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TLR Activation and Allergic Disease: Early Life Microbiome and Treatment

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Abstract

Purpose of Review Allergy and asthma are growing problems in the developed world. The accelerated increase of these diseases may be related to microbiome modification that leads to aberrant activation of Toll-like receptors (TLRs). Current research supports the concept that changes in microbial communities in early life impact TLR activation, resulting in an altered risk for the development of asthma and allergies.

Recent Findings Prenatal and early childhood events that generate microbiome modification are closely related with TLR activation. Early childhood exposure to a rich array of TLR agonists, particularly lipopolysaccharide, strongly predicts protection against allergic disease later in life even when other lifestyle factors are accounted for. Genetic deletion of TLR signaling components in mice results in reduced function of tolerogenic cell populations in the gut. In contrast, weak TLR signaling can promote allergic sensitization later in life.

Summary This review summarizes the role of TLR signaling in microbiome-mediated protection against allergy.

Keywords TLRs · Allergy · Microbiome · Asthma · TLR sensitization · T_{regs} · Tolerogenic DC

Introduction

Allergic disease is the sixth leading cause of chronic illness in the USA. [1]. The unprecedented rise of allergy and asthma in the developed world, which has occurred in just a couple of generations, cannot be explained by genetic changes in the population over this short time frame [2]. Allergy has been described as a maladaptive immune response to innocuous environmental antigens, such as pollen, peanut, and animal dander [3]. The most likely culprits in explaining the rapid and recent rise of allergic disease incidence are environmental influences that include a loss of microbiota diversity. For example, there is evidence that indicates that gut microbiome modulates the activities of helper T cell subsets (Th1 and Th2) that can impact the development of immune tolerance [4]. Environmental effects such as exposure to pollution, living in environments with low endotoxin (urban environment), or smoking (Fig. 1) can collectively lead to immune activation and epigenetic modification [2, 3].

The molecular basis of the allergic process has not been entirely established. However, several mechanisms have been proposed, among them are disruption in function and structure of epithelial barriers. In normal conditions, dendritic cells (DC) can sample antigens through the intact epithelial barrier by dendritic cell extension across epithelial cells and tight junction proteins [5], while in a damaged epithelial barrier, antigens can penetrate the underlying epithelium and activate the innate and adaptive immune response [5, 6]. Another proposed mechanism is that allergens activate protease receptors on epithelial cells, thereby inducing innate cytokines that drive T helper 2-like immunity [6]. The maturation and activation of innate cells are driven by TLR signaling for correct APC activation and to initiate the proper immune environment [7]. In allergic individuals, APCs capture antigen, migrate to the draining lymph node, and polarize T cells to become reactive against environmental allergens. Antigen-specific T cells infiltrate the tissue and produce

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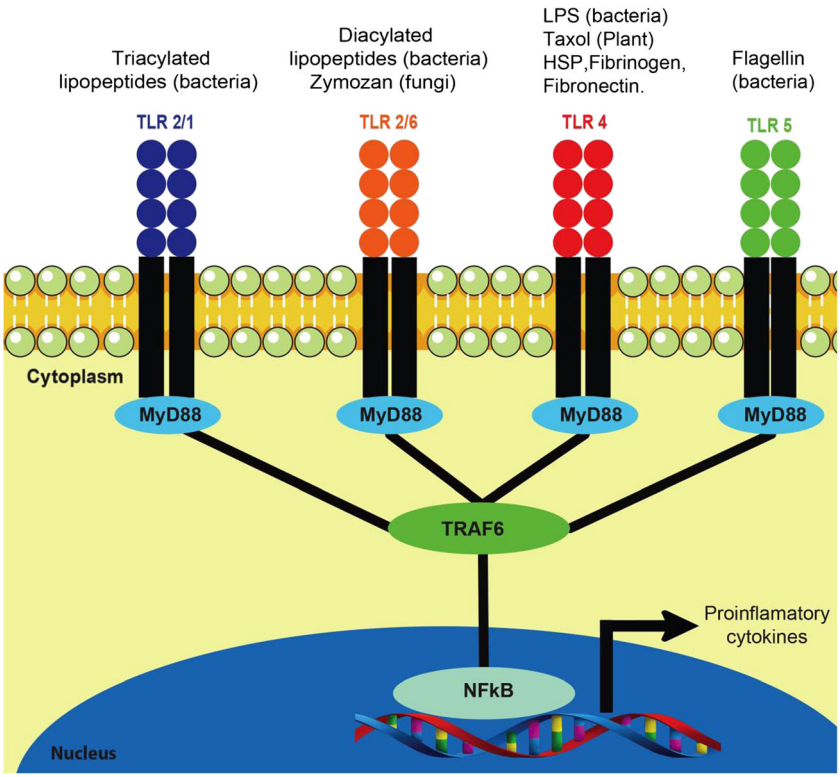

