

Original article

Role of TLR4 in allergic sensitization to food proteins in mice

Allergic sensitization to food proteins and other allergens is increasing in prevalence. One hypothesis for this increase is that the decreased rate of infections or exposure to microbial products leaves the immune system susceptible to inappropriate reactivity to innocuous antigens through the lack of development of regulatory cells. We hypothesized that constitutive Toll-like receptor (TLR)4 signaling (presumably via the commensal flora) could inhibit the development of allergic sensitization to food proteins. We tested this hypothesis by sensitizing TLR4+ and TLR4– mice on two genetic backgrounds, C3H and BALB/c, to two common food allergens [β -lactoglobulin (β LG) and peanut (PN)]. B-cell responses were not significantly influenced by TLR4 status. T-cell responses were Th2 skewed in TLR4-deficient C3H mice compared with TLR4 sufficient C3H mice, but this pattern of Th2 skewing was not observed in TLR4-deficient mice on a BALB/c background. In anaphylaxis-susceptible C3H mice, TLR4 deficiency was associated with increased severity of anaphylaxis to PN, and decreased severity of anaphylaxis to β LG. In anaphylaxis-resistant BALB/c mice, TLR4 deficiency was not sufficient to render mice susceptible to PN-induced anaphylaxis. We conclude that although TLR4 status can influence T-cell responses and anaphylaxis severity, the nature of the influence is highly antigen- and strain-dependent.

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Food allergy and other atopic disorders have been increasing in prevalence in westernized countries, and food allergies alone affect approximately 3.5% of the adult population and up to 6% of the pediatric population (1). One hypothesis that has been proposed to underlie the increased prevalence of allergies in developed countries is the hygiene hypothesis, which proposes that environmental factors associated with an urban western lifestyle promote the development of atopy. Initially it was proposed that infection or exposure to bacterial products would skew the immune system toward a Th1 cytokine response to encountered antigens; however, protective effects of parasitic infections that induce strong Th2 responses refute such a hypothesis (2, 3). More recently it has been proposed that infection, whether bacterial or parasitic, induces the development of interleukin (IL)-10-producing regulatory cells that can suppress cytokine responses of both Th1 and Th2 patterns (4, 5). Studies focusing on the influence of lipopolysaccharide (LPS) and the receptor Toll-like receptor (TLR)4 provided evidence both in support of (6–8) and refuting the hygiene hypothesis (9, 10). That is, studies have shown that LPS/TLR4 signaling can either promote or inhibit allergic sensitization.

Sensitization via the gastrointestinal tract may be a unique situation in that the gastrointestinal-associated lymphoid tissue (GALT) is in close proximity, and is highly influenced by, the microflora. Germ-free mice have a poorly developed mucosal immune system (11–13), and re-colonization with a single strain of bacteria has been shown to have fundamental effects on intestinal gene expression (14). However, it has been shown that the resident immune cells of the gastrointestinal tract are relatively LPS-resistant (15, 16), which is thought to be necessary to maintain a noninflamed state despite the continuous exposure to bacterial products from commensal bacteria. Therefore, the influence of TLR4 on allergic sensitization via the gastrointestinal tract, i.e. to food proteins, may be unique from allergic sensitization via other mucosal sites such as the airways or skin. Bashir et al. recently showed that TLR4-deficient mice were susceptible to sensitization to the peanut (PN) allergen Ara h 1, and this susceptibility was associated with elevated secretion of the Th2 cytokine IL-13 (17).

In the present study, we addressed the role of TLR4 in the development of allergic sensitization to β -lactoglobulin (β LG; a cow's milk allergen), or PN in mouse models of food-induced anaphylaxis. We compared two strains of TLR4-deficient mice with their respective controls in their responsiveness to oral sensitization and challenge. In contrast to the previously reported findings with the PN allergen Ara h 1 (17), we did not find a consistent

Abbreviations: β LG, β -lactoglobulin; CT, cholera toxin; CPE, crude peanut extract; LPS, lipopolysaccharide; PN, peanut; TLR, Toll-like receptor; DIG, digoxigenin; Ct, threshold cycle.

influence of TLR4 on humoral responses, cytokine responses, or susceptibility to anaphylaxis.

