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Session 5: Early programming of the immune system and the role of nutrition
Role of Toll-like receptors in the development of immunotolerance mediated by probiotics

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Commensal bacteria are important in intestinal homeostasis and appear to play a role in early tolerance to foreign antigens. The requirement for homeostatic balance between tolerance and immunity poses a unique regulatory challenge to mucosal immune systems. Dysregulation of this balance can contribute to the pathogenesis of numerous inflammatory conditions such as inflammatory bowel diseases. The primary response to these bacteria is triggered by pattern recognition receptors (PRR), which bind pathogen-associated molecular patterns (PAMP). PRR comprise Toll-like receptors (TLR), nucleotide-binding oligomerization domains, adhesion molecules and lectins. Probiotics are living commensal micro-organisms of the intestinal tract with clinically documented health effects in human subjects. They are known to affect the gastrointestinal tract and the associated immune system and to have numerous effects on intestinal function and immune responses, including immunotolerance. This last effect appears to be mediated via regulatory T-cell activation by intestinal dendritic cells and the low activation of T-helper 1 and 2 (Th1 and Th2) cell inflammatory responses. However, the precise mechanisms of probiotic activity remain poorly understood. The aim of the present work was to review the function of TLR in the development of immunotolerance and examine the specific role of probiotics in the regulation of tolerance to antigens.

Toll-like receptor: Dendritic cells: Immunotolerance: Probiotics

A central property of the mammalian immune system is its ability to generate appropriate immunity against a given pathogen while maintaining tolerance to self-tissues[1]. Immunotolerance, also known as immunological tolerance or immune tolerance, is defined as a mechanism by which the immune system prevents pathological autoreactivity against self-antigens, thereby preventing autoimmune diseases[2].

Most human pathogens enter the body through a mucosal surface, e.g. in the intestine. Indeed, the intestinal immune system is the largest and most complex part of the immune system[3]. In addition to its constant exposure to dietary and environmental antigens, the adult human intestine is home to a huge number of commensal bacteria[4]. The means by which the host distinguishes between commensal and non-pathogenic bacteria is still not well understood. Dogi *et al*. demonstrated that commensal and non-commensal bacteria have a similar capacity to interact with the gut[5]. In a situation of constant immunological stimulation, the preservation of a homeostatic balance between tolerance and immunity poses a unique regulatory challenge to mucosal immune systems. Dysregulation of this balance can contribute to the pathogenesis of numerous inflammatory conditions, including food allergies, inflammatory bowel diseases and intestinal cancer[4]. It has been shown that intestinal homeostasis is regulated by a crosstalk

Abbreviations: INF, interferon; IRAK, IL receptor-associated kinase; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; PAMP, pathogen-associated molecular patterns; PRR, pattern recognition receptors; TGF, transforming growth factor; Th, Treg and Teff, helper, regulatory and effector T-cells respectively; TIR, Toll-IL-receptor; TLR, Toll-like receptors.

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between enterocytes and immune cells, especially with dendritic cells[6].

Dietary antigens are degraded by the time they reach the small intestine, but the degradation is partial and some antigens are absorbed, especially when large doses of antigen are received[2]. Thus, the gastrointestinal tract is constantly in contact not only with microbial agents from the microbiota but also with food antigens and other molecules. The inflammatory response in the intestinal tract is abrogated or avoided by the complex and well-regulated tolerance-inducing mechanisms present in the gut-associated lymphoid tissue[7]. Several cells capable of antigen presentation exist in the GALT, including macrophages, M-cells, DCs, B-cells and ECs. DCs have been shown to be one of the major intestinal antigen-presenting cells[2].

The immune system can be divided between the innate and adaptive systems. The adaptive immune response depends on B- and T-lymphocytes, which are specific for particular antigens. In contrast, the innate immune system responds to common structures, called pathogen-associated molecular patterns (PAMP), shared by the vast majority of pathogens. The primary response to pathogens is triggered by pattern recognition receptors (PRR), which bind PAMP. PRR comprise Toll-like receptors (TLR), nucleotide-binding oligomerization domains, adhesion molecules and lectins[8]. TLR recognise specific microbial components and induce the production of T-helper (Th)1 cytokines through a process dependent on the NF-κB pathway[9].