Fig. 1 TLR activation. TLRs 1, 2, 4, 5, and 6 have been implicated in the development of allergies. Different ligands have been reported to activate a diverse set of TLRs. The principal signaling pathway that TLRs use is through MyD88-TRAF6 cascade that leads to NF-κB activation to regulate the transcription of inflammatory genes. TLRs, Toll-like receptors; MyD88, myeloid differentiation primary response gene 88; TRAF6, TNF receptor associated factor 6; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells.

cytokines (Th2) that promote classic symptoms of allergy [8]. Polarized Th2 T cells support B cell class switching to IgE, and infiltration of mast cells and eosinophils [5, 6]. Initiation of the classic Th2 immune response produces a positive reinforcement cycle of IL-4, IL-5, and IL-13 expression by T cells and innate lymphoid cells (ILCs). The production of innate cytokines IL-25, IL-33, and thymic stromal lymphopoietin (TSLP) from the surrounding epithelial cells can both initiate and perpetuate the ongoing Th2-type responses [9]. The maturation and activation of the innate immune cells are driven in part by TLRs that are necessary to maintain immune homeostasis, but can also promote allergic responses when activated in the improper environment [7].

TLRs are a family of transmembrane protein receptors essential for the appropriate activation of the innate immune system, are evolutionarily preserved, and contain binding domains of leucine-rich repeat motifs. TLRs are one of the first lines of defense and activated by pathogen-associated molecular patterns (PAMPs), which are structurally conserved molecules that occur in many pathogens but not the host. Eleven TLRs have been identified in humans (TLR1 to TLR11), and all are functional except for TLR11 [7, 8]. TLRs are differentially located on the cell surface or in endosomes on both immune and non-immune cells critical to initiate the adaptive immune response, including epithelial cells, neutrophils, natural killer cells, and APC. Following TLR activation by their ligands, APCs increase their expression of costimulatory molecules (MHC, CD80, CD86, and CD40) and cytokines such as IL-6 are produced that can intervene in the suppressor activity of the T_{regs} [10]. Activated dendritic cells can also produce cytokines like IL-12 that help activate the clonal expansion of T cells [11]. TLRs also have an essential role in the maturation of dendritic cells [12] that participate in the development of healthy immune tolerance later in life. As TLR ligands are found both on beneficial and pathogenic microbes, the final result of TLR activation is dependent on cell type, location, and signal strength.

This review will cover how interactions between different TLRs, and their respective ligands, can either protect against or enhance the allergic process depending on location and stage of life (Fig. 1). It is now well documented that the gut microbiome plays an essential role in the development of the immune response, and it is becoming increasingly clear that both maternal influences and the early life microbiome that develops in the neonate have crucial roles in the development of allergies. An important component of microbiome-mediated protection appears to include TLR stimulation necessary to properly activate and mature regulatory APCs and T cells to avoid development of allergy.

Prenatal and Early Childhood Microbiome-TLR Interactions and the Susceptibility to Asthma and Allergy

Exposure to factors that affect the susceptibility of an individual to an allergic disease begin before birth, starting with the

environment and allergy status of the mother. Microbes have been described in the placenta, amniotic fluid, fetal membrane, umbilical cord blood, and meconium [13–16]. The maternal-fetal interface is an active immune site, where the mother must maintain fetal tolerance but still protect against infection. Natural killer cells, dendritic cells, and macrophages infiltrate the endometrium and trophoblast regions and become activated during fetal development [17, 18]. The complex interactions between microbes, the maternal immune system, and developing fetus can have far-reaching consequences on childhood sensitivity to development of atopic disease.