Methods

Mice

C3H/HeJ (TLR4 defective) and C3H/HeOuJ [wild-type (WT) control] were purchased from The Jackson Laboratory (Bar Harbor, ME). C3H/HeJ mice have a point mutation in TLR4 that renders them insensitive to LPS (18). In addition, congenic mice carrying the TLR4 mutation from C3H/HeJ on a BALB/c background were also used (C-C3H-*Tlr4*^{Lps-d}), with BALB/c mice (TLR4+) used as WT controls (The Jackson Laboratory). Animals were kept in the same room under specific pathogen-free conditions in filter-top cages in a *Helicobacter*-free mouse facility. All procedures were approved by the Mount Sinai School of Medicine Institutional Animal Care and Use Committee, following the guidelines outlined in the 'Guide for the Care and Use of Laboratory Animals'.

Sensitization and oral challenge of mice

Mice were sensitized by feeding of antigen plus cholera toxin (CT) every 7 days beginning at 3 weeks of age. Mice were sensitized to β LG by feeding 1 mg β LG (Sigma-Aldrich, St Louis, MO, USA) plus 10 μ g CT (List Biologicals, Campbell, CA, USA) in 0.4 ml 0.2 M bicarbonate for 4 weekly feeds, followed by a challenge with 50 mg β LG given i.g. at day 28. Mice were sensitized to PN by feeding crude peanut extract (CPE) plus CT as described previously (19). Mice were challenged 14 weeks after the start of sensitization with two feeds of 100 mg ground PN in bicarbonate, 30 min apart.

Anaphylaxis score

Symptoms reached a peak within 30 min after β LG challenge, and 50–60 min after the first PN challenge. A symptom score was given based on the following scale: 0 = no symptoms; 1 = scratching and rubbing around the nose and head; 2 = puffiness around eyes and mouth, pilar erecti, reduced activity, and/or decreased activity with increased respiratory rate; 3 = wheezing, labored respiration, and cyanosis around mouth and tail; 4 = no activity after prodding; and 5 = death (20). Rectal temperature was also measured as a further assessment of anaphylaxis severity.

Histamine assay

About 30 (β LG) or 60 min (PN) after challenge, blood was collected in chilled ethylenediaminetetraacetic acid (EDTA)-coated tubes to obtain plasma. Plasma histamine levels were determined using an enzyme immunoassay kit (Immunotech Histamine Kit, Beckman Coulter, Brea, CA).

Measurement of antigen-specific immunoglobulins

The β LG and PN-specific immunoglobulin (Ig)E and IgG1 were measured by enzyme-linked immunosorbent assay (ELISA) as described previously (19). The β LG and PN-specific IgE levels were confirmed using a monoclonal anti-IgE capture ELISA, using digoxigenin (DIG)-labeled β LG or CPE and horseradish peroxidase (HRP)-anti-DIG antibody to detect antigen-specific IgE in serial dilutions of serum.

In vitro cytokine responses

Spleens were removed after challenge and splenocytes plated in 24-well plates at a density of 5×10^6 cells per well. Splenocytes were cultured with LPS (*Escherichia coli* O55; Sigma; 0–1000 ng/ml) or antigen (β LG, or CPE). TLR4 deficiency was confirmed in each experiment by *in vitro* stimulation with LPS. After 72 h, supernatants were harvested and cytokines measured by ELISA (all Opt-EIA ELISA kits; Pharmingen, San Diego, CA, USA).

Intestinal gene expression by real-time PCR

Intestine from naive or PN-sensitized and challenged mice was snap-frozen in Tri-Reagent (Sigma). Tissues were ground and total RNA isolated according to the manufacturer's guidelines. After reverse transcription, cDNA samples were amplified using Quantitect SYBR green master mix (Qiagen, Valencia, CA) in an ABI 7900HT sequence detection system (Applied Biosystems, Foster City, CA). Primers specific for mouse CD14, TLR4, Foxp3, IL-10, and transforming growth factor (TGF)- β were used, with β -actin as housekeeping gene. Target genes were expressed as percentage β -actin based on threshold cycle (Ct) differences between target and β -actin.

Statistical analysis

Comparisons between TLR4+ and TLR4- mice were performed using Student's *t*-test or Mann-Whitney rank sum test. *P*-value of <0.05 was considered statistically significant. Data are expressed as mean $\pm$ SEM.

