TOLL-LIKE RECEPTOR ACTIVATION IN IMMUNITY VS. TOLERANCE

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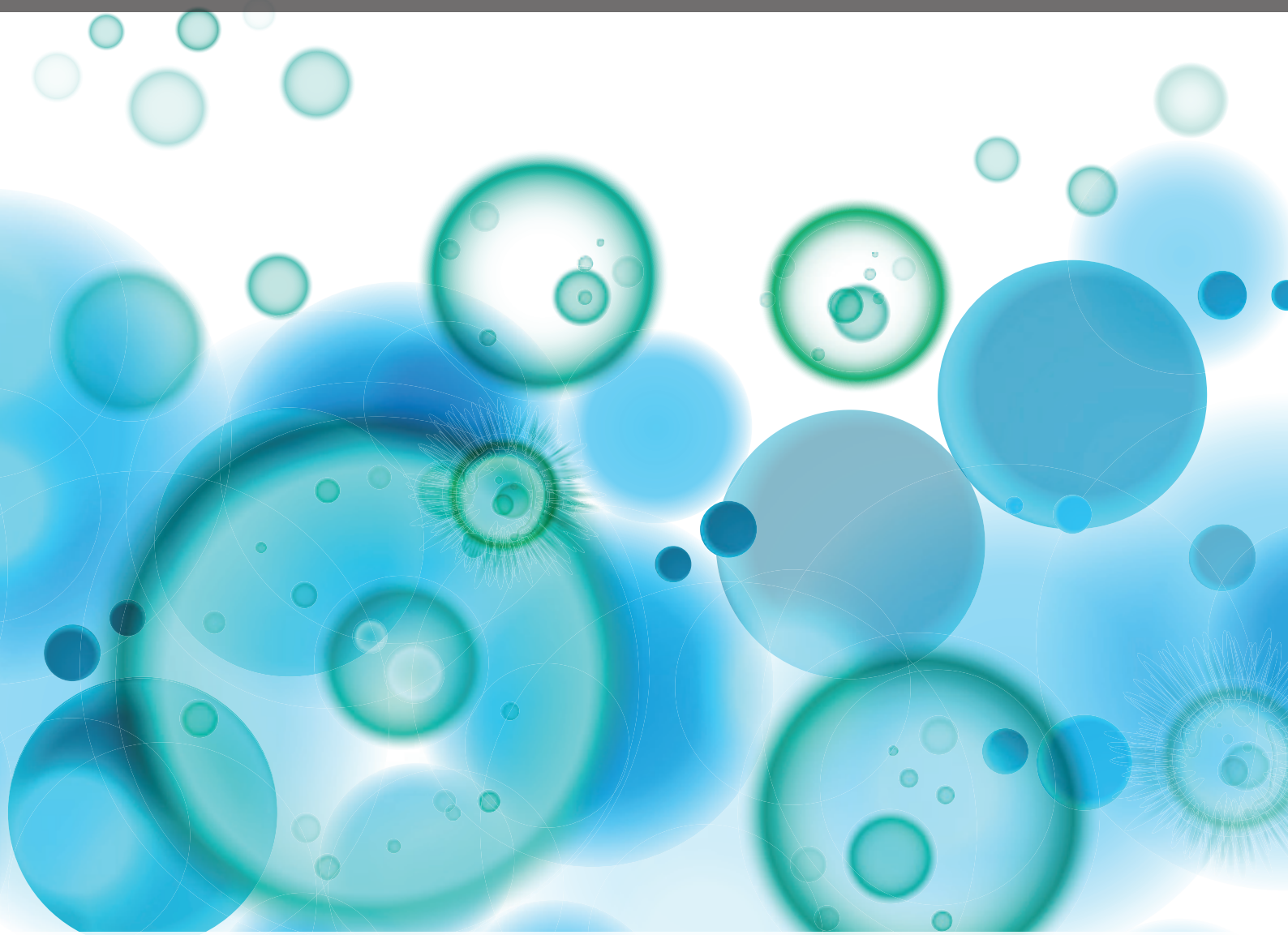

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TOLL-LIKE RECEPTOR ACTIVATION IN IMMUNITY VS. TOLERANCE

Topic Editor:
Christophe M. Filippi, Genomics Institute of the Novartis Research Foundation, USA

The innate immune system has evolved means to recognize and react suitably to foreign entities such as infectious agents. In many cases infectious microorganisms threaten the integrity and function of the target organs or tissues; therefore, consequent to their recognition the immune system becomes activated to ensure their elimination.

Toll-like receptors (TLR) constitute a family of receptors specialized in the recognition of molecular patterns typically associated with infectious agents. Different TLRs exist, each selective for molecular entities and motifs belonging to a specific pathogen group. Consequently, it is thought that the molecular nature of invading microorganisms activates specific TLRs to drive adequate anti-infectious immunity. For instance, nucleic acid-specific, intracellular receptors (TLR3/7/8/9) are used to sense viruses and drive antiviral immunity, while other receptors (such as TLR2 and TLR4) recognize and promote immunity against bacteria, yeast, and fungi.

Yet, it is becoming evident that activation of TLR pathways trigger mechanisms that not only stimulate but also regulate the immune system. For instance, TLR stimulation by viruses will drive antiviral interferon but also immunoregulatory cytokine production and regulatory T cell activation. Stimulation of TLRs by bacteria or using molecular agonists can also trigger both immune stimulatory and regulatory responses. TLR stimulation by infectious agents likely serves to activate but also control anti-infectious immunity, for instance prevent potential immunopathological tissue damage which can be caused by acute immune defense mechanisms. Previous work by us and others has shown that the immunoregulatory arm of TLR stimulation can additionally help control autoreactive processes in autoimmune disease. Hence, it is becoming established that gut commensals, which also play a crucial part in the control of autoimmune disease, establish immune regulatory mechanisms through activation of particular TLRs.

In sum, it appears that TLRs are key immune players that not only stimulate but also regulate immune processes in health and disease. In this Research Topic, we wish to review the dual role of TLRs as activators and regulators of immune responses. We aim to motivate

data-driven opinions as to the importance of context of TLR agonism for determining immune activation vs. regulation.

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Toll-like receptor activation in immunity vs. tolerance

Christophe M. Filippi*

Immunology, Genomics Institute of the Novartis Research Foundation, San Diego, CA, USA
**Correspondence: cfilippi@gnf.org*

Edited and reviewed by:
Herman Waldmann, University of Oxford, UK

Keywords: toll-like receptors, immune stimulation, immunoregulation, autoimmune diseases, cancer immunotherapy, infection, probiotics, microbiome

After the discovery of Toll-like receptors (TLR) in the late 1990s, initial investigations were focused on understanding their role, stimulating immune responses against infectious agents. Yet, the human body is home to a myriad of TLR agonistic bacteria that have not only established symbiosis with the immune system but also likely contribute to the induction and maintenance of immune homeostasis by dampening immune responses. In fact, stimulation of TLRs might be critically involved in this process and ultimately contribute to preventing development of inflammatory and autoimmune diseases. Sander De Kivit and colleagues present an overview of those aspects, notably outlining the role of gut epithelial TLRs in the induction of immunity and the maintenance of tolerance (1). In addition, the authors highlight the mechanisms through which the gut microbiota regulates intestinal immune responses through interaction with TLRs. The capacity of probiotic microorganisms to modulate immunity via specific TLRs is further discussed in two articles by Julio Villena and Haruki Kitazawa. The authors explore the role of TLR interaction with immunobiotics for the regulation of intestinal inflammation in pigs, with a focus on TLR4 and *Lactobacillus jensenii* TL2937 (2). Interaction of probiotics with TLR3 to promote beneficial immunity beyond the gut in the respiratory tract is also examined (3). Specifically, inflammatory and immunoregulatory mechanisms conferred by *Lactobacillus rhamnosus* CRL1505 are presented along with their proposed effects increasing resistance to RSV infection while limiting immunopathology.

While TLRs stimulate and regulate immunity against infectious agents, they are also highly pursued targets for therapy of cancer due to their strong ability to activate multiple arms of the immune system, and in particular to stimulate those specific cellular and cytokine responses critical to anti-tumor immunity. However, the immune potentiating, anti-tumor effects of TLR agonists may be restrained by their parallel ability to trigger immune regulation. Dampening of immune responses may enable control of inflammatory damage or immunopathology, but the downside is a limiting effect on efficacy. The impact of this dichotomy on the use of TLR agonists for immunotherapy of cancer is discussed by Hailing Lu in a review article where key immune regulatory facets of TLR agonists are presented, which may impair their efficacy (4). The dual role of TLRs in cancer likely extends beyond the dichotomy between immune stimulation and regulation. Inflammatory processes triggered by TLR engagement may notably constitute a double-edged sword in elimination vs. development of tumors. In that regard, Erin Burns and Nabiha Yusuf contribute two opinion articles discussing TLR targeting for the treatment of cancer. The use of TLR agonists for skin cancer treatment is presented in the context of their impact on skin carcinogenesis, and the question of TLR tolerance is also discussed (5). Similarly, the dual effect of TLR agonists in breast cancer is examined, where TLR stimulation might beneficially activate the immune system but inflammatory processes may also promote tumor development, while TLR-conferred immune regulation may further curb anti-tumor immunity (6).

The mechanisms that underlie the immune regulatory properties of TLRs are not well understood. It is possible that the "default" response induced by TLR stimulation may vary between cell types, or depending on the microenvironment/anatomical location of TLR-expressing cells (as exemplified in gut mucosal tissue). Alexandra Zanin-Zhorov and colleagues described an immune regulatory mechanism conferred by TLR expression on T cells, which the authors review herein and discuss in the context of TLR-induced T cell effector functions (7). The role played by endogenous TLR ligands in conferring immune regulatory mechanisms is also presented. Nobuhiro Nakamoto and Takanori Kanai focus on a key organ, the liver, where various types of TLR-expressing cells are faced with continuous exposure to foreign antigens (8). The authors review immune stimulatory and regulatory effects of TLR signaling that coexist in the liver and influence liver health and disease. Elke Gülden and Li Wen concentrate on another organ, the pancreas, where TLR engagement also ultimately controls health and disease (9). Here, the beneficial and detrimental effects of TLR stimulation on type-1 diabetes are discussed, and notably the authors explore how endogenous TLR agonists can confer immune activation vs. regulation of autoreactive T cells. To close on the question of immune regulation conferred by TLRs, Himanshu Singh Chandel and colleagues contribute an opinion article investigating an alternative immune modulatory aspect that may influence the outcome of parasitic infection (10). The authors review mechanisms that underlie cross-talk between TLRs and CD40, and discuss how this interaction may determine the nature of anti-leishmanial immune responses and ultimately parasite elimination.

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Regulation of intestinal immune responses through TLR activation: implications for pro- and prebiotics

***Sander de Kivit*[1]*, *Mary C. Tobin*[2], *Christopher B. Forsyth*[1], *Ali Keshavarzian*[1,3] *and Alan L. Landay*[2,3]**

[1] *Division of Digestive Diseases and Nutrition, Rush University Medical Center, Chicago, IL, USA*
[2] *Department of Immunology/Microbiology, Rush University Medical Center, Chicago, IL, USA*
[3] *Division of Pharmacology, Utrecht Institute for Pharmaceutical Sciences, Faculty of Science, Utrecht University, Utrecht, Netherlands*

Edited by:
Christophe M. Filippi, Genomics Institute of the Novartis Research Foundation, USA

Reviewed by:
Ari Waisman, University Medical Center of Johannes Gutenberg University Mainz, Germany
Muriel Moser, Université Libre de Bruxelles, Belgium

***Correspondence:**
Sander de Kivit, Division of Digestive Diseases and Nutrition, Rush University Medical Center, 1735 West Harrison Street, Chicago, IL 60612, USA
e-mail: sander_dekivit@rush.edu

The intestinal mucosa is constantly facing a high load of antigens including bacterial antigens derived from the microbiota and food. Despite this, the immune cells present in the gastrointestinal tract do not initiate a pro-inflammatory immune response. Toll-like receptors (TLRs) are pattern recognition receptors expressed by various cells in the gastrointestinal tract, including intestinal epithelial cells (IEC) and resident immune cells in the lamina propria. Many diseases, including chronic intestinal inflammation (e.g., inflammatory bowel disease), irritable bowel syndrome (IBS), allergic gastroenteritis (e.g., eosinophilic gastroenteritis and allergic IBS), and infections are nowadays associated with a deregulated microbiota. The microbiota may directly interact with TLR. In addition, differences in intestinal TLR expression in health and disease may suggest that TLRs play an essential role in disease pathogenesis and may be novel targets for therapy. TLR signaling in the gut is involved in either maintaining intestinal homeostasis or the induction of an inflammatory response. This mini review provides an overview of the current knowledge regarding the contribution of intestinal epithelial TLR signaling in both tolerance induction or promoting intestinal inflammation, with a focus on food allergy. We will also highlight a potential role of the microbiota in regulating gut immune responses, especially through TLR activation.

Keywords: toll-like receptors, intestinal epithelial cells, food allergy, microbiota, probiotics, prebiotics, circadian rhythm

THE MUCOSAL IMMUNE RESPONSE IN THE INTESTINE – AN OVERVIEW

The mucosal tissue of the intestines contains the largest part of the immune system present in the human body, and is constantly exposed to many antigens, which are derived from amongst others food and micro-organisms including the commensal microbiota or invading pathogens. Approximately, 70% of the cells of the immune system are present in the gut and are continuously discriminating between harmless and pathogenic antigens. Nevertheless, the majority of oral foreign antigens do not result in inflammatory responses in healthy individuals. This phenomenon is known as oral tolerance. Local or systemic pathological inflammation may occur when oral tolerance toward some harmless luminal antigens is lost. This is seen for instance in food allergy, which is characterized by an inflammatory immune response toward generally harmless food-derived antigens.

Intestinal epithelial cells (IEC) provide a physical and chemical barrier between the intestinal lumen and the lamina propria. The expression of tight junction proteins by IEC, production of mucus by goblet cells and Paneth cell-derived antimicrobial peptides prevent translocation of luminal antigens and micro-organisms into the lamina propria (1, 2). Nevertheless, antigens are actively sampled into the gut-associated lymphoid tissue (GALT). Understanding of the GALT is essential to gain insight in both disease pathogenesis and to design new therapeutic strategies to prevent or cure inflammatory diseases of the intestine. As an antigen ends up in the lumen of the intestine, it is generally recognized by dendritic cells (DC) present in Peyer's patches, after the antigen has been transported into the Peyer's patch via specialized IEC known as M cells (3, 4). Antigen sampling also occurs via dendrites of DC that protrude between the IEC (5, 6). Upon antigen recognition, DC migrate toward the draining mesenteric lymph nodes (MLN) and activate T cells, which migrate back toward the intestinal lamina propria to carry out their effector functions (7).

Intestinal epithelial cells have been described to suppress DC activation as well and contribute to tolerance induction by secreting amongst others TSLP and TGF-β, and metabolize vitamin A into retinoic acid to induce the development of $CD103^+$ DC (8–12). These $CD103^+$ DC induce antigen-specific regulatory T cells (T_{reg}) as well as the expression of the specific gut-homing molecules α4β7 integrin and CCR9 on T cells in the MLN (13). T_{reg} cells suppress adaptive immune responses through cell–cell contact dependent mechanisms or secretion of the anti-inflammatory cytokines IL-10 or TGF-β. Indeed, induction of T_{reg} cells results in abrogation of food hypersensitivity responses (14, 15). A higher frequency of allergen-specific T_{reg} cells is observed in children that have outgrown cow's milk allergy and allergen-specific immunotherapy has been shown to induce T_{reg} cells (16, 17), implicating that the induction of T_{reg} cells is essential for mucosal tolerance.

REGULATION OF INTESTINAL IMMUNITY AND TOLERANCE BY TLRs EXPRESSED BY IEC

Toll-like receptors (TLRs) recognize a wide range of microbial fragments and therefore recognize both antigens derived from the microbiota as well as invading pathogens. TLRs are expressed by a variety of cells, including IEC. TLR2 can dimerize with TLR1 or TLR6 to recognize bacterial cell wall lipoproteins. LPS produced by Gram-negative bacteria is recognized by TLR4 in conjunction with CD14 and MD2, whereas unmethylated CpG motifs of bacterial DNA are recognized by TLR9. In addition, flagellin is recognized by TLR5, which is expressed at the basolateral membrane by IEC. TLR2, 4, and 5 are generally expressed at the cell membrane, whereas TLR9 is expressed intracellularly. However, in IEC, TLR9 has been reported to be expressed at the cell membrane as well (18, 19).

Under homeostatic conditions, IEC show low expression of TLR2 and TLR4 and are therefore unresponsive to TLR stimuli (20, 21). However, under inflammatory conditions, epithelial TLR expression is increased, which contributes to both inflammation as well as immune tolerance (19, 22, 23). Increased epithelial TLR2 and TLR4 expression is associated with inflammatory bowel disease (24). In contrast, apical TLR9 stimulation has been described to contribute to intestinal homeostasis (18). Interestingly, TLR activation of IEC appears to be important in regulating adaptive immune responses. Using an *in vitro* co-culture system, it was shown that TLR4 and basolateral TLR9 activation on IEC is important in driving an inflammatory response, whereas apical TLR9 activation supported the differentiation of an anti-inflammatory response (25). The underlying mechanisms by which TLR9 promotes tolerance are not well understood, but it has been described that apical but not basolateral TLR9 ligation on IEC prevents degradation of IκB-α, and therefore suppresses NF-κB-induced pro-inflammatory cytokine production by IEC (18). In addition, it has recently been indicated that apical TLR9 activation supports the expression and secretion of galectin-9, a soluble protein of the lectin family, which supports the differentiation of T_{reg} cells potentially by supporting the development of tolerogenic DC (26, 27). Though IEC are important in driving the development of tolerogenic $CD103^+$ DC and suppress DC activation (8), it is not known whether TLR activation on IEC influences the generation of $CD103^+$ DC. Recently, it has been shown that gut bacteria stimulate the recruitment of $CD103^+$ DC into the epithelium potentially via TLR-dependent mechanisms in both IEC and hematopoietic cells (28). Altogether, TLR stimulation in the intestinal epithelium plays an important role in regulating mucosal immune responses in the intestine.

In addition to regulating intestinal immunity, TLR activation on IEC is also known to modulate the expression of tight junction proteins. In many inflammatory disorders, including food allergy, epithelial tight junctions are impaired and increased bacterial translocation occurs (29). This increased bacterial translocation into the lamina propria may sustain the inflammatory response. In particular, epithelial TLR2 activation has been described to protect against barrier disruption by enhancing zonula occludens (ZO)-1 expression in IEC in a protein kinase C-dependent manner (30). In contrast, activation of TLR4 increases intestinal permeability and results in enhances bacterial translocation (31). NF-κB signaling as a result of TLR4 activation by LPS appears to play a major role in LPS-mediated barrier disruption (32, 33). Similarly, apical *Campylobacter jejuni* infection of T84 cell monolayers results in a rapid decrease in the transepithelial resistance of the monolayer involving NF-κB signaling (34). Activation of TLR9 apically on IEC prevents TLR4-induced gut leakiness and infection of IEC monolayers with *Campylobacter jejuni* disrupts the intestinal epithelial barrier function by reducing TLR9 expression at the surface membrane of IEC (33). In this similar study, the authors also indicate an increase in the intestinal barrier function upon apical, but not basolateral TLR9 stimulation with a synthetic CpG DNA (35). Preliminary data from our group also report a potential protective effect of apical TLR9 activation in T84 cell monolayers co-cultured with CD3/28-activated PBMC. Hence, paracellular transport of antigens as well as bacterial translocation under pathological conditions may be affected by TLR activation on IEC.

With respect to food and environmental allergens, the contribution of TLR activation on IEC is not well studied. Recently, TLR4 activation by wheat α-amylase trypsin inhibitors, a recognized plant-derived allergen (36), has been described to drive intestinal inflammation (37). The percentage of α-amylase trypsin inhibitors is markedly higher in genetically modified grain seeds that are more resistant to infection than traditional seeds (38–40), which might explain why a wheat-free diet could be beneficial in a wide range of inflammatory and allergic disorders. Similarly, the house dust mite allergen Der p 2 as well as the major cat allergen Fel d 1 enhance signaling through TLR2 and TLR4 (41). Although these studies were carried out on innate immune cells, this does not exclude that these allergens may interact with TLR expressed by IEC as well. Especially, since TLR activation on IEC affects the mucosal barrier function and potentially shapes mucosal immune responses in the intestine, interactions of allergens with TLR expressed by IEC may facilitate their entry into the gut mucosa and sustain the allergic inflammatory response. Interestingly, treatment with CpG oligodeoxynucleotides improved the intestinal barrier function and increased the percentage of T_{reg} cells in the spleen and MLN (42). Since epithelial TLR may interact with the gut microbiota and luminal antigens, further understanding of the role of epithelial TLR activation in food allergy is necessary.

INTERACTIONS BETWEEN THE MICROBIOTA AND TLRs

The microbiota is the largest source of microbial stimulation in the gut. Furthermore, the microbiota is necessary for development of the intestinal immune system (43). The "hygiene hypothesis," currently the most popular theory of deregulation of the microbiota, theorizes that specific microbial stimulation is necessary for gut health. Originally, it states that microbial stimulation polarizes the immune response toward T_h1, while lack of microbial stimulation maintains a T_h2 polarized immune response, which is characteristic for atopy (44). Recently, a specific microbiota signature was linked to oral allergic sensitization in mice exhibiting a gain-of-function mutation in the IL-4 receptor α chain, which rendered these animals more prone to developing food allergy. This microbiota signature was characterized by a reduction in *Firmicutes* spp. and increase in *Proteobacteria* spp. (45). Another example that

indicates the importance of the gut microbiota composition in the development of food allergy is a recent study showing that colonization of germ-free mice with the fecal microbiota of a healthy infant rich in *Bifidobacterium* spp. and *Bacteroides* spp. protected against the development of cow's milk allergy following sensitization to β-lactoglobulin (46). This was associated with lower T cell reactivity toward the allergen, an increase in $Foxp3^+$ T_{reg} and lower bacterial translocation into the lamina propria. *Bifidobacterium breve* potentially activates $CD103^+$ intestinal DC to produce IL-10 and IL-27 in a TLR2-dependent fashion to induce IL-10-producing T_r1 cells (47), whereas colonization of germ-free mice with *Bacteroides fragilis* restores the T_h1/T_h2 balance and prevents intestinal inflammation through induction of IL-10 producing $CD4^+$ T cells. This was dependent on recognition of *B. fragilis* polysaccharide A by gut DC (48, 49).

Disturbances in the commensal bacterial composition in the gut, reflected by increased colonization with *Escherichia coli* or *Clostridium difficile*, is associated with an increased risk in the development of allergic disease and IBD in humans (50, 51). The fecal microbiota of allergic infants shows a higher prevalence of *Clostridium* spp. and *Staphylococcus aureus*. In parallel, lower levels of *Bifidobacteria*, *Enterococci*, and *Bacteroides* were found in the stool of allergic infants compared to healthy individuals (52, 53). Bacterial colonization early in life has been shown to affect cytokine production by T helper cell subsets, implicating that dysbiosis at an early age may increase the risk of developing food allergy (54). Likewise, infants that have developed eczema by the age of 12 months show a lower diversity in the gut microbiota during the early postnatal period (55). Thus, it appears that low abundance of *Bifidobacteria*, *Enterococci*, and *Bacteroides* and a higher abundance of *Clostridium* spp. and *Staphylococcus* are associated with loss of tolerance and an exaggerated allergic response toward food-derived antigens. However, it was recently shown that *Clostridium butyricum* can induce IL-10 producing macrophages in the gut in a TLR2-dependent manner and suppresses TLR4 expression by colonic IEC (56, 57). Hence, host–microbiome interactions not only promote a normal T_h1/T_h2 balance, but support the development of T_{reg} responses as well. Whether changes in microbiota composition are a factor to promote an allergic response to food or are a consequence of food allergy remains to be studied.

It is important to note that not only changes in the microbiota are present in individuals with food allergy, but the response of immune cells toward the microbiota has also been described to be different. The so-called beneficial bacteria are not necessarily associated with anti-inflammatory responses in allergic patients. For example, although an increased prevalence of *Bifidobacteria* is rendered as beneficial, specific *Bifidobacterium* strains isolated from the feces of allergic infants were shown to induce increased production of the pro-inflammatory cytokines IL-1β, IL-6, and TNF-α (58). This is supported by the observation that the allergic infants showed an increased IL-6 and TNF-α response toward TLR2, TLR4, and TLR5 stimuli (59).

Using *in vitro* models it was shown that IEC play an important role in discrimination between different bacterial strains at the apical membrane (60, 61). In addition, commensal bacteria have the capacity to enhance TLR expression by IEC (62–66). This suggests that TLR responses toward microflora constituents may be important. However, not all bacterial strains are equally effective in suppressing food allergy. This is reflected by the selective capacity of bacterial strains to induce $Foxp3^+$ T_{reg} cells in a murine model for OVA-induced asthma and OVA-induced food hypersensitivity (67). Similarly, only specific *Lactobacillus* strains attenuate T_h2 responses by inducing $CD103^+$ tolerogenic DC (68). Both *Lactobacillus* and *Bifidobacterium* strains have been shown to induce T_{reg} type immune responses, thereby suppressing allergy (47, 69–72). Recently, it has been shown that the bacterial DNA from *Lactobacillus* spp. or probiotics contain a higher frequency of immunoregulatory CpG motifs – potentially stimulating TLR9 – when compared to pathogenic bacteria like *E. coli*, which is important for T_{reg} conversion in the intestinal mucosa (73). Exposure of IEC to DNA derived from *E. coli* or *S. dublin* induces high IL-8 production by IEC (19, 74), whereas DNA from *Lactobacillus rhamnosus GG* prevents NF-κB-induced IL-8 production by IEC (66). Similarly, apical exposure of IEC to genomic DNA from *B. breve* M-16V was found to enhance IFN-γ and IL-10 secretion by PBMC in an HT-29/PBMC co-culture model (26). In line with this study, it was shown that DC cultured in the conditioned medium of IEC apically exposed to *S. Dublin* DNA, but not from *B. breve*, produced increased amounts of pro-inflammatory cytokines (75). This suggests that not all probiotic bacterial strains are potentially effective in treating allergic diseases. Selection of probiotic bacterial strains should possibly be based on their richness in CpG motifs, targeting TLR9, and bacterial strains high in these motifs may be considered for clinical trials.

PREBIOTICS SHAPE THE INTESTINAL MICROBIOTA

Breast feeding also affects the microbiota composition by increasing the amount of *Bifidobacteria* as shown by higher fecal *Bifidobacteria* counts (76). Human milk contains a high amount of non-digestible oligosaccharides with over 1000 different oligosaccharide structures and it has been shown that human milk, as well as specific dietary fibers like chicory-derived inulin and lactose-derived short-chain galacto-oligosaccharides (scGOS), selectively support the growth of *Lactobacillus* and *Bifidobacterium* strains (77). Therefore, these oligosaccharides have prebiotic effects in the intestine. Based on the basic structure and size of neutral non-digestible oligosaccharides present in human milk, a specific prebiotic mixture consisting of scGOS and long-chain fructo-oligosaccharides (lcFOS) in a 9:1 ratio has been developed. Oral supplementation of scGOS/lcFOS has been shown to reduce allergic symptoms in mice and humans (78–80). Especially dietary supplementation with a combination of scGOS/lcFOS and *B. breve* M-16V (GF/*Bb*) is effective in reducing allergic symptoms (81, 82). In a colitis model in rats, inulin, and FOS reduced colitis, which was associated with increased *Bifidobacterium* species and reduced *Enterobacteriaceae* and *C. difficile* in the feces (83). The underlying mechanisms are not known. However, exposure of IEC to GF/*Bb* may result in the generation of tolerogenic DC and consequently T_{reg} polarization in the GALT. In addition to supporting T_{reg} conversion, stimulation of the growth of *Lactobacillus* and *Bifidobacterium* strains may also improve the intestinal barrier function in a TLR2 and potentially TLR9 dependent manner (84, 85).

CIRCADIAN CLOCK AND TLR

Although the type of microbiota composition is a critical factor for the state of TLR activation in the gut of patients with allergic disorders, other environmental factors can also influence TLR activation. It has recently been shown that the expression of TLRs is under regulation of the circadian clock. This implicates that the expression of TLRs is not temporally fixed in a 24-h day and night cycle. Recently, the expression of TLR9 as well as other TLRs were shown to be regulated by the circadian clock (86, 87). Interestingly, the severity of TLR9-mediated induction of sepsis is associated with the time-dependent expression of TLR9 (86). Moreover, further studies have indicated that the interaction between the microbiota and TLRs expressed by the gut epithelium is dependent on the circadian rhythm as well (88). Besides the observation that the expression of TLRs is under circadian control, cytokine production by macrophages and $CD4^+$ T cells, the suppressor function of $Foxp3^+$ T_{reg} cells, leukocyte trafficking, and antibody production also show a circadian pattern (89–97). Furthermore, it was recently shown that the circadian clock is critical for regulation of intestinal permeability as well, as disruption of the circadian rhythm led to increased microbial translocation and disruption of the epithelial tight junctions (98). Hence, interactions between the microbiota and the intestinal mucosal immune system may not only be dependent on the type of bacterial species present in the microbiome, but are also temporally regulated, which may contribute to regulation of immune responses in the intestine. These data may explain why many allergic reactions like asthma attacks occur in the early morning (99, 100). Recently, it was shown that the expression of the FcεRI by mast cells and IgE-mediated mast cell degranulation is temporally regulated by the circadian clock (101, 102). Also, it might, at least partially, explain the rapid rise of incidence of (food) allergies in western societies where disruption of normal circadian patterns and stress is a consequence of modern day society (103).

IMPLICATIONS FOR THE USE OF PRO- AND PREBIOTICS

There is still controversy about the effectiveness of probiotic and prebiotic treatment in food allergy (104). However, given the data that alteration of the gut microbiota influences mucosal immune responses in the gut indicates that treatment using

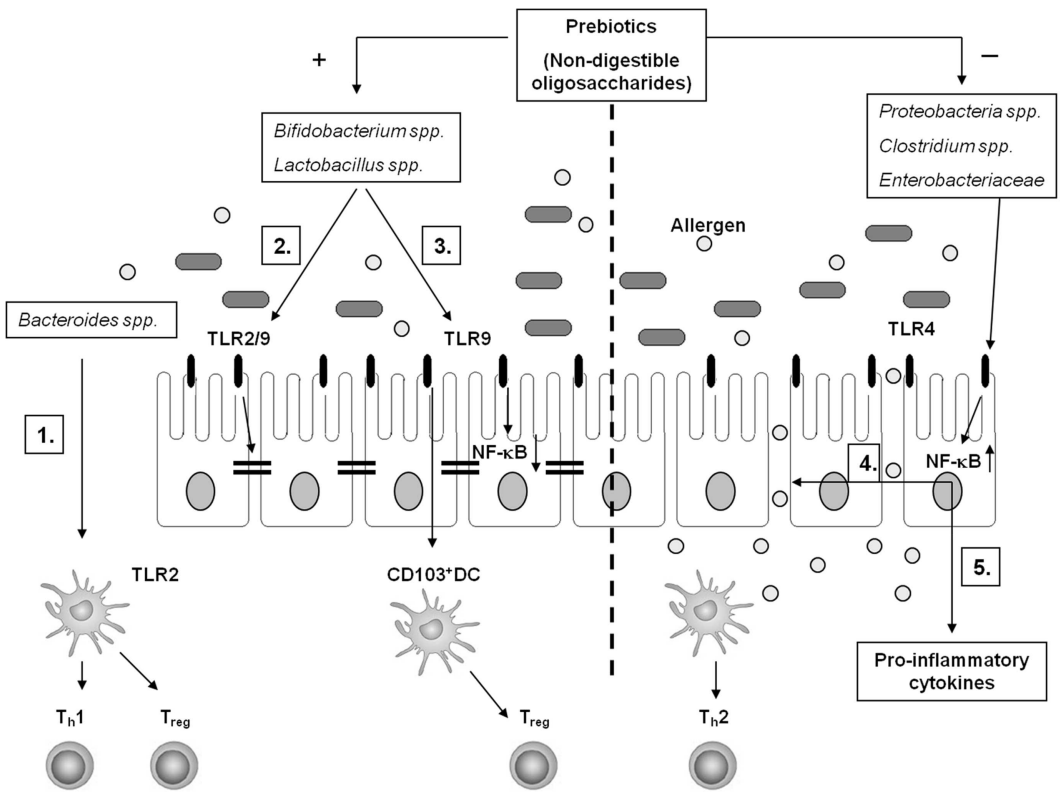

FIGURE 1 | Schematic overview of potential interactions between the gut microbiota and the intestinal mucosal immune system. A healthy gut microbiota composition is high in the frequency of *Bacteroides* spp., *Lactobacillus* spp., and *Bifidobacterium* spp. (1) In particular, *Bacteroides fragilis* supports T_h1 and T_{reg} polarization in a TLR2-dependent manner through recognition of polysaccharide A by gut DC. Genomic DNA of *Bifidobacterium* spp. and *Lactobacillus* spp. – rich in unmethylated CpG motifs – potentially interact with TLR2 and/or TLR9 to enhance the intestinal epithelial barrier function (2) and to support T_{reg} conversion via $CD103^+$ DC (3). Furthermore, apical TLR9 activation by IEC suppresses NF-κB activation (3). In food allergy, the microbiota composition shifts toward a higher frequency in *Proteobacteria* spp., *Clostridium* spp., and *Enterobacteriaceae*. This may favor TLR4 mediated barrier disruption facilitating allergen translocation in the gut mucosa (4) and pro-inflammatory cytokine production (5) in a NF-κB-dependent fashion, sustaining an allergic inflammation. Specific non-digestible oligosaccharides (prebiotics) support the growth of *Bifidobacterium* spp. and *Lactobacillus* spp. and suppresses the growth of *Clostridium* spp. and *Enterobacteriaceae*, which may contribute to induction of tolerance toward allergens in the intestines.

specific probiotic bacterial strains as well as prebiotics may be useful in treatment for food allergy (**Figure 1**). Selection of the right bacterial strains appears key to the effect of treatment using probiotics. Especially, characterization of specific probiotics based on CpG rich motifs in the DNA may improve the selection of potential beneficial strains. Hence, studies aimed at the interaction between probiotic bacteria and epithelial expressed TLRs may be warranted. In addition, timing of treatment may play an essential factor in the effectiveness of treatment using pro- and prebiotics as expression of TLRs and immune cell functions appears to be regulated by the circadian clock. In conclusion, more studies are necessary focusing on interaction between the gut epithelium and gut bacteria, both in terms of selecting potential beneficial bacterial strains as well as appropriate timing of intervention.