Prenatal exposure to an environment rich in microbial compounds (including endotoxin), such as is found in rural and farm communities, reduces the prevalence of childhood allergy [19, 20•, 21]. Children of mothers with allergic disease who were supplemented with *Lactobacillus rhamnosus* and *Bifidobacterium longum* during pregnancy and the first 2 months of breastfeeding demonstrated decreased incidence of eczema and skin prick sensitivity to allergens at the age of 2 compared to mothers given placebo [22]. A decreased allergy incidence was found in similar studies, where prenatal probiotic supplementation was associated with microbial alteration detected in infant meconium compared to placebo control [22, 23]. These studies imply that a healthy microbiome during pregnancy can impact the development of allergy in the offspring. While the mechanism of probiotic-mediated protection is incompletely understood, studies in mice suggest that maternal TLR signaling is required to convey protective effects to the offspring. Conrad and colleagues demonstrated that maternal exposure to *Acinetobacter lwoffi* F78 protected the murine offspring against an allergic airway disease model, but protection was lost entirely if the challenged mothers were TLR2, TLR3, TLR4, TLR7, and TLR9 deficient (−/−) [24••]. Even though the offspring carried one functional allele of the TLRs from the father, they were not protected, demonstrating that TLR signaling in the mother was required for a protective effect. These studies suggest that the interaction between microbial products and the maternal immune system is essential to induce protective effects to the offspring.

Regulation of immune responses early in life appears to be central to controlling the development of allergic diseases. Since T_{regs} are involved in the early stage of the immune programming and their suppressive activity is needed to maintain a balance of the immune response, understanding the regulation of their development is essential to understanding the allergic disease. It has been described that the TLR2 and TLR4 pathways upregulate T_{reg} development and activation [25] and that TLRs are expressed in both human and murine T_{regs}, suggesting direct TLR signaling for modulating T_{regs} function [26]. Previous studies using human cord blood mononuclear cells (HCBMC), which are reported to contain functional T_{regs} [26, 27], showed that cells obtained from infants of allergic mothers had decreased numbers of T_{regs}, and lower expression of T_{reg}-associated genes accompanied by decreased suppressive function of the T_{regs} [28•]. HCBMC obtained from infants of allergic mothers had decreased production of IL-10 and IFN-γ, but increased production of IL-13, when stimulated by TLR2 ligand peptidoglycans (PPG) [28•]. Thus, maternal allergy could impair T_{reg} development and function via TLR2 and TLR4 in the infants and increase susceptibility to allergic diseases [28•]. These latter findings were confirmed in studies using HCBMC from neonates stimulated with TLR2 or TLR4 ligands that found infants who developed eczema had a decreased percentage of T_{regs} and IL-10 cytokine production compared to HCBMC samples from non-allergic infants [27]. The altered cytokine response was accompanied by a lower percentage of T_{regs} in the samples from infants with atopic sensitization after, but not before, TLR2 stimulation. It was proposed that deficient T_{reg} responses to microbial stimuli at the time of birth might contribute to increased risk of allergic diseases in the first year of life [27]. Thus, it appears that early activation of the immune system via TLR contributes to the development of T_{reg} cells that can modify the immune environment.

Early Establishment of a Rich Microbiome Protects Against Allergic Disease

The microbiome composition and its diversity increase within the first years of life and determine the characteristics of the adult microbiome [29]. Since microbial components can provide strong TLR activating signals, they provide the earliest environmental stimuli to shape the immune responses. Microbial communities can be influenced by the use of pharmaceuticals, nutrients, microbial exposure, and infection [29, 30]. In a study that used human data from multiple countries, the prevalence of atopic sensitization and asthma was significantly lower in children who grew up on farms compared to those who did not with a stronger protective effect observed if mothers were also active on the farm. Interestingly, farm children had higher leukocyte gene expression of TLR2, TLR4, and CD14 (multifunctional receptor for endotoxin and other bacterial wall components) [21]. The importance of environmental microbial exposure was exemplified in a recent study, which compared allergy and asthma incidence in two closely related communities, the Amish and Hutterites. Despite sharing many similarities such as genetic ancestry, diet, family size, and long intervals of breastfeeding, Amish communities had much lower rates of childhood asthma and allergy compared to Huttertie communities. Protection in the Amish communities was closely associated with farming and increased levels of household endotoxin, diverse microbiome, and enhanced innate immune signaling; in contrast, Hutterite households had low endotoxin and reduced microbiome due to the purposefully separation of the community from the farm

[31••]. The innate immune signals that were associated with protection are the same signals driven by TLR activation. Thus, understanding how the microbiome and their products (TLR ligands) influence the development of appropriate responses in susceptible populations will be critical.