Results

Effect of TLR4 deficiency on B-cell responses to food allergens

Mice having a normal or deficient TLR4 on C3H and BALB/c backgrounds were sensitized to the milk protein β LG or CPE by intragastric administration with the mucosal adjuvant CT. All mice fed antigen plus CT generated antigen-specific IgE and IgG1 antibodies over the course of the sensitization protocol. As illustrated in Fig. 1, mice generated levels of anti- β LG and anti-PN IgE and IgG1 antibodies independent of their TLR4 status. Naive mice had no detectable anti- β LG or anti-PN IgE or IgG1 antibodies. There was a trend toward increased β LG-specific IgE and IgG1 antibody levels in TLR4+ mice compared with TLR4- mice, but this did not reach the level of statistical significance. As we have observed previously, C3H/HeJ mice generated more vigorous anti-PN IgE and IgG1 responses than BALB/c mice (*P* < 0.05), but this did not correlate with TLR4 status. We confirmed the relative β LG and PN-specific IgE levels between groups using a monoclonal anti-IgE capture ELISA system, and found that β LG- and PN-specific IgE titers were not significantly different between TLR4+ and TLR4- groups (data not shown). These results indicate that humoral responses to food proteins are not significantly influenced by TLR4 status.

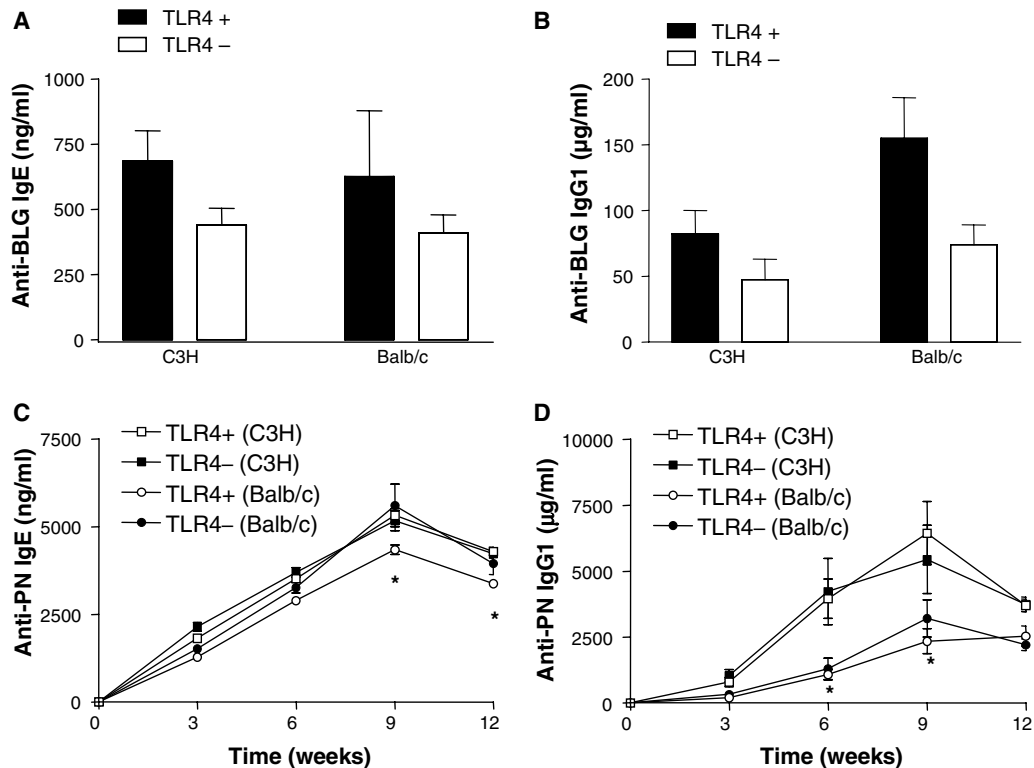


Figure 1. Antigen-specific immunoglobulin (Ig)E and IgG1 responses. Mice were sensitized to β -lactoglobulin (β LG; A and B) or peanut (PN; C and D). Antibody levels are shown for a single time point (26 days after sensitization) for β LG-sensitized mice, as they were challenged and killed at day 28. A time course is shown for PN-sensitized mice throughout the 14-week experimental period. Antigen-specific IgE (A and C) and IgG1 (B and D) levels were measured, and values are mean $\pm$ SEM for $n = 5$ –10 per group (* $P < 0.05$ compared with C3H/HeJ TLR4 $^-$ mice).

Influence of TLR4 deficiency on cytokine responses to food allergens

Cytokine response to β LG. Splenocytes were isolated from β LG-sensitized TLR4 $^+$ and TLR4 $^-$ mice on C3H and BALB/c backgrounds. After *in vitro* stimulation with β LG for 72 h, cytokines were measured in culture supernatants by ELISA. As illustrated in Fig. 2, splenocytes from TLR4 $^-$ C3H/HeJ mice produced significantly more IL-5, and IL-13, and significantly less interferon (IFN)- γ , than TLR4 $^+$ C3H/HeJ mice. Naive mice did not release cytokines above background level in response to β LG stimulation (data not shown). Thus, in β LG-sensitized C3H mice, TLR4 deficiency was associated with skewing of the cytokine response to a 'Th2' phenotype. Mice on a BALB/c background sensitized to β LG did not release significant levels of Th2 cytokines in response to *in vitro* restimulation with β LG, but IFN- γ production was suppressed in TLR4-deficient mice (Fig. 2). IL-4 and IL-10 were below the level of detection in all mice.

