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Toll-Like Receptor 4 Signaling by Intestinal Microbes Influences Susceptibility to Food Allergy[1]

Mohamed Elfatih H. Bashir, Steve Louie, Hai Ning Shi, and Cathryn Nagler-Anderson[2]

The mechanisms by which signaling by the innate immune system controls susceptibility to allergy are poorly understood. In this report, we show that intragastric administration of a food allergen with a mucosal adjuvant induces allergen-specific IgE, elevated plasma histamine levels, and anaphylactic symptoms in three different strains of mice lacking a functional receptor for bacterial LPS (Toll-like receptor 4 (TLR4)), but not in MHC-matched or congenic controls. Susceptibility to allergy correlates with a Th2-biased cytokine response in both the mucosal (mesenteric lymph node and Peyer's patch) and systemic (spleen) tissues of TLR4-mutant or -deficient mice. TLR4-mutant mice are not inherently impaired in their ability to regulate Th1 cytokine production because they respond to stimulation via TLR9. Coadministration of CpG oligodeoxynucleotides during sensitization of TLR4-mutant mice with allergen plus CT abrogates anaphylactic symptoms and Ag-specific IgE, and results in a Th1-polarized cytokine response. When the composition of the bacterial flora is reduced and altered by antibiotic administration (beginning at 2 wk of age), TLR4 wild-type mice become as susceptible to the induction of allergy as their TLR4-mutant counterparts. Both allergen-specific IgE and Th2 cytokine responses are reduced in antibiotic-treated mice in which the flora has been allowed to repopulate. Taken together, our results suggest that TLR4-dependent signals provided by the intestinal commensal flora inhibit the development of allergic responses to food Ags. ***The Journal of Immunology,*** **2004, 172: 6978–6987.**

Both environmental and genetic factors play a role in susceptibility to allergy. Its increasing prevalence has been linked to a concomitant reduction in childhood infectious disease. The relationship between microbes and allergy is of particular interest in the digestive tract, which is colonized by commensal bacteria. The Th2/T regulatory cell-dominant tone of the gut-associated lymphoid tissue (GALT)[3] is shaped, in part, by antigenic stimulation by these luminal bacteria and protects against the development of intestinal inflammation (reviewed in Ref. 1). By mechanisms only poorly understood, antigenic stimulation of the GALT by the noninvasive commensal flora has a profound impact on lymphocyte responsiveness and on the generation of Th1-biased memory effector cells (2, 3). In the absence of such stimulation, tolerance to orally administered Ags cannot be induced and Th2 hyperresponsiveness ensues (4).

The innate immune system's initial response to microorganisms involves the detection of broad microbial "signatures" by pattern recognition molecules such as Toll-like receptors (TLRs) and influences the nature of the subsequent adaptive response (5, 6). TLR4 has been identified as the receptor for bacterial LPS. Mice of the C3H/HeJ strain have a point mutation in the intracellular domain of TLR4 that blocks LPS signaling and are hyporesponsive to LPS (7, 8). Previous work has shown that repeated oral administration of peanut (PN) extract with the mucosal adjuvant, cholera toxin (CT), induces a systemic allergic response in C3H/HeJ mice (9, 10). The major PN allergen, *Ara h 1*, is an abundant PN protein recognized by most PN-sensitive individuals. Allergic responses to food are a growing threat and are the most common cause of anaphylactic reactions seen in hospital emergency departments (11). In particular, allergic responses to PNs (and other tree nuts) are increasingly prevalent and can have life-threatening consequences.

In this report, we have examined whether the inability to signal via TLR4 influences susceptibility to an allergic response to food. We show that, in three different strains of mice, the inability to signal via TLR4 is associated with an Ag-specific anaphylactic response. A role for the luminal flora in signaling via TLR4 is suggested by the induction of an allergic phenotype in TLR4 wild-type mice by antibiotic decontamination of the gut. Both Ag-specific IgE responses and allergic symptoms are reduced when the flora is allowed to repopulate. Our results suggest that TLR4 signals from the luminal flora influence allergic susceptibility to food.

Materials and Methods

Mice

Three-week-old female C3H/HeJ (H-2^k), C3H/HeOuJ (H-2^k), C3HeB/FeJ (H-2^k), CBA/J (H-2^k), C57BL/10SnJ (H-2^b), C57BL/6J (H-2^b), and B6129PF2/J mice were purchased from The Jackson Laboratory (Bar Harbor, ME). C57BL/10ScNHsd (H-2^b) mice were purchased from Harlan Sprague-Dawley (Indianapolis, IN). B6129-TLR4 knockout mice were generously provided by R. Medzhitov (Yale University, New Haven, CT). All mice were maintained under specific viral pathogen-free conditions at an American Association for the Accreditation of Laboratory Animal Care-accredited facility at Massachusetts General Hospital (Boston, MA).

*Intragastric (i.g.) sensitization with purified PN allergen (*Ara h 1*)*

Crude PN extract was prepared as previously described (10). The major PN allergen, *Ara h 1*, was purified by ammonium sulfate precipitation and anion exchange chromatography, essentially as described (12, 13). Pooled *Ara h 1* containing fractions were dialyzed, sterile-filtered, and stored in

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[3] Abbreviations used in this paper: GALT, gut-associated lymphoid tissue; TLR, Toll-like receptor; PN, peanut; CT, cholera toxin; i.g., intragastric; *Ara h 1*, major PN allergen; ODN, oligodeoxynucleotide; MLN, mesenteric lymph node; PP, Peyer's patch; SFC, spot forming cell.

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aliquots at −80°C until use. The *Ara h 1* preparation used in this study contained <0.4 endotoxin U/mg as assayed by the endochrome *Limulus* amebocyte lysate assay (Charles River Breeding Laboratories, Charleston, SC). Various strains of mice were sensitized by i.g. gavage with two (days 0 and 14) or three (days 0, 14, and 21) doses of 1 mg of *Ara h 1* in PBS with or without 10 μg of CT (List Biological Laboratories, Campbell, CA). In one set of experiments, C3H/HeJ mice sensitized with crude PN extract plus CT were coadministered CpG oligodeoxynucleotides (ODN) (100 μg per mouse) on days 0 and 14. CpG ODN and non-CpG ODN control were generously provided by Coley Pharmaceuticals (Ottawa, Canada). CpG ODN sequence no. 1826 (5′-TCCATGACGTTCCTGACGTT-3′), as well as non-CpG ODN sequence no. 1982 (5′-TCCAGGACTTCTCTCAG GTT-3′) were each synthesized with a nuclease-resistant phosphorothioate backbone (14–16).

All mice were bled weekly, beginning at 1 wk after the initial sensitization with PN allergen. One week after the last sensitization, mice were fasted overnight before two i.g. challenges with PN allergen (2 mg per mouse divided into 2 doses) at 30- to 40-min intervals. The mice were continuously monitored for signs of allergic sensitization. Plasma and serum were harvested from each mouse 30 min after the second challenge with allergen. Plasma samples were used for measuring histamine levels before and after allergen challenge. In each experiment, there were 5–10 mice per group.