The hypothesis unifying these findings is that increased microbiome diversity prepares the immune system for appropriate responses later in life by the recognition of commensal bacteria via TLRs and conditions the immune response to develop a balanced, rather than allergic, immune response. One of the most studied pathways linking the microbiome and immune regulation is microbial lipopolysaccharide (LPS) that activates TLR4. Peripheral blood mononuclear cells (PBMC) collected from children exposed to high levels of LPS in early life had decreased production of IL-10, IFN-γ, IL-12, and TNF-α when stimulated with LPS compared to children exposed to low levels of endotoxin. These results suggest that environmental exposure has a significant role in the development of tolerance to environmental stimuli [32]. It also has been suggested that the difference in TLR4 activation could be due to differences in LPS isoforms; the main difference between isoforms has been described in the TLR4-binding lipid A, which is the primary inducer of immunological response to LPS; and difference between variants is in the extent of acylation of the lipid A [33]. The penta-acylated lipid A isoform confers a degree of TLR-4 inhibition, while the hexa- or hepta-acylated lipid A variants are considered a potent stimulator of TLR4 signaling that leads to a Th1 response [33, 34].

Innate immune development is further impacted by early exposure to environmental triggers that alter molecular mechanisms of TLR-mediated cytokine production. For example, upon an infant's initial exposure to LPS shortly after birth, intestinal epithelial cells become hypo-responsive to subsequent TLR stimulation, presumably to facilitate microbial colonization and host-microbe homeostasis [35]. Together, these studies suggest that increased microbial exposure prevents the development of allergic diseases through TLR regulation, and this effect is stronger if the exposure begins at prenatal stages and continues during the first years of life.

Recognition of Gut Microbes by TLR Supports Treg Expansion

Depletion of the microbiota with antibiotics sensitizes mice to allergy, whereas reconstitution of germ-free mice with protective microbiota protects against allergy [36, 37•, 38]. The beneficial effects of healthy microbiome are complex and diverse but include the production of beneficial metabolites that dampen systemic inflammation, including short-chain fatty acids and polyunsaturated fatty acids [39, 40]. Many of the anti-inflammatory effects of diverse gut microbiome are attenuated in mice with genetic deletions in TLR signaling components, suggesting that TLR-mediated recognition of commensal bacteria is essential for some of the protective effects of the microbiome [38, 41, 42, 43•]. For example, an influential study found that TLR4 and TLR5 ligation induced A proliferation-inducing ligand (APRIL) production in intestinal epithelial cells, which supported B cell class switching from IgM to IgA2 in the intestine, an essential component of tolerance against mucosal antigens [44]. Other groups have reported reduced tolerogenic mechanisms in mice with a genetic deletion in TLR signaling components, such as decreased production of IL-10 in lung interstitial macrophages of MyD88−/− and TLR4−/− mice [45], and reduced numbers of CD25+ regulatory T cells in the blood and spleen of MyD88−/− mice [46]. Current evidence points to a model where host-microbiome interactions through TLR recognition is critical for expanding regulatory subsets which protect against allergy, both in the gut and at distal sites such as the airway and skin [47]. However, the mechanism of how, or which components of the microbiome activate the TLR molecules, is unknown. Thus, a strategy to appropriately activate particular TLRs early in life may provide a protective response to generate a tolerogenic environment.

