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

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

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 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 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 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 disease.

Bacterial flora is allowed to repopulate. Our results suggest that TLR4 signaling by the luminal flora is associated with 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. Taken together, our results suggest that TLR4-dependent signals provided by the intestinal commensal flora inhibit the development of allergic responses to food Ags.

Materials and Methods

Mice

Three-week-old female C3H/HeJ (H-2k), C3H/HeOuJ (H-2k), C3HeB/FeJ (H-2b), CBA/J (H-2b), C57BL/10SnJ (H-2b), C57BL/6J (H-2b), and B6129PF2/J mice were purchased from The Jackson Laboratory (Bar Harbor, ME). C57BL/10ScNHSd (H-2b) 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
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 per mouse) on days 0 and 14. CpG ODN and non-CpG ODN control were

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. 1982 (5’-TCCAGGACTTCTCTCAGGTGTT-3′) was as non-CpG DNA sequence no. 1982 (5′-TCCAGGACTTCTCTCAGGTGTT-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 30–40-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).

![Figure 1](http://www.jimmunol.org/Downloadedfrom)

**FIGURE 1.** Induction of allergen-specific IgE and anaphylactic symptoms is associated with TLR4 deficiency. TLR4-mutant (C3H/HeJ, n = 20), -deficient (C57BL/10ScNsd, 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-2k (A) and H-2b (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/10ScNsd 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-2k wild-type strains (A and C), and C57BL/10ScNsd (TLR4-deficient) mice and MHC-matched C57BL/10SnJ and C57BL/6J H-2b 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 × 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). The gut washes were then incubated for 30 min at 4°C with occasional mixing before centrifugation at 10,000 × g for 10 min. The supernatants were stored at −70°C.

Measurement of cytokine production by PN-stimulated splenocytes in vitro

Splenocytes from individual mice were cultured at 1 × 10⁶ 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 BV64D-1D11, IL-4) and biotinylated second Abs (XMG1.2, IFN-γ; TRFK-4, IL-5 and BV6D6-2G2, 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 ± 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 ± 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 × 10⁵ 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 (10⁵ per well), MLN (10⁴ per well), or PP (10⁵ 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 antimouse IgE (4 μg/ml) and overnight incubation at 4°C. IgE spots were detected by HRP-streptavidin (1 μg/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.

![Graph](https://www.jimmunol.org/)
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-2k haplotype strain, CBA/J. We also examined susceptibility to an allergic response to *Ara h 1* in mice with a different (H-2b) MHC background. The response of TLR4-deficient C57BL/10ScNsd mice was compared with that of the related C57BL/10SnJ strain as well as another H-2b 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 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.

**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).
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-2k 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-2b background, the C57BL/10ScNHsd TLR4-deficient mice showed the greatest susceptibility to anaphylactic symptoms. CD4+ T cells from allergic mice were restimulated with 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 the spleen, MLN, and PP of groups of mice sensitized with two doses of PN plus CT and challenged 1 wk later. 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. 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, B and D) and little IFN-γ (Fig. 3C). By contrast, CD4+ T cells from the nonallergic, C3HeB/FeJ mice produced high levels of IFN-γ, but little or no Th2 cytokines.
response was associated with a Th2-biased (IL-13) cytokine response to in vitro restimulation by spleen cells taken from allergic TLR4 mutant C3H/HeJ mice (Fig. 4 C). By contrast, spleen cells from congenic C57BL/6 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. 5A 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 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. 5C) and correlated with the absence of
PN-specific IgE. IFN-γ (Fig. 5D) 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 $10^9$ to $10^5$ CFU/g); anaerobic bacteria were reduced 100-fold (from $10^{10}$ 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. 6A). 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 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).
Plasma histamine levels and anaphylactic scores were also markedly elevated in the antibiotic-treated C3HeB/FeJ mice (Fig. 6B). 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. 6C), but very low levels of the Th2 cytokines, IL-13 (Fig. 6D) and IL-5 (Fig. 6E). 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 days), the serum PN-specific IgE response was significantly reduced (Fig. 7A) 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. 7B). In the PP, PN-IgE SFC were not increased by antibiotic treatment of TLR4 wild-type mice (Fig. 7C). 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 mutant mice increased by antibiotic treatment of TLR4 wild-type mice (Fig. 8C), but very low levels of IgA were detected in 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

**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-2k (C3H/HeJ) than on an H-2b (C57BL/10ScNd) 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 lipoproteinmannan), 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 STAT1αβ 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 thatendotoxin 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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