AUTHOR CONTRIBUTIONS

Sander de Kivit wrote the manuscript; Mary C. Tobin, Christopher B. Forsyth carefully reviewed the manuscript; Ali Keshavarzian and Alan L. Landay reviewed the manuscript and provided overall supervision.

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Modulation of intestinal TLR4-inflammatory signaling pathways by probiotic microorganisms: lessons learned from *Lactobacillus jensenii* TL2937

Julio Villena*[1,2] *and Haruki Kitazawa*[3]*

[1] *Immunobiotics Research Group, Tucuman, Argentina*
[2] *Laboratory of Immunobiotechnology, Reference Centre for Lactobacilli (CERELA-CONICET), Tucuman, Argentina*
[3] *Food and Feed Immunology Group, Laboratory of Animal Products Chemistry, Department of Science of Food Function and Health, Graduate School of Agricultural Science, Tohoku University, Sendai, Japan*

Edited by:
Christophe M. Filippi, Genomics Institute of the Novartis Research Foundation, USA

Reviewed by:
Dominique M. A. Bullens, KU Leuven, Belgium
Shomyseh Sanjabi, Gladstone Institutes, USA; University of California, San Francisco, USA

****Correspondence:***
Haruki Kitazawa, Food and Feed Immunology Group, Laboratory of Animal Products Chemistry, Department of Science of Food Function and Health, Division of Bioscience and Biotechnology for Future Bioindustries, Graduate School of Agricultural Science, Tohoku University, Tsutsumidori-Amamiyamachi 1-1, Aoba-ku, Sendai 981-8555, Japan
e-mail: haruki@bios.tohoku.ac.jp

The intestinal mucosa plays a critical role in the host's interactions with innocuous commensal microbiota and invading pathogenic microorganisms. Intestinal epithelial cells (IECs) and gut associated immune cells recognize the bacterial components via pattern-recognition receptors (PRRs) and are responsible for maintaining tolerance to the large communities of resident luminal bacteria while being also able to mount inflammatory responses against pathogens. Toll-like receptors (TLRs) are a major class of PRRs that are present on IECs and immune cells which are involved in the induction of both tolerance and inflammation. A growing body of experimental and clinical evidence supports the therapeutic and preventive application of probiotics for several gastrointestinal inflammatory disorders in which TLRs exert a significant role. This review aims to summarize the current knowledge of the beneficial effects of probiotic microorganisms with the capacity to modulate the immune system (immunobiotics) in the regulation of intestinal inflammation in pigs, which are very important as both livestock and human model. Especially we discuss the role of TLRs, their signaling pathways, and their negative regulators in both the inflammatory intestinal injury and the beneficial effects of immunobiotics in general, and *Lactobacillus jensenii* TL2937 in particular. This review article emphasizes the cellular and molecular interactions of immunobiotics with IECs and immune cells through TLRs and their application for improving animal and human health.

Keywords: *Lactobacillus jensenii* TL2937, TLR4, intestinal immunity, inflammation, immunobiotics

INTRODUCTION

The mammalian gastrointestinal tract harbors trillions of beneficial commensal bacteria, a population composed of at least 1,000–5,000 species (1). Studies probing the composition and function of the endogenous microbiota in the normal gastrointestinal tract have greatly expanded our appreciation for an understanding of how the microbiota shape mucosal immune responses, as well as how commensal bacteria in the gastrointestinal tract regulate the production of immunoregulatory, diet-dependent nutrients and metabolites (2). In fact, recent studies have highlighted that alterations in the composition of commensal bacterial populations are linked to multiple metabolic and inflammatory diseases in humans including but not limited to inflammatory bowel disease (IBD), obesity, type 2 diabetes, atherosclerosis, allergy, and colon cancer.

Mammals have an evolutionary partnership with the microbiota that is critical for host defense. In the gastrointestinal tract, part of the local immune response is aimed at maintaining a peaceful coexistence with the resident microbiota. Abundant experimental and clinical data support the idea that commensals residing in the gastrointestinal tract can calibrate both innate and adaptive responses (3, 4). Unique groups of commensals as well as defined metabolites of commensals also can have key roles in the control of mucosal responses (4). Additionally, despite being contained by the intestinal mucosa, the gut microbiota can also modulate immune responses at distal sites in the steady-state and during inflammation (5).

In recent years, the study of microbe-intestinal cell interactions has unraveled several molecular mechanisms and cellular pathways, showing that these interactions play a crucial role in the regulation of several immunological functions in the gut. Moreover, better understanding of the host-microbe interactions in the gut has provided new opportunities for preventing and treating a number of inflammatory disorders such as the use of specific probiotic strains to beneficially modulate the intestinal immune system. Probiotic bacteria that are able to modulate the immune system (immunobiotics) are demonstrably beneficial for treating a variety of mucosal disorders, including inflammatory diseases (6).

Weaning-associated intestinal inflammation occurs in various animal species including the pig. Intensification of the pig industry has brought increased risks of both clinical and sub-clinical enteric disease. Piglets are vulnerable to potentially harmful microorganisms such as *Escherichia coli*, *Salmonella* spp., and *Clostridium perfringens* (7). Antibiotics have been applied widely in animal husbandry to prevent and treat the gastrointestinal infection caused by pathogens (8). However, the promiscuous use of antibiotics has resulted not only in the emergence and spread of resistant

bacteria in humans but also in animals (9). Early weaning of piglets is often accompanied by a high susceptibility to diarrhea. It has been established that this process is multi-factorial and that post-weaning inflammation and malnutrition are major etiological factors. Pigs coexist with a dense and diverse microbiota in their gut. As observed in humans, the microbial colonization of the porcine intestine begins at birth and follows a rapid succession during the neonatal and weaning period (10, 11). Following the withdrawal of sow's milk the young piglets are highly susceptible to enteric diseases partly as a result of the altered balance between developing beneficial microbiota and the establishment of intestinal bacterial pathogens. In addition to the changes in microbiota composition, the intestinal immune system of the newborn piglet undergoes a rapid period of maturation, expansion, and specialization that is not achieved before commercial weaning (10, 11).

Various nutritional approaches for optimizing the weaning transition and minimizing gut inflammation and enteric diseases have been tested in the past decade. Among the novel dietary strategies investigated that are focused on improving gut health in pigs, prebiotics and probiotics are clear nutritional options. This review aims to summarize the current knowledge of the beneficial effects of probiotic microorganisms with the capacity to modulate the immune system (immunobiotics) in the regulation of intestinal inflammation in pigs. We discuss the role of toll-like receptors (TLRs), their signaling pathways, and their negative regulators in both the inflammatory intestinal injury and the beneficial effects of immunobiotics in general, and *Lactobacillus jensenii* TL2937 in particular. This review article emphasizes the cellular and molecular interactions of immunobiotics with intestinal epithelial cells (IECs) and immune cells through TLRs and their application for improving animal health and also human's because the pigs are expected to be a better human model than rodents.

TLR4 SIGNALING PATHWAY AND INFLAMMATION IN THE GUT

Toll-like receptor-4 is expressed by epithelial and immune cells and might play a role in the intestinal mucosal host defense against Gram-negative bacteria. However, since many body surfaces are colonized by the physiological microflora, activation of epithelial TLR4 must be tightly controlled to avoid unintended stimulation and mucosal inflammation.

Upon recognition of its cognate ligand, TLR4 dimerizes and initiates a signaling cascade that leads to the activation of a pro-inflammatory response (**Figure 1**). Ligand binding can induce two signaling pathways, the myeloid differentiation primary response gene 88 (MyD88)-dependent and MyD88-independent pathways, which induce the production of pro-inflammatory cytokines and type I IFNs (12). These two distinct responses are mediated via the selective use of adaptor molecules recruited to the TIR domains of the TLRs after ligand recognition and binding. Four adaptor molecules have been identified so far: MyD88, TIR-associated protein (TIRAP), TIR domain-containing adaptor protein-inducing IFN-β (TRIF), and TRIF-related adaptor molecules (TRAM) (13). MyD88 and TIRAP are responsible for the induction of pro-inflammatory genes, and TRIF and TRAM induce IFNs. In MyD88-dependent signaling, upon ligand recognition, MyD88

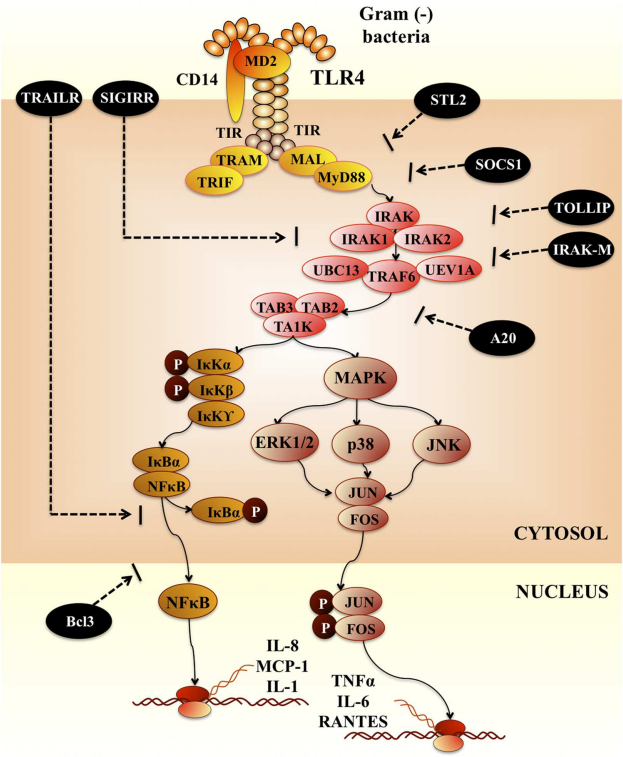

FIGURE 1 | Toll-like receptor-4 signaling pathway.

is recruited to and associates with the cytoplasmic domain of the TLRs. Then IL-1R-associated kinase 4 (IRAK-4) and IRAK-1 are recruited and activated by phosphorylation. Activated IRAK-4 phosphorylates IRAK-1, which subsequently associates with tumor necrosis factor receptor (TNFR)-associated factor 6 (TRAF6). TRAF6 activates transforming growth factor (TGF)-activating kinase 1 (TAK1) (**Figure 1**). TAK1 phosphorylates IKK-b and mitogen-activated protein kinase (MAPK) kinase 6 (MKK6), leading to degradation of I-κB and thereby leading to the nuclear translocation of NF-κB, which results in the induction of genes involved in inflammatory responses (**Figure 1**). Activation of the MyD88-dependent pathway also results in the activation of MAPKs such as p38 and JNK, which leads to the activation of AP-1 (13). For the MyD88-independent signaling TLR4 activation triggers the induction of a type I IFN response, leading to the induction of IFN-α and IFN-inducible genes.

Various negative regulatory mechanisms have evolved to attenuate TLR signaling and maintain the immune balance. At least six levels of negative regulation have been discovered so far (14, 15): (i) degradation of TLRs; (ii) down-regulation of transcription of TLRs and related genes; (iii) post-transcriptional repression by microRNAs (miRNAs); (iv) production of soluble TLRs functioning as decoy receptors; (v) intracellular inhibitors and; (vi) membrane-bound suppressors that inhibit TLR signaling pathways after TLR and ligand interactions have occurred. TLR signaling pathways can be tightly regulated by transmembrane proteins ST2, single immunoglobulin interleukin-1-related receptor

(SIGIRR), and TNF-related apoptosis-inducing ligand receptor (TRAILR). SIGIRR is an orphan receptor that does not induce NF-κB activation. It interacts with IRAK and TRAF6 and inhibits TLR signaling. In contrast, TRAILR suppresses NF-κB activation at downstream TLR signaling events, perhaps by stabilizing IκB and preventing its degradation (14) (**Figure 1**). Another important negative regulatory mechanism for TLR signaling involves the endogenous intracellular negative regulators such as sMyD88 (the short form of MyD88), interleukin-1 receptor-associated kinase M (IRAK-M), suppressor of cytokine signaling 1, NOD2, phosphatidylinositol 3-kinase, Toll interacting protein (TOLLIP), and A20. The intracellular negative TLR regulators can act at multiple levels. For example, IRAK-M can heterodimerize with IRAK-1 or -2 and bind both MyD88 and TRAF6. Upon TLR-TIR-ligand engagement and formation of the MyD88 adaptor complex, IRAK-M is thought to bind MyD88/IRAK-4 and inhibit IRAK-4 phosphorylation of IRAK-1. This prevents formation of TRAF6/IRAK-1 complexes, which initiate IκB kinase and MAPK signaling pathways (16) (**Figure 1**). Some intracellular regulators are constitutively expressed to control TLR activation at a physiological level, whereas others are up-regulated by TLR signaling during infection to attenuate the TLR response in a negative feedback loop. Therefore, regulation of TLR signaling pathways constitutes a complex network.

Toll-like receptor signaling in IECs and immune cells has been shown to be involved in three important mechanisms that are crucial for maintaining a healthy epithelial barrier: (i) epithelial cell proliferation and maintenance of tight junctions; (ii) expression of antimicrobial factors; and (iii) modulation of immune responses [reviewed in Ref. (15)]. In a healthy individual, intestinal colonization stimulates these mechanisms that in turn contain the microbiota within the intestinal lumen and neutralize MAMPs. Moreover, these mechanisms protect the host from the systemic translocation of bacteria or bacterial products and from the outburst of pro-inflammatory cascades in intestinal epithelial and innate cells (17).

MODULATION OF INTESTINAL INFLAMMATION BY COMMENSAL BACTERIA

Several studies have identified a role for pattern-recognition receptors (PRRs) in mediating non-inflammatory immune responses to the microbiota, challenging the paradigm that PRRs have evolved solely to recognize and respond to pathogens. MyD88-deficient mice are more susceptible to DSS-induced colitis, suggesting that commensal bacteria may be directly recognized by TLRs under steady-state conditions to mediate host-protective responses (18). To corroborate this notion, depletion of gut bacteria with antibiotics results in increased susceptibility to DSS; remarkably, oral feeding of lipopolysaccharide and lipoteichoic acid corrects this predisposition to colitis, revealing that TLR ligands have beneficial effects on the host (18). As DSS induces intestinal injury, these findings suggest that TLR signaling by the microbiota leads to maintenance of intestinal epithelial homeostasis in the absence of enteric pathogens. The polysaccharide from *Bacteroides fragilis* is a unique TLR2 ligand found in the human microbiome, which orchestrates anti-inflammatory immune responses that ameliorate diseases mediated by the immune system. This polysaccharide is ingested by intestinal DCs, which then stimulate responses of $Foxp3^+$ Treg cells (19). Interestingly, TLR2-deficient mice are not protected by the polysaccharide against colitis (20). TLR2-deficient DCs do not promote responses of $Foxp3^+$ Treg cells and production of IL-10, demonstrating that specific gut bacterial molecules have evolved to promote benefits to the host via PRR signaling in antigen presenting cells. Studies have also demonstrated that commensal organisms may target and inhibit NF-κB activation to suppress inflammation. By analyzing the composition of the intestinal microbiota of Crohn's disease patients, Sokol et al. (21) identified *Faecalibacterium prausnitzii*, which is greatly reduced in Crohn's disease patients, as an anti-inflammatory commensal bacterium in the gut by showing that the supernatant of *F. prausnitzii* inhibits NF-κB activation in a human IEC line and suppresses the production of pro-inflammatory cytokines both *in vitro* and in a mouse colitis model. However, the molecular mechanism by which this process occurs was not elucidated. Several studies have also highlighted the importance of TLR-MyD88 signaling among lymphocytes. In B cell–specific MyD88-deficient mice, bacteria disseminate to systemic sites, such as liver or lung, after DSS-induced damage of the colon, but not in epithelial cell–specific or dendritic cell–specific MyD88-deficient mice (22). Further, it has recently been appreciated that T cell subsets express functional TLRs (23). Transfer of MyD88-deficient T cells into RAG-deficient mice results in less intestinal inflammation (24). Conversely, whereas TLR signaling by T cells was classically thought to promote immunity, it now appears that this process can restrain inflammatory responses. For example, treatment of $CD4^+$ T cell subsets with a TLR4 agonist increases suppressive activity and enhances protection from colitis (25). Therefore, TLRs represent a dynamic signaling system that triggers various immune outcomes, and TLR signaling directly by adaptive immune cells mediates reactions in the absence of innate immune cells.

A growing list of inhibitors for TLR signaling in the intestinal mucosa, including IRAK-M, TOLLIP, SIGIRR, A20, and peroxisome proliferator-activated receptor-γ (PPARγ), ensure that chronic inflammatory and potentially destructive TLR responses to MAMPs do not occur (26). In this regard, IECs deficient in SIGIRR are more susceptible to commensal-dependent intestinal inflammation, indicating that the intrinsic expression of SIGIRR by IECs regulates the communication between commensal bacteria and the host immune system (27). Additionally, an anti-inflammatory mechanism activated by commensal *B. thetaiotaomicron* that attenuates pro-inflammatory cytokine expression in IECs by promoting nuclear export of the NF-κB subunit RelA through a PPARγ-dependent pathway has been reported (28). Furthermore, the contact time between IECs and commensal bacteria seems to be critical, as short-term stimulation with LPS leads to activation of pro-inflammatory signaling cascades in IECs, including phosphorylation of IRAK and MAPK and increased IL-8 secretion, whereas prolonged incubation results in a state of hyporesponsiveness with minimal reaction by the IECs. Up-regulation of inhibitory TOLLIP contribute to this hyporesponsiveness (29).

In addition to TLRs, other PRRs have been involved in the anti-inflammatory effects of gut microbiota. The peptidoglycan recognition protein (PGRP) family is involved in the regulation of commensal microbiota in mice. Mice deficient in any one

of the four PGRPs harbor a microbiota that promote increased sensitivity to DSS-induced colitis (29). Indeed, germ-free mice inoculated with stool from PGRP-deficient donor mice are more sensitive to DSS-induced colitis compared to mice that received stool from wild-type mice and exhibit greater mortality, weight loss, and colitis scores. Thus, mammalian PGRPs are important in shaping a homeostatic commensal microbiota and preventing intestinal inflammation (29). It is probable that in the near future studies will demonstrate that other PRRs are involved in the complex bidirectional cross-talk between commensal gut bacteria and the host.

MODULATION OF INTESTINAL INFLAMMATION BY PROBIOTIC BACTERIA

Several studies have shown that immunobiotics can beneficially modulate the PRRs-mediated inflammatory response in the gut by modulating the functions of IECs and APCs (30, 31).

Probiotics inhibit excessive NF-κB-induced pro-inflammatory cytokine production by IECs. Immunobiotics suppress TNF- or *S. typhimurium*-induced IL-8 gene expression and secretion by IECs in an NF-κB-dependent manner (32, 33). A study in Caco-2 cells demonstrated that *Lactobacillus rhamnosus* GG counteracts the enterotoxigenic *Escherichia coli* (ETEC)-induced up-regulation of IL-1β and TNF-α and the down-regulation of TGF-β1 expression, consequently blocking cytokine deregulation (30). In addition, comparative studies between *L. rhamnosus* GG and *Bifidobacterium animalis* MB5 demonstrated that individual strains of probiotics have a different impact on the inflammatory response triggered in IECs (30). Others studies evaluating the effect of immunobiotic yeasts have shown that *Saccharomyces cerevisiae* CNCM I-3856 decreases the expression of the pro-inflammatory mediators IL-6, IL-8, CCL20, CXCL2, and CXCL10 in porcine intestinal epithelial IPI-2I cells cultured with $F4^+$ ETEC (34). Moreover, the CNCM I-3856 strain inhibits ETEC-induced expression of pro-inflammatory cytokines and chemokine transcripts and proteins, and this inhibition is associated with a decrease in ERK1/2 and p38 MAPK phosphorylation and an increase in the mRNA level of anti-inflammatory PPARγ (35).

Additionally, the importance of direct stimulation of DCs by immunobiotics to promote tolerance was illustrated by some studies. Comparative studies using *Lactobacillus plantarum* NIZO B253, *Lactobacillus casei* NIZO B255, and *Lactobacillus reuteri* ASM20016 showed that *L. reuteri* and *L. casei*, in contrast to *L. plantarum*, prime DCs to promote the development of Treg cells. Experiments with TLR transfectants showed that none of the three lactobacilli tested substantially activated TLRs. However, *L. reuteri* and *L. casei* both potently induce the development of Treg cells and are recognized by DC-SIGN on DCs, an interaction that appears to be crucial for the priming of regulatory DCs (36). Another study showed that the direct interaction between DCs and *Lactobacillus acidophilus* NCFM is sufficient to induce IL-10 production and low IL-12p70 production by these cells. This acquisition of a non-inflammatory phenotype by the DCs was dependent on the activation of DC-SIGN that recognizes surface layer protein A (SlpA) of the bacterium. *L. acidophilus* with mutated SlpA fails to induce Th2 polarization of the DCs, and instead, promotes IL-12p70, TNF-α, and IL-1 production (37). Additionally, it was reported that transfer of LAB-treated bone-marrow-derived DCs protects mice from 2-4-6-trinitrobenzenesulfonic acid-induced colitis. This effect is mediated by TLR2 and NOD2 activation of the DCs and depends on the activation of Treg cells (38). Teichoic acid, a cell wall component of the Gram-positive bacteria *L. plantarum* NCIMB8826, is involved in the anti-inflammatory activity of this strain. A mutant with enhanced anti-inflammatory capacity incorporates much lower levels of D-Ala in its teichoic acids than the wild-type strain and induces dramatically reduced secretion of pro-inflammatory cytokines by blood monocytes, resulting in a significant increase in IL-10 production. The effects observed were clearly TLR2 dependent. This mutant was also more protective in a murine colitis model than its wild-type counterpart (39). Some probiotics activate anti-inflammatory and regulatory immune effects in the settings of enteric infections and mucosal inflammation. *Lactobacillus paracasei* CNCM I-4034 and its supernatant dramatically reduce the production of IL-6, IL-8, IL-12p70, and TNF-α in human intestinal DCs challenged with *Salmonella typhi* (40). These authors demonstrated that *L. paracasei* CNCM I-4034 activates the expression of TLR2 in DCs, up-regulates the expression of TOLLIP, and promotes the stimulation of TGF-β2, whereas the supernatant of the probiotic increases the secretion of TGF-β1.

Lebeer et al. (41) suggested that the final outcome of a host cell response against probiotic bacteria depends on the combination of the distinct MAMPs that can interact with the various PRRs and associated co-receptors that fine-tune signaling; as well as on the concentration of these MAMPs. To date, several MAMPs of immunobiotics have been indentified, that can be connected to specific host responses (41) and these effector molecules are in many cases associated with the bacterial cell surface (42). Although most beneficial effects of probiotics require direct bacterium-cell contact with live bacteria, some reports demonstrated that soluble factors secreted by probiotics are able to modulate the production of cytokines and therefore, to modulate the immune system. In fact, recent investigations have exposed some of the underlying mechanisms in the modulation of gut immune system by probiotic soluble factors. Peña and Versalovic (43) reported that *L. rhamnosus* GG specifically inhibits TNF-α production and reduces TNF-α/IL-10 ratios in a murine macrophage model with an anti-inflammatory net effect. This effect is contact-independent, requiring the presence of a soluble *L. rhamnosus* GG immunomodulin for complete modulatory activity. The putative immunomodulin has a protein or peptide component that inhibits TNF-α production in murine macrophages. Further research work using *L. rhamnosus* GG strain, to investigate molecular mechanisms by which probiotics regulate IECs, reported the purification of two novel *L. rhamnosus* GG-derived soluble proteins, p75 and p40. Each of these purified protein preparations activated Akt, inhibited cytokine-induced epithelial cell apoptosis, and promoted cell growth in human and mouse colon epithelial cells and cultured mouse colon explants. TNF-induced colon epithelial damage was significantly reduced by p75 and p40. Immunodepletion of p75 and p40 reversed the *L. rhamnosus* GG conditioned media activation of Akt and its inhibitory effects on cytokine-induced apoptosis and loss of IECs (44). These findings suggest that probiotic bacterial components may be useful for preventing

cytokine-mediated gastrointestinal diseases. Another example of a secreted protein associated with probiotic activity is the *prt-P*-encoded protease of *L. paracasei* that degrades secreted CXCL10 (also called IP-10), resulting in reduced lymphocyte recruitment in an ileitis model (45). Secreted factors produced by *Lactobacillus casei-rhamnosus* were tested on human lymphocytes, monocytes, and a human monocytic leukemia-cell line (THP-1). The soluble factor(s) present in supernatants effectively induced apoptosis of immune cells. These were mainly soluble heat-stable proteins. For immune cells, pre-treatment with the supernatant significantly promoted apoptosis via a mitochondrial pathway. The supernatant also inhibited the release of LPS-induced pro-inflammatory cytokines TNF-α, IL-1β, IL-6, and IL-8 by immune cells (46). It was also described that in the human gut, *L. plantarum* secretes an extracellular protein that releases an internal fragment (STp) when is cleaved by intestinal proteases. It is characterized by the abundance of serine and threonine residues within its sequence. STp is encoded in one of the main extracellular proteins produced by such species, which includes some probiotic strains. *In vitro* studies using DCs from human peripheral blood showed that STp increased the production of regulatory IL-10 in healthy controls. In addition, T cells stimulated with STp-pulsed DCs decreased the production of pro-inflammatory IFNs and increased anti-inflammatory IL-10 production, suggesting that these T cells acquired an immunoregulatory phenotype (47).

INTESTINAL INFLAMMATION IN PIGLETS AFTER WEANING: IMPACT OF PROBIOTICS IN IMMUNE HEALTH AND PRODUCTIVITY

The weaning transition is a complex period during which the piglets have to face an abrupt separation from their mother, mixing with other litters in a usually new environment, and switch from milk to a solid feed which involves a change from a highly digestible to a less-digestible and more-complex feed. In consequence, several physiological changes occur in the intestine of pigs during the process of weaning [reviewed in Ref. (11)]. Early studies of Pluske et al. (48) showed that weaning induces several modifications in the intestinal tissue including changes in villus and crypt architecture and reduced activities of brush-border digestive enzymes. Moreover, these histological and physiological modifications have been implicated in a higher susceptibility to intestinal pathogens such as *E. coli* and rotaviruses (48). Changes in the gut microbiota have been also described. The gut of piglets is sterile at birth and is then colonized by microbes from the mother and the environment, starting with lactic acid bacteria, enterobacteria, and streptococci. After weaning and the introduction of solid feed obligate anaerobes increase in number and diversity until an adult-type pattern is achieved (11, 49). These modifications in microbial communities has a great impact in the gastrointestinal health of piglets, considering that microbial activity is important for improvement of energy yield, vitamin production, fermentation of carbohydrates, gut motility, production of volatile fatty acids, and water and Na^+ absorption [reviewed in Ref. (11)].

In addition, it has to be considered that the piglet is not immunocompetent at birth. Piglet is dependent on a supply of several specific and non-specific immune factors present in maternal colostrum and milk for immune protection, resistance against pathogens, development, and survival. Clearly, development of immunocompetence is an absolute requirement for optimum growth and performance. Early weaning at 3 weeks of age is associated with a transient reduction in the ability of intraepithelial lymphocytes to respond to mitogens and splenic T cells to secrete IL-2. Furthermore, tolerance to fed proteins introduced at weaning is not fully achieved until 8 weeks of age (11).

A growing body of experimental and clinical evidence supports the therapeutic and preventive application of probiotics for several gastrointestinal inflammatory disorders in pigs. In this regard, Qiao et al. (50) conducted experiments to evaluate the effects of a complex Lactobacilli preparation on performance, resistance to *E. coli* infection and gut microbial flora of weaning pigs. The mix of four lactobacilli (*Lactobacillus gasseri*, *L. reuteri*, *L. acidophilus*, and *Lactobacillus fermentum*) isolated from weaning pigs was able to reduce *E. coli* and anaerobe counts in the gut, and decrease diarrhea. Additionally, lactobacilli treatment significantly improved average daily feed intake of pigs compared to controls during the first 2 weeks after weaning and the average daily gain (50). It is known that the ratio of Bacteroidetes and Firmicutes bacterial groups in the gut can affect the ability to absorb nutrients. Therefore, Cui et al. (51) investigated the effect of probiotic *Bacillus subtilis* on Bacteroidetes and Firmicutes in cecal contents and growth performance and fat deposition in weaning piglets. The study found that the addition of *B. subtilis* improves growth performance and affects lipid metabolism through regulation of the proportion of Bacteroidetes and Firmicutes in the gut. Herfel et al. (52) examined the impact of a novel probiotic strain of *Bifidobacterium longum* AH1206 on the health, growth, and development of neonatal pigs. Authors found that ileal IL-10 expression increased progressively with AH1206 supplementation, which indicated the potential for modulation of the inflammatory tone of the intestinal mucosa of suckling piglets. However, no differences were found between AH1206-treated and control piglets when comparing body weight gain, feed efficiency (gain:intake), and histological and physiological modifications in intestines. Another recent study evaluated the effect of the co-administration of *B. subtilis* RJGP16 and *Lactobacillus salivarius* B1 on intestinal immunity in piglets (53). Authors demonstrated that probiotic administration increased the expression of IL-6, porcine beta-defensins, and IgA producing cells in the intestine, clearly showing that co-administration of RJGP16 and B1 strains strongly enhances the intestinal mucosal immunity of piglets.

Some recent studies have specifically evaluated the capacity of probiotics to improve the resistance of piglets against ETEC. It was shown that the probiotic strain *L. plantarum* CJLP243 may serve as a potential alternative to antibiotic supplementation to improve the growth and health performance of weaning pigs because of its capacity to reduce the severity of ETEC-induced diarrhea (54). Li et al. (55) showed that pre-treatment of piglets with *L. rhamnosus* ATCC7469 ameliorates $F4^+$ETEC-induced diarrhea. In piglets exposed to $F4^+$ETEC, jejunal TLR4 and IL-8 expression were increased; however, these increases were attenuated by administration of *L. rhamnosus*. Notably, expression of jejunal TLR2, ileal TLR9, NOD1, and TNF-α was up-regulated in the ATCC7469-treated piglets after $F4^+$ETEC challenge (55). These

results indicate that probiotic treatments would be able to beneficially modulate the overwhelming inflammatory response in infected piglets.

Although these studies demonstrated that is possible to modulate piglets' gut microbiota and immunity and improve growth performance by using appropriate probiotics strains, the true efficacy of probiotics in agricultural animals remains unclear because of inconsistent experimental results. Explanations for the disparities between studies include differences in experimental conditions, animal age, genetics, and health status. Additionally, the inconsistent results could be attributed to a lack of understanding of detailed cellular and molecular mechanism of action, as well as unknown interactions among these bacteria, the host, and the intestinal microbiota (56).

MODULATION OF TLR4-MEDIATED INFLAMMATION IN INTESTINAL EPITHELIAL CELLS BY *LACTOBACILLUS JENSENII* TL2937

Intestinal epithelial cells are a central component of the immune system of the gut. Several works have demonstrated that microbial recognition by IECs is an integral aspect of first-line host responses. Then, current observations point to the idea that more than simply a physical barrier separating luminal contents from mucosal APCs, the intestinal epithelium is increasingly recognized as playing an essential role in immune homeostasis, through the promotion of tolerogenic and regulatory responses. These findings have important implications for the regulation of mucosal homeostasis by probiotic bacteria. To study the mechanisms by which IECs induce an immune response to pathogens and the potential immunoregulatory effect of immunobiotics in pigs, we established a clonal porcine intestinal epitheliocyte cell line (PIE cells) (58). Studies of TLRs expression in PIE cells demonstrated that TLR4 is expressed most strongly. It was confirmed that PIE cells, which preferentially express TLR4/MD-2, undergo inflammatory responses regarding cytokine expression in response to LPS stimulation (58). Moreover, stimulation of PIE cells with porcine-specific ETEC significantly increases the levels of IL-6, IL-8, and monocyte chemotactic protein (MCP)-1 (57). It was also found that damage to PIE cells correlates with the levels of pro-inflammatory cytokines produced after stimulation with ETEC and LPS (57), which is consistent with reports demonstrating that challenging human intestinal Caco-2 cells with ETEC causes strong up-regulation of pro-inflammatory mediators that lead to membrane damage (59, 60). We selected lactobacilli strains that regulate the inflammatory response induced by ETEC and LPS in PIE cells by evaluating the levels of IL-1α, IL-6, IL-8, and MCP-1. The challenge of PIE cells with the intestinal pathogen significantly increased levels of pro-inflammatory cytokines in lactobacilli-untreated control cells (61). However, IL-6 and IL-8 levels in PIE cells stimulated with some lactobacilli strains, especially *L. jensenii* TL2937, were significantly lower than those in the control (61). Interestingly, *L. jensenii* TL2937, a strain with a high capacity to activate TLR2, was also the strain with the highest capacity to down-regulate IL-6 and IL-8 production by PIE cells in response to ETEC and LPS. For this reason, we became interested in *L. jensenii* TL2937 and examined the mechanisms behind the anti-inflammatory effect mediated by this strain, and demonstrated that *L. jensenii* TL2937 inhibits NF-κB and MAPK signaling pathways in ETEC- and LPS-challenged PIE cells (**Figure 2**).