DC require microbial ligand activation via TLR signaling in order to generate regulatory T cells in the gut and the periphery. Transgenic mice engineered to have dendritic cell-specific TRAF6 deficiency (a primary component of TLR signaling) unexpectedly developed spontaneous Th2 inflammation and fibrosis in the gut that was associated with fewer intestinal T_{regs} in vivo. In vitro, TRAF6-deficient DC induced low levels of IL-2 and had a reduced capacity to induce Foxp3 expression in CD4 T cells [42••]. CD103+ DC play a critical role in homeostasis by capturing allergens, migrating to lymph nodes, and inducing tolerance. Intestinal CD103+ DC from TLR4−/− mice showed a reduced capacity to migrate to lymph nodes following oral antigen challenge and had a reduced ability to convert T_{regs} in vitro [48••]. These findings are complementary to other studies that show CD103+ DC sample the luminal contents of the gut in a TLR-dependent manner [49]. Thus, gut DC have a reduced capacity to promote tolerance to the antigen if they cannot recognize gut bacteria through TLRs.

Several groups have reported that T cell intrinsic TLR signaling plays a role in suppressing inflammation [35]. Transgenic mice with Foxp3-specific MyD88 deletion had reduced T_{reg} and T follicular helper populations in the intestine and reduced total gut IgA and showed that TLR1/2-STAT3 signaling was necessary for optimal T_{reg} cells [43•]. Two additional papers reported that T cell intrinsic TLR2 signaling actively promotes the production of IL-10 and the expression of CD25 and FoxP3 on T cells, and implicated both microbial (lipoproteins and peptidoglycan) and endogenous (human 60-kDa heat shock protein) ligands [50, 51]. These murine studies have been corroborated by in vitro analysis of

T cells collected from asthmatic humans, where it was reported that increased expression of TLR2 and TLR4 on the surface of T_{regs} was associated with the enhanced suppressive ability [52]. These mechanistic studies suggest that early exposure to microbial ligands is crucial for developing regulatory immune cell populations toward potential allergens encountered later in life. Individuals who did not develop these protective populations are vulnerable to sensitization against innocuous antigens.

TLR and Food Allergy

In the past two decades, there has been an unprecedented rise in children that develop a food allergy. Currently, this statistic is at 1 in 13 children. The increased incidence of food allergy closely mirrors the expansion of other atopic diseases including allergy, eczema, and wheeze, suggesting a shared mechanism. The appropriate immune response is for gut DC and macrophages to induce strong tolerance to soluble antigens found in the diet by activating T_{regs}. It has long been known that antigens delivered orally induce tolerance at secondary challenge sites such as the lung and skin. Food allergy, therefore, represents a severe disruption of normal tolerogenic mechanisms [53••]. It has been proposed that sensitization against food allergens may occur at a different site, such as the skin. Like other allergy models, healthy gut flora protects against food allergy in mice through a complex and multifactorial mechanism [38]. Gnotobiotic studies in mice have demonstrated that colonization of the gut with protective strains of bacteria, notably from the Clostridia class, protects against food allergy, whereas depletion of bacteria with antibiotics sensitizes mice to food allergy [54].

Recent work supports an active role in TLR signaling in the microbiome-dependent protection against food allergy. Oral supplementation of pregnant mice with the probiotic *Lactobacillus rhamnosus* was found to protect pups against an OVA food allergy model and was associated with increased IgA+ cells in the intestine [55]. Pups from probiotic-supplemented mothers also had increased TLR2 expression in the small intestine, suggesting a protective role in TLR2-mediated immune activation. Consistent with this, a study reported that TLR4 deficiency increases the susceptibility to food allergy in numerous strains of mice, although the mechanism of susceptibility was unclear [56]. More recent work suggests that T_{reg} maturation is central to the protective effects of the microbiome and TLR signaling. Wild-type mice that were orally gavaged with the skin contact sensitizer dinitrofluorobenzene (DNFB) were protected from a subsequent topical challenge, but protection was reduced in both germ-free and TLR4-deficient mice [48••]. Furthermore, MyD88−/− and TLR2−/− mice have reduced capacity to imprint gut-homing of T_{regs}, a critical step in developing intestinal tolerance [54]. Together, these studies suggest that TLR-APC interactions are critical for inducing tolerance to oral antigens.