Antibiotic treatment of weanling mice

In a series of experiments, groups of mice were treated with a mixture of antibiotics using a modification of a protocol previously described (17). C3H/HeJ and C3HeB/FeJ female mice with litters of 2-wk-old pups were purchased from The Jackson Laboratory. Beginning 1 day after their arrival, groups of C3H/HeJ and C3HeB/FeJ mice (litters and mother) received a daily i.g. gavage with 200 μl of a mixture of antibiotics: kanamycin (4 mg/ml), gentamicin (0.35 mg/ml), colistin (8500 U/ml), metronidazole (2.15 mg/ml), and vancomycin (0.45 mg/ml) (all purchased from Sigma-Aldrich, St. Louis, MO). Antibiotic treatment i.g. was continued for 1 wk, until the day before sensitization with *Ara h 1* plus CT. After oral sensitization with *Ara h 1* plus CT, antibiotics were administered to some groups of mice by addition to the drinking water until sacrifice (2000 μl of antibiotic mixture per 100 ml water). The mice were housed with sterile food, water, and bedding. The efficacy of the antibiotic-treatment protocol was evaluated by periodic bacteriologic examination of feces. One gram of feces per cage was collected in 10 ml of 1% tryptone broth beginning 3 days after the start of antibiotic treatment and on odd-numbered days thereafter. Cages were changed daily. Fecal samples (no older than 24 h) were homogenized, diluted in 1% tryptone broth, and plated on Luria-Bertani agar (Difco, Detroit, MI). Plates were incubated for 18–24 h at 37°C before being counted. Anaerobic growth conditions were created using BBL GasPak pouches (BD Microbiology Systems, Sparks, MD).

The activation marker status and proportions of T and B cells in TLR4-mutant C3H/HeJ and wild-type C3HeB/FeJ mice before and after antibiotic treatment was assessed by flow cytometric analysis. Single cell suspensions were prepared from the spleen, mesenteric lymph node (MLN), and Peyer's patch (PP) of C3HeB/FeJ mice after 0, 1, and 3 wk of antibiotic treatment. Samples of each tissue were pooled from 2 to 3 mice and stained with PE-conjugated anti-CD45R/B220 (RA3-6B2), anti-CD69-PE (H1.2F3),

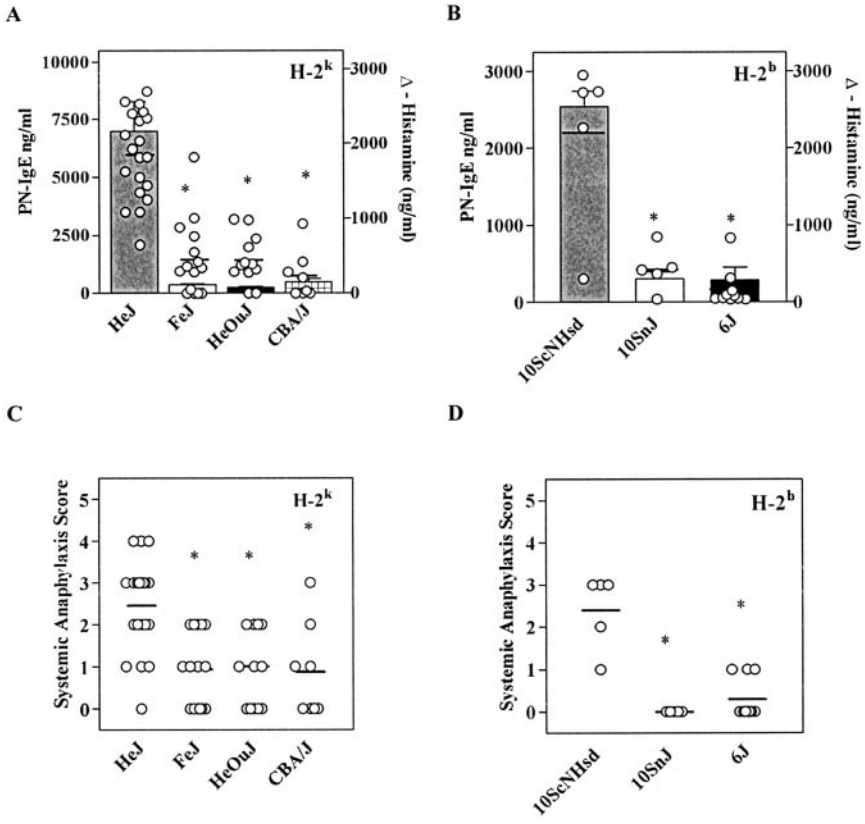

FIGURE 1. Induction of allergen-specific IgE and anaphylactic symptoms is associated with TLR4 deficiency. TLR4-mutant (C3H/HeJ, $n = 20$), -deficient (C57BL/10ScNHsd, $n = 5$), and age-matched TLR4 wild-type (C3HeB/FeJ, $n = 15$; C3H/HeOuJ, $n = 12$; CBA/J, $n = 10$; C57BL/10SnJ, $n = 5$ and C57BL/6J, $n = 5$) mice were sensitized by three i.g. administrations of 1 mg of PN allergen (*Ara h 1*) plus the mucosal adjuvant, CT, on days 0, 14, and 21. On day 28, the mice were challenged with 2 i.g. doses of *Ara h 1* and monitored for 30–40 min before sacrifice. Sera were collected for determination of *Ara h 1*-specific IgE levels by ELISA. Challenge with PN allergen induced much higher levels of both allergen-specific IgE and plasma histamine in TLR4-mutant or -deficient mice than in TLR4 wild-type mice on both the H-2^K (*A*) and H-2^b (*B*) MHC backgrounds. For each group, the mean *Ara h 1*-specific IgE is presented as a bar graph ± SEM. The difference in histamine levels from samples taken before, and 30 min after, allergen challenge was measured using an enzyme immunoassay kit. Individual Δ-histamine levels for each mouse per group are shown (nanograms per milliliter, ○). Allergen challenge after *Ara h 1* plus CT sensitization induces pronounced clinical symptoms of anaphylactic shock in TLR4-mutant mice from the C3H/HeJ strain (*C*) or TLR4-deficient mice from the C57BL/10ScNHsd strain (*D*). Each symbol represents one mouse. The data presented in this figure are pooled from three independent experiments. Statistically significant differences between C3H/HeJ (TLR4-mutant) mice and MHC-matched C3HeB/FeJ, C3H/OuJ, and CBA/J H-2^k wild-type strains (*A* and *C*), and C57BL/10ScNHsD (TLR4-deficient) mice and MHC-matched C57BL/10SnJ and C57BL/6J H-2^b wild-type strains (*B* and *D*) are indicated by an asterisk ($p < 0.001$).

anti-CD45RB-PE (16A), anti-CD25-PE (3C7), anti-CD62L-PE (MEL-14), anti-CD86-PE (GL1), anti-CD44-PE (1M7), and anti-CD40-PE (3/23) (all purchased from BD PharMingen, San Diego, CA). FITC-conjugated rat anti-mouse CD4 was obtained from Caltag Laboratories (Burlington, CA). Analysis was performed on a FACScan flow cytometer using CellQuest software (BD Immunocytometry Systems, San Jose, CA).

*Measurement of PN allergen (*Ara h 1*)-specific IgE and IgA*

Ara h 1-specific IgE and IgA in the sera or gut washings of sensitized mice were measured by ELISA. To prepare gut washes, the small intestine of each mouse was removed and rinsed with 2 ml of ice cold PBS containing the protease inhibitors, aprotinin (10 μg/ml), leupeptin 50 μg/ml, and 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride (25 μg/ml), all purchased from Sigma-Aldrich. The gut washes were then incubated for 30 min at 4°C with occasional mixing before centrifugation at 10,000 $\times$ *g* for 10 min. The supernatants were stored at -70°C.

Sera or gut washes from individual mice were added to *Ara h 1*-coated Maxisorp Immunoplates (Nalge Nunc International, Naperville, IL). *Ara h 1*-specific IgE Abs were detected with biotinylated rat anti-mouse IgE (BD PharMingen) and avidin alkaline phosphatase (Sigma-Aldrich), and developed with *p*-nitrophenyl phosphate (Kirkegaard & Perry Laboratories, Gaithersburg, MD). *Ara h 1*-specific IgA was detected with HRP-goat anti-mouse IgA (Southern Biotechnology Associates, Birmingham, AL) and the substrate, *o*-phenylenediamine (Zymed Laboratories, San Francisco, CA). OD values were converted to nanograms per milliliter of IgE or IgA by comparison with standard curves of purified IgE or IgA (BD PharMingen) by linear regression analysis, and are expressed as the mean concentration for each group of mice $\pm$ SEM. Statistical differences in serum Ab levels were determined using a two-tailed Student's *t* test. A p value < 0.05 was considered significant.