Proteins that regulate the intensity and duration of TLR activation are able to modulate the cellular outcome, thereby controlling whether TLR activation leads to homeostatic or inflammatory responses (14). To dissect the mechanism(s) involved in the anti-inflammatory effect of *L. jensenii* TL2937, the effect of this strain on the expression of the negative TLR regulators in PIE cells was evaluated. The expression of SIGIRR, Tollip, A20, Bcl-3, MKP-1, and IRAK-M was studied, and it was found that MKP-1, A20, and Bcl-3 mRNA expression was up-regulated in PIE cells stimulated with *L. jensenii* TL2937 (61) (**Figure 2**). MKP-1 plays a role in the inhibition of pro-inflammatory mRNA expression by inactivating MAPK. MKP-1 desensitizes cells to TLR ligands by inactivating the p38 signaling pathway in enterocytes (62). Moreover, MKP-1 is not induced by TLR2 stimulation, although ligands for TLR3, TLR4, TLR5, and TLR9 induce MKP-1. This is in agreement with our finding that the TLR2 ligand Pam3CSK4 itself does not induce the expression of MKP-1 (61). Bcl-3 functions as an inhibitor of NF-κB activity by stabilizing repressive NF-κB homodimers in a DNA-bound state and preventing the binding of transcriptionally active dimers. In fact, stabilization of repressive complexes through the induction of Bcl-3 expression has been proposed to function during the processes of LPS tolerance (63). Moreover, treatment of macrophages with IL-10 induces the expression of Bcl-3, leading to inhibition of LPS-induced TNF-α production (64).

Lactobacillus jensenii TL2937 also upregulate the expression of A20 in PIE cells. A20 is a zinc finger protein that inhibits activation of NF-kB via inflammatory cytokine receptors (65, 66), TLR (67, 68), and the nucleotide-binding oligomerization domain-containing receptor NOD2 (69). A20 functions via its two ubiquitin-editing activities, an N-terminal deubiquitinase that removes K63-linked polyubiquitin chains and a C-terminal ubiquitin ligase that facilitates target protein degradation via attachment of K48-linked poly-ubiquitin chains (66, 70). These two activities cooperatively down-regulate TRAF6 (71). Therefore, A20 plays an essential role in the termination of NF-kB signaling in response to TNF-α and microbial products such as LPS (72). A20 deficiency in enterocytes renders mice sensitive to TNF-a-induced lethal inflammation, leading to disruption of the epithelial barrier and infiltration of commensal bacteria that initiate a systemic inflammatory response (73). These data suggest that A20 is important for the inhibition of innate immune responses in the gut (26). In addition, gut decontamination with a mixture of antibiotics with limited oral bioavailability in drinking water markedly reduces A20 protein and mRNA levels in the ileal epithelium of mice (74). Moreover, partial rather than complete abrogation of A20 expression is likely due to incomplete elimination of intestinal bacteria by the antibiotic treatment (74). These results show that A20 expression in the epithelium positively correlates with the bacterial load in the lumen. The observations that A20-deficient mice develop severe gut inflammation early in life (75) and that this inflammatory state can be alleviated by antibiotics or knockout of the TLR signaling mediator myeloid differentiation factor MyD88 (76) further support a key role for A20 in intestinal tolerance to the intestinal microbiota.

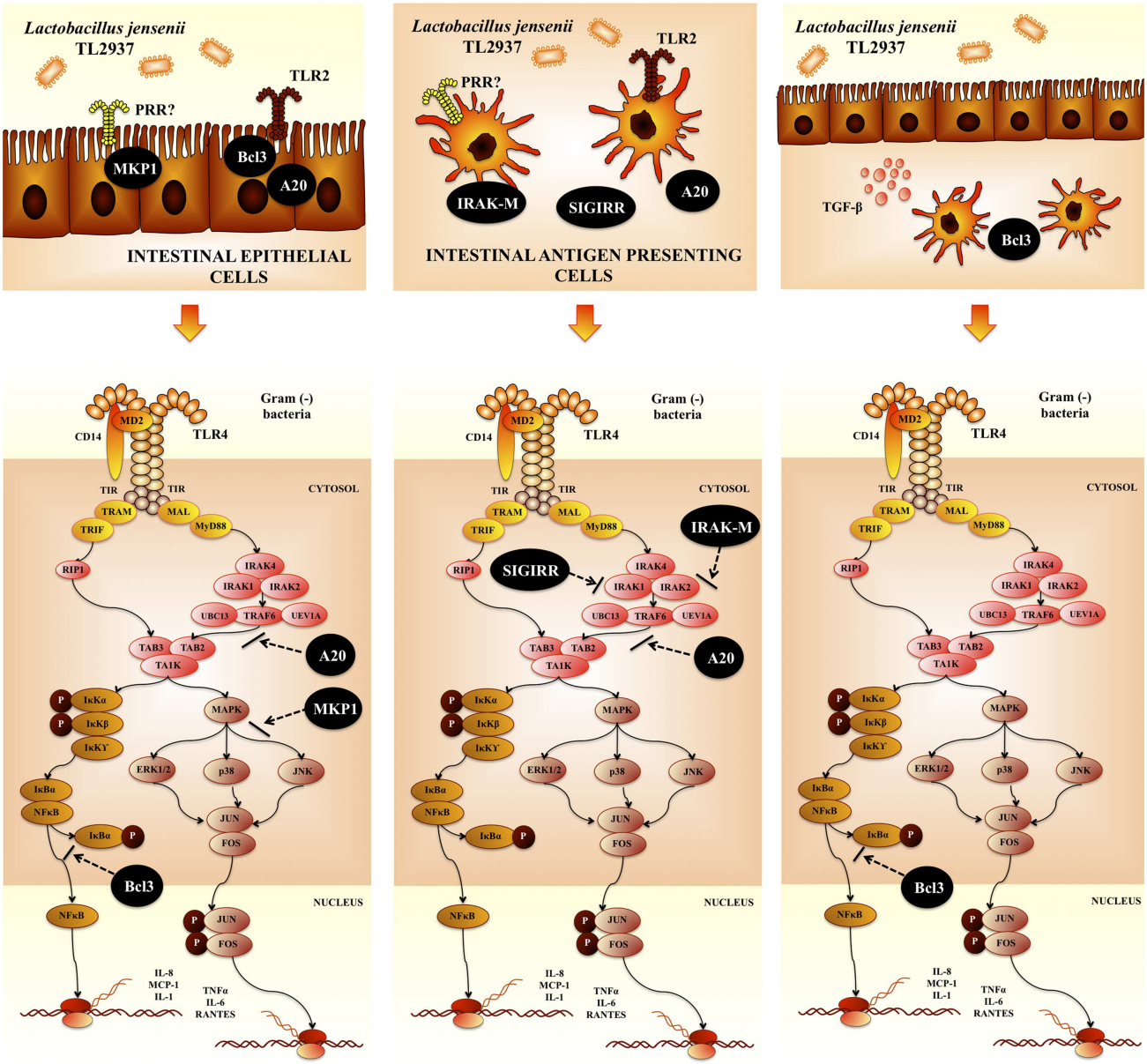

FIGURE 2 | Modulation of negative regulators of toll-like receptor-4 signaling pathway by *Lactobacillus jensenii* TL2937 in porcine intestinal epithelial cells and antigen presenting cells.

Recently, it was demonstrated that *B. longum* BB536 and *Bifidobacterium breve* M-16V significantly down-regulated levels of IL-8, MCP-1, and IL-6 in PIE cells challenged with ETEC by modulating the NF-kB and MAPK pathways (77). Moreover, both bifidobacteria up-regulated A20 in PIE cells. Then, the most effective anti-inflammatory strains evaluated in our laboratory, *L. jensenii* TL2937 and bifidobacteria strains BB536 and M-16V, strongly up-regulated the ubiquitin-editing enzyme A20. This finding is of interest because it not only shows a common mechanism for the anti-inflammatory activity of immunobiotics but also provides a potential biomarker for the screening and selection of new immunoregulatory strains.

MODULATION OF TLR4-MEDIATED INFLAMMATION IN INTESTINAL ANTIGEN PRESENTING CELLS BY *LACTOBACILLUS JENSENII* TL2937

Considering the anti-inflammatory effects of the TL2937 strain in IECs and the critical importance of APC polarization in immunoregulation, it was also examined the effect of *L. jensenii* TL2937 on activation patterns of APCs from porcine Peyer's patches (PPs). In swine, the most frequent marker expressed on DCs and macrophages is CD172a. Additionally, CD11R1 is considered to be a marker that is specifically and differentially expressed on porcine DCs, but not on macrophages (78). Then, in our studies we used CD172a and CD11R1, together

with MHC-II, to define three different populations of APCs in porcine PPs: $CD172a^{+}CD11R1^{high}$, $CD172a^{-}CD11R1^{low}$, and $CD172a^{+}CD11R1^{-}$ cells (79). According to our studies and previously published works (72, 80, 81), $CD172a^{+}CD11R1^{high}$ and $CD172a^{-}CD11R1^{low}$ cells could be DCs, and $CD172a^{+}CD11R1^{-}$ cells could be macrophages; however, functional studies are needed to accurately define each population. Therefore, in our studies, we refer to each of the three populations as APCs.

Ex vivo experiments using the adherent population of PPs APCs showed that the treatment with *L. jensenii* TL2937 increases the expression of IL-10 and TGF-β in $CD172a^{+}CD11R1^{high}$ and $CD172a^{+}CD11R1^{-}$ cells, whereas treatment with this bacterium is associated with increased levels of IFN-γ in $CD172a^{-}CD11R1^{low}$ cells (79). Then, the direct exposure of porcine APCs to *L. jensenii* TL2937 in the absence of inflammatory signals activates $CD172a^{+}$ APCs and causes them to become phenotypically and functionally mature and to display tolerogenic properties (79). Our findings show similarities to previous studies with lactobacilli and APCs from different species. For example, human myeloid DCs exposed to lactobacilli show increased expression of MHC-II and co-stimulatory molecules (39, 82, 83). Moreover, similar to our work, previous studies by Drakes et al. (36) reveal that probiotic lactobacilli induce up-regulation of IL-10 production and cell surface markers of maturation and activation in DCs (36).

On the contrary, *L. jensenii* TL2937 increased the production of IFN-γ in $CD172a^{-}CD11R1^{low}$ cells (79). One possible explanation for the differential immunoregulatory effect of TL2937 may be the levels of expression of TLR2 in distinct APCs. $CD172a^{+}CD11R1^{high}$, $CD172a^{+}CD11R1^{-}$, and $CD172a^{-}CD11R1^{low}$ cells differ regarding TLR2 expression (79), and therefore, they are likely to differ in the degree to which they interact with *L. jensenii.* In support of this hypothesis, it was reported that teichoic acid, a cell wall component of the Gram-positive bacteria *L. plantarum* NCIMB8826, is involved in the anti-inflammatory activity of this strain. A mutant with enhanced anti-inflammatory capacity incorporates much lower levels of D-Ala in its teichoic acids than the wild-type strain and induces dramatically reduced secretion of pro-inflammatory cytokines by blood monocytes, resulting in a significant increase in IL-10 production. The effects observed were clearly TLR2 dependent. This mutant was also more protective in a murine colitis model than its wild-type counterpart (37). Other PRRs would be also involved in the immunoregulatory effect of immunobiotics on APCs. Comparative studies using *L. plantarum* NIZO B253, *L. casei* NIZO B255, and *L. reuteri* ASM20016 showed that *L. reuteri* and *L. casei*, in contrast to *L. plantarum*, prime DCs to promote the development of Treg cells. Experiments with TLR transfectants showed that none of the three lactobacilli tested substantially activated TLRs. However, *L. reuteri* and *L. casei* both potently induce the development of Treg cells and are recognized by DC-SIGN on DCs, an interaction that appears to be crucial for the priming of regulatory DCs (84). Another study showed that the direct interaction between DCs and *L. acidophilus* NCFM is sufficient to induce IL-10 production and low IL-12p70 production by these cells. This acquisition of a non-inflammatory phenotype by the DCs was dependent on the activation of DC-SIGN that recognizes SlpA of the bacterium (85).

Treatment of APCs with *L. jensenii* TL2937 also results in differential modulation of the production of pro- and anti-inflammatory cytokines in response to ETEC or LPS challenges. The differential effects of the TL2937 strain in each PPs APC population persist because increased production of IFN-γ is observed in $CD172a^{-}CD11R1^{low}$ cells and improved synthesis of IL-10 is detected in $CD172a^{+}CD11R1^{high}$ and $CD172a^{+}CD11R1^{-}$ cells (79).

In order to find the mechanism(s) involved in the immunoregulatory effects of the TL2937 strain, the expression of negative regulators of TLRs in porcine APCs was also evaluated. Of the six regulators tested, SIGIRR, A20, and IRAK-M mRNA expression was up-regulated in $CD172a^{+}$ cells stimulated with *L. jensenii* TL2937 (**Figure 2**). It was shown *in vitro* that overexpression of SIGIRR inhibits TLR-induced NF-κB activation and attenuates the production of inflammatory cytokines (86). The LPS-induced inflammatory response is enhanced in SIGIRR-deficient mice (87). As described above, A20 also has an essential role in regulating inflammatory responses in the gut (68, 72). Notably, IRAK-M-deficient cells stimulated with TLR ligands or bacteria produce an increase in NF-κB and MAPK activation and elevated amounts of pro-inflammatory cytokines, such as IL-12, IL-6, and TNF-α (88). IRAK-M expression is induced upon LPS stimulation, and endotoxin tolerance is diminished in IRAK-M-deficient cells; these observations indicate that IRAK-M plays a critical role in regulating innate immunity through a negative feedback loop (89). Therefore, induction of these three negative regulators by *L. jensenii* TL2937 in $CD172a^{+}$ APCs cells from swine PPs may be important for establishing tolerance to LPS and ETEC (**Figure 2**).

Although our studies in PIE cells and APCs demonstrated the ability of immunobiotics to modulate the inflammatory response, these *in vitro* models may be overly simplified and may not account for the effect of cell-cell interactions in a complex organic microenvironment, completely changing the resulting response. As mentioned before, IECs express a broad range of factors that may influence intestinal APCs and lymphocytes (90, 92). Therefore, to further assess the immunoregulatory effect of *L. jensenii* TL2937, in a recent study a co-culture system with a PIE cell monolayer and immunocompetent cells from swine PPs was used to model an *in vitro* PP culture system (91).

A significant up-regulation of pro-inflammatory cytokines was observed in PIE cells co-cultured with PPs APCs and challenged with ETEC or LPS. These results were consistent with findings described for PIE cells monocultures described above. Therefore, PIE cells did not responded differently to TLR4 activation when co-cultured with APCs (91). Moreover, it was confirmed that the pre-treatment of PIE cells with *L. jensenii* TL2937 reduced pro-inflammatory cytokines in response to ETEC or LPS and that this effect was related to up-regulation of the three TLR negative regulators: A20, Bcl-3, and MKP-1 as in PIE cell monocultures (61, 91, 93). In addition, *L. jensenii* TL2937-treated PIE cells were able to significantly upregulate TGF-β expression (91). It is well known that IECs-derived factors are able to condition mucosal DCs to secrete cytokines such as IL-10 and TGF-β in response to commensal microbes, thereby initiating differentiation of Treg immune responses (94). Moreover, conditioning of monocyte-derived DCs with IECs supernatants confer on DCs the capacity to produce

large amounts of IL-10, which is attributable, at least in part, to the release of the IECs-derived factors such as TGF-β and thymic stromal-derived lymphopoietin (TSLP) (95). Therefore, in addition to its direct tolerogenic effects on PIE cells, *L. jensenii* TL2937 could have an indirect anti-inflammatory effect on APCs under the influence of factors produced by PIE cells such as TGF-β (91).

The study of the indirect effect of *L. jensenii* TL2937 on APCs in co-cultures, demonstrated that the response of these cells was completely different to those observed in APCs monocultures. In PIE-APCs co-cultures, no modifications in the levels of TGF-β in $CD172a^{+}CD11R1^{-}$ and $CD172a^{+}CD11R1^{high}$ cells or levels of IFN-γ in $CD172a^{-}CD11R1^{low}$ cells were observed. However, increased levels of IL-10 were found in $CD172a^{+}$ cells co-cultured with PIE cells (91). In addition, no modification in SIGIRR, A20 or IRAK-M expression was observed in those cells. Notably, Bcl-3 expression was up-regulated in APCs cells co-cultured with PIE cells (91) (**Figure 2**). The Bcl-3 protein functions as an inhibitor of NF-κB activity. It was reported that treatment of macrophages with IL-10 induces the expression of Bcl-3, and Bcl-3 expression leads to inhibition of LPS-induced TNF-α production (64). Then it is probable that immunoregulatory cytokines (IL-10) produced by APCs act in an autocrine way and upregulate the expression Bcl-3. Then, the response of PPs APCs to *L. jensenii* TL2937 is significantly modified when the stimulus is mediated indirectly through IECs (91).

IMPACT OF *LACTOBACILLUS JENSENII* TL2937 IN PIGS' IMMUNE HEALTH AND PRODUCTIVITY

Recent *in vivo* data concerning the immunoregulatory effect of *L. jensenii* TL2937 demonstrated that the administration of this immunobiotic strain improved immune health and growing performance and productivity of piglets (91). Feeding the TL2937 strain to 3 week-old LWD piglets significantly increased carcass grading (according to the standards of the Japanese Meat Grading Association) and improved juicy, tenderness, and overall palatability.

As mentioned before, at weaning, piglets are stressed, the food intake is strongly depressed, the structure and function of the gastrointestinal tract are altered, and these conditions can favor bacterial translocation, inflammation, and infection with pathogenic bacteria. It was reported that the optimal gut microbiota significantly improves intestinal health and beneficially affects the efficiency of gastrointestinal and whole body growth throughout the productive life cycle of a pig (11). In this regard, studies on the expression profiles induced by gut microbiota in the ileal epithelium of neonatal piglets showed and enhanced expression of NF-κBIA, a protein associated with the inactivation of NF-κB by sequestration, and the negative regulator of TLRs TOLLIP together with the down-regulation of GATA1 in colonized versus germ-free animals; reflecting the activation of pathways that prevent excessive inflammation. In addition, it is extremely important to direct piglets intestinal immune system toward appropriate immune responses that strives to maintain intestinal homeostasis, not only in the induction of tolerance against harmful antigens, but in effective effectors responses against pathogens. Some studies have associated probiotic bacteria with the improvement of intestinal homeostasis in pigs, albeit with different levels of success as described previously (53, 55). Considering the capacity of *L. jensenii* TL2937 to functionally modulate the response of PIE cells and porcine APCs, it was hypothesized that this strain would significantly impact on piglets' immune heath. The *in vivo* experiments in pigs indicate that *L. jensenii* TL2937 is able to improve immunity and regulate excessive inflammation (91). These effects seem to be related to the complex secretion of cytokines induced by the probiotic strain in the gut. *L. jensenii* TL2937 could strongly induced secretion of IL-10 and IFN-γ that would be related to the beneficial effects achieved by the immunobiotic strain (91). The capacity to modulate inflammation and improve defenses at the same time has been described for several probiotic strains (95, 96). *L. jensenii* TL2937 could be included in the list of probiotic strains with those capabilities.

CONCLUSION

Post-weaning diarrhea mainly occurs within the first week after weaning and affects pigs across the globe, causing great economic loss to the swine industry due to reduced growth performance and considerable morbidity and mortality. Our studies demonstrated that the use of immunobiotics strains as supplemental additives for piglet feedings could be used as a strategy to maintain and improve intestinal homeostasis; that is important for the development of the pig and for health and performance throughout the productive life of the animal.

The scientific research into probiotic mode of actions has come to age and has shown how probiotics are able to induce beneficial changes in the host. Our research work allows us to propose a complete view of the cellular and molecular mechanisms involved in the immunoregulatory effects of *L. jensenii* TL2937 (**Figure 3**). When reaching the porcine intestinal mucosa, *L. jensenii* TL2937 would have the capacity to interact with local cells at three levels (**Figure 3**): (i) the interaction of the TL2937 strain with IECs would induce the up-regulation of MKP-1, Bcl-3, and A20 expression, which would be mostly dependent on TLR2 activation as we have demonstrated for several immunobiotic bacteria including the TL2937 strain; (ii) *L. jensenii* TL2937 could be taken by APCs indirectly through M cell transport or by direct sampling from the intestinal lumen, inducing an increase in the production of the immunoregulatory cytokines IL-10 and TGF-β by $CD172a^{+}$ cells as well as the expression of SIGIRR, IRAK-M, and A20. In addition, through its direct interaction with $CD172a^{-}CD11R1^{low}$ cells, the TL2937 strain would have the capacity to improve Th1 responses by increasing the production of IFN-γ and; (iii) *L. jensenii* TL2937, through its capacity of stimulating the production of immunoregulatory factors such as TGF-β in EICs, would indirectly increase the expression of Bcl-3 and the production of IL-10 in $CD172a^{+}$ APCs reinforcing its effects in these cells. Then, *L. jensenii* TL2937 would functionally modulated IECs and APCs to improve resistance against infections and avoid unproductive inflammation. In fact, experiments using ETEC challenge, clearly demonstrated that the TL2937 strain is able to induce protection against inflammatory damage and improve immunity at the same time (**Figure 3**). It was also demonstrated that the immunological networks induced by *L. jensenii* TL2937 help to maintain intestinal tolerance and improve the development of appropriate protective and controlled immune responses. Then, *L. jensenii* TL2937 has a great potential

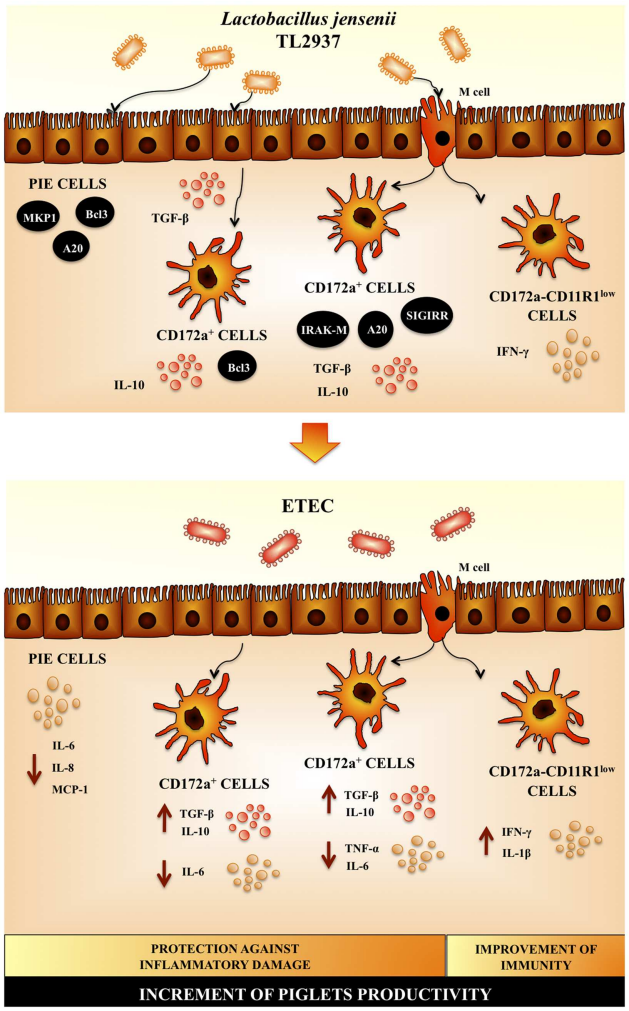

FIGURE 3 | Proposed mechanism for the immunoregulatory effect of *Lactobacillus jensenii* TL2937 in porcine intestinal mucosa.

to be used as a pig probiotic feed. In addition, accumulation of empirical data in pigs may increase the probiotic use in human because the pigs are also expected for development as human model.

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Modulation of respiratory TLR3-anti-viral response by probiotic microorganisms: lessons learned from *Lactobacillus rhamnosus* CRL1505

***Haruki Kitazawa*[1] *and Julio Villena*[2,3] ***

[1] *Food and Feed Immunology Group, Laboratory of Animal Products Chemistry, Department of Science of Food Function and Health, Graduate School of Agricultural Science, Tohoku University, Sendai, Japan*
[2] *Immunobiotics Research Group, Tucuman, Argentina*
[3] *Laboratory of Immunobiotechnology, Reference Centre for Lactobacilli (CERELA-CONICET), Tucuman, Argentina*

Edited by:
Christophe M. Filippi, Genomics Institute of the Novartis Research Foundation, USA

Reviewed by:
Cecile King, Garvan Institute of Medical Research, Australia
Anne Cooke, University of Cambridge, UK

***Correspondence:**
Julio Villena, Laboratory of Immunobiotechnology, Reference Centre for Lactobacilli (CERELA-CONICET), Chacabuco 145 – (T4000ILC) San Miguel de Tucumán, Tucuman, Argentina
e-mail: jcvillena@cerela.org.ar

Respiratory syncytial virus (RSV) is the leading cause of lower respiratory tract illness in infants and young children. Host immune response is implicated in both protective and immunopathological mechanisms during RSV infection. Activation of Toll-like receptor (TLR)-3 in innate immune cells by RSV can induce airway inflammation, protective immune response, and pulmonary immunopathology. A clear understanding of RSV–host interaction is important for the development of novel and effective therapeutic strategies. Several studies have centered on whether probiotic microorganisms with the capacity to stimulate the immune system (immunobiotics) might sufficiently stimulate the common mucosal immune system to improve defenses in the respiratory tract. In this regard, it was demonstrated that some orally administered immunobiotics do have the ability to stimulate respiratory immunity and increase resistance to viral infections. Moreover, during the last decade scientists have significantly advanced in the knowledge of the cellular and molecular mechanisms involved in the protective effect of immunobiotics in the respiratory tract. This review examines the most recent advances dealing with the use of immunobiotic bacteria to improve resistance against viral respiratory infections. More specifically, the article discuss the mechanisms involved in the capacity of the immunobiotic strain *Lactobacillus rhamnosus* CRL1505 to modulate the TLR3-mediated immune response in the respiratory tract and to increase the resistance to RSV infection. In addition, we review the role of interferon (IFN)-γ and interleukin (IL)-10 in the immunoregulatory effect of the CRL1505 strain that has been successfully used for reducing incidence and morbidity of viral airways infections in children.

Keywords: *Lactobacillus rhamnosus* CRL1505, TLR3, respiratory immunity, respiratory syncytial virus, immunobiotics

INTRODUCTION

The first isolation of human respiratory syncytial virus (RSV) was performed in 1955 from a captive chimpanzee. The virus was quickly identified as a major respiratory pathogen in infants and children (1). RSV is a negative-strand, non-segmented RNA pneumovirus of the family *Paramyxoviridae*, and a highly contagious virus. Significant epidemiological studies have characterized RSV to be a relevant human pathogen that causes a major health burden worldwide (World Health Organization, www.who.org).

Respiratory syncytial virus causes cold-like symptoms in most healthy adults and children. In infants and young children predisposed to respiratory illness, however, RSV infection is more likely to move into the lower respiratory tract, leading to pneumonia and bronchiolitis (2). RSV has been also identified as an important cause of morbidity and mortality in the elderly, patients with chronic obstructive pulmonary disease, and transplant patients (3).

During the past years, a great advance in the knowledge of the pathogenesis and the immune response against RSV has been achieved. RSV targets both type I alveolar and non-basilar airway epithelial cells and possibly alveolar macrophages. These changes in the respiratory mucosa results in the damage of respiratory epithelial cells and the impairment of their ciliary actions. Although RSV is not a highly cytopathic virus, peribroncheal mononuclear cell infiltration, submucosal edema, mucus secretion, and sometimes syncytia are observed in the lung of RSV-infected hosts (4). In addition, several studies demonstrated that the host immune response to RSV is implicated in both protective and immunopathological mechanisms. Although inflammation elicited in response to RSV is designed to destroy, dilute, and/or sequester the virus, it can also contribute to the injury of lung tissue as a collateral damage. Indeed, the incapacity of the host to control inflammation in RSV infection correlates with the difficulty to limit virus spread, reduce the extension of lung damage and proceed onward to a phase of resolution. It is likely that understanding the pathogenesis of RSV disease, including the immune response to infection, will help to develop novel immunoregulatory therapeutic strategies and design safe and effective vaccines.

It is clear then, that the inflammatory response to RSV is complex, and refractory to treatments with antivirals and glucocorticoids, which are the standard approaches. The immumodulatory impact of probiotic is of great interest considering that these microorganisms are able to modify the responses of mucosal tissue to subsequent pro-inflammatory challenge. Moreover, several studies have centered on whether probiotic microorganisms with the capacity to stimulate the immune system (immunobiotics) might stimulate the common mucosal immune system to improve respiratory tract defenses. In this regard, it was demonstrated that some orally administered immunobiotics do have the ability to stimulate respiratory immunity and increase resistance to viral infections. During the last decade, scientists have significantly advanced in the knowledge of the cellular and molecular mechanisms involved in the protective effect of immunobiotics in the respiratory tract.

This review examines the most recent work dealing with the use of immunobiotic strains to improve resistance against viral respiratory infections. More specifically, the article review the mechanisms involved in the capacity of the immunobiotic strain *Lactobacillus rhamnosus* CRL1505 to beneficially modulate the immune response triggered by Toll-like receptor (TLR)-3 activation in the respiratory tract and to increase the resistance to RSV infection. In addition, we will discuss the role of interferon (IFN)-γ and interleukin (IL)-10 in the immunoregulatory effect of the CRL1505 strain that has been successfully used for reducing incidence and morbidity of viral airways infections in children (5).

INNATE IMMUNE RESPONSES AGAINST RSV

It is known that the initiation of the mucosal and systemic immune responses to respiratory virus requires the recognition by the immune system of pathogen-associated molecular patterns (PAMPs). Recognition of viral PAMPs is achieved by cellular receptors known as pattern recognition receptors (PRRs) that are expressed in both respiratory epithelial cells and immune cells. PRRs sensors include the TLRs; C-type lectin receptors and; RNA-sensing RIG-I-like receptors (RLRs) including melanoma differentiation-associated protein 5 (MDA5) and, retinoic acid-inducible gene I (RIG-I) (6).

Double-stranded RNA (dsRNA) is a replication intermediate of several virus that is able to sensitize innate immune system through TLR3. dsRNA is observed during most RNA virus replications like RSV. The important role of TLR3 in anti-viral immunity has been experimentally proved using TLR3 knockout mice and an artificial dsRNA, the synthetic dsRNA polyinosinic–polycytidylic acid [poly(I:C)]. TLR3-deficient mice have been found to have their anti-viral immune response impaired in challenge-experiments with dsRNA or poly(I:C) (6). Then, TLR3 is considered a major PRR against virus in animal cells. In fact, epithelial cells from the respiratory mucosa over-express TLR3 when challenged with respiratory viruses and, this overexpression of TLR3 allow cells to detect virus and acquire resistance (7, 8).

Respiratory syncytial virus predominantly infects primary airway epithelial cells, but can also infect other structural airway and immune cells. Upon viral entry and activation of signaling complexes including TLR3 (**Figure 1A**) (6, 9), inflammatory cytokines and chemokines are expressed and secreted in airway cells (10). In addition, respiratory epithelial cells and infiltrating leukocytes produce large amounts of anti-viral molecules, such as type I IFN. Type I IFNs signal through its receptor and induce the transcription of many interferon responsive genes (ISGs). The products of these genes limit virus replication and enhance the immune response (**Figure 1B**) (10).

Proliferation and activation of NK cells, as well as its anti-viral capacities are also important for the protection against RSV. An emerging trend born from multiple clinical studies of severely RSV-infected infants is a failure to generate a robust NK-cell response (11–13). In addition to their anti-viral activities, NK cells play a crucial role in the priming of adaptive immune responses against a variety of viral infections. Indeed, the recruitment and activation of IFN-γ-producing NK cells to the site of inflammation plays a critical role in the subsequent development of effector CD4 Th1 and cytotoxic T lymphocytes (CTLs) responses (14). This may occur indirectly through NK-cell licensing of DCs (**Figure 2A**). During this bidirectional cross-talk, IFN-γ released by NK cells activates DCs to produce IL-12, which in turn feeds back on the NK cell to further amplify IFN-γ secretion (14, 15). Of note, defective NK-cell function is strongly linked with the development of Th2-dominated immune responses in RSV infections (16).

