

Reduced severity of peanut-induced anaphylaxis in TLR9-deficient mice is associated with selective defects in humoral immunity

MC Berin¹ and W Wang¹

Signaling through the innate immune system can promote or suppress allergic sensitization. Toll-like receptor 9 (TLR9) has modulatory effects on the mucosal immune system, and we hypothesized that TLR9 would influence susceptibility to allergic sensitization to foods. We observed that TLR9^{-/-} mice were resistant to peanut-induced anaphylaxis. This was associated with a significant impairment in total immunoglobulin E (IgE) and peanut-specific IgE and IgA, but not IgG1 or Th2 cytokine production. TLR9^{-/-} mice had reduced development of Peyer's patches, but resistance to sensitization was not restricted to oral routes. Rag1-deficient mice were reconstituted with TLR9^{+/+} or ^{-/-} B cells plus CD4⁺ T cells. TLR9^{-/-} B cells regained the ability to produce IgE in the presence of a wild-type environment. Our results demonstrate that TLR9 on an unknown cell type is required for the development of IgE-producing B cells, and we conclude that TLR9 signaling indirectly shapes the immune response for optimal IgE production.

INTRODUCTION

The role of the innate immune system in the development of inappropriate allergic sensitization to innocuous antigens has been of great interest in the field of allergy and immunology. The idea that microbial products promote a regulatory tone in the immune system—and therefore a reduction in exposure to microbial products promotes allergic disease—is the central idea behind the hygiene hypothesis. Alternatively, microbial products such as bacterial toxins^{1,2} or lipopolysaccharide³ can have adjuvant activity that supports allergic sensitization.

There is interest in harnessing the innate immune system therapeutically for the purpose of reprogramming an immune response from a T-helper type 2 (Th2)-biased response to a regulatory or Th1-biased response. Several microbial-based approaches have been tested at the pre-clinical level for the treatment of food allergy. The use of heat-killed listeria as an adjuvant together with peanut allergens, either unmodified or modified to reduce immunoglobulin E (IgE) binding, resulted in significant reductions in peanut-induced symptoms in mice and dogs.^{4,5} Heat-killed *Escherichia coli* containing modified peanut allergens was also shown to tolerize mice to peanut when administered by the rectal route.⁶ Toll-like receptor 9

(TLR9) ligands (cytosine phosphate guanosine (CpG) oligonucleotides) given at the time of sensitization to peanut could suppress sensitization to peanut^{7,8} in mice. Furthermore, TLR9 agonists coupled to ragweed have been used with some success in human trials for allergic rhinitis. House-dust mite has also been conjugated with CpG in virus-like particles for the purpose of immunotherapy, with promising preliminary results.⁹ TLR9 ligands are potent Th1 adjuvants and can be used at mucosal sites to prime for humoral and cellular immune responses.^{10,11} A major source of endogenous TLR9 ligands is the intestinal flora, and a loss of constitutive signaling through the flora in TLR9-deficient mice has been shown to have significant effects on the responsiveness of the mucosal immune system. TLR9^{-/-} mice have a decreased number of effector cells producing interferon γ and interleukin (IL)-17 in the small intestine, and an increased number of Foxp3⁺ regulatory T cells,¹² suggesting that TLR9 ligands function as endogenous adjuvants. Others have found that TLR9 influences the colonic epithelium leading to a suppressed responsiveness to inflammatory signaling, and they observed that TLR9^{-/-} mice exhibit an enhanced susceptibility to experimental colitis.¹³ There is a window of responsiveness of the mucosal immune system to CpG oligonucleotides in the neonatal period,¹⁴ indicating that

¹Pediatric Allergy and Immunology, Immunology Institute, Mount Sinai School of Medicine, New York, New York, USA. Correspondence: C Berin (Cecilia.Berin@mssm.edu)

Received 6 January 2012; accepted 18 May 2012; published online 20 June 2012. doi:10.1038/mi.2012.55

early exposure to this class of TLR ligand may profoundly influence the immune tone of the small intestine in adulthood. This idea is supported by the recent finding of a gene–environment interaction between TLR9 polymorphisms and breast-feeding in the development of sensitization to foods.¹⁵ We hypothesized that constitutive signaling to the mucosal immune system through TLR9 could influence the susceptibility to allergic sensitization to foods. We tested this hypothesis through the use of experimental models of peanut-induced sensitization and anaphylaxis in mice deficient in TLR9, and found that TLR9 was required to indirectly promote the generation of IgE and IgA from B cells.

RESULTS

TLR9-deficient mice have reduced susceptibility to peanut-induced sensitization and anaphylaxis

Mice were orally sensitized to peanut by repeated feeding together with the mucosal adjuvant cholera toxin (CT). Mice on the C57BL/6 background strain do not respond to oral peanut challenge with anaphylaxis, but develop a robust peanut-specific IgE response and will respond to intraperitoneal (IP) peanut challenge. This model of sensitization to peanut utilizing IP re-challenge has been shown to be primarily mast cell and IgE dependent, with minor but detectable contribution from macrophages and IgG.^{16,17} C57BL/6 TLR9 +/+ mice sensitized and challenged with peanut extract underwent systemic anaphylaxis, as measured by a significant drop in the core body temperature (**Figure 1a**). The severity of anaphylaxis was significantly less but not absent in TLR9 –/– mice (mean temperature of 36.4°C in TLR9 –/– mice compared with 33.6°C in TLR9 +/+ mice and 37.8°C in naive mice). This reduced susceptibility to anaphylaxis in TLR9 –/– was associated with significantly lower peanut-specific IgE levels in serum obtained before challenge as compared with wild-type controls (**Figure 1b**). TLR9-deficiency was also associated with a significant reduction in peanut-specific IgA responses in serum (**Figure 1d**), whereas peanut-specific IgG1 responses were similar in TLR9 +/+ and –/– mice (**Figure 1c**). Sensitization to peanut was associated with a mixed cytokine response from allergen re-stimulated spleen cells. Similar levels of IL-13, IL-10, and interferon γ were observed in TLR9 –/– mice, whereas IL-17 levels were significantly elevated in orally sensitized TLR9 –/– mice (**Figure 1e**). IL-4 was routinely below the level of detection.