Measurement of cytokine production by PN-stimulated splenocytes in vitro

Splenocytes from individual mice were cultured at 1×10^6 cells/well in the presence or absence of *Ara h 1* (200 μg/ml) in complete DMEM as previously described (10). At 72 h after the initiation of the culture, culture supernatants were collected for the assessment of IL-4, IL-5, IL-13, and IFN-γ production by ELISA. ELISA capture (R4-6A2, IFN-γ; TRFK-5, IL-5, and BVD4-1D11, IL-4) and biotinylated second Abs (XMG1.2, IFN-γ; TRFK-4, IL-5 and BVD6–24G2, IL-4) were purchased from BD PharMingen. Standard curves were obtained using recombinant murine IFN-γ (Genzyme, Cambridge, MA), IL-5, and IL-4 (BD PharMingen) and are expressed in picograms per milliliter $\pm$ SEM. A DuoSet ELISA development kit for the detection of murine IL-13 was purchased from R&D Systems (Minneapolis, MN). IL-13 was assayed according to the manufacturer's instructions and is expressed in picograms per milliliter $\pm$ SEM.

In some experiments, $CD4^+$ T cells, enriched from the spleen, MLN, and PP of individual mice using T cell CD4 subset columns (R&D Systems), were cultured at 2.5×10^5 cells/well in the presence or absence of 200 μg/ml *Ara h 1*. Culture supernatants were collected 48 h after the initiation of the culture for analysis of cytokine production by ELISA.

ELISPOT for Ab-forming cells

The frequencies of IgA- and IgE-producing cells in the spleen, MLN, and PP of sensitized C3H/HeJ and C3HeB/FeJ mice were analyzed by ELISPOT assay. MultiScreen HA 96-well plates (Millipore, Bedford, MA) were coated overnight at 4°C with the *Ara h 1* (10 μg/100 μl). The plates were blocked with PBS, 10% FCS, and washed three times with PBS-0.05% Tween. Spleen (10^5 per well), MLN (10^4 per well), or PP (10^4 per well) cells were plated in complete DMEM and incubated overnight at 37°C. The plates were then washed again before the addition of HRP-conjugated goat anti-mouse IgA (2 μl per 1 ml) or biotinylated rat anti-mouse IgE (4 μg/ml) and overnight incubation at 4°C. IgE spots were detected by HRP-streptavidin (1 μl/ml; Zymed Laboratories). The spots were visualized by the addition of the substrate, *o*-phenylenediamine. After 10 min, the reaction was stopped by washing the plates with PBS-Tween. The number of spots per well was counted using an Eclipse TE 2000-S microscope (Nikon, Melville, NY).

Assessment of hypersensitivity reaction

Anaphylactic symptoms were evaluated for 30–40 min after the second challenge dose using the following scoring system (9, 10): 0, no symptoms; 1, scratching and rubbing around the nose and head; 2, puffiness around the eyes and mouth, diarrhea, pilar erecti, reduced activity, and/or decreased activity with increased respiratory rate; 3, wheezing, labored respiration, and cyanosis around the mouth and the tail; 4, no activity after prodding or tremor and convulsion; and 5, death.

Measurement of plasma histamine levels

One day before, and 30 min after, the last allergen challenge plasma samples were collected to measure histamine levels using an enzyme immunoassay kit (ImmunoTech, Marseille, France; Refs. 9 and 10). Histamine concentrations were calculated by comparison with a reference standard curve provided by the manufacturer.

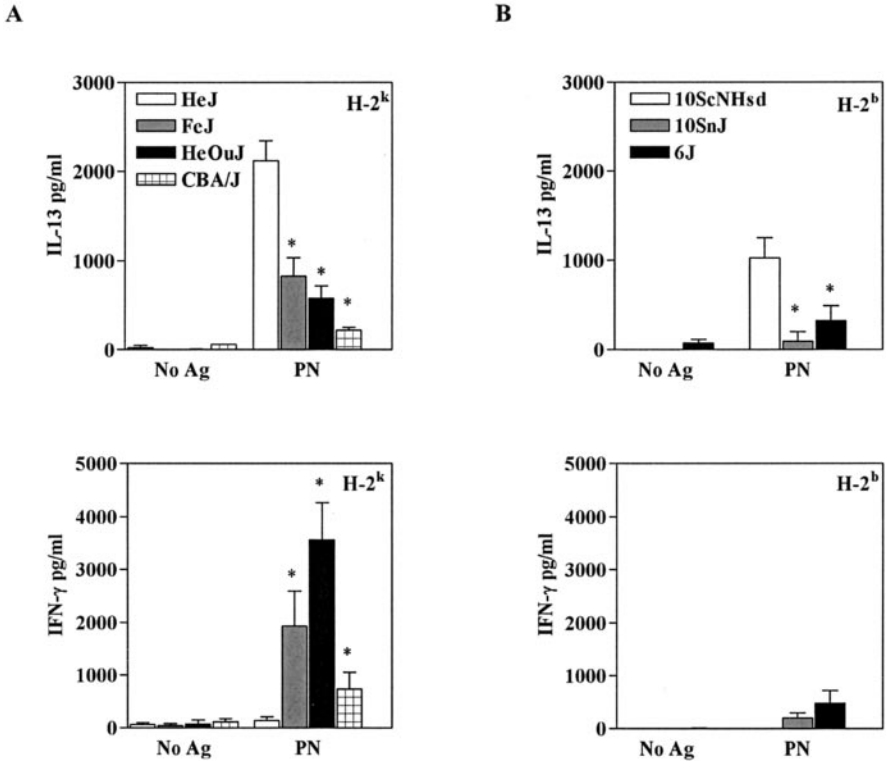

FIGURE 2. TLR4-mutant or -deficient mice make high levels of IL-13 and little IFN-γ, in response to oral sensitization with *Ara h 1*. Splenocytes derived from each sensitized TLR4-deficient mouse of both the C3H (*A*) and C57BL/10 (*B*) strains and their TLR4 wild-type, MHC-matched controls (all shown in Fig. 1) were restimulated in vitro with *Ara h 1* (200 μg/ml, PN) or left untreated (no Ag). IL-13 and IFN-γ secreted into 72-h culture supernatants was measured by ELISA. Results shown are the mean picograms per milliliter of cytokine secreted into the supernatants of cultures prepared from individual mice, and assayed in triplicate $\pm$ SEM. $*$, $p < 0.001$ compared with C3H/HeJ strain (*A*); $*$, $p < 0.001$ compared with C57BL/10ScNhsd strain (*B*).