In addition, recent studies demonstrated an important role for macrophages in providing an immediate pro-inflammatory response (17), and producing type I IFN (18) following RSV infection. Additionally, macrophages clear debris later in infection, and avoid further damage and inflammation (19). There is also evidence of activated granulocytes and inflammatory cytokines the airways of children and infants with severe RSV infection, being neutrophils the most abundant immune cells (**Figure 2B**). It is known that RSV-induced damage is produced mainly by an excessive infiltration of inflammatory cells into the airways and lung. Studies investigating the infiltration of immune cells into the lung and airways of RSV-infected children showed that neutrophils constituted the predominant population of infiltrating cells in nasal and bronchoalveolar (BAL) lavages. Moreover, neutrophils were also the most common cells found in autopsy tissues from infants infected with RSV (12, 13, 20, 21). RSV infection of the respiratory epithelium induces the secretion of pro-inflammatory mediators by epithelial cells and associated immune cells. The release of pro-inflammatory chemokines and cytokines as well as the upregulation of adhesion molecules, such as ICAM-1, induce and mediate the recruitment of leukocytes to the respiratory tract. Cytokines and chemokines, such as IL-1, IL-6, IL-8, IL-18, TNF, CCL2, CCL3, CCL5, CXCL8, and CXCL10 are significantly augmented in blood, BAL, and nasal aspirates from infants infected with RSV (12, 13, 20, 21). In particular, high levels of CXCL10 and CXCL8 that are major chemo-attractants for macrophages, neutrophils, and T cells, are hallmarks of RSV-infected infants (12, 20, 22). Furthermore, the levels of some of these molecules correlated with disease severity.

ADAPTIVE IMMUNE RESPONSES AGAINST RSV

Virus elimination and the recovery from primary viral respiratory infection are primarily mediated by the adaptive immune response. Both cellular and humoral immune responses act

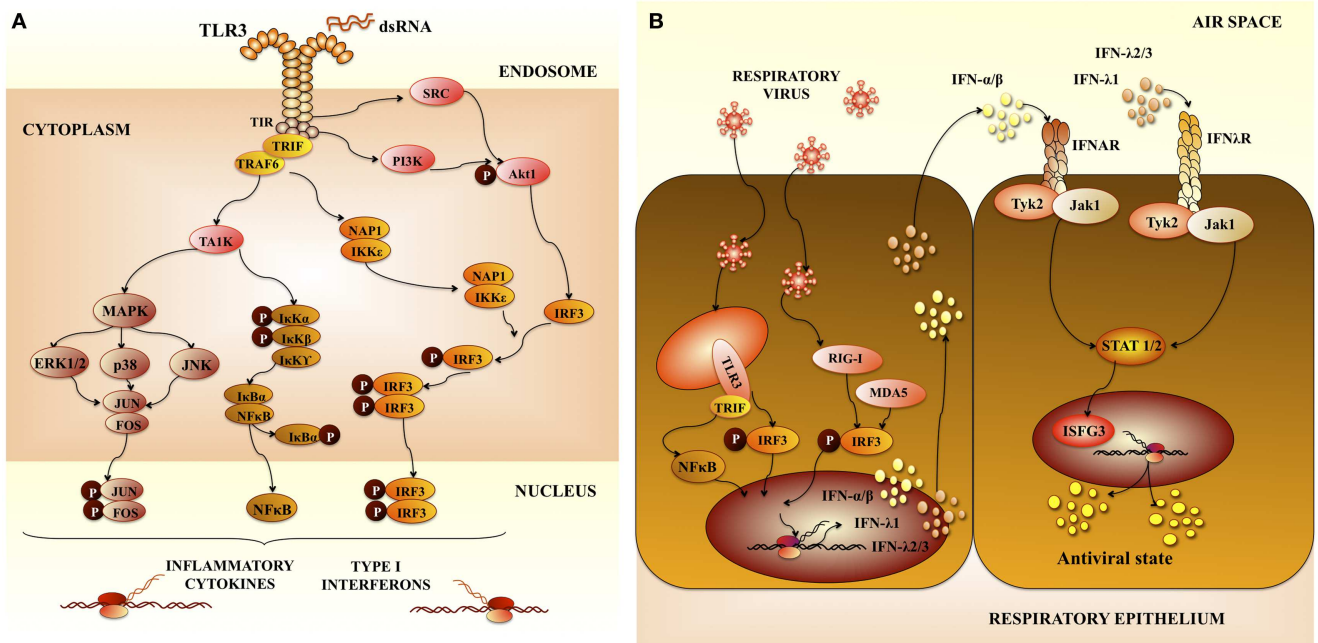

FIGURE 1 | Pattern recognition receptors in respiratory anti-viral immunity. **(A)** Toll-like receptor 3 (TLR3) signaling pathway. TLR3 mediates signaling via the adaptor protein TRIF (TIR-containing adaptor molecule-1). The TIR domain of TRIF is essential for binding to the TIR domain of TLR3. TRIF-1 is localized in the cytoplasm of resting cells, when TLR3 is activated, TRIF co-localizes with endosomal TLR3. Then TRIF dissociates from TLR3 and co-localize with downstream-signaling molecules. The serine-threonine kinases, TANK-binding kinase 1 (TBK1) and IkB kinase-related kinase-e (IKK-e) are activated once TRIF interact with them. As a result of this activation, IRF-3 is phosphorylated. TRAF3 and NF-kB-activating kinase (NAK)-associated protein 1 (NAP1) participates in the recruitment of IRF-3 kinases and in IRF-3 activation. This pathway results in the induction of type I interferons (IFNs). In addition, mitogen-activated protein kinases and (MAPK) and NF-kB pathways are activated, which results in the induction of genes involved in inflammatory responses. **(B)** Anti-viral immune response in airway epithelial cells mediated by pattern recognition receptors and type I interferons (IFNs). Type I IFNs produced are secreted by virus-infected cells and signal in neighboring cells through the IFN-α/β receptor complex (IFNAR). This receptor is constituted by two protein subunits called IFNAR1 and IFNAR2, which are present on the surface of cells. Interaction of type I IFNs with IFNAR in neighboring cells enhance the production of type I IFNs and other inflammatory cytokines. Activation of IFNAR by IFN-α or IFN-β leads to activation of Jak1 and Tyk2 kinases, which phosphorylate the STAT transcription factors. Then, STAT heterodimers (STAT1/STAT2) or homodimers (STAT1) are generated. IRF-9 together with phosphorylated STAT1 and STAT2 form a complex called interferon-stimulated gene factor 3 (ISGF3). This complex activates the transcription of ISGs inducing an anti-viral state in the cell.

directly to eliminate viral pathogens in the respiratory tract (**Figure 3**).

The interaction of RSV with respiratory DCs results in activation and maturation of those cells, being both processes important in establishing virus-specific immunity. The quality and durability of the host immunity as well as the susceptibility to reinfection are significantly influenced by these early events during the initial immune response (23). Respiratory DCs that have acquired RSV antigens maturate and migrate to the lung-draining lymph nodes (LN) where they present antigens and activate antigen-specific T cells (24). In mice, lung DCs can be divided into two major populations: conventional DCs (cDCs) that are $CD11c^{hi}MHC\text{-}II^{hi}$ and plasmacytoid DCs (pDCs) that are $CD11c^{int}B220^{+}$. In addition, cDCs can be further divided into $CD11b^{+}CD103^{-}$ cDCs ($CD11b^{+}$ cDCs) and $CD11b^{-}CD103^{+}$ cDCs ($CD103^{+}$ cDCs) (24). Both populations of cDCs ($CD11b^{+}$ and $CD103^{+}$) have essentially different locations within the lung tissue. $CD11b^{+}$ cDCs are located in the parenchyma of the lung and they promote the recruitment of leukocytes through the production of pro-inflammatory chemokines. In contrast, $CD103^{+}$ cDCs are located in the basal lamina and they are able to extend dendrites into the airway lumen, allowing them to sample potential foreign pathogens from the airway.

After RSV challenge, the total number of lung and lung-draining LN DCs is augmented (25). However, kinetics of DCs mobilizations following acute RSV infection is different in the subepithelial $CD11b^{+}$ cDCs when compared with parenchymal $CD103^{+}$ DCs populations. Whereas the number of $CD11b^{+}$ cDCs increases, the total number of $CD103^{+}$ cDC decreases and remained low during the course of the RSV infection (26). Migrating $CD11b^{+}$ and $CD103^{+}$ cDCs exhibit a mature phenotype, with high expression of CD40, CD80, and CD86 molecules. Moreover, both populations exhibit a similar capacity to stimulate IFN-γ production by CD4 and CD8 T cells (26).

T cells have clear direct and indirect anti-viral effects during RSV infection. Several studies of primary and secondary RSV infections in mice models have demonstrated the central role of T lymphocytes in the pathology of RSV disease. In BALB/c mice, primary infection produces lymphocyte infiltration into the lungs and a strong production of IFN-γ by CD8 cells. Moreover,

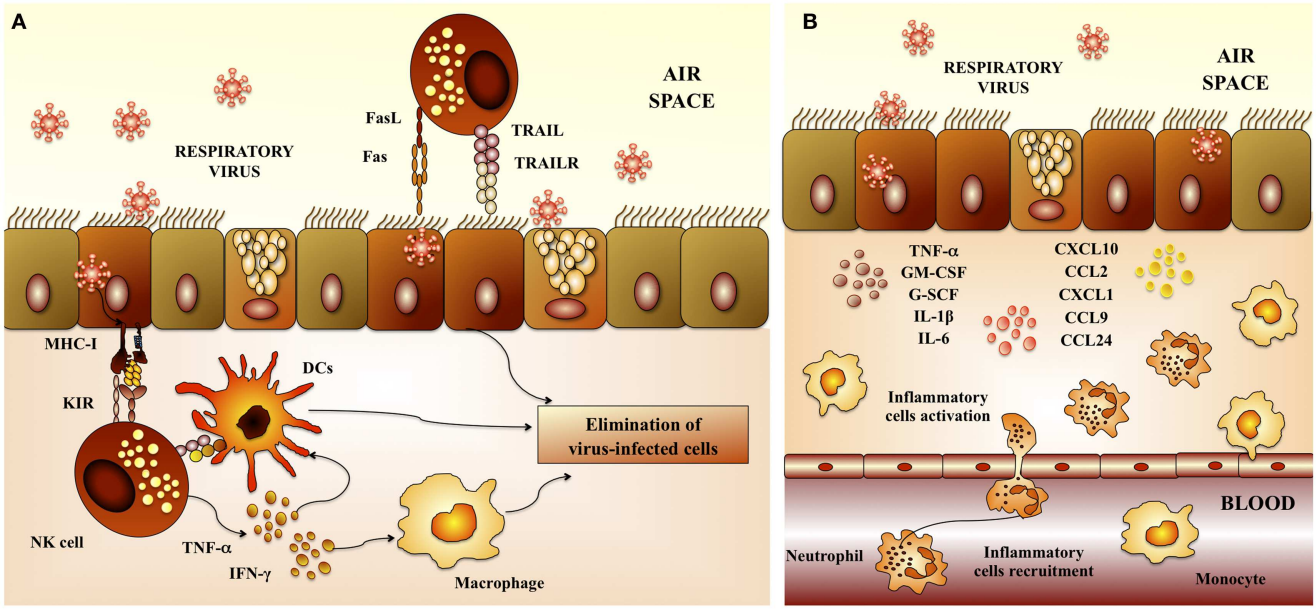

FIGURE 2 | Respiratory anti-viral innate immune response. **(A)** Activity of natural killer cells. Natural killer (NK) cells are involved in the elimination of virus-infected cells because of their cytotoxic capacities. NK cells are recruited to the lungs early after respiratory virus infection. Dendritic cells (DCs) potentiate NK-cell activation and cytotoxicity. In addition, alveolar macrophages are also required to activate NK cells. **(B)** Inflammatory response. Epithelial cells and macrophages are crucial in the innate immune response to respiratory virus. Several chemokines and cytokines including IL-8/CXCL8, IP-10/CXCL10, MCP-1/CCL2, MIP-1a/CCL3, MIP-1b/CCL4, RANTES/CCL5, IL-6, TNF, and IL-1 are produced by epithelial cells and macrophages in response to virus infection. Upregulation of these cytokines and leads to recruitment of neutrophils, which constitute the majority of infiltrating cells. While neutrophils may mediate elimination of virus-infected cells, their high numbers, ability to secrete further cytokines and chemokines, and degranulation products may contribute to respiratory virus-induced immunopathogenesis.

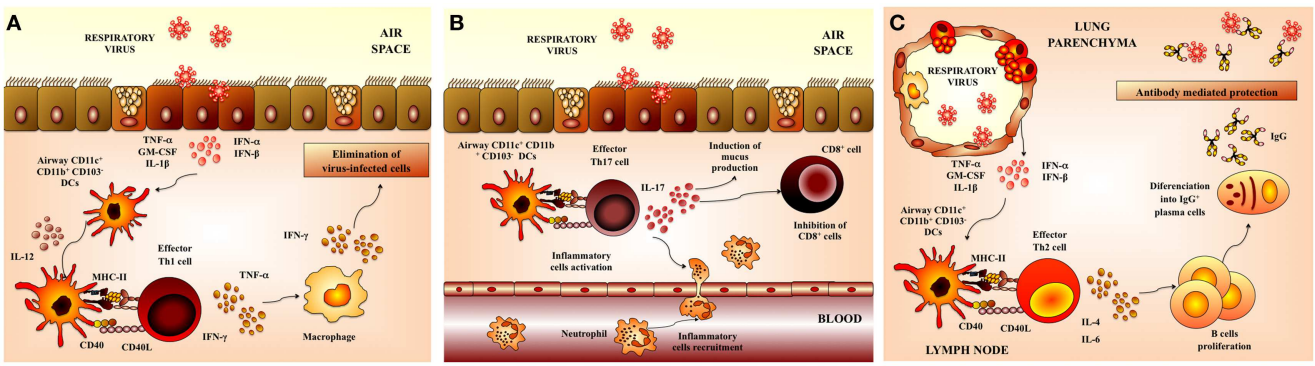

FIGURE 3 | Respiratory anti-viral adaptive immune response. **(A)** Th1 cellular immunity. Upon respiratory virus infection of lungs, $CD11b^+$ and $CD103^+$ dendritic cells (DCs) are matured and migrate to the draining lymph nodes. These DCs prime Th1 cells that return to the lung and promote viral clearance. **(B)** Th17 cellular immunity. Th17 cells produce IL-17 that co-operates with IL-1β and TNF-α to induce the release of chemokines. These changes in the respiratory tract induce neutrophils recruitment and activate inflammatory responses in the lung. **(C)** Antibody-mediated immunity. Neutralizing antibodies have a critical role in protection from respiratory virus infection. Serum antibodies, mainly composed of IgG, gain access to the lungs via transduction and provide partial or complete protection against virus replication in the lungs.

although CD4 T lymphocytes are less frequent, Th1 cells predominate even in BALB/c mice that are naturally Th2-responders (**Figure 3A**). CTLs appear in the lungs at day 4, peak around days 6–14, and are critical for viral clearance but can also contribute to disease (27). Other studies have strongly associated Th2 responses with increased pathology in lungs of RSV-infected mice. Decreased mucus production and lung inflammation were found in acute RSV infection when the Th2 cytokine IL-4 was depleted before viral challenge (28, 29). By contrast, decreased pulmonary pathology is associated with Th1 responses (28, 30, 31). Depletion of IL-12, a Th1 polarizing cytokine, significantly increased production of IL-13, along with increased mucus production,

airway resistance, and pulmonary inflammation (30). Moreover, mice deficient in the IFN-induced transcription factor STAT1 exhibit increased production of Th2 cytokines and delayed viral clearance (31).

It has more recently been shown that Th17 cells may also play a role in effector mechanisms triggered in response to RSV. The production of IL-17 by $CD4^+$ Th17 cells has both positive and negative effects in the respiratory tract. Activated Th17 cells produce IL-17 that induce the recruitment of neutrophils. Additionally, it was described that IL-17 facilitates the development tertiary lymphoid structure in infected lungs, which increase protection against RSV infection (1, 2, 32) (**Figure 3B**). However, IL-17 also acts synergistically with other pro-inflammatory factors and cells to exacerbate inflammatory damage and alter lung function in RSV-infected hosts. Moreover, it was recently described that IL-17 inhibits the ability of $CD8^+$ cells to clear viral particles (1, 2, 32). Furthermore, IL-17 enhances IL-13 production, which promotes the activation of Th2 lymphocytes and excessive mucus production (32). As IL-17 is known to play a role in the development of asthma, its role in RSV pathogenesis was recently examined. Increased IL-6 and IL-17 levels were found in the tracheal aspirate samples from severely ill RSV-infected infants. Furthermore, IL-6, IL-17, and IL-23 were increased in RSV-infected mice, while treatment with anti-IL-17 antibodies reduced inflammation, decreased viral load, and increased antigen-specific $CD8^+$ T cells in the lung (32, 33) (**Figure 3B**).

An effective B-cell response is also essential for resistance against viral respiratory tract infections. B cells response is reflected in the generation of antibodies capable of neutralizing the virus in both the respiratory tract and serum (**Figure 3C**). In this regard, a wealth of evidence indicates that mainly neutralizing antibodies confers protection against RSV infection. The F and G glycoproteins are the only viral antigens able to induce neutralizing antibodies as well as relatively long-lived protection in animal models (1). It was also reported that the prophylactic administration of RSV-neutralizing polyclonal or monoclonal antibodies is able to protect adult and infants from severe RSV disease (1, 34, 35).

ROLE OF TLR3 IN DEFENSE AND PATHOGENESIS OF RESPIRATORY VIRUS

It is known that TLR3 has a complex role in viral infections. Challenge-infection experiments in $TLR3^{-/-}$ animals have demonstrated that the immune response to viruses can be unaffected or impaired depending on the virus type. In fact, TLR3 has been implicated in both protective immunity and inflammatory tissue damage during viral infections. Studies of *Coxsackievirus* group B infection showed that $TLR3^{-/-}$ mice are more vulnerable to the pathogen than wild-type mice, when considering myocarditis severity and mortality (36). In the hearts of coxsackievirus-infected TLR3-deficient mice, there was an impaired expression of IL-12p40, IL-1β, and IFN-γ, but not IFN-β, when compared with wild-type mice. On the other hand, it was reported that $TLR3^{-/-}$ mice are more resistant to the infection with West Nile virus, indicating an important role of TLR3 in viral pathogenesis (37). It was shown that inflammatory responses and neuropathology as well as the viral load in the brain were significantly lower in $TLR3^{-/-}$ mice compared with wild-type animals. The work clearly demonstrated that TLR3-mediated enhanced cytokine production and that this inflammatory response was critical for the alteration of the blood-brain barrier. Moreover, the magnitude of the inflammatory damage in the blood–brain barrier correlated with viral entry into the brain and the severity of lethal encephalitis.

In respiratory viral infections such as influenza virus or RSV, inflammatory response mediated by TLR3 also appears to affect the pathology induced by the virus as well as host survival.

Acute pneumonia is considered one of the most severe complications of influenza virus infection. Pneumonia develops rapidly and often results in respiratory failure and death. Remarkably, it was reported that TLR3-deficient animals are more resistant than wild-type mice to influenza virus A challenge (8). Authors described that lungs of wild-type animals presented a black hemorrhaged lung surface indicating a sever injury, whereas lungs obtained from $TLR3^{-/-}$ knockdown mice showed only diffuse hemorrhagic foci. These results suggested that lesions induced by influenza virus A are reduced in the absence of TLR3. Lung tissue injuries correlated with a strong inflammatory response in the lungs of wild-type mice after influenza challenge, which is critically reduced in $TLR3^{-/-}$ animals (8). Among leukocytes infiltrating the lungs of infected mice, macrophages and $CD8^+$ T cells were the predominant immune cells in infected wild-type animals. However, in $TLR3^{-/-}$ mice the number of $CD8^+$ T lymphocytes was significantly lower than the one found in wild-type animals. Likewise, a significant reduction of the number of macrophages was observed in $TLR3^{-/-}$ mice. On the contrary, neutrophils number in the lungs of $TLR3^{-/-}$ animals was 1.5 times higher than in wild-type mice (8). Remarkably, the study showed that TLR3 deficiency caused a significant reduction of cytokine synthesis, including IL-6, IL-12p40/p70, and RANTES while other cytokines such as IFN-γ, G-CSF, IL-9, eotaxin, MCP-5, and IL-10 were increased in $TLR3^{-/-}$ versus wild-type lungs (8). Overall, it emerges that TLR3-mediated inflammatory response would be a key point in the clinical manifestations of influenza virus-induced pneumonia.

The roles of inflammation, in general, and TLR3 in particular, in the pathogenesis of RSV have been also investigated. BALB/c mice have been used as a suitable animal model to explore the innate and adaptive immune responses to human RSV (38). The first histopathological studies of susceptible BALB/c mice challenged with human RSV were reported by Graham et al. (39). Authors demonstrated that lung injury was severe between days 5–8, resolving by day 10 after inoculation. Lung alterations were characterized by perivascular and peribronchial infiltrates of inflammatory cells. In addition, infiltration of lymphocytes and macrophages in the alveolar spaces were described. Subsequent work identified several pro-inflammatory cytokines and chemokines produced and released after RSV infection, including IP-10, KC, MIP-1α, MCP-1, RANTES, the IFN-γ regulated protein (40–42), and more recently, IL-17 (32, 33).

TLR3 can detect the dsRNA generated during the RSV replication cycle (43). It is thought that TLR3 has no or little effect on RSV clearance from the lungs. However, it is now accepted that TLR3 is necessary to regulate the respiratory immune environment. In fact, the lack of an appropriate regulation of TLR3 activation significantly contributes to the pulmonary immunopathology associated to RSV infection (44–46). It was

reported that RSV-infected cells upregulate TLR3 expression and MyD88-independent chemokines, such as IP-10/CXCL10 and CCL5 after activation of the TLR3 signaling pathways by the virus (44). This increased TLR3 expression in the respiratory epithelial cells sensitizes these cells to subsequent viral dsRNA exposure and increase the production of IL-8 via NF-kB pathway (46). Moreover, it was demonstrated that RSV promotes a predominant Th1-type response when TLR3 is activated during the infection (45). By contrast, increased pathogenic Th2-biased response is generated when TLR3 is deleted, including accumulation of eosinophils in the lung and overproduction of Th2 cytokines and mucus (45).

These results are in line with the notion that the persistent unregulated inflammatory responses induced by RSV in lungs, may provide an environment that facilitates the infection with other respiratory pathogens (47). Therefore, an appropriate modulation of respiratory TLR3 could be an interesting therapeutic target not only for reducing RSV-induced lung inflammatory damage, but for avoiding subsequent infections.

IMPROVEMENT OF RESPIRATORY ANTI-VIRAL IMMUNITY WITH IMMUNOBIOTICS

Certain probiotic lactic acid bacteria (LAB) strains can exert their beneficial effect on the host through their immunomodulatory activity. These strains have been termed immunobiotics (48). Although most research works concerning the immunostimulatory activities of probiotic LAB is focused on their effect in the gastrointestinal tract, several recent studies have clearly demonstrated that immunobiotics are able to improve protection against respiratory pathogens. In fact, research from the last years indicate that immunobiotic bacteria could be effectively used for the development of new prophylactic strategies that could be effective tools to protect against respiratory infections.

There are several lines of evidence that orally or nasally administered immunobiotics are capable of improving resistance against viral infections in the respiratory tract. Different aspects of respiratory anti-viral immunity can be beneficially modulated by immunobiotics, including the production of type I IFNs, the activity of NK cells, the generation of Th1 responses as well as the production of specific antibodies, and the regulation of inflammatory-mediated lung injury (**Table 1**).

Maeda et al. (53) showed that orally administered heat-killed *Lactobacillus plantarum* L-137 augmented the resistance against influenza virus infection by stimulating the production of type I IFN. The study showed that *L. plantarum* L-137 treatment significantly prolonged the mean survival time in mice infected with a mouse-adapted virulent strain of influenza virus H1N1, and that this effect correlated the increased production of IFN-β (53). However, detailed studies to investigate the immune mechanisms involved in *L. plantarum* L-137 activity were not performed.

Other studies emphasized the importance of IFN-γ production and NK cells activation for the protective effect of immunobiotics against influenza infection (55–57). Earlier studies with the known probiotic strain *Lactobacillus casei* Shirota clearly demonstrated the capacity of this bacterium to stimulate NK cells activity and cellular immunity in the respiratory tract. Moreover, the study showed that the Shirota strain was able to improve the resistance of mice to influenza virus challenge (50). It was found that mice receiving *L. casei* Shirota intranasally strongly induced production of IL-12 in mediastinal lymphoid nodes (MLN) cells. In addition, both IFN-γ and TNF-α were augmented in MLN cell cultures from mice receiving *L. casei* Shirota intranasally. These changes in MLN's cytokine profile, induced by the immunobiotic treatment, explain the improvement of NK cells stimulation and the enhancement of the Th1 response (50). A second work of the same group demonstrated that orally administered *L. casei* Shirota activated the systemic and respiratory immune systems and diminished influenza virus infection severity in both aged (51) and infant mice (52). As observed in adult mice, the protective effect of the Shirota strain correlated with augmented NK-cell activity in splenocytes and lungs and enhanced IFN-γ and TNF-α production of nasal lymphocytes. More recently, it was showed that oral administration of heat-killed *L. gasseri* TMC0356 or lyophilized *L. rhamnosus* GG resulted in a higher expression of pulmonary IFN-γ and reduced pulmonary virus titers between control and lactobacilli-treated mice (54).

In an effort to evaluate the capacity of lactobacilli to reduce the pathogenesis of severe pneumovirus infection *in vivo*, Gabryszewski et al. (61), developed a model pneumonia virus of mice (PVM) infection. Authors showed that nasally administered *L. plantarum* or *Lactobacillus reuteri* were highly effective for controlling inflammation induced by PVM infection and for protecting against lethal disease. Lactobacilli treatments reduced virus recovery and diminished granulocyte recruitment, and the expression of pro-inflammatory cytokines including CXCL10, CXCL1, CCL2, and TNF (**Table 1**). Other studies also showed the capacity of immunobiotics to beneficially modulate the balance between pro- and anti-inflammatory mediators during respiratory virus infections. Takeda et al. (58) demonstrated that *L. plantarum* 06CC2, when orally administered, differentially modulated the production of cytokines during influenza infection. The levels of IFN-γ, IL-12, and IFN-α in infected mice administered the 06CC2 strain were significantly higher than those in the controls while the level of TNF-α was significantly lower than that in the control mice (58). Another study investigated whether that sublingual administration of *L. rhamnosus* enhanced protection against influenza virus (63). The work reported that immunobiotic treatment was able to augment T cell and NK-cell activity in the respiratory mucosa, enhancing the resistance against viral infection. Moreover, authors found that *L. rhamnosus*-treated mice had improved levels of IL-12 and reduced IL-6 and TNF-α levels in lungs when compared to controls, indicating that the probiotic treatment modulated cytokine profile in response to the infection. Taking into account that the levels of the pro-inflammatory cytokines IL-6 and TNF-α have a positive correlation with vascular dysfunction and lung inflammation, these results suggest that the reduced concentrations of some pro-inflammatory mediators would be helpful to protect against influenza virus infection (63).

The impact of immunobiotics on anti-viral humoral response has been also evaluated. Early studies from Yasui et al. (71) showed that orally administered *Bifidobacterium breve* YIT4064 augmented the production of anti-viral antibodies including anti-poliovirus, anti-influenza virus, and anti-rotavirus antibodies

Table 1 | Effect of immunobiotics on viral respiratory infections.

Respiratory virus	Immunobiotic treatment	Protective effect	Ref.
Influenza virus H1N1	Orally administered heat-killed *B. breve* YIT4064	Reduction of accumulated symptom rate Improvement of survival rate Improvement of serum IgG	(49)
Influenza virus H1N1	Nasally administered heat-killed *L. casei* Shirota	Reduction of virus titer in nasal wash Improvement of survival rate Improvement of IL-12, TNF-α, and IFN-γ in MLN	(50)
Influenza virus H1N1	Orally administered heat-killed *L. casei* Shirota	Reduction of virus titer in nasal wash Improvement of NK-cell activity in spleen and lung Improvement of TNF-α and IFN-γ in nasal lymphocytes	(51)
Influenza virus H1N1	Orally administered viable *L. casei* Shirota	Reduction of virus titer in nasal wash Reduction of accumulated symptom rate Improvement of NK-cell activity in lung Improvement of IL-12 in MLN	(52)
Influenza virus H1N1	Orally administered heat-killed *L. plantarum* L-137	Reduction of virus titer in lung Improvement of survival rate Improvement of serum IFN-β	(53)
Influenza virus H1N1	Orally administered lyophilized *L. gasseri* TMC0356	Reduction of virus titer in lung Reduction of clinical scores Reduction of lung injury Immune mechanism not studied	(54)
Influenza virus H1N1	Nasally administered heat-killed *L. pentosus* S-PT84	Reduction of virus titer in BAL Improvement of NK-cell activity in lung Improvement of IL-12 and IFN-γ in BAL	(55)
Influenza virus H1N1	Nasally administered lyophilized *L. rhamnosus* GG	Improvement of survival rate Reduction of accumulated symptom rate Reduction of lung injury Improvement of NK-cell activity in lung Improvement of IL-1β, TNF-α, MCP-1, and IFN-γ in lung	(56)
Influenza virus H1N1	Orally administered lyophilized *B. longum* BB536	Reduction of symptom score Reduction of lung injury Reduction body weigh loss Improvement of IL-1β, IL-6, and IFN-γ in lung	(57)
Influenza virus H1N1	Orally administered heat-killed *L. plantarum* 06CC2	Reduction of virus titer in lung Reduction body weigh loss Improvement of NK-cell activity in spleen Improvement of INF-α, IFN-β, IFN-γ, TNF-α, IL-12, and IL-6 in BAL Reduction of infiltrated neutrophils	(58)
Influenza virus H1N1	Orally administered heat-killed *L. pentosus* b240	Improvement of survival rate Reduction of virus titer in lung Improvement of BALF IgA and IgG	(59)
Influenza virus H1N1	Orally administered formalin treated Lactobacilli mixture	Improvement of survival rate Reduction of lung injury Improvement of lung IgA Improvement of lung TNF-α and IL-12	(60)
Influenza virus H1N1	Nasally administered formalin treated Lactobacilli mixture	Improvement of survival rate Reduction of lung injury Improvement of lung IgA Improvement of lung TNF-α and IL-12	(60)

(Continued)

Table 1 | Continued

Respiratory virus	Immunobiotic treatment	Protective effect	Ref.
Pneumonia virus of mice	Nasally administered viable or heat-killed *L. plantarum* ATCCBAA793	Improvement of survival rate Reduction of virus titer in lung Suppression of virus-induced CXCL10, CCL2, CXCL1, CCL9, TNF, and CCL24 in a MyD88-TLR signaling independent manner	(61)
Pneumonia virus of mice	Nasally administered viable or heat-killed *L. plantarum* ATCC23272	Improvement of survival rate Reduction of virus titer in lung Suppression of virus-induced CXCL10, CCL2, CXCL1, CCL9, TNF, and CCL24 in a MyD88-TLR signaling independent manner	(61)
Poly(I:C)	Orally administered viable *L. rhamnosus* CRL1505	Reduction of lung injury Improvement of DCs and CD4$^+$IFN-γ^+ T cells in lung and levels of IFN-γ, IL-10, and IL-6 in BALF	(62)
Influenza virus H1N1	Sublingual administration of lyophilized *L. rhamnosus*	Reduction of virus titer in lung Reduction of lung injury Improvement of lung IgA, IL-12, and NK-cell activity and reduction of IL-6 and TNF-α	(63)
Respiratory syncytial virus	Orally administered viable *L. rhamnosus* CRL1505	Reduction of virus titer in lung Reduction of lung injury Improvement of DCs and CD4+IFN-γ^+ T cells in lung and levels of IFN-γ, IL-10, and IL-6 in BAL	(64)
Respiratory syncytial virus	Nasally administered viable or heat-killed *L. rhamnosus* CRL1505	Reduction of virus titer in lung Reduction of lung injury Improvement of DCs and CD4+IFN-γ^+ T cells in lung and levels of IFN-γ, IL-10, and IL-6 in BAL	(65)
Influenza virus H1N1	Intragastric administration of *L. plantarum* CNRZ1997	Reduction of virus titer in lung Reduction of weight loss and alleviation of clinical symptoms Immune mechanism not studied	(66)
Influenza virus H1N1	Orally administered viable and non-viable *L. acidophilus* L-92	Reduction of virus titer in lung Improvement of NK cells activity in lungs Reduction of infiltrating neutrophils Increase of IL-17 in Peyer's patches	(67)
Influenza virus H1N1	Orally administered lyophilized *L. brevis* KB290	Alleviates clinical symptoms, loss of body weight, and the deterioration of physical conditions Improvement of IgA and IFN-α in BAL	(68)
Influenza virus H1N1	Orally or nasally administered *L. plantarum* DK119	Reduction of virus titer in lung Reduction of body weight loss Modulation of DCs and macrophages activities in lungs	(69)
Respiratory syncytial virus – influenza virus H1N1	Orally administered viable *L. rhamnosus* CRL1505	Reduction of virus titer in lung Reduction of lung injury Modulation of tissue factor and thrombomodulin expression in lungs Improvement of IFN-γ and IL-10 in lungs	(70)

after the challenges with the respective viral pathogens (71). Moreover, a second work of the same group with the YIT4064 strain clearly demonstrated that the immunobiotic treatment significantly improved the protection of mice against influenza infection; and that this protective effect was related to increased anti-influenza virus IgG titers in serum (49). More recently, the ability of non-viable immunobiotics to improve respiratory anti-viral immunity was evaluated. It was reported that orally administered heat-killed lactobacilli enhanced anti-influenza antibodies in the airways. Both IgA and IgG specific antibodies significantly reduced the susceptibility of mice to influenza virus infection (59, 60). Then, immunobiotics are capable to modulate the production of systemic and mucosal antibodies against respiratory viruses (**Table 1**).