Weak TLR Signaling Promotes Allergic Asthma

While early life exposure to microbial ligands is protective against allergy later in life, the same ligands play a critical role in sensitization against antigens encountered in the airway [57]. For example, early childhood exposure to endotoxin protects against allergy, but home endotoxin levels can be positively correlated with asthma in individuals over 18 years of age [58]. Low doses of endotoxin are sufficient to sensitize against allergen in allergic airway disease and a significant component of the house dust extract (HDE) model [6]. Interestingly, TLR4 signaling on both dendritic cells and radio-resistant structural cells contributes cumulatively to HDE allergy [59••, 60]. Furthermore, protease from *Aspergillus oryzae* can generate endogenous TLR4 ligands from fibrinogen cleavage products and can promote allergy by stimulating TLR4 allowing allergic sensitization [61•, 62•]. These studies further suggest that exposure to environmental irritants might promote asthma exacerbations through activation of TLRs.

Analysis of lung microbiome composition garners further evidence that TLR signaling plays a pathogenic role in airway hyperreactivity. The lungs of healthy individuals are colonized with bacteria [63•]. In healthy individuals, *Provetella* is the primary strain which colonizes the lung. In individuals with asthma, there is an outgrowth of *Haemophilus*, *Streptococcus*, and *Moraxella* subspecies [64]. The development of infantile wheezing in neonates delivered vaginally was lower compared to cesarean section (CS) [65] and was associated with decreased production of TNF-α and IL-6 in cord blood mononuclear cells in response to TLR1/2 stimulation in CS infants. Differential microbial communities were observed in the airways of the infants born by CS compared with natural delivery, with increased detection of *Streptococcus pneumoniae*, *Staphylococcus aureus*, and *Moraxella catarrhalis*. A correlation between CS delivery and increased risk of wheezing was observed at 1 year of age, suggesting that CS delivery increased the risk of wheeze by decreasing perinatal infant cytokine responses to TLR1/2 stimulation [65]. The critical role of microbiome diversity during the first month of life in the prevention of allergies has been reported [66], and the decreased microbial exposure that CS generated has been linked with the abnormal development of immunity [67]. Interestingly, in vitro studies demonstrated that asthma-associated pathogens *Haemophilus* spp. and *Moraxella* spp. elicit higher cytokine production from human DC and THP-1-derived macrophages than commensal lung bacteria [68]. The

LPS structure of *Haemophilus* and *Moraxella* elicits 10-fold higher TLR4 signaling compared to commensal bacteria. Bacterial species associated with healthy lung microbiome elicit minimal TLR responses, whereas bacterial species associated with asthma and COPD can elicit more robust TLR responses. Mice experimentally inoculated with the asthma-associated proteobacteria exhibited enhanced neutrophilia and cytokine production compared to mice inoculated with commensal *Provetella* [69]. *Provetella* bacteria promoted leukocyte recruitment to the lungs in a TLR2-dependent manner, but this recruitment was nonpathogenic. The enhanced pro-inflammatory capacity of pathobionts and the inert immunological properties of healthy commensals suggest that minimal TLR signaling in the airway can protect against allergy.

Strong TLR Signaling Protects Against Allergic Airway Disease

While trace levels of TLR ligands in the airway promote airway sensitization and Th2-type immunity, pharmacological doses of the same ligands can protect against allergy [27, 70, 71]. The differential outcome that TLR ligands generate in allergic sensitization has created apparent contradictions in the literature, where some authors report that TLR ligands are pathogenic while others report they are protective. In a seminal study, low-dose LPS exacerbated allergy, whereas high-dose LPS protected against eosinophilia and production of Th2 cytokines in association with enhanced interferon gamma production [71]. Subsequent studies demonstrated that repeated administration of low-dose LPS has a protective effect by suppression of cytokine production in epithelial cells [72•]. While low-dose flagellin (TLR5 ligand) enhanced OVA-induced allergy in mice, a high dose of flagellin protected mice against allergy by generating regulatory dendritic cells and T cells dependent on CD25+ cells T_{reg} cells [73••]. The differential effects of flagellin in allergic airway disease can also be observed in other studies [74] and showed that high dose is associated with accumulation of regulatory cell populations [75]. Probiotics also protect against allergic airway disease using intranasal administration of *Lactococcus lactis* G121 to protect mice in an OVA asthma model [76] through TLR3 and TLR8 activation resulting in IL-10 and IL-12 production [77•]. The distinct response to TLR stimulation has historically been described as a shift from Th2- to Th1-type immunity as the strength of stimulation is increased. More recent work suggests a model where Th2 responses are dampened not only by a competing Th1 response, but by the activation of T_{regs}, and IL-10-producing DC and macrophages [47].