Cytokine responses to peanut. Splenocytes were isolated from PN-sensitized TLR4 $^+$ and TLR4 $^-$ mice on C3H and BALB/c genetic backgrounds, and cultured with CPE.

Splenocytes from TLR4 $^+$ and TLR4 $^-$ C3H mice produced significant levels of IL-4 (not shown), IL-5, IL-13 and IFN- γ , and IL-10 in response to stimulation with CPE *in vitro* (Fig. 3). Splenocytes from naive mice did not respond to CPE stimulation with any increase in cytokine secretion (data not shown). Splenocytes from TLR4 $^-$ C3H/HeJ mice produced significantly higher levels of IL-4 and IL-5, and IL-13 but not IFN- γ or IL-10 compared with TLR4 $^+$ C3H/HeJ mice. However, the pattern of cytokine secretion was reversed on the BALB/c background, such that TLR4 $^-$ BALB/c splenocytes produced significantly less IL-5, IL-13 and also IFN- γ compared with TLR4 $^+$ BALB/c mice. Therefore, although there were significant differences between TLR4 $^+$ and TLR4 $^-$ mice within strains, there was no clear difference in cytokine pattern across different strains, suggesting that although TLR4 may influence cytokine responses to fed proteins, the outcome of that influence is highly background strain- and antigen-dependent.

Influence of TLR4 deficiency on anaphylactic responses to food allergens

Anaphylactic reactions to β LG. Mice sensitized to the cow's milk protein β LG were challenged by intragastric

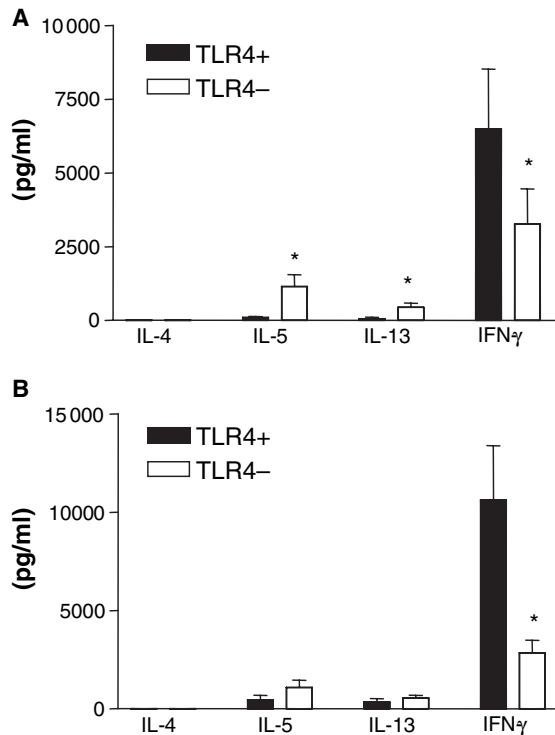


Figure 2. Splenocyte cytokine secretion in response to β -lactoglobulin (β LG). Splenocytes from β LG-sensitized mice were cultured for 72 h in the presence of β -lactoglobulin (β LG; 10 μ g/ml). Values are mean $\pm$ SEM, $n = 5$ spleens per group (* $P < 0.05$ comparing TLR4+ and TLR4- values). (A) C3H background mice, (B) BALB/c background mice.

feeding with 50 mg β LG in 0.2 M bicarbonate solution. Within 20 min of antigen administration, mice developed anaphylactic symptoms characterized by decreased activity, pilar erecti, tachypnea, and drop in body temperature. Mice on the C3H genetic background were more susceptible to anaphylaxis, with 75–100% of mice exhibiting anaphylactic symptoms to β LG, compared with 40% of mice on the BALB/c background (Fig. 4). TLR4+ C3H/HeOuJ mice had significantly higher symptom scores and lower body temperature compared with TLR4- C3H/HeJ mice in response to β LG challenge. This was paralleled by a significantly greater level of plasma histamine in TLR4+ C3H/HeOuJ mice. However, in the more resistant BALB/c background mice, there was no influence of TLR4 on anaphylactic severity. TLR4+ and TLR4- BALB/c mice demonstrated equivalent anaphylaxis scores, drop in body temperature, and plasma histamine levels after oral β LG challenge.