We aimed to evaluate whether a probiotic yogurt containing the immunobiotic strain *L. rhamnosus* CRL1505 was able to beneficially modulate both gut and non-gut related illnesses in humans. For this purpose, we performed a randomized controlled trial in children under 5 years old (62). We demonstrated that the intervention with the immunobiotic strain CRL1505 was able to reduce the frequency and severity of mucosal infections (intestinal and respiratory) in young children; and that this protective effect was related to an improvement of mucosal immunity. It was also found that in children who consumed *L. rhamnosus* CRL1505, the presence of fever and the need for antibiotic treatment were significantly reduced when compared to the placebo control group, indicating less serious infections (63). We did not study the etiology of the respiratory infections in the clinical trial, however previous epidemiological evaluations have shown that viral pathogens including RSV, human metapneumovirus, influenza A virus, parainfluenza viruses, and rhinoviruses are the major viruses responsible of respiratory tract diseases in children in our country (72). Therefore, the findings of our study suggested that administration of *L. rhamnosus* CRL1505 could be an interesting tool for reducing the incidence and severity of common childhood infectious diseases, especially those associated to viral pathogens (62).

DISTAL MODULATION OF RESPIRATORY ANTI-VIRAL IMMUNITY BY *L. RHAMNOSUS* CRL1505

Taking into consideration the results of the clinical studies, we were interested in demonstrating the capacity of *L. rhamnosus* CRL1505 to improve respiratory anti-viral immunity and to gain insight into the immunological mechanism(s) involved in the beneficial effect. Then, we evaluated the effect of the oral administration of *L. rhamnosus* CRL1505 on respiratory anti-viral immunity triggered by TLR3 activation. For this purpose, we used infant and adult BALB/c mice and the nasal administration poly(I:C) that is and artificial dsRNA analog and TLR3 ligand, to induce lung inflammation. This mice model allows us to imitate functional alterations and pro-inflammatory consequences of RNA viral infections in the lung. We showed that after nasal administration of poly(I:C) to BALB/c mice there was an increased inflammatory cell recruitment into the lung and production of pro-inflammatory cytokines, that were accompanied by a marked impairment of lung function (62) in accordance with results published by Stowell et al. (73). Increased levels of albumin concentration and lactate-dehydrogenase (LDH) activity were observed in BAL after poly(I:C) administration indicating impaired epithelial barrier function and respiratory epithelial cell death. Moreover, TLR3 activation by intranasal administration of poly(I:C) resulted in neutrophils and mononuclear cells influx into the lung (43, 62, 73).

Increased levels of respiratory MCP-1, IL-6, TNF-α, and IL-8 were observed in our *in vivo* experiments with BALB/c mice. Previous *in vitro* studies showed that stimulation of respiratory epithelial cells with poly(I:C) increases TLRs and transcription factors expression and induces the secretion of multiple cytokines and chemokines (73). Therefore, the source of pro-inflammatory cytokines and chemokines after poly(I:C) administration may be the airway epithelium. It was described that the profile of pro-inflammatory mediators induced by RSV is similar to the one triggered by poly(I:C) (43, 73), then the experimental model used in our works resembles RSV infection. Moreover, experimental RSV challenge in mice and RSV infection in children is characterized by a prominent secretion of pro-inflammatory mediators in the respiratory tract, as mentioned before. The coordinated actions of these pro-inflammatory mediators promote neutrophils and monocytes/macrophages recruitment and activation in the lung (38), also observed in our mice model (62).

Host's inflammatory response has to be tightly regulated during acute viral lung infection. A regulated inflammatory response enables pathogen elimination without the detrimental effects of inflammation on the delicate lung tissue where gas exchange is produced. Therefore, an appropriate balance between pro-inflammatory and anti-inflammatory factors is crucial for an effective and safe response against RSV. In fact, it was described that excessive IL-10 production can induce a delayed virus clearance while exuberant production of TNF-α/IL-8/MCP-1 can lead to increased immunopathology (74). During the early stages of RSV infection, TNF-α significantly contributes to virus clearance. However, overproduction of TNF-α in the late stages of RSV infection exacerbates tissue injuries and illness (42). Interestingly, it was shown that immunopathology and lethal disease during influenza infection is prevented by IL-10 (75). IL-10 also seems to play an important role in the control of infection severity in RSV challenged hosts (75, 76). IL-10 deficiency during RSV infection did not affect lung viral titers. However, lack of IL-10 significantly increases the severity of RSV disease. Absence of IL-10 allows a greater release of inflammatory cytokines, enhanced influx of inflammatory cells, and delayed recovery (77). Then, the reduction of MCP-1, IL-8, TNF-α, and IL-6 in the lung after the challenge with poly(I:C) could explain, at least partially, the capacity of *L. rhamnosus* CRL1505 to reduce lung injuries (62). Moreover, IL-10 concentrations in the respiratory tract and serum of *L. rhamnosus* CRL1505-treated mice were significantly increased prior the challenge with poly(I:C). IL-10 would be valuable for attenuating TLR3-mediated inflammatory damage in the lungs. Consequently, *L. rhamnosus* CRL1505 treatment could be used to beneficially modulate the balance between pro- and anti-inflammatory cytokines, allowing a reduction of lung tissue damage through an effective regulation of the inflammatory response.

Oral treatment with the CRL1505 strain also increased levels of IFN-γ in the respiratory tract after poly(I:C) challenge (62). The higher levels of respiratory IFN-γ in *L. rhamnosus* CRL1505-treated mice could be related to the increased activity lung DCs that are able to augment $CD3^+CD4^+$IFN-γ^+ T cells numbers. In addition, we found increased levels of $CD11b^{high}$ and $CD103^+$ DCs in lungs of *L. rhamnosus* CRL1505-treated mice after challenge with poly(I:C). Moreover, an improved MHC-II expression was found in both DCs populations when compared with controls. However, only $CD103^+$ DCs showed higher production of IL-12 and IFN-γ in *L. rhamnosus* CRL1505-treated mice (62). In line with our results, it was reported that priming of $CD4^+$DO11.10$CD62L^{high}$ T lymphocytes with lung $CD103^+$ DCs, induced $CD4^+$ T cells that produce preferably IFN-γ rather than IL-4 (78).

These results of our clinical trial and the studies in mice clearly indicated that *L. rhamnosus* CRL1505 could be useful as

a prophylactic agent to control viral respiratory virus since this probiotic strain is a potent inducer of anti-viral cytokines. However, further research was needed to conclusively demonstrate the protective effect of the CRL1505 strain against real viral challenges. Therefore, we next examined whether oral administration of *L. rhamnosus* CRL1505 was able to reduce the susceptibility of infant mice to RSV infection. We demonstrated that oral administration of *L. rhamnosus* CRL1505 contributed to a significant decrease of RSV titers and lung tissue damage after the challenge with the respiratory pathogen (64). The protective effect achieved by the immunobiotic strain was related to its ability to modulate the respiratory anti-viral response. As observed in poly(I:C) challenge-experiments, infant mice orally treated with the CRL1505 strain showed an early increase in the levels of respiratory TNF-α and IL-6 after RSV infection. The early increases of these cytokines together with the improved levels of IFN-γ were probably related to the higher ability of the immunobiotic bacterium to reduce viral loads. In addition, orally administered *L. rhamnosus* CRL1505 significantly augmented IL-10, which contributed to protection against inflammatory damage (64). In fact, we demonstrated that both IFN-γ and IL-10 are necessary to achieve full protection against RSV in infant mice and that these cytokines are differently involved in the immunoprotective effect of *L. rhamnosus* CRL1505. The reduction of RSV titers induced by the immunobiotic strain was abolished when blocking anti-IFN-γ antibodies were used. In addition, the reduction of lung tissue injury induced by the CRL1505 strain was partially abolished with anti-IFN-γ antibodies (64). On the contrary, the use of blocking anti-IL-10R antibodies did not affect the ability of the immunobiotic strain to reduce RSV titers. However, blocking antibodies against IL-10R significantly abolished the protective capacity of *L. rhamnosus* CRL1505 against lung tissue damage (64).

L. rhamnosus CRL1505 also improved lung $CD103^+MHC\text{-}II^+$ and $CD11b^{high}MHC\text{-}II^+$ DCs after RSV challenge (64). Considering that $CD103^+$ and $CD11b^{high}$ lung DCs are able to present RSV antigens to naïve T cells (26), and that both DCs populations are important in the generation of $CD8^+$ and $CD4^+$ effectors T cells, the increase of lung DCs would have a critical role in the immunoregulatory effect of *L. rhamnosus* CRL1505. It could be speculated that the immunobiotic strain would be able to improve protective adaptive immune response by beneficially modulating DCs activity, considering that activation and maturation of antigen presenting cells after RSV arrival to the lung determine the quality and durability of host immunity and influence susceptibility to reinfection (64).

Respiratory syncytial virus infection induces Th2-like inflammation in the lung. Therefore, strategies that improve Th1 responses against RSV are considered beneficial to modulate the outcome of the disease especially in young individuals. IFN-γ augments the expression of MHC-II and MCH-I in DCs and increases the cellular Th1 anti-viral immune response. These changes suppress the proliferation and activation of Th2 T cells (79). Consistent with this notion, *L. rhamnosus* CRL1505 administration to infant mice significantly increased RSV clearance and augmented respiratory IFN-γ levels. Then, modulation of respiratory immunity induced by the immunobiotic strain might contribute to an increase in Th1 response and thereby favor protective immunity against respiratory viral infections such as RSV.

We were particularly interested in gaining insight into the mechanism(s) involved in the immunoprotective capacities of *L. rhamnosus* CRL1505. *In vivo* and *in vitro* experiments demonstrated that the CRL1505 strain significantly augmented the levels of IFN-γ, IFN-α, IFN-β, TNF-α, IL-10, and IL-6 in the intestine and the number of $CD3^+CD4^+IFN\text{-}\gamma^+$ T cells in Peyer's Patches. In addition, *L. rhamnosus* CRL1505 is able to improve these cytokines in blood, particularly IFN-γ, IL-10, and IL-6. The profile of blood cytokines was similar to the one in the intestinal fluid, suggesting that levels of serum cytokines are a reflection of intestinal changes (80). On the contrary, the analysis of respiratory cytokines showed that only IFN-γ, IL-10, and IL-6 were increased by *L. rhamnosus* CRL1505 (62). These same cytokines were augmented by the immunobiotic strain in serum, however, it was not possible to attribute a direct correlation between the increases in the respiratory tract and blood, because TNF-α, IFN-α, or IFN-β levels were not augmented in the airways of *L. rhamnosus* CRL1505-treated mice. Therefore, considering the ability of *L. rhamnosus* CRL1505 to augment the number of intestinal $CD3^+CD4^+IFN\text{-}\gamma^+$ T cells, we hypothesized that the immunobiotic strain would induce a mobilization of these cells into the respiratory tract. We confirmed that this assumption was true after demonstrating increased numbers of $CD3^+CD4^+IFN\text{-}\gamma^+$ T in the lungs mice orally treated with *L. rhamnosus* CRL1505 (62). Furthermore, the mobilization of $CD3^+CD4^+IFN\text{-}\gamma^+$ T cells from the intestine to the airways and the higher production of IFN-γ could be involved in the improved anti-viral state induced by *L. rhamnosus* CRL1505 that was observed in clinical studies (5). Probably, IFN-γ secreted in response to *L. rhamnosus* CRL1505 stimulation would be capable of functionally modulate the innate immune microenvironment in the lung, inducing the activation of DCs (64) and macrophages (81). Additionally, IFN-γ would favor the generation of Th1 immunity with the consequent reduction of the damaging Th2 reactions that are associated to RSV challenge (64) (**Figure 4**). In addition, there is increasing information regarding the involvement of Th17 cells in respiratory virus infections such as influenza and RSV. As mentioned before, cytokines produced by Th17 cells have both positive and negative effects during RSV infections. Considering that some works have demonstrated the capacity of immunobiotics to beneficially modulate the Th17 response in respiratory allergy; it would be an interesting topic for future research to evaluate the effect of *L. rhamnosus* CRL1505 on Th17 response during RSV infection in infant mice.

LOCAL MODULATION OF RESPIRATORY ANTI-VIRAL IMMUNITY BY *L. RHAMNOSUS* CRL1505

Considering that nasally administered antigens induce respiratory and systemic immune responses that are superior to those obtained with oral immunizations, we also analyzed whether the nasal administration of immunobiotics is capable of increasing resistance against poly(I:C)/RSV challenges. In addition to the CRL1505 strain, we also evaluated *L. rhamnosus* CRL1506, a strain with a strong capacity to stimulate the production of type I IFNs in intestinal epithelial cells (62, 82). Our work demonstrated that nasally administered CRL1505 or CRL1506 strains

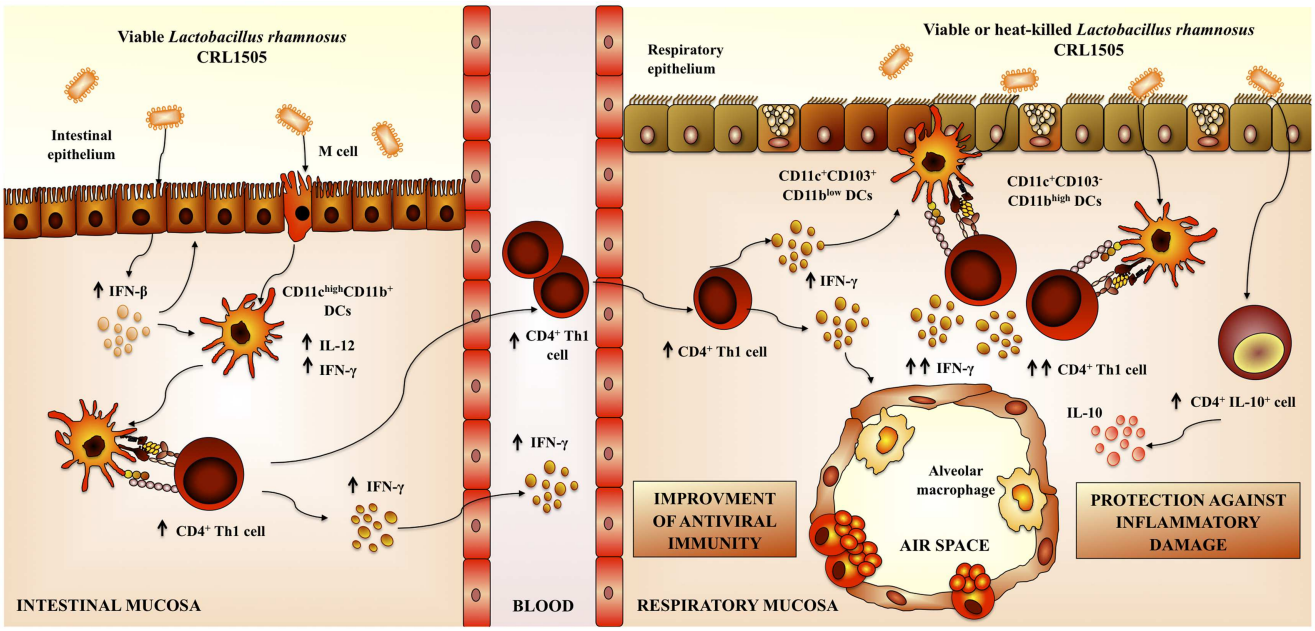

FIGURE 4 | Proposed mechanism for the immunoregulatory effect of *Lactobacillus rhamnosus* CRL1505 on respiratory anti-viral immune responses and resistance against respiratory syncytial virus.

were capable of modulating TLR3-triggered anti-viral respiratory immune response, demonstrating in addition a different immunoregulatory effect for each immunobiotic strain. *L. rhamnosus* CRL1506 significantly modulated the production of type I IFN and IL-6 in the response to poly(I:C) or RSV challenges. On the other hand, priming with *L. rhamnosus* CRL1505 effectively improved levels of IFN-γ and IL-10 in the respiratory mucosa (83).

L. rhamnosus CRL1506 had a significant effect on epithelial cells from the respiratory tract. It is known that type I IFNs increase the expression of genes that are involved in innate anti-viral defenses and the development of a strong Th1 response. Therefore, *L. rhamnosus* CRL1506, through the stimulation of anti-viral defenses in epithelial cells, could play a significant role in the improvement of innate and specific immune responses against respiratory viral infections (83). On the other hand, nasal administration of *L. rhamnosus* CRL1505 augmented levels of BAL IFN-γ and lung $CD3^+CD4^+IFN\text{-}\gamma^+$ T indicating an improvement of the respiratory Th1 response. Moreover, CRL1505 administration significantly activated $CD103^+$ DCs. Those effects were not observed in mice orally treated with the CRL1506 strain. Then, *L. rhamnosus* CRL1505 would be more efficient than *L. rhamnosus* CRL1506 to stimulate $CD103^+$ DCs and improve Th1 response in the lung (83). In line with this notion, recent studies suggested that respiratory $CD103^+$ DCs are more potent at eliciting Th1 responses than $CD11b^{high}$ DCs (78).

Nasal treatment with *L. rhamnosus* CRL1505 and CRL1506 significantly reduced lung injuries caused by poly(I:C). Both lactobacilli augmented IL-10 production in response to TLR3 activation, however, *L. rhamnosus* CRL1505 was more efficient than CRL1506 to increase the levels of this cytokine in the lung. Additionally, the markers of lung damage were lower in CRL1505-treated mice than in those receiving *L. rhamnosus* CRL1506 (83). Therefore, there is a direct connection between the improvement of respiratory IL-10 and the protection against poly(I:C)-induced lung damage after immunobiotic treatment. Moreover, our results indicate that $CD3^+CD4^+IL\text{-}10^+$ T cells would be functionally and quantitatively modulated by *L. rhamnosus* CRL1505 and that these cells would be the source of the IL-10 produced after poly(I:C) challenge (83). Recently, it was reported that the majority of IL-10 produced during acute RSV infections comes from $CD4^+$ T (76). Moreover, it was suggested that this cell population is involved in the protection against lung tissue alterations. Therefore, the improved numbers of lung $CD3^+CD4^+IL\text{-}10^+$ T cells induced by nasally administered immunobiotics could have an important role in the protection against RSV challenge. It should be considered in addition that during respiratory infections, other cell populations are able to produce IL-10 (75, 76). It was described that IL-10 is produced by different $CD4^+$ T cells during RSV infection. These cell populations include $Foxp3^+$ regulatory T cells, IFN-γ producing $Foxp3^-CD4^+$ T cells that coproduce IL-10, and $Foxp3^-CD4^+$ T cells that do not coproduce IFN-γ (76). Moreover, it was described that a small number $CD8^+$ T cells also produce IL-10 after RSV challenge (76). It would be of value to investigate whether immunobiotic treatments influence the production of IL-10 in respiratory $CD4^+Foxp3^+$, $CD4^+Foxp3^-$ $IFN\text{-}\gamma^+$, $CD4^+Foxp3^-IFN\text{-}\gamma^-$, and $CD8^+$ T cells.

Nasal administration of *L. rhamnosus* CRL1505 or *L. rhamnosus* CRL1506 augmented the production of pro-inflammatory mediators and IL-10 in response to RSV infection (83). *L. rhamnosus* CRL1505 was more effective than *L. rhamnosus* CRL1506 to improve the levels of respiratory IL-10, to protect against the inflammatory damage, and to enhance virus clearance, similarly

to our results using poly(I:C). This finding also supports the idea that modulation of IL-10 is an effective way to improve the outcome of RSV disease. In addition, our results demonstrated that the nasal priming with immunobiotics is an interesting alternative to achieve the immunoprotective effect in the respiratory tract; since virus titers and lung alterations were significantly lower in mice nasally treated with *L. rhamnosus* CRL1505 than in those fed with the bacteria (62, 83) (**Figure 4**).

Our results also demonstrated that nasally administered immunobiotics are more effective than orally delivered probiotics to improve anti-viral respiratory defenses and protect against viral infections such as RSV (62, 83).

Finally, we evaluated whether viability of the immunomodulatory lactobacilli was a necessary condition to achieve the protective effect against respiratory viral infection. Some few studies reported that nasally administered heat-killed immunobiotics are capable of improving resistance against respiratory pathogens (50, 61, 84, 85) (**Table 1**). In this regard, studies by Hori et al. (50) showed that the nasal priming with heat-killed *L. casei* Shirota significantly augmented the resistance of adult BALB/c mice to influenza virus by stimulating respiratory tract cellular immunity. *L. casei* Shirota strongly induced production of IL-12 in MLN cells, which stimulates cytotoxic T cells and NK cells, and enhances the Th1 response. Moreover, after influenza virus challenge, both TNF-α and IFN-γ were increased in MLN cell cultures from mice nasally treated with *L. casei* Shirota (50). In addition, it was reported an improved IFN-α production and NK activity as well as a strongly enhanced Th1 immunity in the respiratory tract of mice treated nasally with heat-killed *L. pentosus* S-PT84, which were protected against influenza virus infection (55). More recently, it was shown that nasal priming with both live and heat-killed *L. plantarum* and *L. reuteri* induces a full protection against the lethal pneumovirus infection (61). That work demonstrated that nasally administered heat-killed lactobacilli resulted in a strong regulation of virus-induced pro-inflammatory mediators and diminished virus recovery. The results of our recent experiments are in line with these previous works since administration of both heat-killed *L. rhamnosus* CRL1505 was effective to improve resistance of infant mice to RSV infection and reduce lung injuries, inducing a protective effect that was similar to the observed for the viable strain (83) (**Figure 4**). Interestingly, although both viable and heat-killed *L. rhamnosus* CRL1506 showed a similar capacity to reduce lung RSV titers, the viable bacteria was more effective than the heat-killed ones to reduce lung damage after RSV challenge. These differential effects achieved by viable and heat-killed lactobacilli could be explained by their specific capacities to modulate the production of IL-10 and IFN-γ during RSV infection (83). The four treatments evaluated were capable of increasing the levels of IFN-γ in the respiratory tract and decreasing viral loads. On the other hand, *L. rhamnosus* CRL1505 (viable and heat-killed) and viable *L. rhamnosus* CRL1506 but not the heat-killed CRL1506 strain reduced lung damage by increasing IL-10 concentrations. These results suggest that the immunoregulatory effect of some probiotic bacteria can be changed after heat treatment. Therefore, not all heat-killed bacteria derived from immunobiotic will maintain their immunoregulatory capacities. This fact should be considered when selecting non-viable immunobiotic strains (83).

CONCLUSION

No effective therapy strategies are available at the moment for the prevention and treatment of RSV infections. Findings in RSV biology and immunopathology suggest that only the inhibition replication may not be effective for reducing lung damage during severe infection. It should be considered that once individual experiences the symptoms of RSV infection, the inflammatory response has become uncontrolled and it is not longer linked to the replication of virus directly. Then, the use of replication inhibitors to control lung damage is not useful. Immunoregulatory therapies could be more effective to control the negative sequelae of severe RSV disease.

We have demonstrated that the respiratory immune response triggered by TLR3 activation could be beneficially modulated by mucosal (oral and nasal) administration of immunobiotic lactobacilli. Moreover, those treatments are able to increase the resistance to RSV challenge in both infant and adult hosts. We also showed that the anti-viral capacities of immunobiotic lactobacilli are strain dependent, as it has been reported for other probiotic effects. Comparative studies using two *L. rhamnosus* strains of the same origin (32, 80) allow us to demonstrate that each lactobacilli strain has specific immunoregulatory effects. Each strain differentially modulates the immune response in the respiratory tract after poly(I:C) stimulation. In addition, each lactobacilli confer different degree of protection against RSV challenge and use distinct immune mechanisms (**Figure 4**).

Our research also demonstrated that anti-viral respiratory defenses are beneficially modulated by heat-killed immunobiotics. This implies that non-viable immunobiotics could be an interesting alternative as mucosal adjuvants to improve respiratory defenses and protect against viral infections. The use of non-viable immunobiotics or their cellular fractions could have an important impact in the prevention of viral respiratory infections in immunocompromised hosts in which the use of live bacteria might be dangerous. In addition, heat-killed immunobiotic could have several technological advantages such as easier storage, and transportation and a longer product shelf-life. Therefore, an interesting topic for future research would be the evaluation of non-viable *L. rhamnosus* CRL1505 or its cellular fractions as immunomodulators and anti-viral adjuvants in immunocompromised hosts.

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TLR agonists for cancer immunotherapy: tipping the balance between the immune stimulatory and inhibitory effects

Hailing Lu*

Tumor Vaccine Group, University of Washington, Seattle, WA, USA
**Correspondence: hlu@uw.edu*

Edited and reviewed by:
Christophe M. Filippi, Genomics Institute of the Novartis Research Foundation, USA

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The agonists of toll-like receptors (TLRs) have been actively pursued for their anti-tumor potentials, either as monotherapy or as adjuvants to vaccination or other therapeutic modalities (1). A search on ClinicalTrials.gov using the key words "TLR" and "cancer" returned 34 listings. The idea of using TLR agonist to provide a "danger signal" and break tolerance to tumor antigens has been well embraced by tumor immunologists. However, the promise of TLR agonists-based immunotherapy remains to be realized in the clinic, and only very few TLR agonists have been approved by the FDA. For example, bacillus Calmette–Guerin (BCG) and imiquimod have been approved as standalone therapies, whereas monophosphoryl lipid A (MPL) was approved as a vaccine component. A review of recently published literature on the use of TLR agonists in cancer setting revealed a common mechanism that might have explained the underperformance of TLR agonists as cancer therapeutics: induction of immune suppressive factors that put a break on the TLR agonists-induced inflammation. As shown in **Figure 1**, TLR agonists have immune stimulatory effects through the induction of costimulatory molecules (CD80, CD86, and CD40) on dendritic cells (DCs) and inflammatory cytokines (TNF-α and IL-12) that polarize Th1 immune response. On the other hand, TLR agonists have immune inhibitory effects as evidenced by the induction of several immune suppressive factors, including IL-10, T regulatory cells (Treg), and PD-L1, all of which could dampen anti-tumor immunity. The following is a brief summary on TLR agonists-induced self-regulatory feedback and the indication for cancer immunotherapy.

INDUCTION OF IL-10

IL-10 is an immune suppressive cytokine that inhibits the activity of Th1 cells, thus impeding viral clearance and anti-tumor Th1 immunity. IL-10 could be secreted by different immune cells, including Treg (2), CD4 T cells (3, 4), monocytes, and macrophages (5). The induction of IL-10 by TLR agonists has been demonstrated in infectious disease setting as well as tumor setting. For example, TLR4 signaling with LPS was shown to activate innate IL-10 production in response to *Bordetella pertussis*, which both directly, and by promoting the induction of IL-10-secreting type 1 regulatory T cells (Tr1), inhibit Th1 responses and limit inflammatory pathology in the lungs during infection with *B. pertussis* (6). TLR2 ligation and induction of IL-10 were also shown to suppress immunity against *Candida albicans* (7). Induction of IL-10 also caused the persistence of lymphocytic choriomeningitis virus (LCMV), and IL-10 blockade using a neutralizing antibody can restore T cell immunity and lead to viral clearance (3). IL-10 induction by TLR agonists has been observed in mouse models of breast cancer and melanoma. In MMTV/neu-transgenic mice, a model of human $HER2^+$ breast cancer, topical treatment with TLR7 agonist imiquimod induced IL-10, and the major source of IL-10 was Tr1 cells (4). IL-10 induction was also observed in a mouse model of implanted TSA breast cancer, where topical imiquimod was shown to synergize with radiation and low-dose cyclophosphamide in inhibiting tumor growth (8). In a mouse model of B16 melanoma, the induction of IL-10 has also been shown to limit the anti-tumor effects of TLR2 agonist Pam2 lipopeptide (9). Altogether these publications suggest IL-10 induction is probably a common regulatory mechanism that dampens TLR agonists-induced anti-tumor immunity. IL-10 blockade using anti-IL-10 neutralizing mAb significantly enhanced the anti-tumor effects of topical imiquimod (4). The addition of anti-IL-10R to TLR9 agonist CpG also exhibited robust anti-tumor activity exceeding by far that of CpG alone, and elicited anti-tumor immune memory (10). Thus IL-10 blockade holds promise to augment the anti-tumor effects of TLR agonists.

INDUCTION OF Treg

Although some early studies have reported that TLR agonists could inhibit the function or number of Treg (11), more recent studies have demonstrated that TLR agonists can increase Treg number and function. For example, TLR2 ligation has been shown to promote the survival of Treg (12). Treatment of prediabetic mice with a synthetic TLR2 agonist diminished type 1 diabetes and increased the number and function of Treg, also conferring DCs with tolerogenic properties (13). TLR2 agonist Pam3Cys was also shown to induce Treg expansion in the lungs and result in long-term protection against manifestation of allergic asthma in mice (14). Human plasmacytoid DC activated by TLR9 agonist CpG has been shown to induce the generation of Treg (15). Another study reported that TLR agonists-stimulated allogeneic pDCs induces $CD8^+$ Tregs that inhibit allogeneic T cell responses, including memory T cells (16). Studies from our group demonstrated that TLR7 agonist imiquimod induces Tregs, either as monotherapy or as an adjuvant to vaccination (4, 17). Treg was induced in both

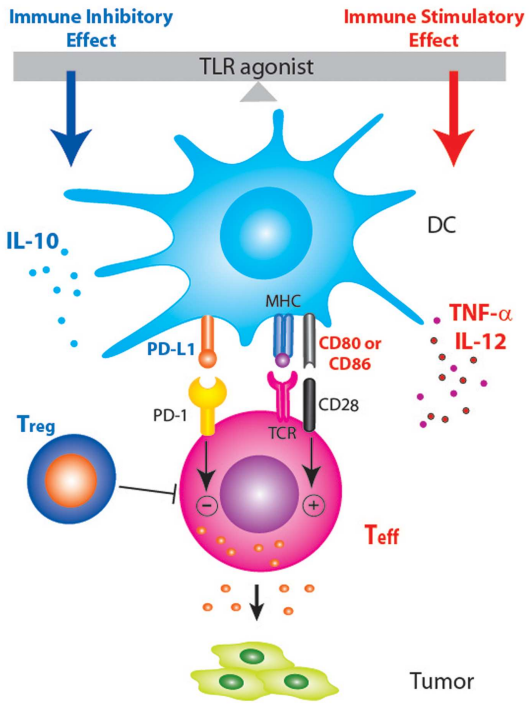

FIGURE 1 | Schematic diagram showing that PD-1/PD-L1 blockade may enhance TLR-based immunotherapy by tipping the balance between the immune stimulatory and inhibitory effects of TLR agonists. Treatment with TLR agonists in tumor-bearing host not only induces pro-inflammatory anti-tumor responses but also induce anti-inflammatory factors (IL-10, Treg, and PD-L1) that dampen anti-tumor immune responses.

the periphery and the tumor microenvironment (4). Another study showed that imiquimod enhanced the suppressor function of Treg cells by sensitizing Treg cells to IL-2-induced activation (18). A study on transcutaneous vaccination using imiquimod as adjuvant showed that Treg and IL-10 act independently to counter-regulate the cytotoxic T lymphocytes (CTL) response induced by vaccination (19). When TLR9 agonist CpG was used as adjuvant to protein vaccination, antigen-specific Treg was induced (20). The ability of CpG to induce Treg has been shown to be mediated by p38 MAPK and inhibition of p38 in DC was shown to attenuate Treg induction by TLR agonists and enhance their efficacy as vaccine adjuvants and cancer immunotherapeutics (21). Thus inhibiting Treg induction represents another opportunity to augment the anti-tumor effects of TLR agonists.

INDUCTION OF PD-L1

PD-L1 (also known as B7-H1 or CD274) is a B7-related protein that inhibits T cell activation via engaging the programed death-1 (PD-1) receptor that is expressed on activated T cells. PD-L1 can be expressed on tumor cells as well as hematopoietic cells. The induction of PD-L1 by TLR ligation has been reported for different TLR agonists, in both *in vitro* and *in vivo* studies. For example, TLR7/8 agonist resiquimod and TLR4 agonist LPS have been shown to induce PD-L1 on DC and contribute to the development of tolerogenic APCs (22). TLR4 ligation by MPL was also shown to enforce the tolerogenic properties of oral mucosal Langerhans cells (23). Topical imiquimod was shown to induce PD-L1 as well as CD86 in different subsets of skin DC (24). Another study showed that TLR4 agonist LPS and TLR7/8 agonist CL097 induced PD-L1 expression on macrophages and the induction appeared to be dependent on IL-10 (25). Induction of PD-L1 has been linked to the lack of protective immunity to bacteria (26). In mouse tumor models, the induction of PD-L1 has also been shown to be an important mechanism that limits the anti-tumor efficacy of TLR agonists (27, 28). The TLR3 agonist poly I:C up-regulated PD-L1 on DC, and depletion or blockade of PD-L1 on activated DCs increased the magnitude of effector CD8 T cell expansion (28). PD-L1 also collaborates with Treg to impair the recall responses of tumor-specific memory T cells (27). Combination of PD-L1 blockade, CD4 T cell depletion, and tumor cell vaccination resulted in complete regression of large established RENCA tumors and established long-lasting protective immunity (27). Thus, blocking PD-1/PD-L1 signaling represents another opportunity to augment the anti-tumor effects of TLR agonists.