Impact of TLR9 deficiency on IgE-expressing B cells

To investigate the source of IgE production in peanut-sensitized mice, we performed flow cytometry to detect IgE-expressing cells. We examined cells in the bone marrow, spleen, mesenteric lymph node, and Peyer's patch (PP). A significant population of IgE+ cells with B-cell markers (CD19, B220, but not the plasma cell marker CD138) was found in the spleen and bone marrow, but not in mesenteric lymph node or PP (see **Supplementary Figure S1** online). Mature IgE transcripts in the spleen and bone marrow were confirmed by RT-PCR (data not shown). Permeabilization of the cells was required for the detection of these cells, indicating minimal surface IgE expression. The

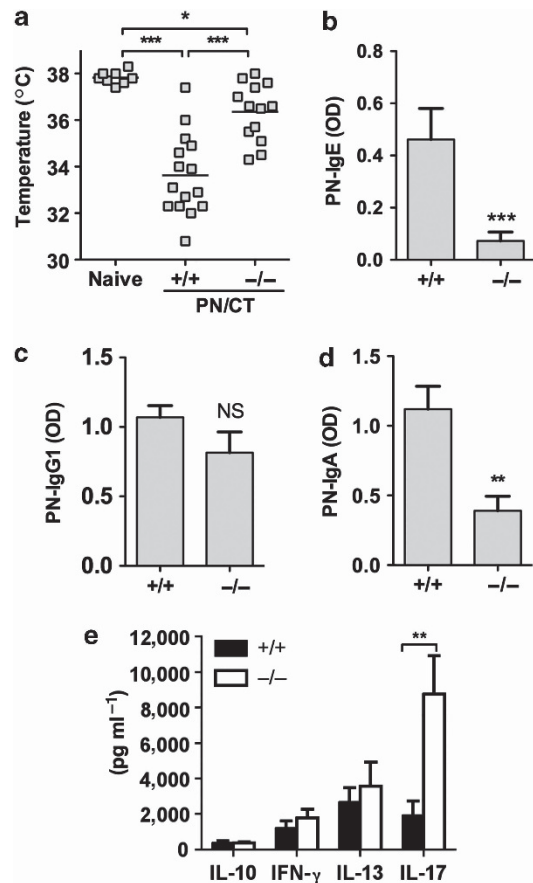


Figure 1 Impact of Toll-like receptor 9 (TLR9) deficiency on peanut (PN)-induced anaphylaxis and antibody and cytokine responses. TLR9 +/+ or –/– mice were orally sensitized to PN with cholera toxin (PN/CT), followed by intraperitoneal challenge with crude PN extract. (a) Body temperature measured 30 min after PN challenge. (b) PN-specific immunoglobulin E (IgE), (c) IgG1, and (d) IgA as measured by enzyme-linked immunosorbent assay in serum obtained 1–2 days before challenge. (e) Cytokine secretion measured after re-stimulation of spleen cells with PN extract. Spleen cells were harvested immediately after allergen challenge. (a) is combined data from two independent experiments; (b–e) are from one representative experiment of two, with $n=10$ (+/+) and 8 (–/–). *** $P<0.001$, ** $P<0.01$, * $P<0.05$ comparing the indicated groups. IFN, interferon; IL, interleukin; NS, not significant; OD, optical density.

median fluorescence intensity of IgE expression was elevated after sensitization. TLR9 –/– mice had a marked reduction in the fluorescence intensity of IgE expressed by the B cells, even in the naive state (**Figure 2**). This was confirmed by measuring total serum IgE in the naive and sensitized TLR9 +/+ and –/– mice, which demonstrated a significant reduction in total IgE at baseline and after sensitization in TLR9 –/– mice. By contrast, total IgA as measured in either serum or feces at baseline was not affected by TLR9 deficiency.

Mast cells from TLR9 –/– mice become functional upon passive sensitization with IgE

Peanut-induced anaphylaxis is dependent primarily on activation of mast cells by cross-linking IgE, although minor contributions

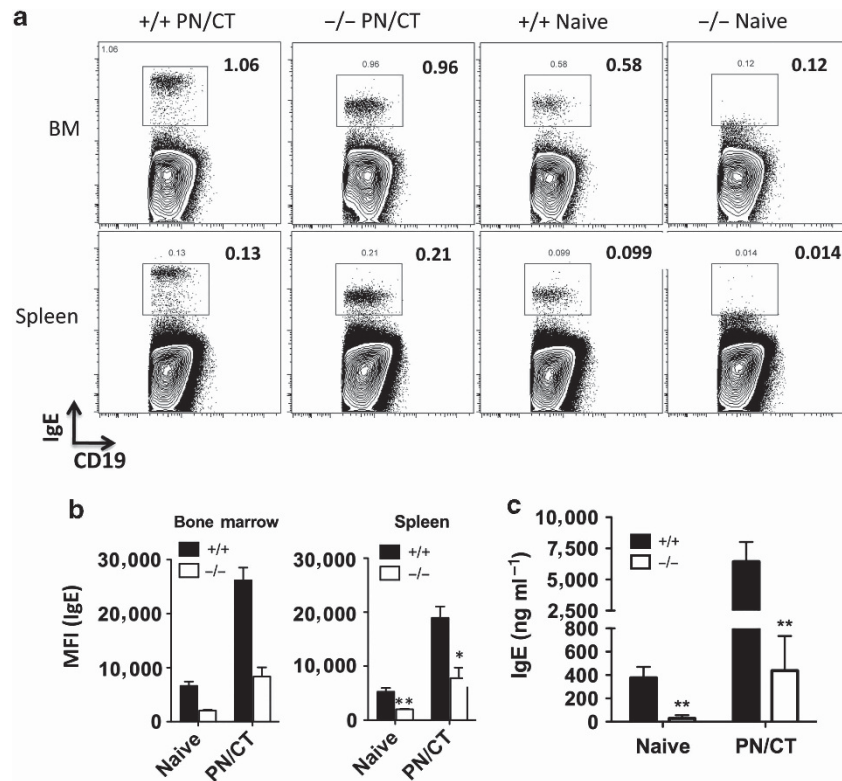


Figure 2 Detection of immunoglobulin E (IgE) + B cells. Mice were left naive as control, or sensitized with peanut (PN) with cholera toxin (PN/CT) for 6 weeks. Sensitization was verified by measurement of PN-specific IgE in serum. Mice were not allergen challenged before collection of tissues. **(a)** Representative staining in bone marrow (BM) and spleen cells. Live CD19⁺ cells were gated, and plots show IgE vs. CD19 in TLR9^{+/+} (Toll-like receptor 9) and TLR9^{-/-} mice orally sensitized to PN (PN/CT) or naive. **(b)** Median fluorescence intensity (MFI) of IgE in IgE⁺ B cells gated as above. *N*=3/group (spleen) and 2/group (BM). **P*<0.05. **(c)** Total IgE measured by enzyme-linked immunosorbent assay in serum of naive or PN-sensitized (PN/CT) TLR9^{+/+} or ^{-/-} mice. ***P*<0.01, *n*=8–15 mice/group.