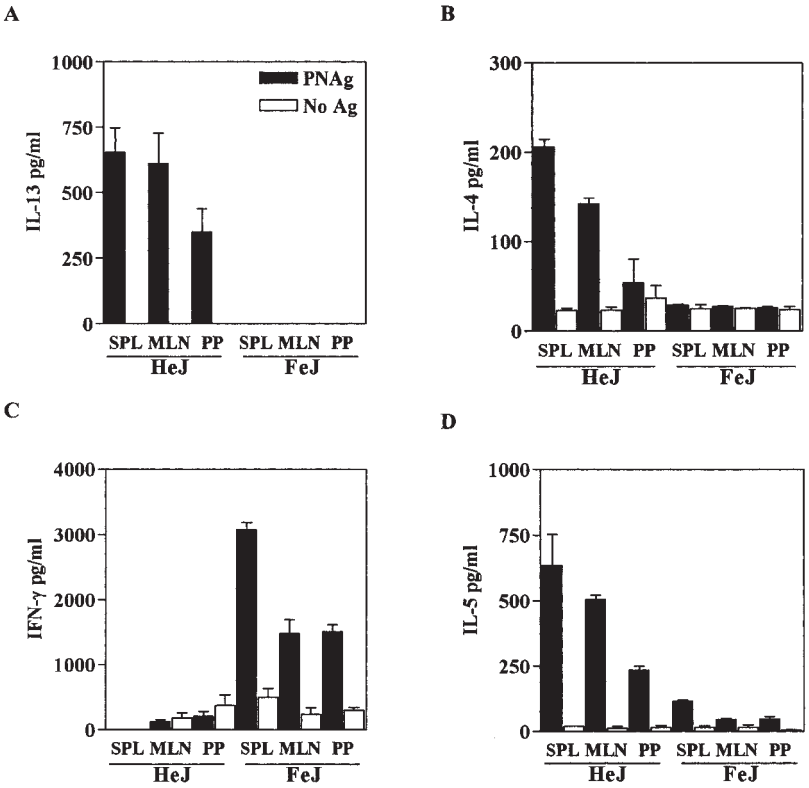

FIGURE 3. $CD4^+$ T cells enriched from both mucosal and systemic tissues of allergen-sensitized TLR4-mutant mice make a Th2-biased cytokine response upon restimulation in vitro. TLR4-mutant C3H/HeJ ($n = 5$) and wild-type C3HeB/FeJ ($n = 5$) mice were sensitized by two i.g. administrations of 1 mg of *Ara h 1* plus CT on days 0 and 14. Mice were challenged on day 21 with two oral doses of *Ara h 1*, as described in the legend to Fig. 1, and sacrificed. $CD4^+$ T cells were enriched from the spleen, MLN, and PP of each strain, pooled for each group, and restimulated in vitro with *Ara h 1*. IL-13 (*A*), IL-4 (*B*), IFN-γ (*C*), and IL-5 (*D*) was measured in 48 h culture supernatants by ELISA. The results shown are the mean ± SEM of triplicate assays.

Results

TLR4 mutation is linked to susceptibility to allergy

To determine whether the susceptibility of C3H/HeJ mice to the development of food allergy is linked to this strain's mutation in TLR4, the PN allergen *Ara h 1* plus CT was administered i.g. to C3H/HeJ mice, the closely related C3H/HeOuJ and C3HeB/FeJ strains, and an unrelated H-2^k haplotype strain, CBA/J. We also examined susceptibility to an allergic response to *Ara h 1* in mice with a different (H-2^b) MHC background. The response of TLR4-deficient C57BL/10ScNHsd mice was compared with that of the related C57BL/10SnJ strain as well as another H-2^b strain, C57BL/6. On both MHC backgrounds, only the TLR4-mutant or -deficient strains mounted an allergen-specific IgE response, which correlated with elevated plasma histamine levels (Fig. 1, *A* and *B*) and the development of anaphylactic symptoms (Fig. 1, *C* and *D*).

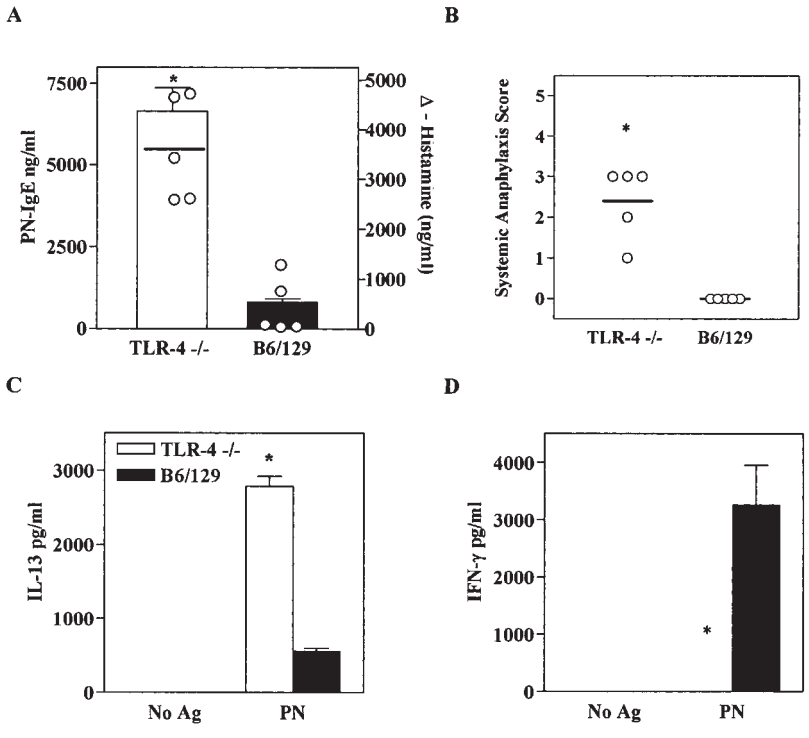

FIGURE 4. TLR4 knockout mice are also susceptible to food allergy. B6129F2 TLR4 knockout ($n = 5$) and control B6129PF2/J ($n = 5$) mice were sensitized with *Ara h 1* plus CT on days 0, 14, and 21 and challenged at day 28. *A*, Challenge with PN allergen induced much higher levels of both allergen-specific IgE and plasma histamine in TLR4 knockout mice than in TLR4 wild-type controls. For each group, the mean *Ara h 1*-specific IgE response is presented as a bar graph ± SEM. Individual Δ-histamine levels for each mouse/group are shown (nanogram per milliliter, ○). *B*, High levels of PN-specific IgE correlated with anaphylactic symptoms. Splenocytes were restimulated in vitro with *Ara h 1* (200 μg/ml, PN) or left untreated (no Ag). IL-13 (*C*) and IFN-γ (*D*) were assayed in 72-h culture supernatants by ELISA. Each bar graph represents the average cytokine secretion from five individual mice and is expressed as the mean ± SEM. Statistically significant differences between the $TLR4^{-/-}$ mice and the B6129 wild-type controls are indicated by an asterisk ($p < 0.001$).