Anaphylactic reactions to peanut. Mice sensitized to PN were challenged by intragastric feeding with two doses of 100 mg ground PN in bicarbonate solution, administered 30 min apart. Anaphylaxis severity was assessed at 60 min after the first dose of PN (Fig. 5). Mice on the

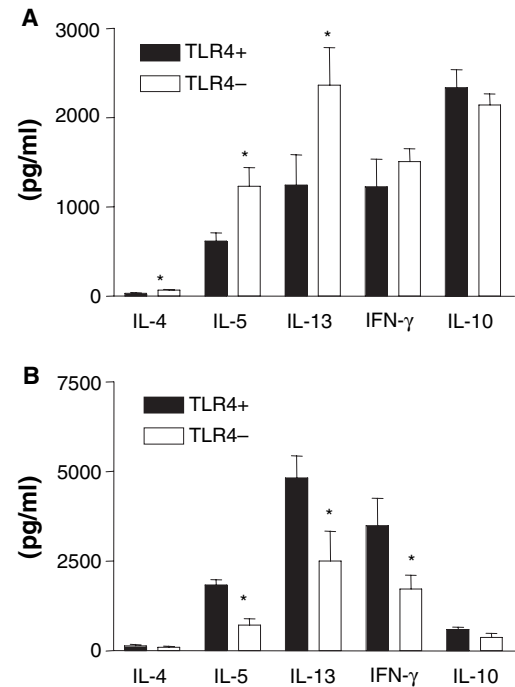


Figure 3. Splenocyte cytokine secretion in response to peanut. Splenocytes from peanut-sensitized mice were cultured for 72 h in the presence of crude peanut extract (CPE; 10 μ g). Values are mean $\pm$ SEM, $n = 8$ –10 per group (* $P < 0.05$ comparing TLR4+ and TLR4- values). (A) C3H background mice, (B) BALB/c background mice.

C3H background were susceptible to PN-induced anaphylaxis, while mice on the BALB/c background were completely resistant. In contrast to the results with β LG sensitization, TLR4+ C3H/HeOuJ mice had significantly lower anaphylactic scores and reduction in body temperature compared with TLR4- C3H/HeJ mice (Fig. 5). Only five of nine C3H/HeOuJ TLR4+ mice developed anaphylaxis in response to oral PN challenge, compared with nine of 10 C3H/HeJ TLR4- mice. Mice on the BALB/c genetic background were completely resistant to oral PN-induced anaphylaxis, and this was not influenced by TLR status. Plasma histamine paralleled the anaphylaxis score and body temperature findings.

Impact of sensitization and challenge on expression of TLR4 and regulatory markers

TLR4 status appeared to influence the development of allergic sensitization in C3H background strain mice. We examined whether expression of TLR4, or its LPS-binding partner CD14, is altered by sensitization and allergen challenge by quantitative polymerase chain reaction (PCR). TLR4 was constitutively expressed in the small intestine, and was not significantly upregulated by sensitization and challenge with PN (0.15 ± 0.03 vs $0.12 \pm 0.03\%$ β -actin in naive vs sensitized TLR4+ C3H mice). CD14 was similarly not regulated by sensi-