from macrophages and IgG have also been demonstrated.^{16,17} To assess the function of mast cells in TLR9^{-/-} mice, we first examined peritoneal mast cells by flow cytometry. The number of c-kit⁺ IgE⁺ cells in the peritoneal cavity was not significantly different in TLR9^{+/+} and ^{-/-} mice (data not shown); however as shown in **Figure 3a**, there was a marked reduction in the level of IgE on the surface of peritoneal mast cells from the sensitized TLR9^{-/-} mice as compared with TLR9^{+/+} mice. To test the function of mast cells in TLR9^{+/+} and ^{-/-} mice, mice were passively sensitized with serum containing high levels of peanut-specific IgE by injection into the ear pinna. As control, the other ear was injected with ovalbumin-IgE⁻ positive serum. The next day, mice were injected intravenously with Evans Blue and peanut extract, and the extent of extravasation in the ear was measured (**Figure 3b**). TLR9^{+/+} and ^{-/-} mice responded to peanut challenge with a similar level of extravasation in the ear passively sensitized with peanut-IgE⁺ serum, indicating that mast cells of TLR9^{-/-} mice are functional and the major defect is at the level of IgE production.

TLR9 influences PP development

It has been described that TLR9^{-/-} mice have elevated numbers of regulatory T cells in their small intestinal lamina propria and within PPs¹² that could potentially explain the suppressed

response to oral sensitization. In addition, the defect in IgA suggested a mucosal immunoglobulin production defect. In our colony of co-housed TLR9^{+/+} and ^{-/-} mice, we did not observe any difference in the number of intestinal CD4⁺ Foxp3⁺ cells (in lamina propria, mesenteric lymph node, or PP) (see **Supplementary Figure S2** online), nor did we observe any quantitative difference in CD4 or CD8 T cells, B cells, or dendritic cells (see **Supplementary Figure S2** online). However, we did observe that the PPs were reduced in size in TLR9^{-/-} mice. Quantification of the number of grossly visible PPs per small intestine and the total yield of cells from the PPs per small intestine showed that there was a significant reduction in number of visible PPs as well as the size of PPs in TLR9^{-/-} mice. In addition, histological examination showed that the number of follicles per patch was also reduced in TLR9^{-/-} mice (**Figure 4**).

Resistance to sensitization to peanut in TLR9^{-/-} mice is not restricted to the gastrointestinal tract

It has been described that crude peanut contains insoluble particles that are readily taken up by M cells overlying the PP,¹⁸ and we have previously observed that site of uptake in the gastrointestinal tract has a significant role in the allergic response to food allergens.¹⁹ To determine whether resistance to sensitization was due to the reduced PP development, we sensitized

mice to peanut by non-oral routes, either IP injection with alum adjuvant, or by the epicutaneous route by applying peanut and CT topically to the skin.²⁰ As shown in **Figure 5**, TLR9^{-/-} mice were resistant to peanut-induced anaphylaxis whether

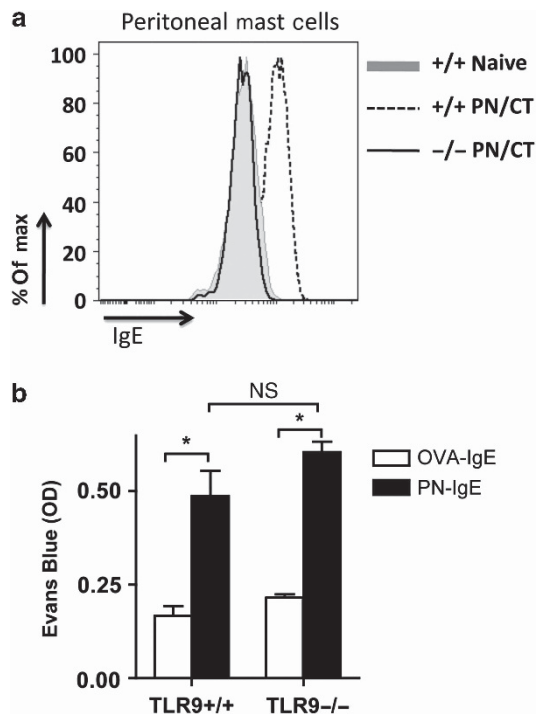


Figure 3 Impact of Toll-like receptor 9 (TLR9) deficiency on mast cells. **(a)** Peritoneal cells were harvested from naive or orally peanut (PN)-sensitized (PN with cholera toxin (PN/CT)) TLR9^{+/+} or ^{-/-} mice. Peritoneal cells were harvested from sensitized un-challenged mice. Mast cells were gated based on c-kit and immunoglobulin E (IgE) staining, and the level of IgE positivity was compared in naive and sensitized ^{+/+} and ^{-/-} mice. **(b)** Naive TLR9^{+/+} or ^{-/-} mice were passively sensitized in the ear with serum from PN-sensitized mice or ovalbumin (OVA)-sensitized mice as controls. The next day, mice were intravenously injected with Evans Blue and PN extract, and extravasation in each ear was quantified by spectrophotometry. **P* < 0.05, NS, not significant. (*n* = 5/group). OD, optical density.

sensitization occurred through the IP route or the epicutaneous route. There was no significant difference between body temperatures in epicutaneously sensitized TLR9^{+/+} and ^{-/-} mice after challenge, but ^{+/+} mice had a significant drop in body temperature compared with pre-challenge temperatures, whereas TLR9^{-/-} mice did not. By both routes of sensitization, there was a significant suppression of IgE in TLR9^{-/-} mice. Peanut-specific IgA was elevated in response to cutaneous, but not IP sensitization, and this was also significantly suppressed in TLR9^{-/-} mice. These data indicate that the resistance of TLR9^{-/-} mice to peanut sensitization is systemic, and not restricted to the induction of responses within the intestinal mucosa.