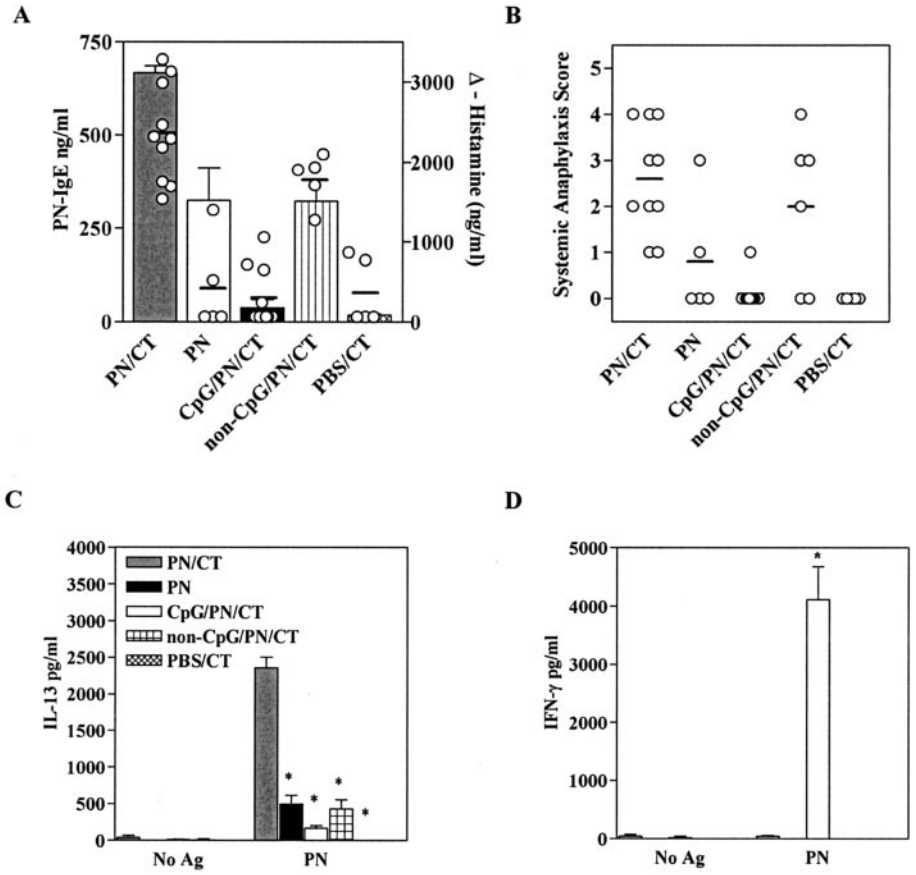

FIGURE 5. Coadministration of CpG ODN during PN/CT sensitization of TLR4-mutant mice abrogates allergic symptoms and PN-specific IgE and results in a Th1-polarized cytokine response. Groups of PN plus CT-sensitized C3H/HeJ mice were treated with either CpG or non-CpG ODN. At day 21 after the initial sensitization, mice were challenged with two i.g. doses of PN extract, monitored for 30–40 min and sacrificed. Sera from two independent experiments (each with $n = 5$ per group) were assayed as described in the legend to Fig. 1 and are expressed as the means ± SEM (*A*). Individual Δ-histamine levels before and after challenge are presented for each mouse per group (nanograms per milliliter, ○). *B*, Clinical anaphylactic symptoms were abrogated by treatment of PN-sensitized mice with CpG ODN. Each symbol represents one mouse. Production of IL-13 (*C*) and IFN-γ (*D*) by splenocytes derived from each group, restimulated in the presence (PN) or absence (no Ag) of 200 μg/ml PN extract was assayed in 72-h culture supernatants by ELISA. Each bar graph represents the average cytokine secretion from 5 to 10 individual mice and is expressed as the mean ± SEM. ∗, $p < 0.001$ compared with PN/CT-sensitized mice.

However, MHC background clearly contributed to a strain's ability to make an allergen-specific IgE response. The TLR4-mutant C3H/HeJ strain made the highest levels of allergen-specific IgE and demonstrated the greatest susceptibility to anaphylactic symptoms. Interestingly, all of the H-2^k haplotype strains showed some tendency toward allergic symptoms and elevated plasma histamine levels. This may be attributable to a marked allergen-specific IgG1 response that was similar in all three C3H strains (data not shown). By contrast, on the H-2^b background, the C57BL/10ScNHsd TLR4-deficient mice made higher levels of both allergen-specific IgE and IgG1 compared with their TLR4 wild-type H-2^b controls. There was no evidence for anaphylactic symptoms or elevated plasma histamine levels in the TLR4 wild-type H-2^b strains.

Susceptibility to allergy correlates with a Th2-biased cytokine response in both mucosal and systemic sites

In a previous study using the C3H/HeJ model, we found that induction of PN-specific IgE by i.g. administration of PN plus CT correlated with the presence of Ag-specific T cells making IL-13 (10). When T cells from allergic mice were restimulated with PN in vitro, large amounts of IL-13, and little or no IFN-γ, was detectable in the culture supernatants. This is also evident from the data presented in Fig. 2. T cells from C3H/HeJ mice made high levels of allergen-specific IL-13 and low levels of IFN-γ. Unlike the allergic C3H/HeJ mice, nonallergic C3HeB/FeJ and C3H/HeOuJ mice made significantly lower levels of IL-13 and higher levels of allergen-specific IFN-γ. Indeed, allergen-specific IL-13 levels inversely correlated with the IFN-γ response (Fig. 2*A*). The correlation of allergic symptoms and IgE with a high IL-13 response and little or no IFN-γ was also seen in the TLR4-deficient C57BL/10ScNHsd strain (Fig. 2*B*).

In our model, systemic allergic symptoms are induced by mucosal sensitization with PN, using CT as a mucosal adjuvant. Previous work with other Ags has shown that the efficacy of CT as a mucosal adjuvant is optimized when two oral doses of Ag plus CT are administered at weekly intervals (18). Accordingly, to examine both mucosal and systemic cytokine responses in TLR4-mutant (C3H/HeJ) and wild-type (C3HeB/FeJ) mice, $CD4^+$ T cells were enriched from the spleen, MLN, and PP of groups of mice sensitized with two doses of PN plus CT and challenged 1 wk later. Fig. 3 shows that a striking Ag-specific Th2 bias is apparent in the response of $CD4^+$ T cells enriched from both peripheral and mucosal tissues of TLR4-mutant mice. $CD4^+$ T cells made high levels of IL-4 and IL-13, the Th2 cytokines necessary to support switching to IgE (Fig. 3, *A*, *B*, and *D*) and little IFN-γ (Fig. 3*C*). By contrast, $CD4^+$ T cells from the nonallergic, C3HeB/FeJ mice produced high levels of IFN-γ, but little or no Th2 cytokines.

TLR4 knockout mice are also susceptible to food allergy

As is apparent in Figs. 1–3, we, and others (19), have noted genetic differences in susceptibility to allergy and the Th1/Th2 cytokine bias in the response to PN plus CT. To rule out a potential contribution of other background genes to susceptibility to allergy in the naturally occurring TLR4-mutant and -deficient mice, we examined the response to PN plus CT in TLR4 knockout mice (created by gene targeting) and their congenic controls. Fig. 4 clearly shows that TLR4 $^{-/-}$ mice (on a C57BL/6 × 129 background) are highly susceptible to an allergic response to PN plus CT. As in the other TLR4-deficient strains, high levels of PN-specific IgE correlated with elevated changes in plasma histamine levels (Fig. 4*A*) and systemic anaphylactic scores (Fig. 4*B*). The PN-specific IgE