It should be noted that the list above is not exhaustive. Other immune suppressive factors such as TGF-beta (TGF-β), mostly notably secreted from Treg, could also be induced after TLR agonist treatment, especially with agonists of TLR2 and TLR4 (13, 29). It should also be noted that the various immune suppressive factors listed above don't function separately but in an interactive manner. For example, induction of PD-L1 is crucial to the induction of Treg (30), and the induction of PD-L1 has been shown to be dependent on IL-10 (25). TLR ligation could induce these different suppressive factors simultaneously, and blocking one factor may decrease the other factors as well.

In summary, the immune responses are highly controlled. Once a T cell response is initiated, it needs to be dampened to prevent collateral damage. TLR ligation not only initiates immune response, but also triggers negative regulatory pathways. The above discussion about TLRs is equally true for the normal existent immune response to cancer as well as cancer vaccine-induced response. The induction of these regulatory pathways represents a major obstacle in developing TLR agonists as cancer immunotherapeutics, and the promise of using TLR agonists to eradicate tumor cells probably won't be realized unless we block the negative regulators (IL-10, Treg, and PD-L1, etc) and tip the balance toward an overwhelming pro-inflammatory response. Cancer immunologists are now exploring novel combinational therapies that combine TLR ligation with blockade of the negative regulators. For example, the cancer immunotherapy trial network (CITN) has considered a pilot clinical trial testing the combination of topical imiquimod and IL-10 blockade

in breast cancer patients with chest wall metastasis (per communication with Dr. Martin Cheever, CITN director). Unfortunately this idea did not move forward due to the unavailability of anti-human IL-10 mAb. Currently a clinical trial combining topical imiquimod and PD-1/PD-L1 blockade for treating breast cancer cutaneous metastasis is also being planned in the University of Washington.

The early data from clinical trials of anti-PD-1 and anti-PD-L1 are harbingers of a radical change in immunotherapy as well as cancer therapy in general. Based in large part on data from the check-point inhibitor trials, it's been predicted that cancer immunotherapy will provide the backbone of up to 60% of cancer therapy within the next decade. There are at least seven companies competing in the anti-PD-1/PD-L1 space. The agents will be tested with every conceivable combination. Combinations with immunotherapies that augment existent immune responses or vaccine-induced immune responses, such as TLR therapies will be tested soon with anti-PD-1/PD-L1 and are highly likely to be more effective in that context. Not only will the blockade of PD-L1 signaling likely enhance the immune stimulatory effects of TLR agonists, but we also expect TLR ligation to enhance the effect of PD-1/PD-L1 blockade by increasing tumor infiltrating immune cells (TIL). The presence of TIL has been associated with good clinical response in the check-point blockade therapy. In addition to anti-PD-1/PD-L1, there will be other check-point inhibitors that will also become available for testing with TLR agonists. The world of cancer immunotherapy is on a predictable course to become a major component of cancer therapy and TLR agonists will likely play an important role.

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Toll-like receptors and skin cancer

Erin M. Burns and Nabiha Yusuf*

Department of Dermatology, University of Alabama at Birmingham, Birmingham, AL, USA
**Correspondence: nabiha@uab.edu*

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Christophe M. Filippi, Genomics Institute of the Novartis Research Foundation, USA

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The skin, the largest organ in the body, provides the first line of defense against the environment both as a physical barrier and as a key immunological component. Toll-like receptors (TLRs) serve as signaling molecules that recognize pathogen-associated molecular patterns (PAMPs) as well as damage-associated molecular patterns (DAMPs), and are expressed by various skin cells including keratinocytes and melanocytes, which are the main cell types involved in both non-melanoma and melanoma skin cancers. TLRs induce inflammatory responses meant for clearing pathogens, but can ultimately contribute to skin carcinogenesis. In contrast, TLR agonists, specifically targeting TLR7, 8, and 9, have been successfully used as therapeutics for melanoma and basal cell carcinoma (BCC), functioning by recruiting dendritic cells and inducing T-cell responses. Here, we discuss the role TLRs play in skin carcinogenesis as well as the use of TLRs as targets for skin cancer treatment options.

SKIN AND TLRs

Non-melanoma skin cancer (NMSC) includes BCC and squamous cell carcinoma (SCC). With over 3.5 million new diagnoses annually, NMSC is the most common cancer in the United States (1). Risk factors for developing NMSC include ultraviolet (UV) light exposure, skin color, sunburns, age, and immunosuppressive status (2). NMSCs account for over 3,000 deaths each year (3) and also contribute to over $1.4 billion annually for the treatment and management of these skin tumors (4). Melanoma contributes to approximately 5% of all skin cancer diagnoses, with 76,000 new cases diagnosed in 2012 (5). Importantly, melanoma leads to over 9,000 deaths annually, which accounts for the majority of skin cancer deaths. Risk factors for melanoma include UV exposure, sunburn, nevi, immunosuppressive status, and family history.

The most common treatments for SCC include excision, Mohs micrographic surgery, and cryosurgery, which, when the lesion is detected early and promptly removed, are effective and cause minimal damage (2). If left untreated, the tumors are able to grow exponentially or metastasize, leading to more invasive procedures. For melanoma, surgical excision is the most common treatment, with recent preferences for Mohs surgery (5). However, in the case of recurring lesions or lesion patches, surgery may not be an option due to extensively damaged skin or lack of tissue for removing clear margins, resulting in the need for alternative treatment options.

The skin is the largest organ in the body and contains three major cell types, which include melanocytes, Langerhans cells, and keratinocytes. Keratinocytes are the major cell type of the epidermis and provide defense against the environment both as a physical barrier and a key component of the innate immune response (6, 7). Epidermal keratinocytes, as the outmost environmental barrier, are responsible for the production of antimicrobial peptides (8), which are up-regulated by various stimuli through both the mitogen-activated protein (MAP) kinase and nuclear factor (NF) kappaB pathways (9). TLRs are expressed by various skin cells including keratinocytes and melanocytes (10), which are the main cell types involved in both non-melanoma and melanoma skin cancers. Human keratinocytes have been shown to express TLRs 1–6 and 9 (10–14). Recently, it has been reported that TLR2–5, 7, 9, and 10 are constitutively expressed in human melanocytes (15).

Toll-like receptors serve as signaling molecules that recognize PAMPs, or pathogen-associated molecular patterns, as well as DAMPs and thus, activate the innate immune response through the transcription factor NF-kB (16). The 10 human TLR family members are characterized by the leucine-rich repeat domain content in both their extracellular region and the intracellular Toll-IL-1 receptor (TIR) domain (17), which can therefore interact with adaptor molecules that contain appropriate adaptor molecules (18).

Toll-like receptors have been demonstrated to be important for both innate immune response specificity (19, 20) as well as for adaptive immune responses such as dendritic cell maturation and costimulatory molecule expression and the promotion of Th-1 cell-mediated responses through increased production of IL-12 by activated TLRs on dendritic cells (21, 22). It also has been reported that innate inflammatory responses localized to the epidermis may be affected by TLR expression in human melanocytes (23). TLRs are activated in melanocytes, as a consequence of the inflammatory response to tissue injury, sunburn or skin infection, and constitute a natural defense to recruit innate immune cells.

TLR STIMULATION AND SKIN CARCINOGENESIS

Besides their function of recognizing exogenous PAMPs, TLRs also recognize endogenous ligands, which are often referred to as alarmins and function to recognize cell or tissue damage and alert the innate and adaptive immune systems (24, 25). Expression association studies have revealed potential functions of TLR endogenous ligands in tumorigenesis. For example, high-mobility group box-1 protein (HMGB1) can function as a DAMP and is released in response to tissue or cellular damage. It is over-expressed in several human neoplasms including lung, pancreatic, breast, liver, and colorectal cancers,

and, importantly, melanoma (26). HMGB1 is either passively released by injured or necrotic cells (27) or actively secreted by monocyte/macrophages, neutrophils, and dendritic cells [reviewed in Ref. (28)].

With the exception of TLR3 that signals through Toll/IL-1R domain containing adaptor inducing IFN (TRIF), TLRs signal through myeloid differentiation factor 88 (MyD88). TLR signaling has been reviewed extensively elsewhere (29). MyD88 is an adaptor protein that is ultimately responsible for initiating NF-kB activation (30), and therefore the amplification of inflammation and the promotion of tumor development (31). Importantly, chronic inflammation has been linked to tumor development in animal models of both spontaneous and chemically induced carcinogenesis (32, 33).

Tumor cells expressing TLRs may be able to evade immune surveillance processes, thus promoting tumor development. The activation of TLR4 and subsequent signaling molecules have been shown to upregulate immunosuppressive cytokines such as IL-10 as well as pro-inflammatory cytokines and chemokines including IL-6, IL-18, and TNF-α, which have been shown to contribute to tumor development, growth, and even metastasis (34). In human melanoma A375 cells, the inhibition of TLR4/MyD88 signaling effectively decreased both VEGF and IL-8 levels with paclitaxel and icariside II combination treatment (35). TLR2-4 are expressed and up-regulated in several human metastatic melanoma cell lines (36), with recent data indicating that melanoma cells also express TLR7, 8, and 9 (37), which are abnormally up-regulated in cells from melanoma biopsies (38). The over-expression of TLR4 within melanoma tumors triggers an inflammatory response leading to tumor development (39). TLR9 activation has also been shown to enhance invasion as well as promote proliferation in several cancer cell lines via NF-kB and Cox-2 activation (40), as well as the secretion of IL-8 and IL-1α (41), and TGF-β (42). Recent studies in head and neck cancer have revealed that TLR3 expression and signaling affects the migration and metastatic potential of tumors as evidenced in oral SCC by inducing CCL5 and IL-6 secretion (43).

Importantly, TLR inhibition can exert anti-cancer effects. TLR4 pathway inhibition reversed tumor-mediated suppression of both natural killer cell activity as well as T-cell proliferation *in vitro* and *in vivo*, resulting in increased tumor latency and survival of tumor-bearing mice (44). TLR2 plays an important role in the induction of tumor regression, which has been demonstrated in a mouse model of glioblastoma multiforme where blocking HMGB1-mediated TLR2 signaling via tumor-infiltrating myeloid DCs resulted in a loss of therapeutic efficacy (45).

TLR3 activation on immune cells results in anti-cancer activities, where T cell-mediated responses are promoted (46). Specifically, upon stimulation with TLR3 agonist poly(I:C), CD8 T cell responses are enhanced, leading to the production of IFNγ and TNF-α and ultimately, the generation of memory CD8 T cells.

TLR-TARGETED THERAPY

Although TLR expression on tumor cells may allow tumors to evade surveillance, TLRs are also considered to be targets for anti-cancer interventions that result in the recognition and ultimate destruction of tumor cells using a tolerant immune system. This idea is further illustrated by the fact that recent studies have demonstrated a dual nature of immune responses in the context of cancer therapies, highlighting the importance of considering conditions, TLR targets, and combinations of immune interventions and TLR ligands (47).

There are studies and case reports that show that 5% imiquimod cream treatment is an effective therapeutic option for actinic keratosis (AK), BCC, Bowen's disease, and lentigo maligna (48–53). The mechanism of action of imiquimod is through the activation of TLR7 (54), and imiquimod has been approved to treat both premalignant actinic keratoses, and malignant superficial BCC (55). The mechanism may also involve Th1-response promotion, the recruitment of macrophages, anti-tumor cytotoxic CD8 T cells, and NK cells to the lesion, as well as induce apoptosis of tumor cells (55, 56). Imiquimod has also been shown to induce IFN-α and IL-12 production, resulting in a heightened immune response (49, 57, 58). The suggested mechanism for exertion of anti-tumor effects on UVB-induced SCC by imiquimod is through the activation of Th17/Th1 cells as well as cytotoxic T lymphocytes (59). Five percent topical imiquimod has been effective in several clinical trials (49, 53, 57, 60). The related drug, resiquimod, has been demonstrated as a safe and effective topical intervention for AK and is a potential treatment option for patients who have large patches of AK (61).

Several cancer types including melanoma have been successfully treated with Taxol, CpG, or otherTLR ligands (62, 63). PF3512676, a synthetic CpG ODN, uses a TLR9-targeted approach to effectively treat BCC (64). TLR 7 and 8 agonists activate a pro-inflammatory response for SCC treatment (65). Additionally, IL-1, 6, 8, and 12 modulation along with a promotion of a Th1-response have been shown to exert anti-tumor and antiviral behavior (65).

Previous studies have demonstrated TLR3 agonists to be promising adjuvants for cancer vaccines, especially in regards to their immunostimulatory properties (46). A recent study has demonstrated that human melanoma cells express TLR3, which in combination with TLR3 agonists, results in tumor cell death via caspase activation when cells are pretreated with cycloheximide or IFN-α (38), suggesting that TLR3 agonists may be multifunctional adjuvants offering more clinical treatment options. Therefore, TLRs and their signaling pathways may be potential therapeutic targets to control tumor progression, especially in diseases such as cutaneous malignant melanoma, which is an aggressive tumor that is not effectively managed with current treatments (66).

It is important to note that, especially in the case of TLR7 agonists such as imiquimod and resiquimod, though quite effective when applied topically to AKs and BCCs, systemic therapeutic interventions have not been as successful. This TLR tolerance has previously been demonstrated with TLR4 agonists, which resulted in decreased NF-kB activation (67). The suggested mechanism for TLR7 tolerance is the diminished capacity for IL-12 secretion as well as IFN-α secretion by plasmacytoid DCs (68). Recent studies have found that local and systemic TLR-targeted therapies have different modes of action and require further investigation, especially into the timing and dosage of treatments to reach

maximum efficacy without inducing TLR tolerance (69).

CONCLUSION

In summary, TLRs are an important immunological component expressed by keratinocytes and melanocytes, which are the main cell types involved in both non-melanoma and melanoma skin cancers. TLRs induce inflammatory responses meant for clearing pathogens, but their activation can also potentiate chronic inflammation, which can ultimately contribute to skin carcinogenesis. In contrast, TLR agonists, specifically targeting TLR7, 8, and 9, have been successfully used as therapeutics for melanoma and BCC, functioning by recruiting dendritic cells and inducing T-cell responses. It is important to consider local versus systemic applications of TLR therapies and the balance between efficacy and inducing TLR tolerance. TLR3 agonists have been shown to be well-tolerated and effective in both directly killing cancer cells and directing immune responses in melanoma. TLR-targeted therapies may be potential treatment options for large or reoccurring skin tumors that may be difficult to treat with surgery or for other skin tumors that are not responsive to current therapies.

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Toll-like receptor mediated regulation of breast cancer: a case of mixed blessings

Nabiha Yusuf*

Department of Dermatology, University of Alabama at Birmingham, Birmingham, AL, USA
**Correspondence: nabiha@uab.edu*

Edited by:
Christophe M. Filippi, Genomics Institute of the Novartis Research Foundation, USA

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INTRODUCTION

Breast cancer is the most common type of cancer in American women after skin cancer. It is also the second deadliest cause of fatalities in women, exceeded only by lung cancer (1). There is a lot of variation within the tumors of an individual and also between the tumors of different individuals. These differences account for assessing therapeutic resistance and progression of disease. It is a challenge to characterize these tumors and design effective therapies to control their progression (2). There is a dynamic interaction between the tumors and the immune system, which determines the fate of their existence (3). Most tumors arise as a result of genetic and epigenetic changes that occur within an individual. These changes are often followed by inflammation that helps in the recruitment of inflammatory cells, fibroblasts, and endothelial cells in the microenvironment of host tumor cells. The interaction of tumor cells and the cells of the tumor microenvironment determines the invasive potential of the tumors (4).

INNATE IMMUNE RESPONSES AND CANCER

The cells of the innate immune system, namely macrophages, dendritic cells (DCs), natural killer (NK), NKT, and γδT-cells, play a critical role in hot immune responses against host tumors by various mechanisms (5). Adaptive immune responses play a critical role in elimination of tumor cells by generating more specific tumor immunity and immunological memory (5). Thus, there is a constant interaction between the innate and adaptive arms of the host immune system to generate a strong immune response to detect and eliminate the pathogens and mutated cells before they become tumors (6). Pathogen-associated molecular patterns (PAMPs) are recognition molecules that are associated with groups of pathogens. Damage-associated molecular patterns (DAMPs) are endogenous molecules created upon tissue injury. Both these patterns signal the threat of either infection or injury to the organism and are recognized by a family of innate immune system called the Pattern recognition receptors (PRRs) (7–10). Toll-like receptors (TLRs) are the most well studied among the members of the cellular receptors and are known to play an important role in bridging innate and adaptive immune responses in cancer (6). The innate immune responses generated by TLRs are known to suppress the function of regulatory T-cells (T-regs) by breaking tolerance and enhancing immune responses against cancers (5, 11–13). Signaling through TLR activates DCs and macrophages to secrete IL-12, a cytokine that directs the adaptive immune response toward a Th1 phenotype (14). TLRs are not only expressed on immune cells but they are also expressed on cancer cells. The expression of TLRs on the cell type can have different consequences (15). Studies suggest that TLR signaling in tumor cells promote tumor angiogenesis and metastasis. Activation or TLRs by DAMPs released by tissue damage can attract more inflammatory cells to cause chronic inflammation leading to tumor progression (16–18).

TOLL-LIKE RECEPTORS AND IMMUNE SURVEILLANCE IN BREAST CANCER

Several TLR agonists have been demonstrated to produce anti-tumor effects in breast cancer (11). Some analogs of nucleic acids that activate TLR7 and TLR9 have been used in clinical trials to improve anti-tumor immune response against solid tumors. Additionally, TLR signaling has been shown to decrease or de-repress the effects of regulatory T-cells on DCs or CD8+ T-cells (19, 20). Among the tumor-infiltrating lymphocytes, a major population (70–90%) of gammadelta (γδ) cells called γδ1 T-cells was shown to inhibit naive and effector T-cell responses and block DC maturation and function (21). γδ1 regulatory T-cells reside naturally in the epithelial tissues and can easily migrate to normal or malignant epithelium. These cells can possibly expand by direct presentation of antigens by tumor cells (21). The immunosuppressive activity of these cells could be reversed by human TLR8 ligands both *in vitro* and *in vivo*. These cells required MyD88, TRAF6, IKKalpha IKKbeta, and p38alpha molecules in gammadelta1 cells to respond to TLR8 ligands (21). In a model of human HER-2/neu(+) breast cancer (neu-transgenic mice), topical treatment with a TLR7 agonist, imiquimod, showed significant regression of spontaneous breast cancers. Analysis of gene expression data from the tumors of these mice revealed that treatment with imiquimod resulted in high levels in addition to TNF-alpha and IFN-gamma. The anti-tumor effect of imiquimod was significantly enhanced by blocking IL-10, thereby increasing survival in these mice. Thus, IL-10 induction maybe a self-regulatory mechanism used by the TLR agonists to control excessive inflammation (22).

Other TLRs expressed on the immune cells have also been reported to improve the efficacy of tumor vaccines or enhancement

of chemotherapy of breast tumors by enhancing anti-tumor immune responses. When polysaccharide krestin (PSK), a TLR2 agonist was orally consumed in neutransgenic mice, it significantly inhibited breast cancer growth by its action on the CD8 (+) T-cell and NK cells but not CD4 (+) T-cells (23). In another study, another TLR2 agonist polysaccharide A (PSA) was shown to cause inhibition of immune responses by production of IL-10 and regulatory T-cells (24). Thus, TLR2 stimulation on immune cells may also have opposing immune effects as in the case of PSA and PSK. Nitrogen bisphosphonates (NBPs) have been shown to cause a rapid influx of neutrophils and monocytes that was dependent via myeloid differentiation primary response gene 88 (MyD88), a downstream adaptor molecule involved in TLR and IL-1 signaling. Using bone marrow chimeras, it was demonstrated that this acute inflammatory response was partially dependent on TLR4 expressed by hematopoietic cells and the IL-1 receptor on radioresistant cells (25). Studies from our laboratory on carcinogenic polyaromatic hydrocarbon 7,12-dimethylbenz(a)anthracene (DMBA) have demonstrated that cell mediated immunity to DMBA was dependent on TLR4 and had a protective effect against mammary tumor development. This effect was primarily mediated by IL-12 secreted by CD11c+ cells in TLR4 proficient mice, which lead to an IFN-γ mediated response resulting in fewer tumors (26).

TOLL-LIKE RECEPTORS AND IMMUNOTHERAPY OF BREAST CANCER

Vaccination strategies using DC/breast carcinoma fusions were effective in generating anti-tumor immune responses patients with metastatic breast cancer, but tumor regression was observed in a minor group of these patients. This was due to the expansion of both activated and regulatory T-cell populations by DC/breast carcinoma fusions, primarily leading to suppression of T-cell responses. TLR9 agonist CpG oligodeoxynucleotides along with IL-12 and IL-18 was able to reduce the level of fusion-mediated regulatory T-cell expansion. The regulatory T-cell response was inhibited by using TLR agonists that enhanced effector T-cell responses, thus increasing the efficacy of vaccine (27). Effective immunotherapy using combination of HER-2/neu genetic vaccine and novel agonist of TLR9 has been reported for breast cancer. This therapy has been reported to be associated with antibody isotype switch and antibody-dependent cellular cytotoxicity activity of the of DNA-EP/Ad-based cancer vaccines (28). Vaccination with Ad-BD2-E1A (E1B-deleted oncolytic adenovirus expressing beta-defensin-2) vaccine inhibited primary breast tumor growth and blocked metastasis in a TLR4 dependent manner, thus suggesting the critical role of TLR4 in the induction of anti-tumor immunity by Ad-BD2-E1A (29).

TOLL-LIKE RECEPTORS AND CHEMOTHERAPY OF BREAST CANCER

The efficacy of chemotherapy is defined by their ability to perturb the division of tumor cells. A successful outcome of chemotherapy or radiotherapy also involves inclusion of an adjuvant that would enhance the efficacy of chemo- or radiotherapy. One such adjuvant, high mobility group box 1 protein (HMGB1) has been successfully used in therapy of breast cancer. Dying tumor cells release HMGB1 that has been shown to activate TLR4 on DCs. It was shown that TLR4 expressed on DCs was required for the cross presentation of tumor antigens and the promotion of tumor specific cytotoxic T-cell responses. Breast cancer patients harboring the loss-of-function Asp299Gly polymorphism of TLR4 relapsed earlier after receiving anthracycline-based chemotherapy. These data suggests that HMGB1- and TLR4-dependent immune responses elicited by conventional cancer treatment may increase the probability of achieving a lasting therapeutic success (30). When small nucleotide polymorphisms (SNPs) in TLR2, TLR3, TLR4, and TLR9 were assessed for their association with breast cancer, no association was found. However, population genetics data has revealed that a hypomorphic variant of TLR4 (p.Asp299Gly) allele was found with no specific allelic frequency (8.4%) in the Croatian population compared to other Caucasians (6.5–10%) (31). The development of drugs targeting TLRs is an emerging area, and there are about 20 drugs that are in pre-clinical and clinical trials (32).

TOLL-LIKE RECEPTORS AND BREAST CANCER CELLS

In addition to their expression on immune cells, TLRs are also expressed on tumor cells (15). Activation of TLR expressed on tumor cells may enhance tumor growth by increasing pro-survival signals, anti-apoptotic signals, tumor promoting cytokines, angiogenesis, and invasiveness (33, 34). Among the TLRs (TLR1-10), expressed on human breast cancer cell line MDA-MB-231, expression of TLR4 was found to be the highest, and knock-down of TLR4 gene resulted in significant cell death and inhibition of IL-6 and IL-8 cytokines, compared with vector control (33). In another study, TLR9 was shown to increase invasion of MDA-MB-231 cells, by increasing the activity of matrix metalloproteinase 13 (MMP13) (35). TLR3, TLR4, and TLR9 have been shown to be highly expressed in human breast tumors. There was also an increase in the expression of TLR4 by mononuclear inflammatory cells and TLR9 by fibroblast-like cells in mammary tumors. There was more metastasis in TLR3 expressing tumor cells, TLR4 expressing inflammatory cells but not in TLR9 expressing fibroblasts like cells (36). TLR9 isoforms A and B has been detected in clinical breast cancer, and ERα and sex steroid hormones have been shown to contribute to its invasiveness. TLR9 expression was also found to be affected by the hormonal cancer therapy using bicalutamide (37). In a randomized clinical trial using poly (A:U) dsRNA, TLR3 agonist, chemotherapy was enhanced in patients with TLR3-positive cancers. Chemotherapy using poly A:U was successful only when it was combined with an immunochemotherapeutic regimen of vaccination against tumor antigens (38). In a recent study, it was found that activation of TLR5 on breast cancer cells by its agonist flagellin, led to inhibition of cell proliferation and anchorage dependent cell growth. This was further confirmed in mouse xenograft models using human breast cancer cells. This inhibitory activity was further confirmed *in vivo* using mouse xenografts of human

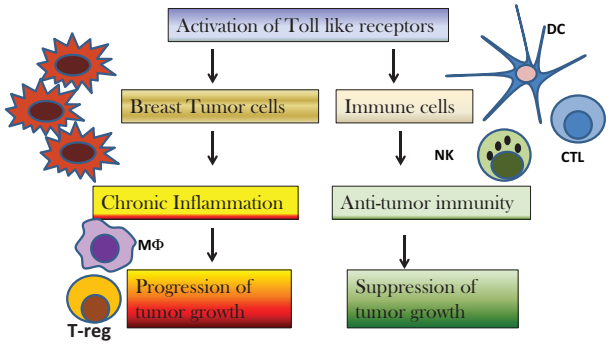

FIGURE 1 | Diagram to depict the effect of activation of toll-like receptors (TLRs) on breast cancer cells versus immune cells. Activation of TLRs on breast cancer cells results in chronic inflammation and recruitment of macrophages (MΦ) and regulatory T-cells (T-reg) in the tumor microenvironment that cause suppression of immune responses and progression of tumor growth. Activation of TLRs by TLR agonists cause infiltration of dendritic cells (DC), natural killer (NK) cells, and cytotoxic T-cells (CTL) that result in suppression of tumor growth.

breast cancer cells (39). Inflammatory signals generated by TLR signaling have also been reported to increase expression of chemokines, thus causing an influx of Th17 cells by tumor cells and tumor derived fibroblasts (40).

CONCLUSIONS AND PERSPECTIVES

Discovery of the role of TLRs in cancer biology have paved the way for development of new therapies targeting TLRs. There is a lot of interest to study the relation between inflammation and cancer as it has been termed as the seventh hallmark of cancer. TLRs play an important role in inflammation mediated cancers as well as cancer related inflammation. Activation of TLRs for therapy may be an exciting proposition, but one has to be careful as over activation of TLRs can also lead to development of tumors (**Figure 1**). Thus, regulatory mechanisms should also be taken into account before using TLRs for cancer therapy. Furthermore, molecular and genetic analysis of breast cancer subtypes should be considered before deciding the course of therapy with TLRs. There are some reports on the role of genetic polymorphisms in TLRs in the outcome of breast cancer therapy. More studies need to be conducted to determine whether the loss or gain of function polymorphisms in TLRs is an indicator of disease outcome or therapy.

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Signaling via TLR2 and TLR4 directly down-regulates T cell effector functions: the regulatory face of danger signals

Alexandra Zanin-Zhorov [1]* ***and Irun R. Cohen*** [2]

[1] *Kadmon Research Institute, New York, NY, USA*
[2] *The Department of Immunology, The Weizmann Institute of Science, Rehovot, Israel*

Edited by:
Christophe M. Filippi, Genomics Institute of the Novartis Research Foundation, USA

Reviewed by:
Sylvie Lesage, University of Montreal and Maisonneuve-Rosemont Hospital, Canada
Lauren A. Zenewicz, The University of Oklahoma Health Sciences Center, USA

***Correspondence:**
Alexandra Zanin-Zhorov, Kadmon Research Institute, 450 East 29th Street, New York, NY 10016, USA
e-mail: alexandra.zanin-zhorov@kadmon.com

Toll-like receptors (TLRs) are widely expressed and play an essential role in the activation of innate immune cells. However, certain TLRs are also expressed on T cells, and TLR ligands can directly modulate T cell functions. Here, we discuss findings indicating that T cells directly respond to Heat Shock Protein (HSP) 60, a self molecule, or to the HSP60-derived peptide, p277, via a TLR2-dependent mechanism. HSP60 has been considered to be a "danger signal" for the immune system because of its ability to induce pro-inflammatory phenotypes in innate immune cells – in this case via TLR4 activation; nevertheless, TLR2 engagement by HSP60 on T cells can lead to resolution of inflammation by up-regulating the suppression function of regulatory T cells and shifting the resulting cytokine secretion balance toward a Th2 phenotype. Moreover, T cell TLR4 engagement by LPS leads to up-regulation of suppressor of cytokine signaling 3 expression and consequently down-regulates T cell chemotaxis. Thus, TLR2 and TLR4 activation can contribute to both induction and termination of effector immune responses by controlling the activities of both innate and adaptive immune cells.

Keywords: HSP60, TLR2, TLR4, direct signaling, T cell inhibition, inflammation, LPS

TOLL-LIKE RECEPTORS FUNCTION IN INNATE AND ADAPTIVE IMMUNE CELLS

A key issue in immunology is to understand how the immune system is able to discriminate between self and non-self, inhibiting autoimmune responses, but allowing effective immune responses against microbial antigens (1, 2). One of the mechanisms evolved by the immune system is expression of pathogen recognition receptors, such as Toll-like receptors (TLRs) on immune cells that encounter pathogen-associated molecular patterns (PAMPs) (3). TLRs are a highly conserved class of receptors that are involved in regulation of both innate and adaptive immunity. All TLR belong to the type 1 trans-membrane glycoprotein receptor family with molecular weights ranging between 90 and 115 kDa and containing 16–28 extracellular leucine-rich repeat domains (4). The intracellular C-terminal domain is known as the Toll/IL-1 receptor domain, which shows homology with that of the IL-1 receptor. This domain is required for the interaction and recruitment of various adaptor molecules to activate the down-stream signaling pathway, including the transcription factors NF-κB, AP-1, and IRF (5).

Both humans and mice express TLR1-9; in addition humans, but not mice, express TLR10 and mice exclusively express TLR11-13 (6). TLR are expressed in two distinct cellular compartments (7). In humans, TLR1, TLR2, TLR4, TLR5, and TLR6 are located on the outer membrane and recognize mainly bacterial surface-associated PAMPs like peptidoglycan and lipopeptides (TLR1, 2, 6), lipopolysaccharide (TLR4), and flagellin (TLR5). The other human TLRs are expressed on the membrane of intracellular endosomes, where they bind viral dsRNA (TLR3), ssRNA (TLR7 and 8) or unmethylated bacterial DNA (TLR9) (8). Also, as we shall discuss below, endogenous host molecules can also function as TLR ligands.

Toll-like receptors are widely expressed in innate immune cells, such as macrophages, dendritic cells (DCs), but also in non-immune cells, such as endothelial and epithelial cells (3, 6). In DCs, TLR signaling triggers a maturation program that includes up-regulation of MHC and co-stimulatory molecules, and expression of pro-inflammatory cytokines, such as TNF-α, IL-1, and IL-6. This maturation of DCs significantly increases their ability to prime naïve T cells (9).

More recent TLR expression profiling studies have revealed that certain TLRs are expressed not only in innate immune cells but also in various adaptive immune cells, such as B cells (10, 11), $CD4^+$ and $CD8^+$ (12, 13), γδ T cells (14), and the $CD4^+CD25^+$ regulatory T cell population (15–17); TLR ligands can directly modulate the function of these adaptive immune cells. When TLR4 signaling induces proliferation and cytokine secretion in naïve mouse B cells (10); several natural and synthetic ligands, including bacterial lipopeptides Pam_3CSK4 (TLR1/TLR2), flagellin (TLR5), and R-848 (TLR7/8) were found to co-stimulate proliferation and cytokine secretion in human memory $CD4^+$ T cells (18, 19). In addition, the TLR3 ligand poly(I:C) and TLR2 ligands increase IFN-γ and IL-6 secretion in TCR-stimulated γδ T cells (20, 21). Furthermore, TLR ligands have been reported to promote the survival and modulate the suppressive capacity of regulatory T cells (17, 22, 23). Thus, the involvement of TLR signaling in modulation of immune response is not limited to innate immune cells.

TLR2 SIGNALING MEDIATES THE INNATE EFFECTS OF HSP60 ON T CELLS

Heat shock proteins (HSP) are highly conserved proteins induced in response to cellular stress, such as heat shock or nutrient deprivation (24, 25), and function as an endogenous danger signal of the immune system. Inside cells, HSP molecules assist the folding of newly synthesized proteins, participate in protein transport across membranes and refold proteins denatured during cell stress (26). However, HSPs, and in particular, HSP60 interests immunologists because, in addition to serving as a chaperone, extracellular HSP60 could directly activate innate immune cells, including macrophages and DCs (27, 28), through binding to various cell-surface receptors such as CD14, CD40, TLRs and the scavenger receptors CD36 and CD91. However, it was argued that many of the reported pro-inflammatory effects that result from exposure of cells to HSP60 are actually mediated through LPS or other microbial compounds contaminating the HSP60 (29, 30). Nevertheless, highly purified HSP60 was shown to be able trigger inflammatory responses *in vivo* via TLR2 and TLR4 signaling (31). Indeed, it is becoming clear that the self-HSP60 molecule and its synthetic peptides are able to activate TLR signaling (32).