TLR have been implicated in the development of T regulatory (Treg) cell responses and immunotolerance[1]. Commensal bacteria are important in intestinal homeostasis, and appear to play a role in early tolerance to foreign antigens. The addition, to food, of living probiotic bacteria capable of colonising the intestine may also contribute to the development of immunotolerance.

The aim of the present study was to review the function of TLR in the development of immunotolerance and examine the specific role of probiotics in the regulation of tolerance to antigens.

DCs

DCs are a family of bone marrow-derived antigen-presenting cells that are uniquely capable of inducing the differentiation of naive T-cells (Th0 cells). Microbes activate DCs directly via their PRR or indirectly, such as by the capture of apoptotic/necrotic products of other cells dying in response to microbial exposure. Microbes can also induce a wide repertoire of cells (e.g. ECs, fibroblasts and innate immune system cells) to secrete cytokines that can activate DCs[10]. DCs are thought to be critical in the 'decision' to mount a tolerant or protective immune response.

In human subjects, two major subsets of DCs have been described, myeloid and plasmacytoid DCs. Myeloid DCs express all TLR except TLR7 and 9, and plasmacytoid DCs express several TLR, such as TLR1, TLR6, TLR7 and TLR9[1,10,11]. The intestine and associated lymphoid tissues are home to an extensive network of innate immune cells, including $CD11c^{hi}$ DCs and plasmacytoid DCs. Various subpopulations of DCs are present in the organised lymphoid structures of the intestinal immune system (e.g. Peyer's patches and mesenteric lymph nodes) and throughout the small intestinal and colonic lamina propria. DCs are frequently classified into subsets on the basis of cell surface receptor expression. The myeloid DCs $CD11c^{hi}CD11b^{+}CD8\alpha^{-}$, $CD11c^{hi}CD11b^{-}CD8\alpha^{+}$ and $CD11c^{hi}CD11b^{-}CD8\alpha^{-}$ and the plasmacytoid DC $CD11c^{int}$ are present in Peyer's patches and mesenteric lymph nides. Mesenteric lymph nides contain migratory DCs arriving from the intestinal lamina propria in the steady state and resident DCs developed from blood-borne precursors[12,13].

DCs can acquire antigens that have been transported across the intestinal epithelium via various different routes. Thus, specialised M-cells that are present in the follicle-associated epithelium of Peyer's patches can transcytose luminal antigens, which are then taken up by nearby DCs. Moreover, antigens can be transported into the intestinal lamina propria via a mechanism involving the neonatal Fc receptor for IgG. Finally, DCs can also sample antigens directly from the intestinal lumen by forming tight-junction-like structures with ECs and projecting dendrites through the epithelial-cell layer and into the lumen. It is possible that this last process contributes to the sampling of antigen from the commensal microbiota, since DC extensions are readily detected under normal conditions[12].

Upon activation, DCs up-regulate co-stimulatory molecules and migrate to secondary lymphoid organs (i.e. spleen and lymphoid nodes), where they activate antigen-specific T-cells[10]. The types of cytokines and other factors secreted by DCs and other innate immune cells programme the differentiation of naive Th0 into Th1, Th2 or Th17 effector cells or Treg cells[1].

Th1 immune responses critically depend on the ability of DCs to produce IL-12 and are characterised by the production of interferon (INF)-γ and IL-2, which induce cell-mediated immunity. Th2 immune responses involve IL-4, IL-5, IL-6 and IL-13 and induce humoral immunity[14]. Th17 cells are induced by IL-6 plus transforming growth factor-β (TGF-β) and secrete large amounts of IL-17 and IL-12[7]. Treg cells originating from the thymus are characterised by the expression of Foxp3 as a key transcription factor for their development and function, and they have a pivotal role in maintaining immunological self-tolerance[15]. One of the most extensively studied mechanisms for the induction of Treg cells by DCs is the release of IL-10 or TGF-β, resulting in Th1 and Th3 regulatory T-cells, which in turn also secrete IL-10 and TGF-β, respectively[14]; IL-10 suppresses Th1 and Th2 immune responses, while TGF-β antagonises Th1- and Th2-type inflammatory responses[14,16] (Fig. 1).