TLR9-dependent promotion of IgE production is through indirect activity on B cells

TLR9 ligands have been shown to have direct activity on IgE production from B cells *in vitro*,^{21,22} although the results were conflicting, with one report showing suppression, whereas the other showed enhancement of IgE. We wanted to test whether the effect of TLR9 deficiency on IgE production was through direct or indirect actions on B cells. To test this, we reconstituted Rag1-deficient mice with B cells from naive TLR9^{+/+} or ^{-/-} mice, together with CD4⁺ T cells from TLR9^{+/+} mice primed with peanut/alum to increase the precursor frequency of peanut-specific T cells. Total and peanut-specific IgE was measured before (4 weeks post transfer) and 6 weeks after sensitization of the recipient mice. Transfer of B cells without peanut-primed T cells did not support peanut-specific IgE production in recipient mice, demonstrating the T-cell dependence of the generation of peanut-specific IgE. Sensitization of the recipient mice was necessary to generate peanut-specific IgE responses. Rag1-deficient mice receiving wild-type or TLR9^{-/-} B cells plus wild-type peanut-primed T cells generated similar levels of total and peanut-specific IgE after sensitization, and were similarly susceptible to peanut-induced anaphylaxis (**Figure 6**), demonstrating that TLR9^{-/-} B cells acquire the capacity to generate robust IgE responses in a wild-type milieu.

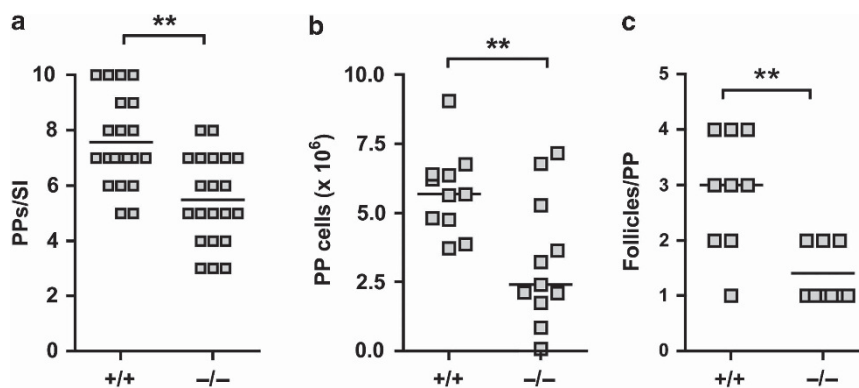


Figure 4 Impact of Toll-like receptor 9 (TLR9) deficiency on Peyer's patches (PPs). **(a)** The entire small intestine (SI) from naive TLR9^{+/+} or ^{-/-} mice was removed and the total number of visible PPs per mouse was counted. **(b)** Cells were isolated by collagenase digestion and the yield of cells per mouse was determined. Segments of intestine containing a PP were fixed, embedded, and cross-sections stained with hematoxylin and eosin. **(c)** The number of follicles per PP was counted. ***P* < 0.01.

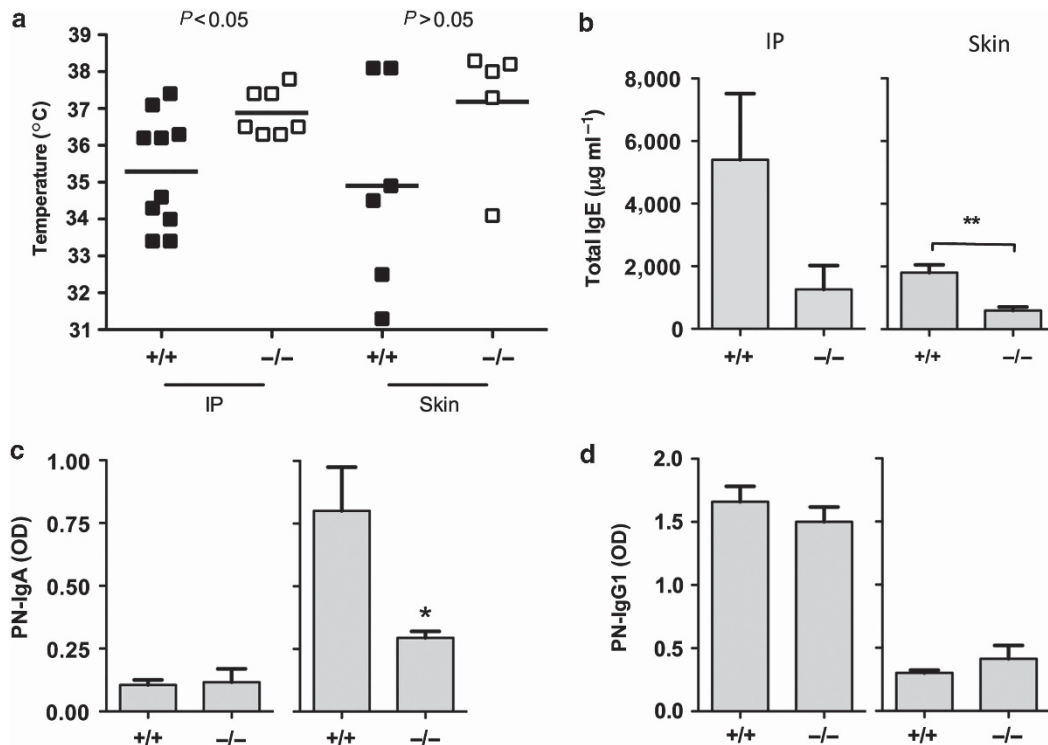


Figure 5 Sensitization to peanut (PN) by non-oral routes. TLR9 $+/+$ (Toll-like receptor 9) and $-/-$ mice were sensitized to PN by intraperitoneal injection with alum (IP), or by repeated epicutaneous exposure (skin). (a) Body temperature measured 30 min after IP challenge with 500 μ g of PN extract. (b) Immunoglobulin E (IgE), (c) IgA, and (d) IgG1 antibodies were measured in serum obtained 1–2 days before challenge. $*P < 0.05$, $**P < 0.01$. OD, optical density.