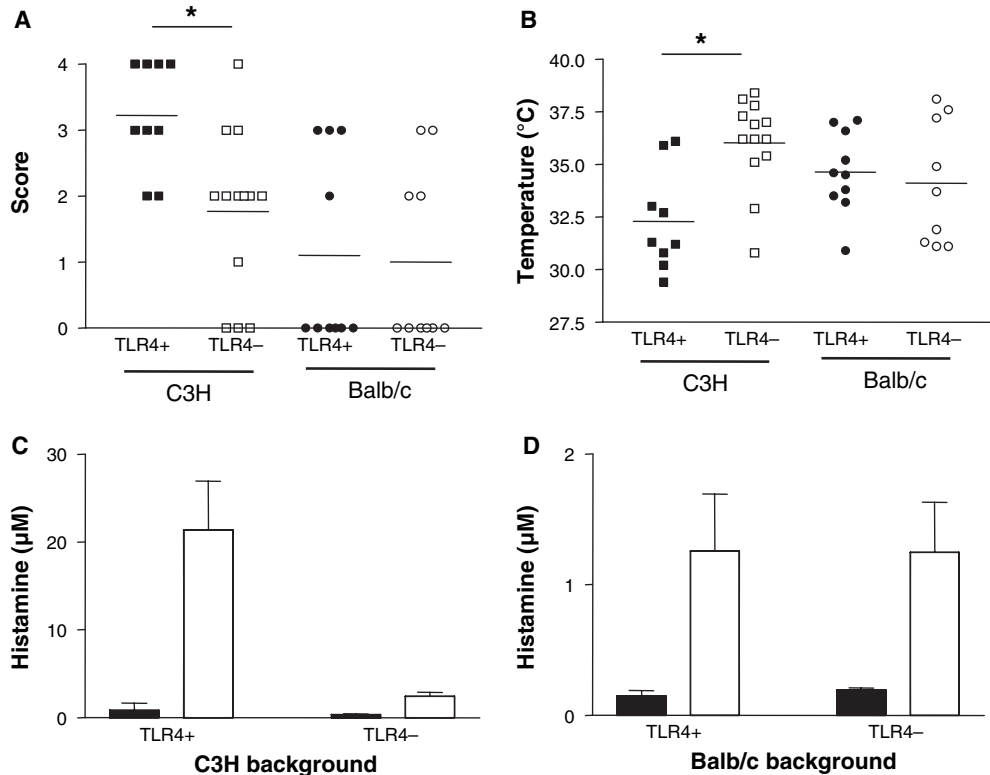


Figure 4. Anaphylaxis to β -lactoglobulin (β LG) in sensitized TLR4⁺ and TLR4⁻ mice. Symptom scores (A) and rectal temperature (B) recorded 30 min after β LG challenge. Histamine in plasma obtained 30 min after challenge (C and D). Histamine is shown as mean $\pm$ SEM for $n = 5$ per group, closed bars indicate values from naive mice (* $P < 0.05$ comparing TLR4⁺ vs TLR4⁻ values).

zation and challenge (33.4 ± 5.5 vs $21.2 \pm 5.9\%$ β -actin and 23.2 ± 10.9 vs $26.7 \pm 10.9\%$ β -actin in naive vs sensitized TLR4⁺ and TLR4⁻ mice, respectively.) The model of food protein-induced anaphylaxis does not result in gastrointestinal inflammation, therefore a recruitment of inflammatory cells bearing TLR4/CD14 was not expected.

We hypothesized that TLR4 deficiency would be associated with a defect in the development of regulatory T cells. We examined the expression of the regulatory T-cell marker Foxp3 in the lamina propria of naive and sensitized TLR4⁺ and TLR4⁻ mice. TLR4⁺ and TLR4⁻ mice did not differ with respect to their baseline level of Foxp3, but unexpectedly TLR4⁻ mice had elevated levels of Foxp3 expression in the mucosa after challenge (Fig. 6). The same pattern was shown for the regulatory cytokines IL-10 and TGF- β (Fig. 6), with elevated levels of cytokines expressed in the intestinal mucosa after allergen challenge in the TLR4⁻ mice, but not the TLR4⁺ mice. Therefore, we found no evidence for a regulatory defect in TLR4⁻ mice, at least within the intestinal mucosa. In addition, increased severity of anaphylaxis in the TLR4⁻ mice was associated with an increase in regulatory markers and cytokines within the intestinal mucosa, perhaps in response to the more vigorous Th2 skewed immune response.

In summary, TLR4⁺ and TLR4⁻ mice on the C3H genetic background differed in their response to β LG and

PN, with TLR4⁺ mice more susceptible to anaphylaxis in response to β LG challenge, and TLR4⁻ mice more susceptible to anaphylaxis in response to PN challenge. However, on the BALB/c genetic background, there was no detectable influence of TLR4 on anaphylaxis susceptibility. Clearly genetic factors other than TLR4 play a key role in the determination of susceptibility to allergic sensitization to fed proteins.

Discussion

The increase of allergic diseases in the westernized world has been suggested to be due to environmental factors, namely the decrease in childhood infection rate and decreased exposure to microbial products in general. This is termed the hygiene hypothesis and has been (and remains) the subject of extensive debate. Several epidemiologic studies support the hygiene hypothesis, with factors such as rural living, large families, attendance at day care, and pet exposure all having been shown to be less frequent in atopic populations. These factors are associated with decreased rate of infection or decreased rate of exposure to microbial products. A negative correlation between endotoxin exposure and asthma has been demonstrated (21). However, a number of studies using murine models of allergic disease have contradicted

