tregs among CD4+ T cells in Abx mice with or without subsequent IL-22-Fc treatment, Clostridia colonization, or Clostridia colonization plus neutralizing antibody to IL-22 (n = 3–7 mice per group). (D) Serum levels of Ara h 6 and (E) Ara h 2 in Abx-treated mice or Abx-treated mice colonized with Clostridia and challenged with PN plus CT. n = 4 mice per group. (F) Serum Ara h 6 and (G) Ara h 2 after PN gavage in Rag−/− Abx-treated mice colonized with Clostridia and injected i.p. with anti-CD90.2 (ILC depleted) or isotype control. n = 8 mice per group.

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group. (H) Quantitative real-time PCR analysis of IL-22 transcripts from lamina propria lymphocytes isolated after colonization from Abx-treated Clostridium-colonized $\text{Rag}^{−/−}$ mice injected with anti-CD90.2 (ILC depleted) or isotype control (from F and G). Data are plotted relative to AbxClostridia+isotype and normalized to $Hprt$. $n = 8$ mice per group. (I) Quantification and representative images of goblet cells in distal colon of sensitized Abx-treated Clostridium-colonized mice treated with anti-IL-22 or an isotype control at 24 h after challenge (mice from Fig. 5 D–I). $n = 5$ mice per group. (Scale bar, 100 μm.) (J) Concentration of IL-13 and (K) IFN-γ in culture supernatants from splenocytes of mice in Fig. 5 D–I. (L) Abundance of Clostridiales and Bacteroidales for mice from Fig. 5 D–I. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ by two-way ANOVA with Bonferroni posttest (C, D, and H) or Student $t$ test (I).