DISCUSSION

Exposure to microbial factors has been hypothesized to have a critical role in the development of allergic sensitization. Epidemiological evidence indicates that lifestyle factors associated with higher rates of microbial exposure or infection are protective against atopy. Studies have also implicated changes in the gastrointestinal flora as a risk factor for eczema in children. Evidence does not indicate that microbial factors are solely protective: bacterial toxins such as CT² and staphylococcal enterotoxin B,¹ lipopolysaccharide,³ and NOD2 (nucleotide-binding oligomerization domain-containing protein 2)²³ have adjuvant activity and promote allergic sensitization and inflammation using experimental models of allergy or asthma. Endogenous signals via TLR9 have been shown to modulate the adaptive immune response in the small intestine,¹² and we hypothesized that signals via TLR9 would alter susceptibility to allergic sensitization. We found that TLR9 $-/-$ mice had reduced susceptibility to allergic sensitization to peanut. This was not due to an altered Th2 cytokine response to peanut but was associated with a selective defect in IgE and IgA production that could be reversed by transferring TLR9 $-/-$ B cells to TLR9 $+/+$ mice.

TLR9 polymorphisms have been found to be associated with wheeze, but not allergen-specific IgE,²⁴ and with atopic eczema.²⁵ In the latter study, a gain-of-function allele was associated with increased risk of atopic eczema. Hong *et al.*¹⁵ recently reported gene–environment interactions between

TLR9 and breast-feeding in IgE sensitization to foods. The finding that the intestine is highly responsive to TLR9 in the neonatal period¹⁴ supports the hypothesis that early exposure to microbial antigens may be decisive in programming the IgE response to food allergens. Our results show that a deficiency in TLR9 signaling can result in protection or resistance in generation of IgE; we speculate that a gain of function in TLR9 signaling could have the opposite effect.

TLR9 is a cytoplasmic pattern recognition molecule that is widely expressed on hematopoietic cells and has also been described as a surface receptor on intestinal epithelial cells.^{13,26} Ligands for TLR9 include unmethylated DNA containing CpG motifs (common in bacterial DNA) and viral DNA. TLR9 ligands are potent stimulators of type-I interferon from plasmacytoid dendritic cells, and promote the activation and proliferation of B cells. Stimulation via TLR9 suppresses class-switching of naive B cells to IgE,^{21,27} and *in vivo* treatment with synthetic oligonucleotides that activate TLR9 have been shown to suppress IgE production.^{8,28} Furthermore, in models of fungal asthma induced by *Aspergillus*, it was found that TLR9 $-/-$ mice had enhanced IgE responses after fungal infection.²⁹ However, in contrast with those findings, it was recently reported that TLR9 ligands could synergize with CD40 and TACI (transmembrane activator and calcium modulator and cyclophilin ligand interactor) to promote B-cell activation and IgE class-switching.²² The authors hypothesized that the difference in responses could be due to sub-optimal

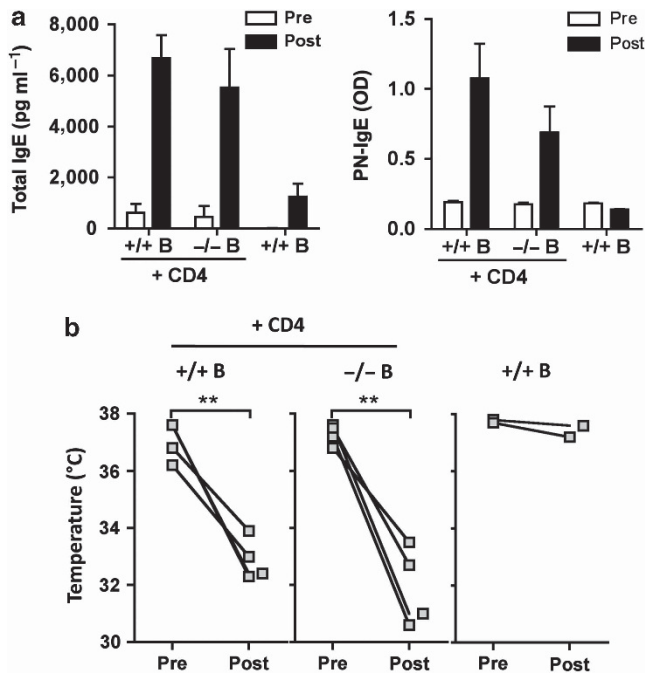


Figure 6 Reconstitution of Rag1-deficient mice with TLR9^{+/+} (Toll-like receptor 9) or TLR9^{-/-} B cells. Rag1^{-/-} mice were reconstituted with B cells from naive TLR9^{+/+} or ^{-/-} mice together with CD4⁺ T cells from TLR9^{+/+} mice primed with peanut (PN). (*n* = 4/group, and two controls receiving no CD4⁺ T cells). (a) Total (left) and PN-specific (right) immunoglobulin E (IgE) was measured 4 weeks after cell transfer (pre-sensitization) and after 6 weeks of oral sensitization with PN/CT. (b) Mice were challenged by the intraperitoneal route with PN extract, and rectal temperature measured before and 30 min after challenge to assess anaphylaxis severity. ***P* < 0.01 using a paired *T* test. OD, optical density.

concentrations of CpG used in their study compared with the doses used in the previous study showing direct inhibitory effects of CpG on IgE production *in vitro*. Our studies with reconstituted Rag1-deficient mice suggest that the modulatory effect of TLR9 deficiency on IgE production is not due to direct effects on the B cell. Our finding of the impact of TLR9 deficiency on IgE production is quite different than the impact of the gut flora as a whole. It was recently shown by Hill *et al.*³⁰ that eradication of the gut flora with antibiotics induced a significant increase in IgE. Total IgE was also elevated in germ-free mice, and in mice lacking the TLR signaling adapter MyD88 (myeloid differentiation primary response gene (88)). This effect of the flora was found to be dependent on MyD88 expression in the B-cell compartment. Treatment with exogenous CpG led to a modest reduction in total IgE levels, consistent with previous reports. This is similar to an earlier finding in a model of peanut allergy, which demonstrated that antibiotic treatment enhanced susceptibility to sensitization to peanut, and exogenous CpG suppressed sensitization to peanut.⁷ Therefore, we think that signaling through TLR9 is important for supportive mechanisms of IgE and IgA production during development, rather than directly influencing

B-cell production of IgE. In addition, peanut may be unique in its dependence on TLR9 for IgE production.