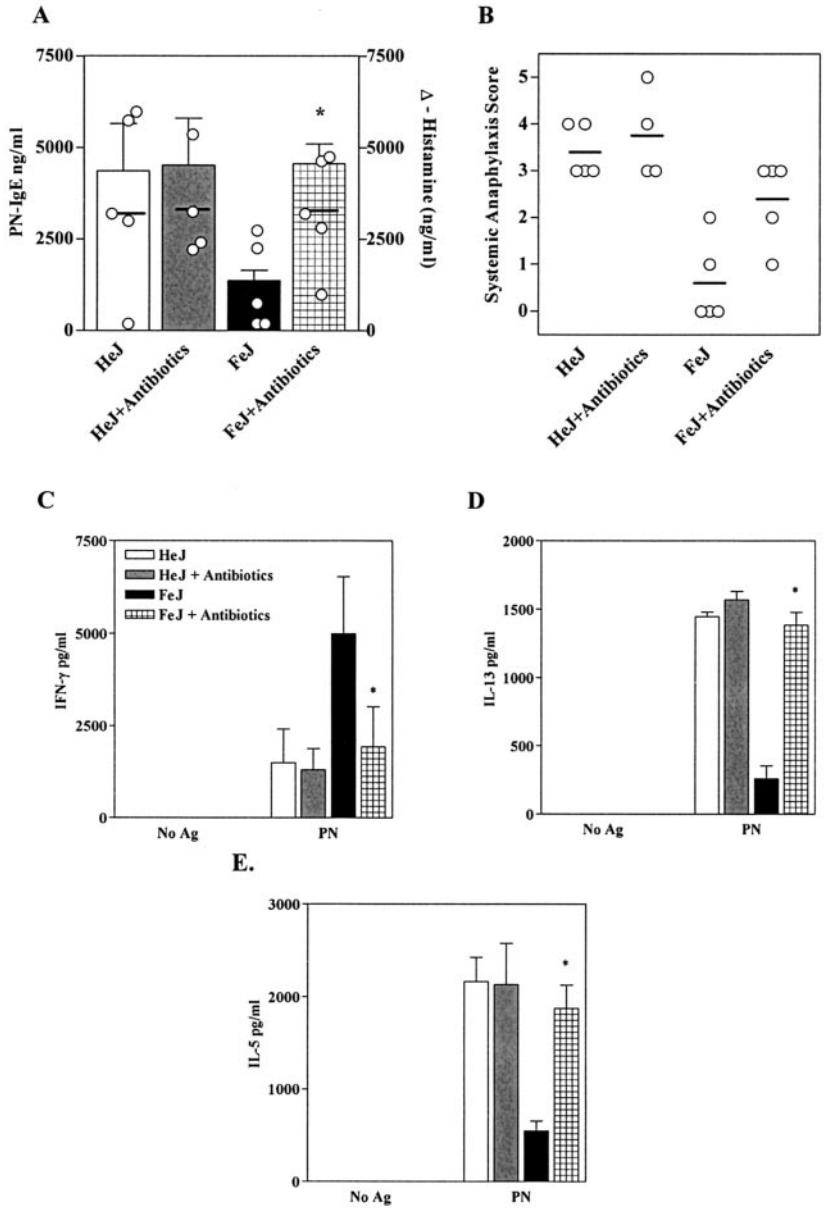

FIGURE 6. Decontamination of the gut by antibiotic treatment of weanling C3HeB/FeJ (TLR4 wild-type) mice prevents TLR4 signaling by the luminal flora and induces an allergic response to *Ara h 1* plus CT. Beginning at 2 wk of age, groups of C3HeB/FeJ (TLR4 wild-type) and C3H/HeJ (TLR4 mutant) mice were untreated, or were given a mixture of antibiotics (see *Materials and Methods*) i.g. for 7 days before PN/CT sensitization. Antibiotics were then administered in the drinking water until sacrifice at day 28. *A*, *Ara h 1*-specific IgE (bar graphs, $n = 5$) and plasma Δ-histamine levels (○) in antibiotic-treated, TLR4 wild-type C3HeB/FeJ mice are similar to those in TLR4-mutant C3H/HeJ mice and correlate with anaphylactic symptoms. *B*, Splenocytes from TLR4 wild-type (C3HeB/FeJ) and TLR4-mutant (C3H/HeJ) mice, either treated or untreated with antibiotics, were stimulated in the presence (PN) or absence (no Ag) of *Ara h 1* (200 μg/ml) for 72 h. Concentrations of IFN-γ (*C*), IL-13 (*D*), and IL-5 (*E*) in the culture supernatants were determined by ELISA. Results shown are the mean ± SEM of triplicate samples. The data shown are representative of three independent experiments with similar results. (*C–E*). *, $p <$ 0.001 compared with the corresponding untreated control.

response was associated with a Th2-biased (IL-13) cytokine response to in vitro restimulation by spleen cells taken from allergic TLR4$^{-/-}$ mice (Fig. 4*C*). By contrast, spleen cells from congenic C57BL/6 × 129 controls made high levels of IFN-γ and little IL-13 when restimulated with PN Ag in vitro (Fig. 4, *C* and *D*).

TLR9 signaling in TLR4-mutant mice abrogates allergic symptoms and PN-specific IgE and results in a Th1-polarized cytokine response

To determine whether cytokine (e.g., IFN-γ) responses to TLR signaling are selectively impaired in TLR4-mutant mice, we examined whether stimulation via another TLR, namely TLR9, altered the PN-specific IgE response typically induced in sensitized mice. TLR9 recognizes the hypomethylated CpG motif present in bacterial DNA. Fig. 5*A* shows that no PN-specific IgE was induced in C3H/HeJ mice that were coadministered CpG ODN during their sensitization with PN plus CT. The PN-specific Ig response in CpG-treated mice was characterized by the production of PN-specific IgG2a (data not shown). CpG ODN coadministration also dramatically reduced the anaphylactic symptoms and elevated plasma histamine levels induced by PN/CT. The abrogation of anaphylactic symptoms by coadministration of CpG ODN in our model is in keeping with the reported efficacy of CpG ODN-based immunotherapeutics in both murine models of allergic disease and the treatment of allergic patients (reviewed in Ref. 20). Interestingly, i.g. administration of non-CpG ODN also led to a partial reduction of the PN-specific IgE response and a concomitant drop in plasma histamine levels and anaphylactic symptoms. In a previous report, oral administration of the same non-CpG ODN sequence (no. 1982) led to immunostimulatory effects not seen when this sequence was used in vitro or administered parenterally (15). The immunostimulatory effect of non-CpG ODN is apparently associated with a response to the phosphorothioate backbone induced when this adjuvant is delivered to a mucosal surface.

To examine whether i.g. treatment with CpG ODN biased the development of PN-specific T cells, splenocytes from PN plus CT-sensitized mice treated with CpG ODN, non-CpG ODN, or without treatment, were restimulated in vitro. The PN-specific IL-13 response characteristic of PN/CT-sensitized mice was abrogated in the CpG-treated group (Fig. 5*C*) and correlated with the absence of