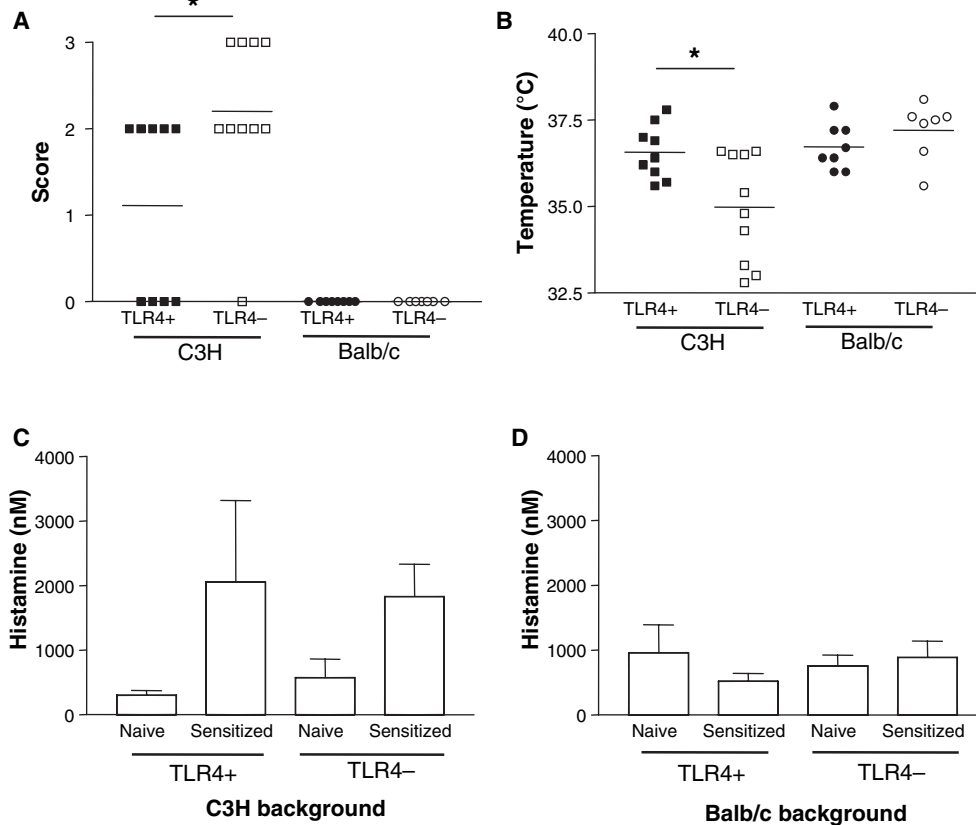


Figure 5. Anaphylaxis to peanut in sensitized TLR4+ and TLR4- mice. Symptom scores (A) and rectal temperature (B) recorded 30 min after peanut challenge. Histamine in plasma obtained 30 min after challenge (C and D). Histamine is shown as mean $\pm$ SEM, $n = 8-9$ per group (* $P < 0.05$ comparing TLR4+ vs TLR4- values).

elements of the hygiene hypothesis, and in fact show that LPS can function as an adjuvant in the development of allergic disorders (10). In addition, a functional LPS receptor (TLR4) has been shown to be required for development of antigen-specific Th2 responses (9). Timing of exposure to LPS has also been shown to be relevant, with exposure prior to sensitization acting as an inhibitory stimulus, while exposure during sensitization or after sensitization acting to enhance or exacerbate allergic sensitization (6). Many of the studies to date have focused on the role of LPS or its receptor in the development of asthma or experimental airway hyper-reactivity. The influence of LPS on immune responses generated in the intestinal mucosa may be significantly different than those generated in the airways, in light of the abundant microflora present within the gastrointestinal tract. Therefore, we sought to determine if the presence or absence of a functional TLR4 influenced the development of an immune response to a fed protein.

We sensitized TLR4+ and TLR4- mice on C3H and BALB/c backgrounds to β LG or PN proteins. When we examined IgE and IgG1 responses to fed proteins, we found that all strains of mice were able to generate robust antigen-specific antibody responses, independent of TLR4 status. Bashir et al. reported that IgE responses

in mice sensitized to the PN allergen Ara h 1 were enhanced in TLR4-deficient mice (17), a finding that we did not observe using whole PN. We did not find that TLR4 status influenced the development of an antigen-specific humoral response to either the milk protein β LG or PN.

The role of TLR4 in the development of antigen-specific cytokine release was examined using splenocytes from sensitized TLR4+ and TLR4- mice. We found that TLR4- C3H mice developed a Th2-biased pattern of cytokine secretion to either β LG or PN [as has been previously reported in Ref. (17)]. However, this finding could not be generalized to other mouse strains, as shown by the absence of Th2 skewing in TLR4- BALB/c mice sensitized to PN. In addition, Th2 skewing did not necessarily correlate with clinical severity, as TLR4+ C3H mice had severe anaphylactic reactions to β LG in the absence of Th2 cytokine secretion from splenocytes.