IgE class-switching in mice is supported by IL-4 and IL-13. IL-4 was routinely below the level of detection in our assays, but IL-13 was secreted in an antigen-specific manner and was not different between TLR9^{+/+} and ^{-/-} mice. We observed a significant increase in peanut-induced IL-17 production in TLR9^{-/-} mice in response to oral sensitization (shown in **Figure 1**). IL-17 and IgE have been shown to negatively correlate in some conditions, such as hyper-IgE syndrome.³¹ However, in data not shown, sensitization by the epicutaneous route was not associated with enhanced production of IL-17 in TLR9^{-/-} mice, yet IgE and IgA suppression was observed. Therefore, we did not find a consistent negative correlation between IgE levels and IL-17 production to support a T-cell basis for the altered immunoglobulin production. Our data do not suggest that the reduced production of IgA and IgE is due to suppressed cytokine responses.

The finding that both IgE and IgA were suppressed suggested a possible deficiency in mucosal isotype switching. We observed that there were developmental changes in the gut-associated lymphoid tissues of TLR9-deficient mice. TLR9^{-/-} mice had a significant decrease in the cellularity of their PPs. Although PPs are programmed lymphoid organs whose development begins prenatally, environmental cues can modulate the enlargement of these tissues. An impairment in PP development has been noted in a number of knock-out mice, including those lacking the chemokine receptor CCR6³² and the chemokine CXCL13.³³ It was shown that neonatal feeding of CpG induced an upregulation of chemokine expression in the small intestine,¹⁴ that we hypothesize would have an important role in the chemoattraction of B cells to the PP leading to enlargement of the PP after microbial colonization. The PP is a major site of IgA production, and has also been described to be a significant induction site of IgE production, even in response to distal systemic immunization.³⁴ However, we could find no evidence for a local mucosal production of IgE. We did not address the impact of TLR9 deficiency on the formation of isolated lymphoid follicles that are highly sensitive to regulation from the gut flora and are also a significant source of IgA production.³⁵ The contribution of isolated lymphoid follicles to IgE production has not yet been addressed. The selective effect of TLR9 deficiency on IgE and IgA in the absence of effects on IgG1 or Th2 cytokines suggests that TLR9 is altering factors that are common to the support of IgE and IgA production *in vivo*. Our data, and the recent finding that TLR9 polymorphisms are associated with increased risk of food sensitization in breast-fed infants¹⁵ highlights the potential importance of constitutive signaling through the TLR9 pathway in the development of sensitization and tolerance to foods early in life. More studies are needed to identify the mechanism of this TLR9-dependent source of support for IgE production from B cells. A greater understanding of the regulation of IgE production is needed to address the increasing health problem of atopic diseases.

METHODS

Mice. TLR9 $-/-$ mice were originally generated by Hemmi *et al.*³⁶ and were maintained as a breeding colony at Mount Sinai School of Medicine, NY. Wild-type C57BL/6 mice were purchased from NCI (Frederick, MD). TLR9 $+/+$ and $-/-$ mice were co-caged in specific pathogen-free conditions for at least 2 weeks before the start of experiments in order to normalize the gut flora. Rag1-deficient mice were obtained from Jackson Laboratories (Bar Harbor, ME) and maintained as a breeding colony at the Mount Sinai School of Medicine, NY. The Institutional Animal Care and Use Committee approved all experimental procedures utilizing mice.

Sensitization. Mice were orally sensitized to peanut by intragastric administration of 10 mg ground peanut (prepared from shelled peanuts) in 0.2 M sodium bicarbonate with 10 μ g of CT (List Biologicals, Campbell, CA) once a week for 6 weeks. Alternatively, mice were injected with 100 μ g of ground peanut mixed 1:1 with Alum Imject (Pierce, Rockford, IL) every 2 weeks for 2 weeks. Mice were sensitized epicutaneously by first removing the abdominal hair with depilatory cream (Veet, Reckitt Benckiser, Parsippany, NJ) under anesthesia. A total of 50 μ l of a solution containing 10 mg of crude ground peanut and 10 μ g of CT was spread topically on the abdominal skin. Sensitization was repeated weekly for 6 weeks as previously described for milk allergens.²⁰ Mice were challenged or used for other experiments 1 week after the last sensitization dose.

Challenge of mice. Mice were challenged with 500 μ g of a defatted crude peanut extract by IP injection. Body temperature was measured 30 min after challenge by intrarectal temperature probe (World Precision Instruments, Sarasota, FL). Immediately after assessment of anaphylaxis severity, mice were euthanized and spleens harvested for *in vitro* re-stimulation.

Antigen-specific cytokine production. Spleen cells were isolated, red blood cells lysed, and cells plated in complete RPMI in the presence or absence of 100 μ g ml⁻¹ of a sterile extract of defatted peanut. Cells were cultured for 72 h before harvesting of supernatants for measurement of cytokines by enzyme-linked immunosorbent assay (all from eBioscience, San Diego, CA).

Immunoglobulins. Serum was obtained 1–2 days before allergen challenge. Peanut-specific IgE in serum was measured by a capture enzyme-linked immunosorbent assay using digoxigenin-labeled peanut extract for detection as described previously.³⁷ Peanut-specific IgG1 and IgA were detected by applying serum dilutions to peanut-coated plates and detecting with biotinylated anti-IgG1 and IgA, respectively (antibodies from BD Pharmingen, San Diego, CA). Total IgE was measured in serum by capture enzyme-linked immunosorbent assay using antibodies from BD Pharmingen. Total IgA was measured in serum and fecal extracts. Fecal pellets were collected and homogenized in phosphate-buffered saline. After centrifugation, total IgA was measured in supernatants by enzyme-linked immunosorbent assay (eBioscience, San Diego, CA) and adjusted to total protein measured by Bradford assay (Pierce, Rockford, IL).

Isolation of cells. Cells were isolated from the intestine using the published protocols.³⁸ The entire small intestine was excised and PPs were removed for separate isolation. After treatment with dithiothreitol and EDTA to remove epithelium, the tissue was incubated with collagenase D (Roche, Indianapolis, IN) and DNase (Roche) before passing through a 70- μ m filter and separation on a discontinuous Percoll gradient (GE Healthcare, Pittsburgh, PA).

Peritoneal cells were harvested by injection of RPMI into the peritoneum before aspiration. Bone marrow cells were harvested by flushing the femurs and tibias with 5 ml of phosphate-buffered saline.