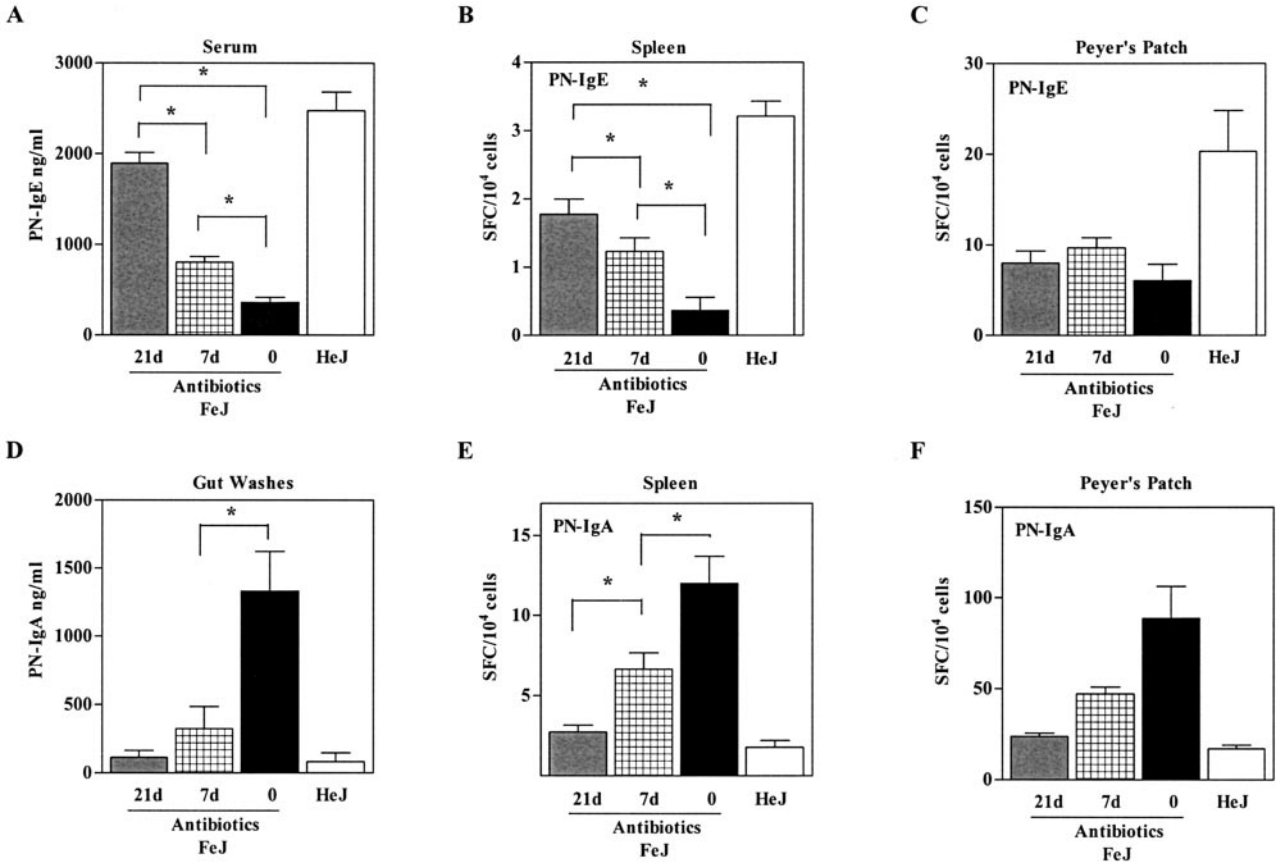

FIGURE 7. Allergen-specific IgE is reduced in flora-reconstituted, antibiotic-treated mice. As in the experiment shown in Fig. 6, groups of 2-wk-old C3HeB/FeJ (TLR4 wild-type) mice were untreated, or were given a mixture of antibiotics i.g. for 7 days before PN/CT sensitization. In this experiment, one group received antibiotics for 7 days only (7 day, $n = 15$, checkered bars). Another group continued to receive antibiotics in the drinking water until sacrifice (an additional 14 days, 21 day, $n = 10$, ▨). Untreated C3HeB/FeJ mice received PBS i.g. for the first 7 days (0, $n = 10$, ■). A group of TLR4-mutant C3H/HeJ mice ($n = 10$, □) were sensitized as a positive control. The microbial content of fecal samples was assessed every other day throughout the antibiotic treatment period. In the 7-day group, bacterial counts reached values close to those seen in the feces of untreated mice between 5 and 9 days after cessation of antibiotic treatment. Serum PN-specific IgE was analyzed by ELISA (*A*). The production of PN-specific IgE (*B* and *C*) and IgA (*E* and *F*) by spleen and PP cells in response to ex vivo stimulation with *Ara h 1* was measured by ELISPOT analysis. PN-specific IgA in gut washes was measured by ELISA (*D*). Each assay was performed in triplicate and is expressed as the mean ± SEM. The results shown are pooled from two separate experiments. Statistically significant differences between treatment groups are indicated by an asterisk ($p < 0.001$).

PN-specific IgE. IFN-γ (Fig. 5*D*) was the major cytokine produced by PN-stimulated spleen cells from the CpG-treated group. CpG coadministration induced a marked PN-specific IFN-γ response in both the presence and absence of CT. The PN-specific IL-13 response was also significantly reduced in mice that received PN/CT with non-CpG ODN, but no IFN-γ response was induced in the absence of the CpG motif (15). Taken together, these results indicate that TLR4-mutant C3H/HeJ mice are not inherently impaired in their ability to regulate IFN-γ and IL-13 production.

Antibiotic treatment induces an allergic response in TLR4 wild-type mice

The data presented thus far suggest that susceptibility to food allergy is linked to an inability to signal via TLR4. We postulated that, in TLR4-mutant or -deficient mice, the microenvironment of the GALT is altered by the absence of immunoregulatory (or Th1-polarizing) signals normally provided by LPS on the luminal commensal flora. Unchecked, the GALT's tendency toward a Th2-biased response is skewed in the direction of allergy. If this hypothesis is correct, removal of the source of the TLR4 ligand from normal mice should induce an allergic phenotype similar to that seen in TLR4-mutant mice.

Both TLR4 wild-type C3HeB/FeJ and TLR4-mutant C3H/HeJ mice were treated with a mixture of five antibiotics by gavage, beginning at 2 wk of age (see *Materials and Methods*). Periodic bacteriologic analysis of the fecal contents showed that this antibiotic treatment protocol greatly reduced and altered the composition of the bacterial flora. Within 5 days of the start of antibiotic treatment, the aerobic bacterial content of the feces was reduced at least 10,000-fold (from $\sim 10^9$ to $< 10^5$ CFU/g); anaerobic bacteria were reduced >100-fold (from $\sim 10^{10}$ to 10^8 CFU/g). Flow cytometric analysis of T and B cell subpopulations confirmed that neither 7 nor 21 days of antibiotic treatment altered the proportions of B and T cells in the spleen, MLN, or PP, or changed their expression of markers of cellular activation (see *Materials and Methods*, data not shown).

When we examined the response to *Ara h 1* plus CT in both antibiotic-treated and -untreated TLR4-mutant and wild-type mice, we found that only a modest, asymptomatic, allergen-specific IgE response was induced when *Ara h 1* plus CT was administered i.g. to C3HeB/FeJ mice (Fig. 6*A*). Antibiotic decontamination of the luminal flora of C3HeB/FeJ mice before, and during, sensitization with *Ara h 1* plus CT induced an allergen-specific IgE response equivalent to that induced in the TLR4-mutant C3H/HeJ strain.

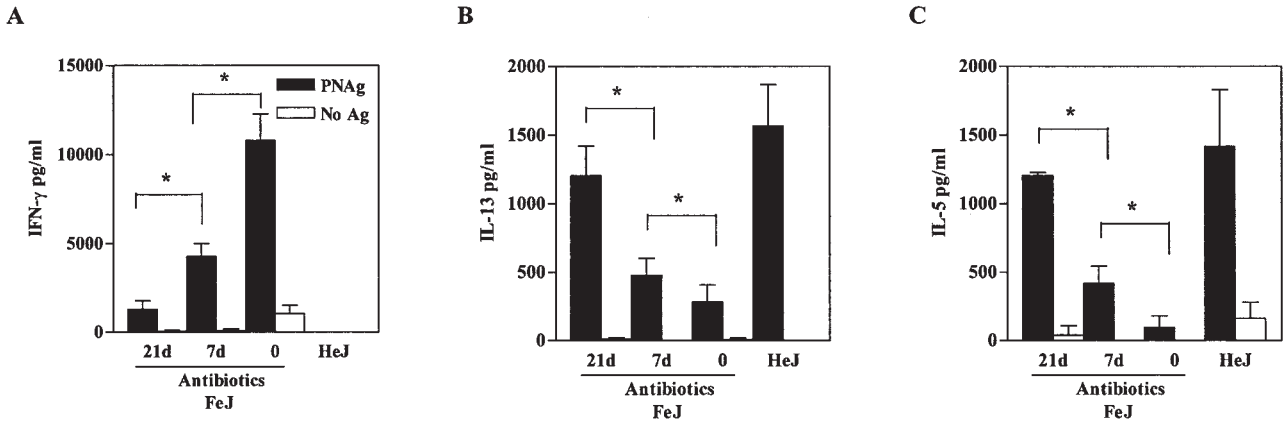

FIGURE 8. Allergen-specific Th2 cytokine responses are reduced, and IFN-γ responses are enhanced, in flora-reconstituted mice. The cytokine response of splenocytes, harvested from the mice analyzed for Ab production in Fig. 7, was assessed by ELISA. Spleen cells from individual mice were restimulated in the presence or absence of *Ara h 1* (200 μg/ml) for 72 h. IFN-γ (*A*), IL-13 (*B*), and IL-5 (*C*) secretion into the culture supernatants was examined by ELISA. Statistically significant differences between treatment groups are indicated by an asterisk ($p < 0.001$).

Plasma histamine levels and anaphylactic scores were also markedly elevated in the antibiotic-treated C3HeB/FeJ mice (Fig. 6*B*). Antibiotic treatment did not alter the allergen-specific IgE response or anaphylactic symptoms induced by sensitization of C3H/HeJ mice with *Ara h 1* plus CT. In vitro restimulation of spleen cells from *Ara h 1*/CT-sensitized C3HeB/FeJ mice induced a marked *Ara h 1*-specific IFN-γ response (Fig. 6*C*), but very low levels of the Th2 cytokines, IL-13 (Fig. 6*D*) and IL-5 (Fig. 6*E*). The reduction in mucosal TLR4 signaling induced by antibiotic treatment of C3HeB/FeJ mice converts the response to in vitro challenge to an allergic Th2-type response characterized by high levels of IL-13 and IL-5. Antibiotic treatment of C3HeB/FeJ mice reduced the *Ara h 1*-specific IFN-γ response to the low levels seen in TLR4-mutant C3H/HeJ mice.