In addition to functioning as a danger signal to innate immune cells, HSP60 also functions as an antigen in host defense and signals through "adaptive" immune receptors, such as T and B cell receptors (33, 34). Autoimmunity to self-HSP60, moreover, does not necessarily cause disease. The cord blood of newborn humans, like the peripheral blood of adults, manifests a relatively high frequency of T cells that can recognize HSP60 (35), and healthy adults manifest T cell reactivity to HSP60 (36). In direct contrast to the function of HSP60 as a danger signal and its involvement in autoimmunity, HSP60 and the HSP60-derived peptide p277 were also found to arrest the destructive inflammation responsible for development of autoimmune diseases such as adjuvant arthritis and type 1 diabetes (37, 38). In a double-blind, Phase II clinical trial the administration of p277 after the onset of clinical diabetes preserved the endogenous levels of C-peptide (a marker of insulin-producing capacity of the pancreas) and was associated with lower requirements for exogenous insulin compared with treatment with a placebo, revealing the arrest of inflammatory β-cell destruction. Treatment with p277 was associated with an enhanced Th2 response to HSP60 and p277 (39). Recently, a large multi-center phase III trial of p277 (DiaPep277) has confirmed the finding of the published phase II study (submitted for publication). Taken together, these results suggest that treatment with HSP60 or its p277 peptide can lead to the induction of HSP60-specific regulators, including anti-ergotypic regulators (40) that can control the collective of pathogenic re-activities involved in the progression of autoimmune diabetes.

The fact that both B and T cells also express TLRs on their surface raised the question about the direct function of these receptors in the regulatory effects of HSP60 on adaptive immune cells. Indeed, recent studies in our laboratory demonstrated that TLR2, but not TLR4 is involved in HSP60-mediated inhibition of T cell chemotaxis via up-regulation of the suppressor of cytokine signaling (SOCS)3 transcription factor (41). In addition, both human and mouse T cells treated with soluble HSP60 or HSP60-derived peptide undergo a signal transduction cascade, activate integrin receptors and induce adhesion to fibronectin via TLR2-dependent signaling (12). Since T cell chemotaxis is a highly coordinated process, which includes the rapid and reversible adhesiveness to extracellular matrix, the ability of HSP60 to induce T cell adhesion via TLR2 may partially contribute to inhibition of T cell chemotaxis.

The involvement of TLR2 in direct effects of HSP60 on T cell function was confirmed in additional studies demonstrating that HSP60 modulates the expression of Th1/Th2 transcription factors (42). It was shown that HSP60 down-regulates expression of the Th1-cell-promoting transcription factor T-bet, the transcription factor NF-κB, and the intracellular-signaling molecule NFATp; HSP60, in contrast, up-regulates the expression of the Th2-cell-promoting transcription factor GATA-3. This leads, in turn, to decreased secretion of TNF-α and IFN-γ and enhanced secretion of IL-10 (42). These innate effects of HSP60 were specifically dependent on intact TLR2, but not TLR4 signaling. In contrast, the ability of HSP60 to induce IL-10 and IL-6 secretion in mouse B cells was largely mediated through TLR4 and MyD88 signaling (10). Thus, HSP60 can directly modulate the adaptive immune cell function via TLR2 and TLR4 signaling pathways, although a direct interaction between HSP60 and these TLRs has not yet been shown.

THE REGULATORY FACE OF DANGER SIGNALS

The involvement of $CD4^+CD25^+$ regulatory T cells (Tregs) in HSP60-mediated suppression of T cell responses seems to be an attractive explanation for the protective effect of the molecule *in vivo* as was mentioned above. Indeed, the HSP60 molecule can function as a co-stimulator of Tregs by way of an innate signaling pathway that involves TLR2 (17). Treatment of Tregs with HSP60, or its peptide p277 before anti-CD3 activation significantly enhanced the ability of relatively low concentrations of the Tregs to down-regulate $CD4^+CD25^-$ or $CD8^+$ target T cells, detected as inhibition of target T cell proliferation and IFN-γ and TNF-α secretion. The enhancing effects of HSP60 co-stimulation on Tregs involved innate signaling via TLR2, led to activation of PKC, PI-3 kinase, and p38, and were further enhanced by inhibiting ERK. HSP60-treated Tregs suppressed target T cells both by cell-to-cell contact and by secretion of TGF-β and IL-10. The down-regulated target T cells manifested inhibited ERK, NF-κB, and T-bet (17). The contribution of TLR2 signaling to the control of Treg suppressive function still remains controversial and various results have been obtained in different species using different ligands. In rabbits, the engagement of TLR2 down-regulates the suppressive ability of Tregs purified from conjunctiva, and leads to the induction of an HSV-specific effector T cell response *in vivo* (43). In mice, the known exogenous agonist of TLR2, Pam_3Cys was shown to reverse Treg function in two studies (15, 16), but had no effect on Foxp3 expression and suppressive activity in murine Tregs in work reported by Chen et al. (44). Interestingly, all three studies agreed on the ability of Pam_3Cys to induce proliferation and promote murine Treg survival. Also, we found that relatively lower concentrations of Pam3Cys as well as polysaccharide A (PSA) of *B. fragilis* could augment Treg inhibition of cytokine secretion by $CD4^+CD25^-$ T cells via TLR2 signaling in humans (17) and mice (22). In contrast, higher concentrations of Pam_3Cys (1 and 5 μg/ml) were reported to down-regulate human Treg function, but controversial data were obtained with regards to the ability

of this TLR2 ligand to down-regulate Foxp3 expression in those two studies (45, 46). Although the precise role of TLR2 signaling in controlling Treg activity needs further characterization, HSP60 via TLR2 acts as a co-stimulator of Treg function.

LPS is known to be involved in activation of macrophages and B cells via TLR4-dependent signaling (3, 6). Although TLR4 expression is detected in T cells (12, 17), LPS was reported not to affect T cell cytokine secretion or proliferation or to activate regulatory T cell function (16, 17, 19, 42, 47). However, certain aspects of LPS-mediated effects on cytokine secretion in T cells via TLR4 *in vitro* did not fully correlate with the pattern seen *in vivo*: TLR4 signaling in $CD4^+$ T cells was shown to be inhibitory in a spontaneous model of colitis (48), whereas Reynolds et al. demonstrated that TLR4 signaling promoted the development of experimental autoimmune encephalomyelitis (EAE) in mice (49). Interestingly, the TLR-4-dependent inhibition of colitis was primarily mediated through Th1, whereas the promotion of EAE involved mainly the Th17 subset suggesting that further investigation is required to clarify the molecular mechanism of TLR4-mediated regulation of different T cell subsets *in vivo*. Consistent with the proposed anti-inflammatory function of direct TLR4 signaling, we demonstrated that LPS can up-regulate human T cell adherence to fibronectin and down-regulate the ability of T cells to migrate toward CXCL12 by way of STAT3-dependent induction of SOCS3 expression *in vitro* (50). This response to LPS was mediated specifically via TLR4, but not TLR2 signaling and required the presence of functional MyD88 (50). Thus, LPS, through TLR4 signaling can affect directly the pro-inflammatory T cell function and lead to termination of effector immune responses.

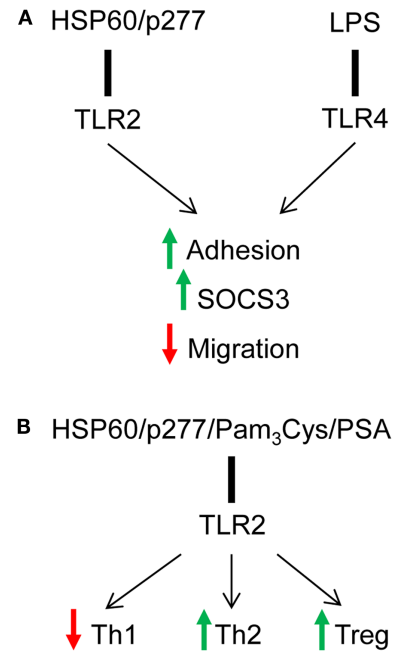

FIGURE 1 | Signaling via TLR2 and TLR4 directly down-regulates T cell effector function. **(A)** HSP60 via TLR2 and LPS via TLR4 induce T cell adhesion and down-regulate T cell chemotaxis in SOCS3-dependent mechanism. **(B)** TLR2 signaling induced by several endogenous and pathogen-derived ligands shifts cytokine profile toward Th2-like phenotype and up-regulates the suppressive function of Tregs.

THE BOTTOM LINE

About two decades ago, Janeway hypothesized that regulation of T cells by APCs must be controlled by receptors with specificity for microbial products; indeed, a class of innate receptors restricted to the recognition of non-self antigens was proposed to mediate the ability of the immune system to discriminate between self and pathogens (51). The function of TLR family appeared to fit this hypothesis, and the results of several studies supported the idea that TLRs do play an important role in controlling adaptive immune responses (3). Although TLRs have classically been studied on innate immune cells, recent reports have demonstrated their expression on adaptive immune cells, T and B cells in both mice and humans. Here we have discussed that the endogenous self-protein HSP60 as well as bacterial components, such as LPS directly signal to T cells and induce adhesion, SOCS3 expression that consequently leads to down-regulation of T cell migration via TLR2 and TLR4 respectively (**Figure 1A**) (12, 41, 50). Interestingly, although LPS-induced signaling through TLR4 had no effect on cytokine secretion in T cells (19, 42, 47), TLR2 signaling induced by both HSP60 and bacterial components, such as Pam_3Cys and PSA, resulted in the down-regulation of Th1 and up-regulation of Th2-like responses, and the induction of Treg function (**Figure 1B**) (17, 22, 42). The difference between TLR-mediated effects of HSP60 and LPS on T cells may result from different levels of sensitivity: T cells are extremely sensitive to HSP60 and respond to concentrations in the 0.1–1 ng/ml range (12, 17, 41, 42); the effects of LPS on T cell adhesion and migration require concentrations of about 100 ng/ml (50). Thus, the involvement of different TLRs, TLR2 for HSP60 vs. TLR4 for LPS as well as different degrees of sensitivity contribute to variation between effects of those TLR ligands on T cell function: notably, TLR2 signaling is involved in direct effects of both endogenous signals (HSP60 and HSP60-derived peptide) and pathogen-derived ligands (Pam_3Cys and PSA). In summary, these findings suggest that direct TLR2 and TLR4 signaling in T cells can modulate decisions dictated by antigen-presenting cells and shift the immune response from a damaging to a healing type.

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Role of Toll-like receptors in immune activation and tolerance in the liver

Nobuhiro Nakamoto and Takanori Kanai*

Division of Gastroenterology and Hepatology, Department of Internal Medicine, Keio University School of Medicine, Tokyo, Japan

Edited by:
Christophe M. Filippi, Genomics Institute of the Novartis Research Foundation, USA

Reviewed by:
Sylvie Lesage, University of Montreal, Canada; Maisonneuve-Rosemont Hospital, Canada
Maria Grazia Roncarolo, San Raffaele Scientific Institute, Italy

****Correspondence:***
Takanori Kanai, Division of Gastroenterology and Hepatology, Department of Internal Medicine, Keio University School of Medicine, Tokyo 160-8582, Japan
e-mail: takagast@z2.keio.jp

Liver has a unique vascular system receiving the majority of the blood supply from the gastrointestinal tract through the portal vein and faces continuous exposure to foreign pathogens and commensal bacterial products. These gut-derived antigens stimulate liver cells and result in a distinctive immune response via a family of pattern recognition receptors, the Toll-like receptors (TLRs). TLRs are expressed on Kupffer cells, dendritic cells, hepatic stellate cells, endothelial cells, and hepatocytes in the liver. The crosstalk between gut-derived antigens and TLRs on immune cells trigger a distinctive set of mechanisms to induce immunity, contributing to various acute and chronic liver diseases including liver cirrhosis and hepatocellular carcinoma. Accumulating evidence has shown that TLRs stimulation by foreign antigens induces the production of immunoactivating and immunoregulatory cytokines. Furthermore, the immunoregulatory arm of TLR stimulation can also control excessive tissue damage. With this knowledge at hand, it is important to clarify the dual role of disease-specific TLRs as activators and regulators, especially in the liver. We will review the current understanding of TLR signaling and subsequent immune activation and tolerance by the innate immune system in the liver.

Keywords: Toll-like receptor, Kupffer cell, dendritic cell, liver tolerance, microbiota

INTRODUCTION

The liver faces continuous exposure to many pathogens and commensal bacterial products, and the innate and adaptive immune responses of the liver favor the induction of immunological activation and tolerance as appropriate (1–5). Although various immune compartments, such as T cells including $CD4^+CD25^+Foxp3^+$ regulatory T cells (Tregs), natural killer (NK) cells, natural killer T (NKT) cells, macrophages [Kupffer cells (KC)], conventional or classical dendritic cells (cDCs), and plasmacytoid DCs (pDCs), reside in the normal liver (1, 2), it is unknown which types of cells induce inflammation and tolerance and how these cells work together to maintain immunological balance. The innate immune system is thought to play a major role in maintaining homeostasis in the liver. Gut-derived bacterial products enter the liver through the portal vein. However, liver inflammation usually does not occur because the intact mucosal barrier system of the healthy intestine prevents translocation of microbial products. When this barrier is disrupted, bacteria translocate to the liver in large quantities, resulting in the activation of the hepatic innate immune system. Cells within the hepatic sinusoids express a receptor that recognizes lipopolysaccharide endotoxin (LPS), expressed in the outer membrane of Gram-negative bacteria, and effectively remove this molecule. The Toll-like receptors (TLRs) recognize pathogen-associated molecular patterns (PAMPs) as part of innate immune defenses against foreign pathogens, including bacteria, DNA and RNA viruses, fungi, and protozoa (6, 7). Thirteen mammalian TLRs have been identified, and TLR1–10 are expressed in humans. TLRs, their ligands, and downstream signaling pathways are shown in **Figure 1**. In general, the healthy liver contains low mRNA levels of TLRs and their downstream signaling molecules, such as myeloid differentiation primary response gene-88 (MyD88), compared with other organs (8, 9). The continuous antigen exposure and recognition via TLRs in the liver may trigger a distinctive set of mechanisms to maintain self-tolerance and induce immunity against infection depending on the particular situation. Here, we will review the dual role of TLRs as activators and regulators of immune responses in the liver.

TLR SIGNALING IN THE LIVER

In the liver, hepatocytes account for 60–80% of the total cell population (10). Non-parenchymal cells consist of KCs, DCs, lymphocytes, hepatic stellate cells (HSCs), liver sinusoid endothelial cells (LSECs), and biliary cells. Each cell population exhibits a different TLR expression.

KUPFFER CELLS

Kupffer cells are hepatic-resident macrophages and account for about 20% of the non-parenchymal cells in the liver. KCs engage in phagocytosis and antigen presentation, and they are the primary cells that encounter gut-derived toxins such as LPS and orchestrate immune responses within the liver (11). Accordingly, KCs express TLR4 and are responsive to LPS (12). KCs also express TLR2, TLR3, and TLR9 and respond to their ligands (13–15). Following LPS stimulation, KCs produce tumor necrosis factor α (TNFα), interleukin (IL)-1β, IL-6, IL-12, and IL-18 (16). However, KCs also release anti-inflammatory cytokines such as IL-10 in response to continuous stimulation with low levels of LPS, a phenomenon known as LPS tolerance (17). Similarly, several murine experiments demonstrated a role for macrophages in restricting inflammatory responses during the recovery phase of liver injury

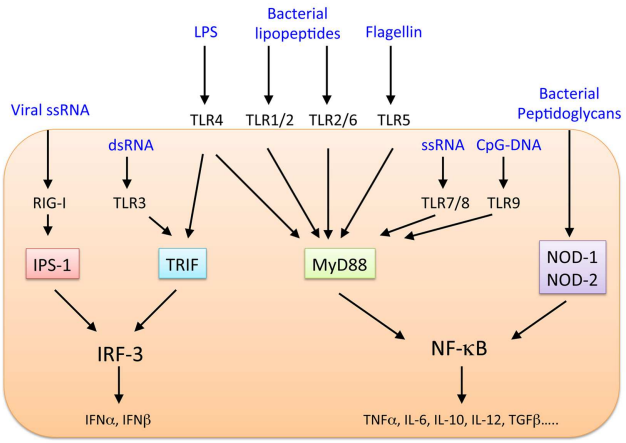

FIGURE 1 | TLRs and downstream signaling pathways.

(18). These results indicate that KCs act as both immune activating and immune regulatory cells depending on the specific situation.

DENDRITIC CELLS

Hepatic DCs account for a very small proportion (<1%) of non-parenchymal cells in the liver. DCs in lymphoid and non-lymphoid tissues are classified into two major subsets: pDCs and cDCs. Murine lymphoid-resident cDC subsets can be further divided into $CD8\alpha^+$ DCs and $CD8\alpha^-$ DCs (19, 20). Likewise, two distinct migratory cDC subsets are subcategorized based on CD103 and CD11b expressions in non-lymphoid tissues: $CD103^+CD11b^-$ cDCs and $CD103^-CD11b^+$ cDCs (21, 22). In humans, pDCs express TLR1, TLR7, and TLR9, while other DC subsets express all other TLRs except for TLR9 (23). In mice, both pDCs and cDCs express TLR2, TLR4, TLR7, and TLR9. In response to signaling through TLR2, TLR3, and TLR4, hepatic cDCs produce TNFα and IL-6 (24). However, recent reports showed that murine cDCs can produce an anti-inflammatory cytokine, IL-10, through TLR9 following ischemia/reperfusion injury (25). Hepatic pDCs produce inflammatory cytokines in response to TLR7 and TLR9 (24, 26, 27). Of note, a new subset of $CCR9^+$ pDCs was identified as tolerogenic pDCs in an acute graft-versus-host disease model (28). Our group demonstrated $CCR9^+$ pDCs exist abundantly within the murine liver, produce IL-10, and transforming growth factor β (TGFβ) and differentiate naïve T cells to a regulatory phenotype through TLR7 and TLR9 signaling (29).

LYMPHOCYTES

Intrahepatic lymphocytes account for about 25% of the non-parenchymal cells in the liver. They consist of NK, NKT, γδ T, αβ T, and B cells. Hepatic NK cells express TLRs1, 2, 3, 4, 6, 7, 8, and 9 and respond to the corresponding TLR ligands (30). TLR3 ligands negatively regulate liver regeneration via activation of NK cells (31). In general, T cells are indirectly activated by TLR signaling, but direct activation of T cells by TLR signaling through TLR2, 3, and 9 has been reported (32, 33).

HEPATIC STELLATE CELLS

Hepatic stellate cells account for a very small proportion (<1%) of non-parenchymal cells in the liver. Following liver injury, activated HSCs produce extracellular matrix components in the liver, such as collagen types 1, 3, and 4, leading to liver fibrosis (34). Activated human HSCs express TLR4 and CD14, and respond to LPS with the secretion of proinflammatory cytokines (35). Activated mouse HSCs express TLR2, TLR4, and TLR9, and respond to the corresponding ligands with the secretion of IL-6, vascular cell adhesion molecule 1 (VCAM-1), TGFβ1, and monocyte chemoattractant protein-1 (MCP-1) (36–38).

LIVER SINUSOIDAL ENDOTHELIAL CELLS

Liver sinusoidal endothelial cells account for about 50% of non-parenchymal cells in the liver. LSECs express mRNAs for TLR1–9 and respond to the corresponding ligands except for that of TLR5. LSECs respond to TLR1, 2, 4, 6, 9 ligands by producing TNFα, and respond to TLR3 ligands by producing TNFα, IL-6, and interferon (IFN) (27). After repetitive LPS challenge, sinusoidal endothelial cells reduce NF-κB activation and mediate liver tolerance to maintain hepatic homeostasis (39). In the same way, LSECs play a role in maintaining the homeostasis of the liver through induction of antigen-specific T cell tolerance (40). A recent report demonstrated that LSECs mediate angiogenesis and subsequent liver fibrosis via TLR4 signaling (41).

HEPATOCYTES

Primary cultured hepatocytes express TLR1–9, but only respond to TLR2 and TLR4 ligands (42). In the steady state, the responses to TLR2 and TLR4 are weak, while the expression of TLR2 and responsiveness to ligands is enhanced under inflammatory conditions (43, 44). Of note, hepatocytes, in concert with TLR4, CD14, and MD-2 play a role in the uptake and removal of LPS from the systemic circulation (45–47).

ROLE OF TLRs IN MURINE AND HUMAN LIVER INJURY

EXPERIMENTAL ACUTE LIVER INJURY (CONCANAVALIN A)

A single intravenous injection of Con A triggers acute liver injury in mice. It is accepted that Con A-induced acute liver injury is mediated mainly by $CD3^+CD4^+NK1.1^+$NKT cells and $CD3^+CD4^+NK1.1^-$ T cells (48–50). However, liver antigen-presenting cells (APCs) including KCs and DCs might be critically involved in the pathogenesis of Con A-induced liver injury, since it is significantly suppressed in KC-depleted mice (51–53). Signaling through TLR2, TLR3, TLR4, and TLR9 has been reported to contribute to liver injury in this model, especially in the early phase (54–56). We recently reported that TNFα-producing $CCR9^+CD11b^+CD11c^-$ macrophages expressed TLR2, TLR4, and TLR6 mRNAs and had a major role in the pathogenesis of acute liver injury in this model by activating Th1 and NKT cells (25). Of note, in the inflamed liver the number of tolerogenic $CCR9^+CD11b^-CD11c^+$ pDCs that express TLR7 and TLR9 mRNAs decreases following Con A injection, suggesting the balance between inflammation and tolerance might be regulated by distinct immune cell subsets and TLRs in this model (**Figure 2**). Following Con A injection, up-regulation of TLR3 expression is observed in liver mononuclear cells and LSECs. The pathological role of TLR3 in this model was confirmed as $TLR3^{-/-}$ mice

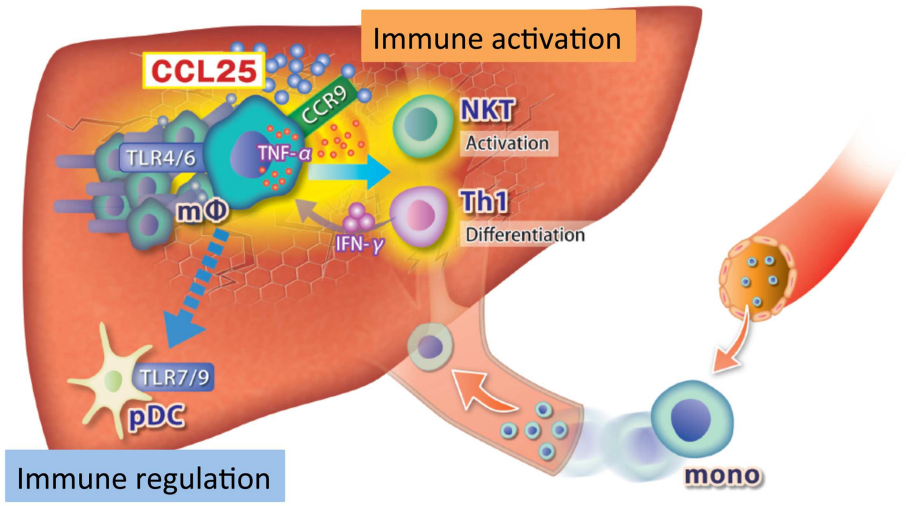

FIGURE 2 | Role of innate immune cells in the pathogenesis of Con A-induced acute liver injury. Following Con A administration, CCL25 expression is up-regulated in the inflamed liver and CCR9$^+$ macrophages accumulate to this site, while pDCs are down-regulated. CCR9$^+$ macrophages produce TNFα and promote proliferation of IFNγ-producing Th1 and NKT cells via TLR4/6.

were protected from Con A-induced hepatitis (57). In contrast, it was reported that Poly-I:C pretreatment activated NK cells and subsequently protected against Con A-mediated liver injury via down-regulation of T/NKT cells (58). Importantly, the protective effect of TLR3 was also reported in an LPS/D-GaiN-induced acute liver injury model (14). These results collectively indicate that TLR3 signaling has pleiotropic functions and is involved in inflammation, regeneration, and tolerance during the course of acute liver injury. The contribution of TLR9 in this model is controversial. TLR9 activation by CpG oligodeoxynucleotides (CpG-ODN) can exacerbate Con A-induced liver injury by promoting the activation of hepatic CD4$^+$ NKT cells. The effect of TLR9 signaling on hepatic NKT cells was dependent on KCs and IL-12 (59). However, another report showed that pretreatment with CpG-ODN protected mice from Con A-induced hepatic injury by attenuating the activation of inflammatory cells (60). These contradictory findings could have resulted from differences in the DNA sequences used, because a different DNA sequences might trigger TLR9 signaling with different consequences, such as the release of potentially harmful (TNFα) or beneficial (IL-12) cytokines (61). Immunological tolerance to Con A was demonstrated as repeated Con A injection within 8 days after an initial Con A injection significantly reduced hepatic injury (62). The authors of that study concluded that CD4$^+$CD25$^+$ Tregs, KCs, and IL-10 were required for Con A tolerance. Further studies are required to clarify the contribution of specific TLRs and their downstream signaling to Con A tolerance.

ISCHEMIA–REPERFUSION AND LIVER TRANSPLANTATION

Ischemia–reperfusion (I/R) injury is most commonly seen in the early period after liver transplantation. Recipients transplanted with livers from TLR4-deficient mice exhibited less I/R injury than those transplanted with wild-type livers (63), suggesting the inflammatory response seen in I/R injury is mainly mediated by TLR4. An alternative explanation is that TLR4 plays an indirect role by exacerbating I/R, as opposed to initiating the pathology. TLR4 expression on non-parenchymal cells is up-regulated by damage-associated molecular pattern molecules (DAMPs), such as high-mobility group box 1 protein (HMGB1) released from damaged hepatocytes during I/R (64, 65). Regarding allograft rejection and tolerance in liver transplantation, hepatic TLR4 expression has a distinctive role in CD8 T cell apoptosis and memory T cell generation (66). Increased TLR4-mediated expression of adhesion molecules in LSECs and KCs following continuous LPS exposure promoted trapping of T cells within the liver, resulting in lower numbers of circulating primed CD8 T cells and weak immune responses (39). The balance between alloimmune responsiveness and tolerance might be mediated by the level of TLR ligands that act as PAMPs or DAMPs, in association with clinical events such as I/R injury and infection after transplantation (67). It was recently reported that IL-10-producing cDCs reduced liver I/R injury in mice via TLR9 (25). Although the liver can mount an appropriate and sometimes excessive immune response to eliminate invading organisms, the overall balance appears to favor a state of immune permissiveness. As critical regulators of both innate and adaptive immunity, hepatic cDCs might play a role in orchestrating immune responses to limit undesirable inflammation and promote tolerance via TLR9. It is still unclear how the immune system can distinguish between threats from pathogens and endogenous danger signals, and contribute to both immune activation and tolerance through TLR9 signaling.

HEPATITIS B AND HEPATITIS C VIRAL INFECTION

The gene expression of TLR1, TLR2, TLR4, TLR6, TLR7, and TLR9 was decreased in peripheral blood mononuclear cells (PBMCs) from chronic Hepatitis B virus (HBV) infected patients, compared with healthy controls (68, 69). Impaired cytokine production with TLR2 and TLR4 ligands was also observed in PBMCs from chronic

Hepatitis B (CHB) patients (68). Several TLR signaling pathways induce antiviral effects by up-regulating IFNs. Activation of TLR3, TLR4, TLR5, TLR7, and TLR9 by ligands mediates the inhibition of viral replication in HBV transgenic mice (70, 71). Importantly, HBV infection also induces immunosuppressive effects through TLR signaling. Overexpression of TLR2 and TLR4 on monocytes is reported to account for persistent HBV infection by modulating Treg functions (72). In PBMCs from chronic Hepatitis C virus (HCV) infected patients, the gene expression of TLR2, TLR3, TLR4, TLR6, TLR7 was increased (73, 74). HCV activates innate immune receptors including TLRs and retinoic acid-inducible gene 1 (RIG-I) to induce a chronic inflammatory state. Concurrently, HCV suppresses specific intracellular signaling to evade the host immune control (75). HCV core and NS3 proteins trigger TLR1, TLR2, and TLR6 on monocytes to enhance the production of inflammatory cytokines (76, 77). However, NS3/4a proteins degrade TIR-domain-containing adapter-inducing IFN-β (TRIF) and inhibit TLR3-mediated TRIF-dependent IFN-β production (78, 79). Furthermore, NS5 inhibits the recruitment of IL-1 receptor-associated kinase 1 (IRAK1), resulting in a decrease in TLRs-Myd88-dependent signals (80). An appropriate T cell response is required to eradicate HBV and HCV, while exhausted HCV-specific T cells with inhibitory immune receptors, such as PD-1 and CTLA-4, account for persistent viral infection within the liver (3, 4, 81, 82). LSECs with up-regulated PD-L1 expression were reported to induce antigen-specific T cell tolerance (40), and recent reports indicated that stimulation of LSECs with TLR1/2 ligands, but not TLR3 or TLR4 ligands could overcome liver-specific tolerance (83). Further study is required to clarify the effect of TLR1/2 ligands on the function of tolerant HBV- and HCV-specific T cells.

ALCOHOL-INDUCED LIVER DISEASE

Excessive alcohol intake induces elevated levels of LPS in the liver through the portal circulation (84). The mechanism involved in the elevation of LPS is thought to be as follows. First, ingested alcohol disrupts the intestinal mucosal barrier and causes enhanced permeability (85, 86). Second, alcohol consumption leads to changes in the intestinal flora (87), and they migrate to liver sinusoids through the portal vein. KCs are a major target of LPS in various liver injuries including alcohol-induced liver injury (35, 88), as demonstrated by reduced liver inflammation following KC depletion (89). Recent reports indicated that TLR4 signaling in alcoholic liver injury was mediated through a MyD88-independent, but TRIF-dependent pathway (90, 91).

NON-ALCOHOLIC STEATOHEPATITIS

Accumulating evidence indicates that LPS/TLR4 is also involved in the development of non-alcoholic steatohepatitis (NASH). A role for LPS in NASH was demonstrated by the finding that genetically obese *ob/ob* mice were sensitive to low-dose LPS (92). Furthermore, when fed a methionine/choline-deficient (MCD) diet, the most widely accepted experiment model of NASH, TLR4-deficient mice exhibited less severe hepatic injury and less accumulation of intrahepatic lipids compared with wild-type mice (93). These findings indicated activated TLR4 signaling pathways were critically involved in the pathogenesis of NASH. Recently, up-regulation of CD14 in KCs and hypersensitivity against low-dose LPS were observed in mice with high-fat diet (HFD)-induced steatosis (94). Hypersensitivity against low-dose LPS leads to accelerated NASH progression, including liver inflammation and fibrosis. In contrast, TLR2-deficient mice were not protected from steatohepatitis induced by MCD diet, affirming the TLR4 dependence of disease progression in this model (95). Notably, probiotics relieve the severity of NASH in leptin-deficient *ob/ob* mice, suggesting alterations of the intestinal flora might affect proinflammatory responses by disease-specific immune components through TLRs (96, 97).

LIVER FIBROSIS

Studies demonstrated elevated plasma LPS levels in experimental liver fibrosis induced by carbon tetrachloride (CCl_4), thioacetamide, and bile duct ligation (BDL). TLR4 is expressed on both parenchymal and non-parenchymal cells in the liver, and several animal studies support the contribution of TLR4 in the development of liver fibrosis (36, 98, 99). Mice deficient for TLR4, CD14, MyD88, or TRIF exhibit reduced liver fibrosis in experimental fibrosis models (36, 98). In a recent study, Seki et al., clearly demonstrated that TLR4 on HSCs, but not on KCs or hepatocytes, was crucial for inducing liver fibrosis (36). Low concentrations of LPS can activate HSCs via TLR4 and downstream signaling to secrete a number of chemokines and adhesion molecules. These chemokines not only induce the migration of macrophages into the liver but also directly activate HSCs, leading to liver fibrosis. The role of chemokine receptors CCR1, CCR2, CCR5, CCR8, and CCR9 in liver fibrosis has been reported (100–104). A human study analyzing a large patient cohort demonstrated that certain single nucleotide polymorphisms (SNPs) in TLR4 were associated with reduced risk of liver cirrhosis in patients with chronic hepatitis C (105). The participation of TLR9 during liver fibrosis has been demonstrated in several mouse models of liver fibrosis, such as CCl_4 and BDL models, in which TLR9-deficient mice exhibited significant reductions in liver fibrosis (106). Endogenous DNA from damaged hepatocytes is reported to enhance HSC activation through TLR9, thereby promoting liver fibrosis (37). TLR3 participates in the early stages of liver fibrosis but not during advanced liver fibrosis. Treatment with the TLR3 ligand Poly-I:C enhanced the activation of NK cells for killing HSCs, leading to attenuation of liver fibrosis (107). Recently, impaired TLR3 and TLR7/8 function was reported to affect rapid fibrosis progression post-liver transplantation with HCV infection (108).