Flow cytometry. All reagents were from eBioscience (La Jolla, CA) unless otherwise stated. Cells were blocked with FcBlock before staining with surface antibodies and live/dead violet viability reagent (Invitrogen, Carlsbad, CA). Cells were fixed and permeabilized (using Foxp3-specific buffers from eBioscience for Foxp3 staining, or general fixative and permeabilization buffer for IgE). Intracellular staining for Foxp3 or IgE was performed, and cells were acquired on a LSR II flow cytometer (BD Biosciences, San Diego, CA). Analysis was performed using FlowJo software (Tree Star, Ashland, OR).

Histology. Rings of small intestine containing a PP were fixed in 10% formalin, and embedded in paraffin. Tissue sections (4 μ m) were cut as rings and stained with hematoxylin and eosin. The number of visible follicles per PP were quantified and averaged per mouse.

Passive cutaneous anaphylaxis. Mice were injected in the ear pinna with 25 μ l of pooled serum from peanut-sensitized mice in one ear or from ovalbumin-sensitized mice in the other ear as control. The next day, mice were intravenously injected with 0.2 ml of 1% Evans Blue containing 500 μ g of peanut extract. After 60 min, at the time when there was obvious blueing of the positive ear, the mice were euthanized and entire ears were harvested, minced, and extracted with dimethylformamide at 56°C for 18 h. The supernatant was collected, and the absorbance of the extract was measured at 655 nm.³⁹ Anaphylaxis was localized to the ear injected with serum from peanut-sensitized mice.

Reconstitution of Rag-deficient mice. Resting B cells were purified from the spleen and mesenteric lymph nodes of naive TLR9 $+/+$ and $-/-$ mice by negative selection (Miltenyi Biotec, Auburn, CA). CD4+ T cells were purified by negative selection (StemCell, Vancouver, BC) from the spleen of TLR9 $+/+$ mice primed twice with peanut and alum on days 0 and 14, and harvested on day 28. In all, 5×10^6 $+/+$ or $-/-$ B cells were transferred together with 5×10^6 CD4+ T cells. Mice were allowed to recover for 4 weeks, and then orally sensitized to peanut with CT adjuvant for 6 weeks as described above. Peanut-specific and total IgE levels in serum were measured before and after sensitization to peanut.

Statistics. Difference between TLR9 $+/+$ and $-/-$ mice were measured by two-tailed *T* test, unless indicated otherwise.

SUPPLEMENTARY MATERIAL is linked to the online version of the paper at <http://www.nature.com/mi>

ACKNOWLEDGMENTS

This work was funded by U19AI66738 and U19AI044236 (from NIAID) and R834064 (from EPA). We thank the CoFAR basic science group (Kim Bottomly, Michael Caplan, Pierre Pochard, and Brian Vickery) as well as Hugh Sampson and Lloyd Mayer for helpful discussions.

DISCLOSURE

The authors declared no conflict of interest.

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REFERENCES

- Ganeshan, K., Neilsen, C.V., Hadsaitong, A., Schleimer, R.P., Luo, X. & Bryce, P.J. Impairing oral tolerance promotes allergy and anaphylaxis: a new murine food allergy model. *J. Allergy Clin. Immunol.* **123**, 231–238 (2009) e4.
- Snider, D.P., Marshall, J.S., Perdue, M.H. & Liang, H. Production of IgE antibody and allergic sensitization of intestinal and peripheral tissues after oral immunization with protein Ag and cholera toxin. *J. Immunol.* **153**, 647–657 (1994).
- Eisenbarth, S.C., Piggott, D.A., Huleatt, J.W., Visintin, I., Herrick, C.A. & Bottomly, K. Lipopolysaccharide-enhanced, toll-like receptor 4-dependent T helper cell type 2 responses to inhaled antigen. *J. Exp. Med.* **196**, 1645–1651 (2002).