PN-specific IgE and Th2 cytokine responses are reduced in flora-reconstituted, antibiotic-treated mice

If the absence of signaling by the luminal flora is critical for the induction of allergy in this model, reconstitution of the flora in antibiotic-treated wild-type mice should lead to a reduction in the PN-specific IgE response and the amelioration of anaphylactic symptoms. In the experiment shown in Fig. 7, C3HeB/FeJ mice were untreated, treated with antibiotics for 3 wk (as in Fig. 6), or treated with antibiotics for 1 wk, after which the flora was allowed to repopulate. Bacterial counts reached values close to those seen in the feces of untreated mice between 5 and 9 days after cessation of antibiotic treatment. In these flora-reconstituted mice (7 day), the serum PN-specific IgE response was significantly reduced (Fig. 7*A*) and correlated with a reduction in changes in plasma histamine levels and anaphylactic symptoms (data not shown). PN-IgE spot forming cells (SFC) in the spleen were also reduced in the flora reconstituted mice (Fig. 7*B*). In the PP, PN-IgE SFC were not increased by antibiotic treatment of TLR4 wild-type mice (Fig. 7*C*). Interestingly, when signaling by the luminal flora is chronically impaired, as in the TLR4-mutant C3H/HeJ mice, elevated numbers of PN-IgE SFC are detectable in the PP as well as in the spleen. Since, as mentioned earlier, there is a dynamic relationship between the flora and the development of secretory IgA responses, we examined PN-IgA SFC in both TLR4-mutant C3H/HeJ mice and antibiotic-treated and -untreated TLR4 wild-type C3HeB/FeJ mice. PN-IgA levels in gut washes of TLR4 wild-type mice were >1000 times higher than in the allergic TLR4-mutant mice (Fig. 7*D*). This correlated with greatly reduced PN-IgA SFC in the spleen (Fig. 7*E*) or PP (Fig. 7*F*) of TLR4-mutant mice. Three weeks of antibiotic treatment reduced the IgA response in TLR4 wild-type mice to levels similar to those in the TLR4-mutant mice. PN-IgA SFC in the spleen and PP were partially restored in flora-reconstituted mice.

As in previous experiments, elevated levels of PN-specific IgE and allergic symptoms correlated with a Th2-biased PN-specific cytokine response. As shown in Fig. 8, spleen cells from C3H/HeJ mice made high levels of the Th2 cytokines IL-13 (Fig. 8*B*) and IL-5 (Fig. 8*C*), but no IFN-γ (Fig. 8*A*), when restimulated with PN in vitro. Spleen cells from sensitized C3HeB/FeJ mice made little Th2 cytokine response, but high levels of IFN-γ. Antibiotic treatment of TLR4 wild-type mice led to an elevated Th2 cytokine response, comparable to that seen in TLR4-mutant mice, and a greatly reduced IFN-γ response. Flora-reconstituted mice made intermediate levels of both Th2 (IL-13 and IL-5) and Th1 (IFN-γ) cytokines.

Discussion

In this report, we present evidence for greatly heightened susceptibility to allergic responses to a food Ag in strains of mice lacking a functional receptor for TLR4. Intragastric administration of *Ara h 1* and the mucosal adjuvant CT to TLR4-deficient mice led to high levels of PN-specific IgE, elevated plasma histamine, and anaphylactic symptoms that mimic those seen in patients with food allergy. Susceptibility to allergy correlated with a Th2-biased cytokine response in both mucosal and systemic sites. We show that the commensal bacterial flora is the likely source of the TLR4 signals relevant to this model. TLR4 wild-type mice were treated with a mixture of antibiotics by gavage beginning at 2 wk of age. When the composition of the flora is greatly reduced and altered by antibiotic treatment before, and during, allergen sensitization, PN-specific IgE and allergic symptoms similar to those seen in TLR4-mutant mice are induced in TLR4 wild-type mice. Reconstitution of the flora, after antibiotic treatment, resulted in markedly reduced PN-specific IgE and Th2 cytokine responses in the flora-reconstituted mice.

That the effects of the flora are due to signaling by TLR4 was initially demonstrated in two different strains of naturally occurring TLR4-mutant and -deficient mice. We confirmed these results by demonstrating that TLR4-knockout mice, created by gene targeting, are also highly susceptible to allergy when compared with their congenic controls. However, susceptibility in this model is

clearly influenced by the genetic background upon which the allergen is presented, as also noted by others (19). We noted a greater susceptibility to an allergic response when PN allergen was presented on an H-2^k (C3H/HeJ) than on an H-2^b (C57BL/10ScNHsd) background. Other molecules are critically involved in the initiation of TLR4 signaling by LPS. LPS initially binds to LPS-binding protein and this complex is recognized by the GPI-linked protein, CD14. CD14 then associates with TLR4, which delivers the transmembrane signal. Interestingly, in human patients, promoter region polymorphisms of the *CD14* gene (but not TLR4) have been associated with an atopic phenotype (21–23). Other genes in the C3H background probably also contribute to the allergic phenotype.

The commensal flora is comprised of both Gram-positive and Gram-negative bacteria. TLR4-deficient mice should therefore retain the ability to signal via other pattern recognition receptors for bacterial pathogen-associated molecular patterns, notably TLR2 (Gram-positive bacterial products such as lipopeptides, peptidoglycan, and lipoarabinomannan), TLR5 (flagellin), and TLR9 (hypomethylated CpG DNA). Although signaling via all known TLRs have certain common outcomes such as activation of the nuclear transcription factor, NF-κB, differential patterns of gene expression, and varied functional responses, are induced. For example, signaling via TLR4, but not TLR2, induces IFN-β mRNA and the activation of genes in the STAT1 $\alpha\beta$ pathway (24). The ability of each TLR to elicit distinctive cellular responses is achieved through the use of different combinations of adaptors that interact with the Toll/IL-1R domains that are critical to TLR signaling (25). Although TLR2, TLR5, and TLR9 are solely dependent on the MyD88 adaptor protein for TLR signaling, TLR4 uses both MyD88-dependent and -independent signaling pathways (26, 27). Moreover, a recent report has shown that CpG/TLR9 (but not LPS/TLR4) signaling directly induces the expression of T-bet, the transcription factor involved in Th1 gene regulation (28). Our data suggest that some functional consequence of signaling by the flora through TLR4 plays a role in inhibiting allergic hyperreactivity to food allergens that cannot be substituted by signaling via other receptors recognizing bacterial pathogen-associated molecular patterns.

The subcomposition of the gut microflora can also impact the development of an allergic response and can differ in allergic and nonallergic individuals (29). Various microbial products have been shown to be efficacious for the treatment and/or prevention of allergy (reviewed in Ref. 30). In addition to CpG ODN, studies in both experimental models (31) and clinical trials (32) have shown that certain types of bacteria, notably Lactobacilli, can act as immunomodulatory "probiotics" to ameliorate allergic inflammation. Whether this is related to differential signaling by TLRs, or groups of TLRs, remains to be determined.

The increasing incidence of asthma and allergic disease has been attributed to reduced exposure to childhood Th1-polarizing infections brought about by vaccination and improvements in sanitation. Originally formulated as the "hygiene" hypothesis (33), recent studies have specifically implicated childhood exposure to bacterial endotoxin (LPS) in determining susceptibility to asthma and allergic disease (34–37). Paradoxically, however, other studies indicate that endotoxin exposure can exacerbate asthma and allergic disease (38). The influence of LPS on the immune system is clearly complex. Genetic predisposition, age at exposure, dose, route of exposure, and activation status of the immune system are all likely to impact the response induced. Our study implicates signaling via TLR4 in determining susceptibility to food allergy in weanling mice and suggests one mechanism by which the luminal flora can influence the response to a food Ag.

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