TLR4 is expressed on a wide range of cell types, including mast cells (22), and could potentially influence the effector phase of allergic sensitization. We challenged β LG-sensitized TLR4+ and TLR4- mice with 50 mg β LG i.g. and monitored symptoms of anaphylaxis. As we have reported previously (23), C3H/HeJ (TLR4-) were more susceptible to anaphylaxis than BALB/c mice

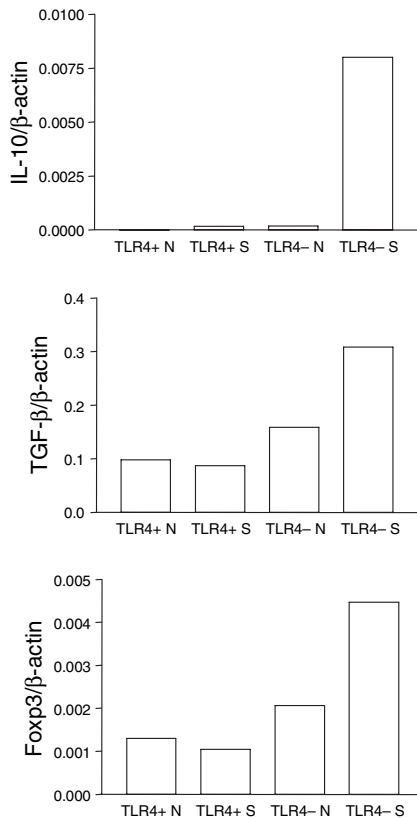


Figure 6. Small Intestinal expression of interleukin (IL)-10, transforming growth factor (TGF)- β , and Foxp3. RNA was isolated from jejunum of naive (N) or peanut-sensitized and challenged (S) TLR4+ and TLR4- mice on the C3H background. Quantitative real-time polymerase chain reaction (PCR) was performed and level of expression of Foxp3, IL-10, and TGF- β was assessed relative to the housekeeping gene β -actin. Data are expressed as percentage β -actin ($n = 5$ per group).

(TLR4+). However, this could not be explained by the TLR4 deficiency in C3H/HeJ mice, as we observed that C3H/HeOuJ (TLR4+) mice developed even more severe anaphylactic symptoms than C3H/HeJ mice, and congenic BALB/c mice carrying a mutant TLR4 developed anaphylactic reactions of an equivalent severity as WT BALB/c mice. Using PN as an antigen, we found that the severity of anaphylactic reactions followed an opposite pattern, with TLR4- C3H/HeJ mice demonstrating a greater severity than TLR4+ C3H/HeOuJ mice, as was recently reported using the PN allergen Ara h 1 as a

sensitizing antigen (17). Mice on a BALB/c genetic background were completely resistant to PN-induced anaphylaxis, and the absence of a functional TLR4 did not confer sensitivity to anaphylaxis in BALB/c mice. Therefore, we conclude that although TLR4 may influence anaphylaxis severity in C3H background strain mice, there is no clear pattern of susceptibility or resistance associated with TLR4 status. The differences observed between C3H/HeOuJ and C3H/HeJ mice could potentially be due to differences developed since the divergence of the strains. This has been noted in infection models, where differences in host susceptibility to pathogens such as *Citrobacter rodentium* (24), *Mycobacterium tuberculosis* (25), and *E. coli* (26) strains have been noted in the C3H strains independent of TLR4 status. Therefore, factors other than TLR4 that remain to be identified may underlie the susceptibility of C3H mice to anaphylactic reactions. Alternatively, TLR4 status may influence the development of the intestinal microflora in a strain-dependent manner. We cannot rule out differences in the intestinal microflora as an important factor in susceptibility to allergic sensitization.

It is likely that the resident microflora can influence the mucosal immune system through TLRs other than TLR4, via microbial products such as flagellin (TLR5; 27), peptidoglycan (TLR2; 28) or bacterial DNA (TLR9; 29). Anaphylactic responses to food proteins have been shown to be inhibited by treatment with TLR9 ligands (17), and we have previously demonstrated that administration of heat-killed *E. coli* bearing modified PN proteins inhibits PN-induced anaphylaxis in a TLR4-independent manner (19). The redundancy of signaling through different TLRs in the gastrointestinal mucosa may prevent a dominant role by a single TLR in susceptibility to allergic sensitization to food proteins. We conclude that TLR4 may influence cytokine responses and anaphylactic severity to food proteins in mice; however, this influence may be positive or negative depending on the individual genetic background and the nature of the antigen.

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