4. Frick, O.L., Teuber, S.S., Buchanan, B.B., Morigasaki, S. & Umetsu, D.T. Allergen immunotherapy with heat-killed *Listeria monocytogenes* alleviates peanut and food-induced anaphylaxis in dogs. *Allergy* **60**, 243–250 (2005).
5. Li, X.M., Srivastava, K., Huleatt, J.W., Bottomly, K., Burks, A.W. & Sampson, H.A. Engineered recombinant peanut protein and heat-killed *Listeria monocytogenes* coadministration protects against peanut-induced anaphylaxis in a murine model. *J. Immunol.* **170**, 3289–3295 (2003).
6. Li, X.M. *et al.* Persistent protective effect of heat-killed *Escherichia coli* producing “engineered,” recombinant peanut proteins in a murine model of peanut allergy. *J. Allergy Clin. Immunol.* **112**, 159–167 (2003).
7. Bashir, M.E., Louie, S., Shi, H.N. & Nagler-Anderson, C. Toll-like receptor 4 signaling by intestinal microbes influences susceptibility to food allergy. *J. Immunol.* **172**, 6978–6987 (2004).
8. Adel-Patient, K., Ah-Leung, S., Bernard, H., Durieux-Alexandrenne, C., Creminon, C. & Wal, J.M. Oral sensitization to peanut is highly enhanced by application of peanut extracts to intact skin, but is prevented when CpG and cholera toxin are added. *Int. Arch. Allergy Immunol.* **143**, 10–20 (2007).
9. Senti, G. *et al.* Use of A-type CpG oligodeoxynucleotides as an adjuvant in allergen-specific immunotherapy in humans: a phase I/IIa clinical trial. *Clin. Exp. Allergy* **39**, 562–570 (2009).
10. McCluskie, M.J. & Davis, H.L. Oral, intrarectal and intranasal immunizations using CpG and non-CpG oligodeoxynucleotides as adjuvants. *Vaccine* **19**, 413–422 (2000).
11. Demento, S.L. *et al.* TLR9-targeted biodegradable nanoparticles as immunization vectors protect against West Nile encephalitis. *J. Immunol.* **185**, 2989–2997 (2010).
12. Hall, J.A. *et al.* Commensal DNA limits regulatory T cell conversion and is a natural adjuvant of intestinal immune responses. *Immunity* **29**, 637–649 (2008).
13. Lee, J. *et al.* Maintenance of colonic homeostasis by distinctive apical TLR9 signalling in intestinal epithelial cells. *Nat. Cell. Biol.* **8**, 1327–1336 (2006).
14. Lacroix-Lamande, S., Rochereau, N., Mancassola, R., Barrier, M., Clauzon, A. & Laurent, F. Neonate intestinal immune response to CpG oligodeoxynucleotide stimulation. *PLoS One* **4**, e8291 (2009).
15. Hong, X. *et al.* Gene polymorphisms, breast-feeding, and development of food sensitization in early childhood. *J. Allergy Clin. Immunol.* **128**, 374–381 (2011) e2.
16. Sun, J. *et al.* Impact of CD40 ligand, B cells, and mast cells in peanut-induced anaphylactic responses. *J. Immunol.* **179**, 6696–6703 (2007).
17. Arias, K. *et al.* Distinct immune effector pathways contribute to the full expression of peanut-induced anaphylactic reactions in mice. *J. Allergy Clin. Immunol.* **127**, 1552–1561 (2011) e1.
18. Chambers, S.J., Wickham, M.S., Regoli, M., Bertelli, E., Gunning, P.A. & Nicoletti, C. Rapid *in vivo* transport of proteins from digested allergen across pre-sensitized gut. *Biochem. Biophys. Res. Commun.* **325**, 1258–1263 (2004).
19. Roth-Walter, F. *et al.* Pasteurization of milk proteins promotes allergic sensitization by enhancing uptake through Peyer’s patches. *Allergy* **63**, 882–890 (2008).
20. Dunkin, D., Berin, M.C. & Mayer, L. Allergic sensitization can be induced via multiple physiologic routes in an adjuvant-dependent manner. *J. Allergy Clin. Immunol.* **128**, 1251–1258 (2011).
21. Liu, N., Ohnishi, N., Ni, L., Akira, S. & Bacon, K.B. CpG directly induces T-bet expression and inhibits IgG1 and IgE switching in B cells. *Nat. Immunol.* **4**, 687–693 (2003).
22. Ozcan, E., Rauter, I., Garibyan, L., Dillon, S.R. & Geha, R.S. Toll-like receptor 9, transmembrane activator and calcium-modulating cyclophilin ligand interactor, and CD40 synergize in causing B-cell activation. *J. Allergy Clin. Immunol.* **128**, 601–609 (2011) e1–4.
23. Duan, W. *et al.* Innate signals from Nod2 block respiratory tolerance and program T(H)2-driven allergic inflammation. *J. Allergy Clin. Immunol.* **126**, 1284–1293 (2010).
24. Genuneit, J. *et al.* A multi-centre study of candidate genes for wheeze and allergy: the International Study of Asthma and Allergies in Childhood Phase 2. *Clin. Exp. Allergy* **39**, 1875–1888 (2009).
25. Novak, N. *et al.* Putative association of a TLR9 promoter polymorphism with atopic eczema. *Allergy* **62**, 766–772 (2007).
26. Ewaschuk, J.B., Backer, J.L., Churchill, T.A., Obermeier, F., Krause, D.O. & Madsen, K.L. Surface expression of Toll-like receptor 9 is upregulated on intestinal epithelial cells in response to pathogenic bacterial DNA. *Infect. Immun.* **75**, 2572–2579 (2007).
27. Lin, L., Gerth, A.J. & Peng, S.L. CpG DNA redirects class-switching towards “Th1-like” Ig isotype production via TLR9 and MyD88. *Eur. J. Immunol.* **34**, 1483–1487 (2004).
28. Spiegelberg, H.L., Tighe, H., Roman, M., Broide, D. & Raz, E. Inhibition of IgE formation and allergic inflammation by allergen gene immunization and by CpG motif immunostimulatory oligodeoxynucleotides. *Allergy* **53** (45 Suppl), 93–97 (1998).
29. Ramaprakash, H., Ito, T., Standiford, T.J., Kunkel, S.L. & Hogaboam, C.M. Toll-like receptor 9 modulates immune responses to *Aspergillus fumigatus* conidia in immunodeficient and allergic mice. *Infect. Immun.* **77**, 108–119 (2009).
30. Hill, D.A. *et al.* Commensal bacteria-derived signals regulate basophil hematopoiesis and allergic inflammation. *Nat. Med.* **18**, 538–546 (2012).
31. Milner, J.D. *et al.* Impaired T(H)17 cell differentiation in subjects with autosomal dominant hyper-IgE syndrome. *Nature* **452**, 773–776 (2008).
32. Varona, R. *et al.* CCR6-deficient mice have impaired leukocyte homeostasis and altered contact hypersensitivity and delayed-type hypersensitivity responses. *J. Clin. Invest.* **107**, R37–R45 (2001).
33. Luther, S.A., Ansel, K.M. & Cyster, J.G. Overlapping roles of CXCL13, interleukin 7 receptor alpha, and CCR7 ligands in lymph node development. *J. Exp. Med.* **197**, 1191–1198 (2003).
34. Auci, D.L., Chice, S.M., Heusser, C., Athanassiades, T.J. & Durkin, H.G. Origin and fate of IgE-bearing lymphocytes. II. Gut-associated lymphoid tissue as sites of first appearance of IgE-bearing B lymphocytes and hapten-specific IgE antibody-forming cells in mice immunized with benzylpenicilloyl-keyhole limpet hemocyanin by various routes: relation to asialo GM1 ganglioside+ cells and IgE/CD23 immune complexes. *J. Immunol.* **149**, 2241–2248 (1992).
35. Bouskra, D. *et al.* Lymphoid tissue genesis induced by commensals through NOD1 regulates intestinal homeostasis. *Nature* **456**, 507–510 (2008).
36. Hemmi, H. *et al.* A Toll-like receptor recognizes bacterial DNA. *Nature* **408**, 740–745 (2000).
37. Berin, M.C., Zheng, Y., Domaradzki, M., Li, X.M. & Sampson, H.A. Role of TLR4 in allergic sensitization to food proteins in mice. *Allergy* **61**, 64–71 (2006).
38. Lefrancois, L. & Lycke, N. *Isolation of Mouse Small Intestinal Intraepithelial Lymphocytes, Peyer’s Patch, and Lamina Propria Cells*, Current Protocols of Immunology: Wiley & Sons, 1996.
39. Thurston, G. *et al.* Leakage-resistant blood vessels in mice transgenically overexpressing angiopoietin-1. *Science* **286**, 2511–2514 (1999).