TLRs AND MICROBIOTA

The translocation of intestinal microbiota into the liver and their recognition by TLRs results in both immune activation and tolerance under specific conditions. Importantly, this process is also critically involved in the development of a variety of liver diseases (109–112). Thus, targeting components of innate immune signaling, such as intestinal microbiota and TLRs may be an effective therapeutic approach to chronic liver diseases including viral hepatitis, alcoholic liver disease, NASH, and subsequent liver fibrosis. In particular, the mechanism of how endogenous TLR ligands associated with bacterial translocation contributes to immune activation and regulation, and subsequent chronic liver disease, should be comprehensively studied. Recent advances

in gnotobiotic technology have enabled analysis of the role of specific bacterial strains in immunological responses (113–116). Using these techniques, a recent study reported that a complex mixture of 46 strains of *Clostridium* induced TGFβ in intestinal epithelial cells, which promoted the subsequent accumulation of IL-10-producing induced T regulatory cells, which in turn suppressed colitis in a dextran sodium sulfate colitis model (117). Very recently, our group reported that a single strain of *Clostridium butyricum* induced intestinal IL-10-producing macrophages via TLR2 and suppressed a mouse model of acute experimental colitis (118). Furthermore, butyrate-producing probiotics reduced the severity of murine NASH (119). These results clearly indicate that a single strain of microbiota can trigger immune activation and regulation via signaling through distinct TLRs. Further research should address in detail the crosstalk between disease-specific microbiota and the innate and adaptive immune system that occurs via specific TLRs signaling pathways in chronic liver diseases.

CONCLUSION AND PERSPECTIVES

The liver is continuously exposed to food antigens and PAMPs from the gastrointestinal tract via the portal vein. TLR signaling has a critical role in maintaining a balance between immune activation and tolerance. Following exposure to foreign antigens, TLRs are immediately activated and promote the induction of inflammatory cytokines and antimicrobial peptides to remove foreign microorganisms from the host. Concurrently, overactivation of TLRs that causes fetal events such as sepsis and acute liver failure should be controlled, which in turn might result in persistent infections in the liver. As described in this review, the following mechanisms have substantial roles in organ-specific tolerance: (1) hyporesponsiveness of individual TLR signaling due to the continuous exposure to ligands as seen in LPS tolerance (TLR4 on macrophages and LSECs) (17, 39), (2) the induction of other TLR signaling by DAMPs and host DNAs released from injured host cells and subsequent immunosuppressive cytokine production as seen in liver I/R injuries (TLR9 on cDCs) (25), and (3) dysfunctional antigen presentation by PD-L1-expressing APCs and the subsequent antigen-specific T cell exhaustion that can be reversed by TLR1/2 ligand stimulation as seen in chronic viral infections (TLR1/2 on LSECs) (83). Further studies, especially in humans, are required to clarify the interaction of each ligand-TLR signaling pathway on individual immune cell subsets that causes both immune activation and tolerance depending on severity and phase of the injury, and which eventually results in liver diseases such as chronic hepatitis, liver cirrhosis, and liver cancer. Understanding the underlying mechanisms in this area can aid the development of new therapeutic strategies in the future.

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Toll-like receptor activation in immunity vs. tolerance in autoimmune diabetes

Elke Gülden and Li Wen*

Section of Endocrinology, Yale University School of Medicine, New Haven, CT, USA
**Correspondence: li.wen@yale.edu*

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Christophe M. Filippi, Genomics Institute of the Novartis Research Foundation, USA

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INTRODUCTION

TOLL-LIKE RECEPTORS: KEY RECEPTORS OF THE INNATE IMMUNE SYSTEM

The human body constantly encounters a diverse spectrum of pathogens. To defend itself, a complex immune system has evolved consisting of two subdivisions: the innate and the adaptive immune systems. The innate immune system constitutes the so-called "first line of defense" and acts through highly conserved germline-encoded pattern recognition receptors (PRRs) (1). These receptors bind to common pathogen-associated molecular patterns (PAMPs), which are vital for the survival of the microorganisms and cannot be altered by mutations.

Pattern recognition receptors were first discovered in the fruit fly *Drosophila melanogaster*. The PRRs in *Drosophila*, named Toll, play an important role in the recognition of microorganisms such as fungi as well as coordinating embryonic development of the dorso-ventral axis.

Homologs of Toll in vertebrates are called Toll-like receptors (TLRs). The first TLR in humans was described in 1997 (2). To date, another 9 TLRs have been identified in humans and 13 in mice. Among these, only murine TLR10 is non-functional due to retrovirus insertion (3). Except for TLRs 3, 7, 8, and 9, which are expressed intracellularly, TLRs are located on the surface of innate and adaptive immune cells as well as on non-immune cells such as muscle cells, epithelial cells, adipocytes, and pancreatic beta cells (1). Cell-surface TLRs detect exogenous lipids, lipoproteins, and proteins from microbes; intracellular TLRs recognize bacterial and viral nucleic acids (3). Recognition of their cognate ligand triggers a complex signaling cascade that ultimately results in the induction of various pro-inflammatory chemokines and cytokines and the activation of the adaptive immune system.

However, the activation of TLRs and the subsequent induction of inflammatory immune responses are not always beneficial to the host. TLRs are involved in the pathogenesis of various autoimmune and non-infective inflammatory diseases such as systemic lupus erythematosus, multiple sclerosis, arteriosclerosis, inflammatory bowel disease, diabetes, allergy, and cancer (4, 5). Their activation can either attenuate or boost the course of disease by inducing tolerance or triggering autoreactivity, respectively.

The role of the TLRs in the pathogenesis of autoimmune-mediated diabetes, also referred to as type 1 diabetes (T1D), has been extensively studied (**Table 1**). T1D is a T-cell-mediated metabolic disorder with progressive destruction of insulin-producing pancreatic β cells (6). During the course of disease development, diabetogenic T-cells, macrophages, and dendritic cells will infiltrate the pancreatic islets and cause islet inflammation and eventually β cell loss.

This opinion letter focuses on the beneficial and detrimental aspects of TLR induction in the course of T1D development.

TLR AND THEIR ROLE IN AUTOIMMUNE-MEDIATED TYPE 1 DIABETES

TLR-MEDIATED INITIAL EVENTS IN THE INDUCTION OF ISLET-DIRECTED IMMUNE RESPONSES

One of the major functions of the immune system is to distinguish self from non-self in order to fight invaders (pathogens) while sparing endogenous structures. PAMPs, the ligands of TLRs, are conserved molecular patterns that are exclusively expressed by pathogens. Therefore, the innate immune system is able to distinguish self and non-self. This beneficial "discrimination" is further enhanced by the adaptive immune system, in which most of the self-reactive T- and B-cells are deleted by central tolerance through negative selection. However, increasing evidence suggests that TLRs also recognize endogenous molecules including self-DNA released by injured or dying cells. These endogenous molecules act as danger signals and are therefore called "danger-associated molecular patterns (DAMPs)." The exposure of DAMPs by necrotic cells is considered to be a potential trigger of autoimmune diseases such as T1D (24). Studies have shown a defective clearance of dying cells in the NOD mouse (25), currently the best characterized model of human T1D (26). Due to the diabetes-prone genetic background, this defect might essentially contribute to the induction of autoimmunity in these mice. The accumulation of apoptotic β cells, which may undergo secondary necrosis (so-called "late state apoptosis"), could result in the activation of antigen-presenting cells (APC) via TLR engagement by released endogenous molecules and thereby contributing to the induction of diabetogenic T-cells (8, 27). Diabetogenic T-cells are then recruited to the pancreatic islets by chemokines like CCL2 (28, 29), CCL5, CXCL9, and CXCL10 (30) that can be released from β cells upon TLR ligation.

Consistent with this hypothesis is the fact that TLR2 induces apoptosis (31) and promotes diabetes in a streptozotocin-induced diabetic model following activation via the synthetic ligand Pam3CSK4

Table 1 | TLR-related studies in the field of T1D.

TLR/adaptor protein	TLR/adaptor protein		Effect on the course of T1D			Reference
	deficiency/ blockade	sufficiency	attenuating/ protective	promoting/ boosting	dispensable	
TLR2		+	+			Filippi et al. (7)
TLR2	+	+		+		Kim et al. (8)
TLR2	+				+	Wen et al. (9)
TLR2	+		+			Devaraj et al. (10)
TLR2		+	+			Karumuthil-Melethil et al. (11)
TLR2	+			+		Al Shamsi et al. (12)
TLR2	+		+			Kim et al. (13)
TLR2, 3, 4, 7		+	+			Aumeunier et al. (14)
TLR3	+				+	Fallarino et al. (15)
TLR3	+				+	Wong et al. (16)
TLR4	+			+		Gülden et al. (17)
TLR4	+				+	Wen et al. (9)
TLR4	+		+			Devaraj et al. (18)
TLR4		+		+		Li et al. (19)
TLR7		+		+		Lee et al. (20)
TLR9	+		+			Zhang et al. (21)
TLR9	+		+			Tai et al. (22)
TLR9	+			+		Fallarino et al. (15)
TLR9		+		+		Zipris et al. (23)
TLR9	+		+			Wong et al. (16)
MyD88	+		+			Wen et al. (9)

The table displays only studies that addressed a TLR directly. The studies that used TLR ligands but did not directly demonstrate the role of a specific TLR are not listed. '+' denotes the composition (TLR or adaptor protein investigated; usage of wild type or knockout mice) and outcome of the study.

(12). The observation of delayed or reduced diabetes onset in TLR9-deficient mice further supports the theory (16, 21, 22). However, in a chemically induced diabetes mouse model, Fallarino et al. found that TLR9-deficient C57BL/6 mice were more susceptible to diabetes induction (15). This opposing finding is most likely due to the different choice of animal model. Other investigators have used the NOD mouse model (16, 21, 22) whereas Fallarino and colleagues induced diabetes by injections of β cell-toxic drug streptozotocin to C57BL/6 mice.

DISEASE PROTECTION VS. DISEASE INDUCTION

As key receptors of the innate immune system, TLRs trigger inflammatory immune responses upon binding of cognate ligands. On a predisposed genetic background, this event might initiate islet inflammation followed by progressive β cell destruction and finally overt T1D.

However, TLR activation is not necessarily causative for T1D development. T1D-related studies summarized in **Table 1**, reveal either a protective or detrimental effect on the induction of islet-directed autoimmunity. One reason for this dichotomy might be the point in time during the prediabetic phase when TLR activation is induced. Moreover, the presence or absence of β cell antigens and/or endogenous DAMPs released by late state apoptotic β cells possibly plays a critical role. Studies showed that activation of TLR2 (8), TLR3 (32), or TLR9 (33) by their cognate ligands in the presence of β cell antigens or DAMPs give rise to T1D development, whereas TLR stimulation in the absence of β cell antigens results in tolerogenic immune responses (11, 34–36). Observations by Filippi et al. suggest that the reason for this outcome might be the capacity of immunostimulatory factors to augment immune regulation (7).

THE ROLE OF TLRs IN MODULATING Treg FUNCTIONS

Regulatory T-cells (Treg) are TLR-expressing adaptive immune cells which control immune responses in order to prevent aberrant immune reactions which could be harmful (37). In the presence of β cell antigens, TLR2 signaling could, while inducing a pro-inflammatory immune response, simultaneously promote suppressive Treg functions.

Dasgupta et al. demonstrated that engagement of TLR2 reverses the suppressor function of conjunctiva Treg in rabbits (38). In mice, in some cases, TLR2 stimulation resulted in a temporary abrogation of the regulatory capacity of Treg (39, 40), while other studies reported that the Treg function was either unchanged (41) or improved (7, 11). On the other hand, studies by other investigators showed a decreased number of Tregs following TLR2 stimulation (12).

One of the explanations for these contrary findings might be the different concentrations of the TLR ligand used in their experimental systems. It has been reported that the concentration of TLR ligands influences the regulatory activity

of Tregs (40, 42, 43). For example, Zanin-Zhorov and colleagues showed that low doses of the TLR2 ligand, Hsp60, resulted in enhanced suppression without increased Treg proliferation (42, 43).

Studies by Round and Mazmanian investigating the immunomodulatory effect of polysaccharide A (PSA), a microbial molecule of the commensal bacterium *Bacteroides fragilis*, reveal that the PSA can signal directly on Treg cells via their TLR2 and promote immune tolerance (44, 45). It is possible that the effect may be accounted for by the anatomical site where TLR2 is engaged. Specifically in the intestine, TLR2 signaling induced by PSA is required for Treg induction and IL-10 expression.

TLR2 ligation does not merely exert influence on Treg; studies also reveal an effect on effector T-cells (46, 47). TLR2 signaling via Pam3Cys achieves resistance of T effector cells toward Tregs (47).

Besides TLR2, other TLRs also modulate Treg functions. TLR4 and TLR5 ligands are capable of boosting the suppressive function of Treg on T effector cells (48, 49). In line with these observations is the finding that TLR4-deficient NOD mice exhibit significantly accelerated diabetes development and impaired suppressive function of Tregs, although the frequency of Tregs remains unchanged (17).

Taken together, TLRs act as important modulators of Treg proliferation and function. Treg function can either be enhanced or attenuated depending on the concentration of TLR ligands and the anatomical site of TLR engagement.

TOLERANCE INDUCTION BY TLR SIGNALING

As potent activators of inflammatory immune responses, the activation of TLRs must be tightly controlled since overactivation or loss of negative regulation can lead to detrimental or even life-threatening effects as seen in the condition of sepsis (50, 51). Repeated exposure to the same ligand can therefore result in hyporesponsiveness or tolerance through down-regulation of TLRs and simultaneous up-regulation of a negative feedback loop (50, 52).

In contrast to short term TLR stimulation, which results in initiation of immune responses, repeated exposure to TLR ligands might lead to the abrogation of inflammatory immune responses. In such a manner, TLR2 tolerance and inhibition of T1D development in NOD mice could be achieved by repeated administration of the TLR2 agonist Pam3CSK4 or zymosan (11, 13). Decreased T1D incidence was also accomplished following treatment with LPS, poly (I:C) (34), or CpG oligonucleotides (36).

As systemic chronic TLR stimulation suppresses the development of T1D in NOD mice (14, 53), it is possible that the absence of TLR stimulation might facilitate T1D development. This possibility is supported by the so-called hygiene hypothesis (54). The hygiene hypothesis coincides with the increase in allergy and autoimmune diseases over the past decades, possibly due to less exposure to microbial products as the hygiene standard has been significantly improved. Supporting the hygiene hypothesis is a recent study revealing that NOD mice deficient in the innate adaptor protein MyD88 are protected from diabetes development in non-germ-free conditions but the protection is abolished in germ-free conditions (9). Furthermore, introducing gut commensals into germ-free mice re-establishes the protection. This study supports the crucial role of environmental (TLR) stimuli in modulating the pathogenesis of diabetes through commensal bacteria.

CONCLUDING REMARKS

The important role of TLRs in the pathogenesis of T1D manifests mainly in their ability to induce APC maturation and to produce inflammatory chemokines and cytokines. These two features will contribute to the priming of autoreactive T-cells, which cause islet cell destruction. However, TLR signaling can also induce immune tolerance that results in diabetes prevention depending on the genetic background and the environment.

Increasing evidence suggests that TLRs also express on tissue cells including pancreatic beta cells. The role of TLRs on islet beta cells is largely unknown. Due to the complexity of the T1D pathogenesis, there is still no cure or ultimate prevention from the disease development. TLRs are critical modulators of islet-directed immune responses and are, therefore, important targets for anti-diabetogenic therapies. However, many functions of TLRs and causal relations are still unknown. Many questions must be answered before we can generate novel and effective therapeutic approaches that target TLRs in treating T1D. Since therapeutic targeting of TLRs can also increase the susceptibility toward infections, safety and efficacy have to be thoroughly balanced when modulating TLRs.

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TLR–CD40 cross-talk in anti-leishmanial immune response

Himanshu Singh Chandel[1†], Surya Prakash Pandey[1†], Sayoni Roy[1], Noelle Doyen[2] and Bhaskar Saha[1]*

[1] *National Centre for Cell Science, Pune, India*
[2] *Pasteur Institute, Paris, France*
**Correspondence: sahab@nccs.res.in*
[†] *Himanshu Singh Chandel and Surya Prakash Pandey have contributed equally to this work.*

Edited by:
Christophe M. Filippi, Genomics Institute of the Novartis Research Foundation, USA

Reviewed by:
Lauren A. Zenewicz, The University of Oklahoma Health Sciences Center, USA
Hans Acha-Orbea, Center for Immunity and Infection Lausanne, Switzerland

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Toll-like receptors (TLRs) recognize pathogen-associated molecular patterns (PAMPs) and activate innate immune cells to induce cytokines and co-stimulatory molecules such as CD40 and to enhance antigen presentation to T cells (1) that, upon activation, can either eliminate or support the pathogen (2). Herein, we propose that this duality in TLR functions results from their cross-talk with CD40. While all TLRs enhance CD40 expression, CD40 augments the expression of only TLR9 (3). As both CD40 and TLR9 induce expression of IL-12, a cytokine that induces the IFN-γ secreting Th1 cell differentiation (4), the CD40–TLR9 cross-regulation implies a positive feedback loop. By contrast, TLR1–TLR2 heterodimer down-regulates TLR9 expression (5) and antagonizes the development of Th1 response but favors the differentiation of regulatory T (T-reg) cells (Pandey et al., unpublished observation). Low CD40 expression levels in dendritic cells also promote T-reg cell differentiation (6). This duality can emerge from the sharing of signaling molecules. CD40 induces TRAF6-mediated, ERK-1/2-dependent IL-10 (7), which can inhibit the TLR-induced p38-MAPK activation and IL-12 production, antagonizing Th1 development. CD40-induced TRAF3-dependent p38-MAPK activation (7) can synergize with the TLR-activated p38-MAPK-dependent IL-12 production and Th1 differentiation. Using *Leishmania* infection, we show that the TLR–CD40 cross-talk can induce contrasting anti-leishmanial immune responses.

Leishmania, a protozoan parasite, lives in macrophages. *Leishmania* expresses lipophosphoglycan (LPG), proteoglycans, flagellin, and profilin for possible recognition by the host cell-expressed TLRs. Recognition of the *Leishmania*-expressed PAMPs results in differential immune responses, which can either reduce or exacerbate *Leishmania* infection. As TLRs modulate the expression of CD40, a co-stimulatory molecule whose expression levels modulate anti-leishmanial T cell responses, we propose that TLR–CD40 cross-talk significantly regulate the outcome of an anti-leishmanial immune response.

TOLL-LIKE RECEPTORS PRESENT SIGNIFICANT DIVERSITY TO IMMUNOREGULATION

A pathogen is perceived as a "danger" when specific molecular patterns associated with it [PAMPs or damage-associated molecular patterns (DAMPs)] are recognized by a set of TLRs, the mammalian homologs of toll, the anti-fungal resistance-mediating receptor in *Drosophila* (8, 9). Of the 13 TLRs, TLR10 is not expressed in mice whereas TLR11, TLR12, and TLR13 are absent from human (10). The extracellular domain of TLRs contains leucine-rich repeats (LRRs) arranged in an alpha-helix and a beta-pleated sheet. The LRR-rich loops impart the flexibility to this domain required for accommodating wide variety of chemically different PAMPs (11). The intracellular C-terminal domain has a toll/interleukin-1 receptor motif responsible for TLR signaling (12). Some TLRs – TLR1, TLR2, TLR4, TLR5, TLR6, TLR10, TLR11, TLR12 g – are located on cell surface to recognize the PAMPs on pathogen surface. Other TLRs – TLR3, TLR7, TLR8, TLR9, TLR13 – are located intracellularly on endosomes, lysosomes, and endoplasmic reticulum (13) to recognize the nucleic acids from the degraded pathogen (14). Thus, PAMPs on pathogen surface are first recognized by the TLRs on host cell surface. Once the pathogen is internalized and degraded, the released nucleic acids are recognized by the intracellular TLRs.

Recognition of the PAMPs by the TLRs on host cell surface triggers intracellular signaling that may result in one of the two contrasting outcomes (**Figure 1**, top panel). The *Leishmania major* parasites that express low levels of LPG are unable to reduce TLR9 expression and are eliminated by the macrophages (5). By contrast, the virulent parasites express higher levels of LPG, reduce TLR9 expression, and survive in macrophages (5). The lipoprotein analogs with modified acylations are preferentially recognized by TLR1 (15). Thus, pathogens may modify PAMPs that differentially bind to the TLRs on host cell surface and signal to modulate the expression and function of intracellular TLRs. Differential signaling may result in either elimination or growth of the intracellular pathogen. Isolation of different strains expressing modified PAMPs and assessments of immune response to those modified PAMPs are required to verify this hypothesis.

CD40 PLAYS DUAL IMMUNOREGULATORY ROLES IN *LEISHMANIA* INFECTION

CD40 is expressed on macrophages, dendritic cells, inflamed tissue histiocytes, and endothelial cells (16). CD40 signals though NF-κB to regulate the production of IL-12 (17), a pro-inflammatory cytokine required for Th1 differentiation (4). IL-4 is the cytokine that is required for the

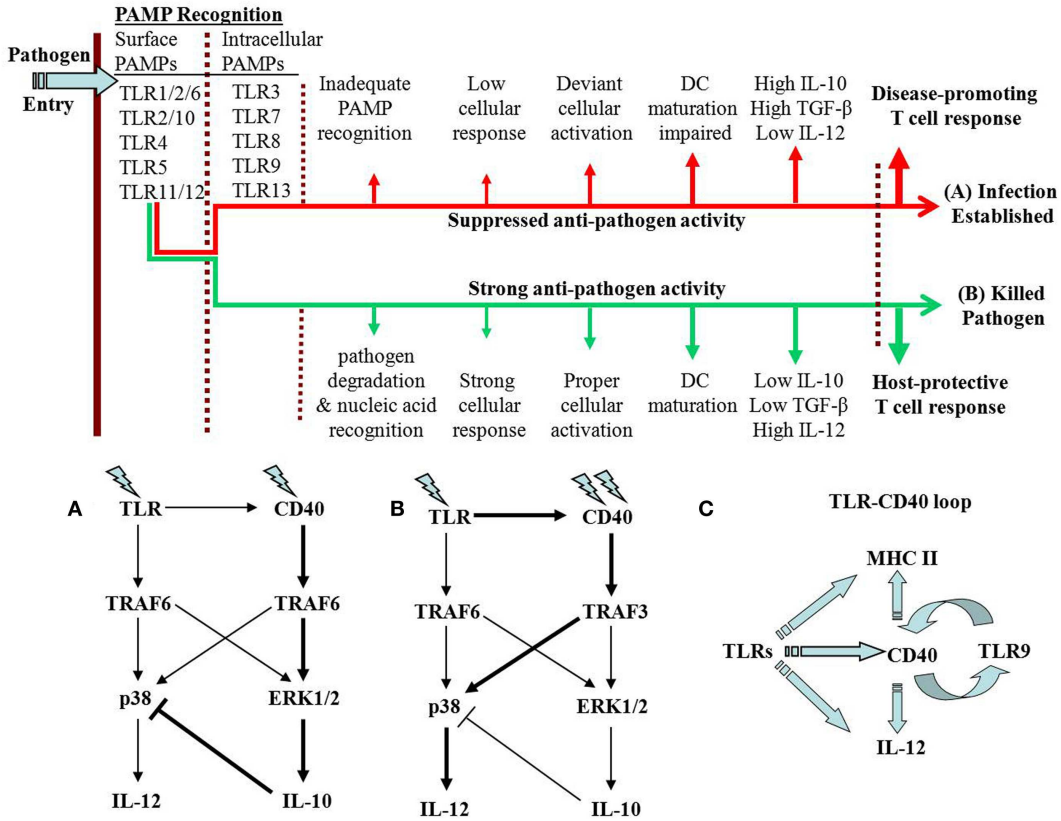

FIGURE 1 | TLR–CD40 cross-talk may result in one of two alternative possibilities. As the pathogen enters its host, it is recognized by cell surface TLRs. Depending on the PAMP–TLR interaction, the TLR may trigger signals with one of two possible fates of the pathogen. **(A)** In case of anti-inflammatory responses, the pathogen degradation inside the cell is impaired leading to less release of the pathogen nucleic acids. As a result, the intracellular TLRs are not optimally activated. The immune response against the pathogen is suppressed causing establishment of the infection. In case of *Leishmania* infection, CD40 signaling through p38-MAPK is suppressed. **(B)** Alternatively, where the signaling results in pro-inflammatory response, the intracellular pathogen is degraded and the intracellular TLRs are optimally activated. In *L. major* infection, the host-protective function of CD40 involves strong p38-MAPK activation with resultant IL-12-dependent Th1 response. The thicker arrows in **(A,B)** represent the dominant signaling. **(C)** Possible feedback loops are proposed. TLRs increase CD40 expression but CD40 enhances the expression of only TLR9, an intracellular TLR that recognizes CpG motifs in pathogen DNA. This can be viewed as a positive feedback loop for enhancing IL-12 production and Th1 response. The other arm of the loop is the TLR-activated MHC-II expression, which is linked to CD40 expression and DC maturation. This is also required for a stronger and prolonged immune response against a pathogen.

differentiation of Th2 cells, which produce IL-4, IL-5, and IL-13 (18). As a function of the strength of its stimulation, CD40 induces ERK-1/2-dependent IL-10 production (7). IL-10 expressed under MHC class-II promoter but not under IL-2 promoter aggravated *Leishmania* infection suggesting that the macrophage or the dendritic cell expressed IL-10 inhibited Th1 response (19). In *L. major* infection, the fate of the parasite is determined not only by Th1/Th2 balance but also by T-reg cells (6, 20–22) that produce IL-10, inhibiting Th1 differentiation but promoting infection (20–22). While low levels of CD40 expression on dendritic cells are required for T-reg cell expression (6), blockade of CD40–CD40L interaction on myeloid-derived suppressor cells (MDSCs) suppresses expansion of T-reg cells (23) suggesting CD40-induced dual regulation of T-reg cells.

It is shown that in response to higher doses of its ligand, CD40 signals from the cholesterol-rich domain through lyn, PKC-β, and p38-MAPK to induce IL-12 production whereas in response to lower doses of the ligand, the same receptor signals from the cholesterol-poor domain through syk, PKC-ζ, and ERK-1/2 to induce IL-10 production; ERK-1/2 inhibition results in enhanced activation of p38-MAPK and vice versa (7, 24–26). CD40 signals reciprocally through a bimodularly arranged cascade of kinases, wherein the reciprocity is incorporated by two feedback loops between p38-MAPK and syk and between ERK-1/2 and lyn (26). Thus, although the mechanism of the duality in CD40 functions is established, how CD40 regulates the contrasting fates of T-reg cells remains to be elucidated.

FUNCTIONAL PLASTICITY IN TLRs

Toll-like receptors bind to their respective ligands and dimerize before recruiting the adaptor molecules – MyD88, TIRAP/MAL, TRIF, and TRAM. MyD88 and TIRAP/MAL belong to the MyD88-dependent pathway and signal through NF-κB. TRIF and TRAM constitute the

MyD88-independent pathway. Only TLR3 signals through MyD88-independent pathway and only TLR4 signals through both pathways (27). TRAF6 is another adaptor that mediates the TLR signals (27). The signals finally converge on MAPKs and activate different transcription factors that effectuate the gene expressions (27). TLRs are differentially involved in T cell activation and T-reg cell development. For example, the T cell-expressed TLR4 promotes the suppressive function of T-reg cells whereas TLR6 abrogates its suppressive function (28). Thus, TLR4 and TLR6 act antagonistically to each other in regulating T-reg cell functions. By contrast, TLR2 alone plays contradictory roles in T-reg cell expansion and in its suppressive function (29–31).

Among the TLRs, TLR2 represents a unique receptor, as it heterodimerizes with TLR1 or TLR6 or with TLR10, in human (32). The heterodimers broaden the repertoire of PAMPs recognized and may elicit different effector functions, which can even be counteractive. Some TLR2 ligands – arabinosylated lipoarabinomannan and lipoteichoic acid – induce pro-inflammatory responses (33, 34) but LPG, another TLR2 ligand, induces anti-inflammatory responses (5, 35, 36). The difference may result from the nature of the heterodimers recognizing the PAMPs. The TLR1/TLR2 heterodimer induces pro-inflammatory response whereas the TLR2/TLR6 heterodimer induces anti-inflammatory response or *vice versa* [(37); Pandey et al., unpublished results]. Besides forming heterodimers, TLR2 may form homodimer too. For example, SitC, a triacylated lipoprotein from *Staphylococcus aureus*, can induce cytokine response in the TLR1/TLR6-deficient macrophages (38). Although TLR2–TLR10 heterodimerization is a theoretical possibility, it appears unlikely because neither mice nor macrophages express TLR10. Although TLR1 and TLR6 cannot possibly recognize ligands or trigger signals on their own, their relative levels of expressions in a cell can determine the constitution of the predominant TLR2 heterodimer. The increased TLR2 expression in *L. major*-infected macrophages promotes TLR2 homo-dimerization, which is accentuated due to reduced TLR2–TLR6 association (Pandey et al., unpublished observation). In this case, because TLR2 homodimers are predominant and recruit primarily MyD88, TLR1–TLR2 and TLR2–TLR6 heterodimers may not be able to recruit enough MyD88. As the MAPKs and the transcription factors mediate TLR signaling (3, 39–41), the specificity, amplitude, and nature of the response will thus depend on the relative usage of these signaling intermediates. Thus, the plasticity in the TLR2-mediated recognition of PAMPs and elicitation of immune responses depend on the variations in the chemical structures of PAMPs, nature of TLR–PAMP interaction, recruitment of adaptor molecules, and competition between the TLRs for the available adaptor molecules.

TLR AND CD40 CROSS-TALK DETERMINES THE NATURE OF IMMUNE RESPONSES

The response to an infection starts with the recognition of the PAMPs, perhaps, by multiple TLRs in tandem. Given the wide variety of PAMPs they recognize, the most probable TLRs to operate in tandem are TLR1, TLR2, TLR6, TLR10, and TLR4. In case of flagellated pathogens, TLR5 may recognize flagellin. The advantage of simultaneous trigger from TLR2 and TLR3 or TLR4 is that both MyD88-dependent and MyD88-independent pathways are involved increasing the overall strength and repertoire of TLR-derived signals. The combinations of TLRs may thus decide the nature of the signal and final effector functions (42) such as CD40 expressions that link the innate immune response to the adaptive immune response.

In peritoneal macrophages, CD40 expression in response to poly-I:C, LPS, and CpG, the TLR3, TLR4, and TLR9 ligands, respectively, is substantially enhanced, whereas CD40 stimulation enhances the expression of only TLR9 (3). *L. major* DNA induces IL-12 through TLR9 (43). CpG and CD40-ligand induced more IL-12 production from macrophages (3) and splenic dendritic cells (44) than that induced by either agent alone. On the other hand, low strength CD40 signal may synergize with the signal from TLR1–TLR2 heterodimers to strongly induce IL-10, which can inhibit p38-MAPK activation (**Figure 1A**). The CD40-induced IL-10 self-limits the CD40-induced p38-MAPK activation and anti-leishmanial functions (24). A possible feedback that comes into play in this CD40–TLR synergy is the quenching of TRAF6 availability to CD40 to result in less CD40-induced IL-10 production and relieving the autocrine IL-10 mediated inhibition of CD40-induced p38-MAPK activation and IL-12 production. Alternatively, exhaustion of TRAF6 by simultaneous signaling by multiple TLRs may divert a strong CD40 signaling primarily through TRAF3 to result in p38-MAPK activation and IL-12 production (**Figure 1B**). Thus, the enhanced IL-12 production as a result of TLR9 and CD40 synergy may represent a positive feedback loop between TLR9 and CD40 (**Figure 1C**). These reports imply that the TLR–CD40 cross-talk modulates the ensuing adaptive immune response.

Several reports support that TLRs can modulate CD40-mediated activation of adaptive immune system. PAMPs induce DC maturation by up-regulating MHC-II, CD40, and CD80/CD86 expressions (45) that are required for robust T cell responses. Because the binding of intracellular MHC-II with Btk via CD40 is required for sustained TLR activation, MHC-II deficiency impaired the TLR-induced production of pro-inflammatory cytokines and type-I interferon in macrophages and DC (46). CpG supported the survival and maturation of human plasmacytoid DC and, in synergy with CD40, induced T cells polarization to Th1 cells (47). Combined stimulation through TLR7 and CD40-induced $CD8^+$ T cells expansion more than that observed with either agent alone (48). These reports indicate that CD40 and TLRs synergize to affect DC maturation, activation, survival, antigen presentation, and differentiation of $CD4^+$ and $CD8^+$ T cells.

TLR–CD40 CROSS-TALK AS A NEW PARADIGM FOR IMMUNOREGULATION

The TLR–CD40 cross-talk exemplifies that one of the fundamental physiological principles of maintaining homeostasis is the plasticity in receiving and processing signals. The signals from TLRs and CD40 modulate each other's expression. Both receptors possess signaling plasticity modulating a range of effector functions (**Figure 1**) that affect both innate and adaptive immune systems. As pathogens sequentially involve cell surface and intracellular TLRs, the collective TLR

activation or inhibition determines the CD40 expression levels. These evidences prompt a new model for the evolution of immune response. According to this model, TLR activation influences CD40 expression and signaling, resulting in both TLR and CD40 simultaneously signaling in the later phase of PAMP-induced innate immune response. As CD40 enhances TLR9 expression, TLR9, perhaps, through induction of IL-12 or further increase in CD40 expression, may further modulate the T cell response. Thus, a continued feedback between the TLR and CD40 during an immune response may finally decide the outcome of an infection. However, further verification of this model awaits detailed investigation.

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