The use of strong TLR ligands to treat allergic asthma has also been examined in pre-clinical models and in clinical trials. In particular, unmethylated CpG was shown to have a particularly strong effect in blocking the onset and maintenance of allergic responses as well as reversing established disease in experimental allergic asthma [78, 79]. However, clinical trials using TLR9 stimulatory CpG compounds have been variable and mixed with the overall phase 2 studies showing little efficacy or benefit in moderate to severe asthmatics [80–83]. Additional studies using other endosomal TLRs, TLR3, and TLR7 agonists have also shown promising effects in vivo in animal models [84–86, 87]. Thus, there continues to be significant interest in using individual or combinations of TLR agonists to provide strong activation to modulate asthmatic/allergic diseases.

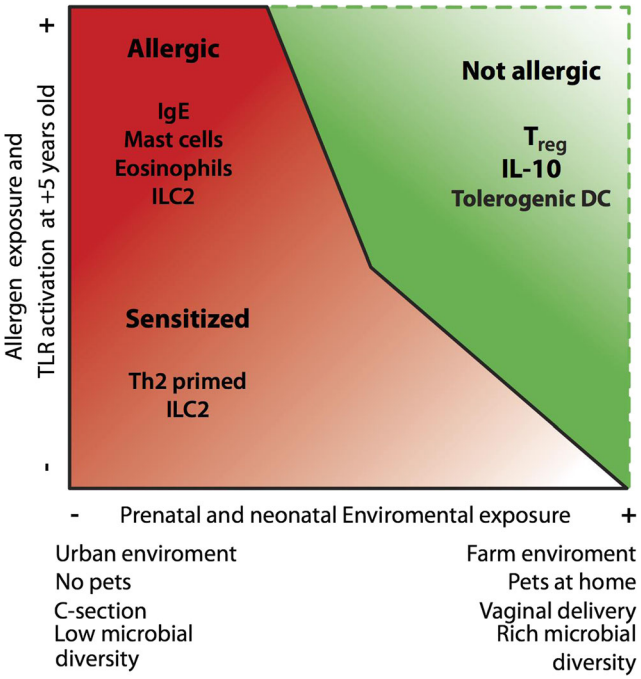

Fig. 2 Proposed relationship between TLR activation, age, and susceptibility to allergy. Individuals who are exposed to a rich variety of TLR agonists, such as living on a farm or growing up with pets, during early childhood are driven toward a tolerogenic immune response that protects against allergy later in life. Peripheral T_{regs}, mucosal IgA production, and IL-10 production appear to regulate allergic disease development. In contrast, individuals with low exposure to microbial products lack these protective regulatory subsets and are vulnerable to allergic disease. Later in life, diverse TLR signaling from the environment can shift this pro-inflammatory state of the immune system into pathogenic allergic disease

Conclusions

It appears that the maternal and early childhood microbiome can generate long-lasting alterations in the TLR responses that can lead to either a decreased or increased predisposition to asthma and allergies. The loss of microbiota diversity may be a significant cause of the rise of allergy, atopy, and asthma in industrialized countries. Mechanistic studies in mice

demonstrate that immune cell subsets that protect against allergy fail to fully develop if protective microbiota or the microbe-TLR signaling pathways are compromised. Individuals who lack early, rich microbiota exposure and the associated protective immune subsets become vulnerable to sensitization against innocuous antigens in the environment. Thus, the timing, intensity, and type of stimuli are likely to have a significant impact on the development of protective vs. pathogenic responses. The same antigens and allergens that promote tolerance in early life can become pathogenic later in life in vulnerable individuals (Fig. 2).

Compliance with Ethical Standards

Conflict of Interest The authors declare no conflicts of interest relevant to this manuscript.

Human and Animal Rights and Informed Consent This article does not contain any studies with human or animal subjects performed by any of the authors.

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