Pattern recognition receptors: Toll-like receptors

TLR are transmembrane proteins expressed on various immune and non-immune cells, such as B-cells, natural killer cells, DCs, macrophages, fibroblast cells, epithelial cells and endothelial cells[17]. They are members of a

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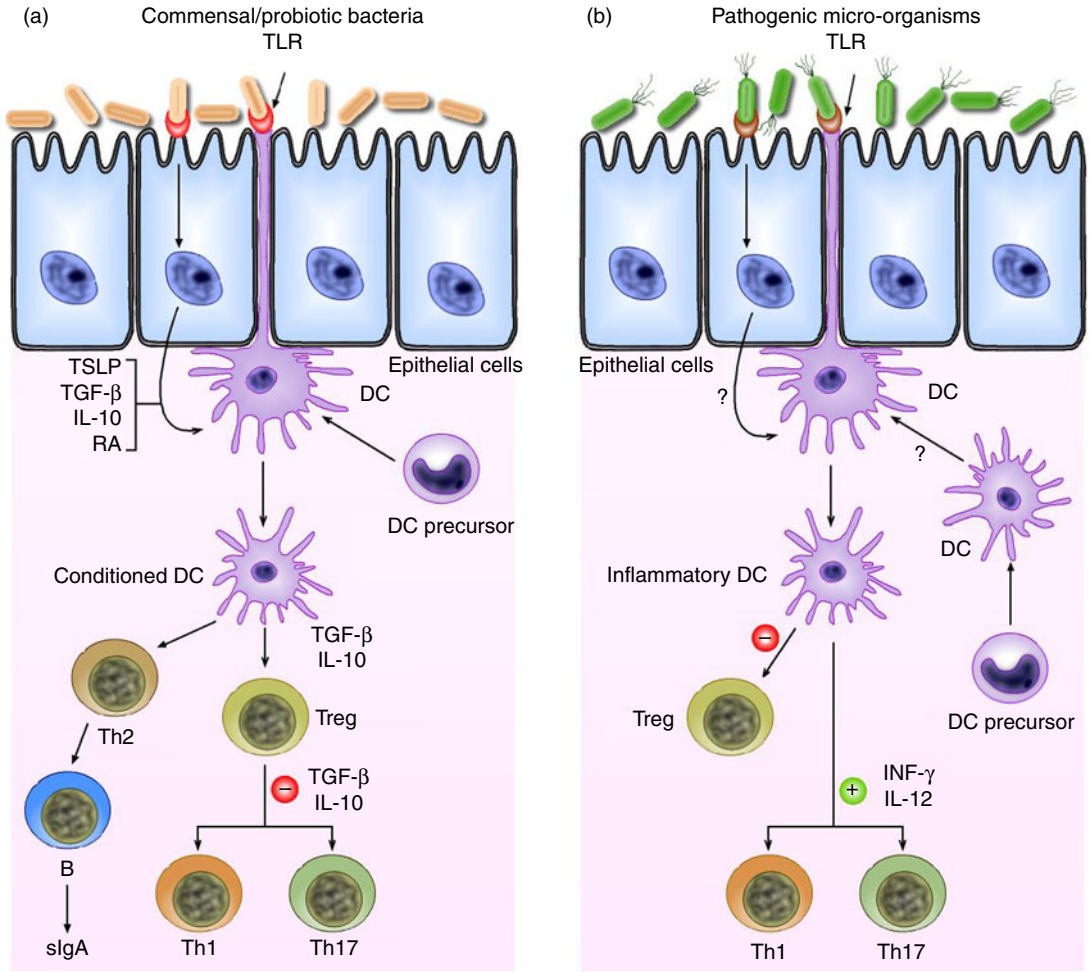

Fig. 1. Schematic view of the potential mechanism of action by which commensal bacteria and pathogenic bacteria interact with Toll-like receptors (TLR) and elicit different immune responses. (a) Commensal and probiotic bacteria interact with intestinal epithelial-cell barrier and dendritic cells (DC) resident in the intestine. Some cytokines, including IL-10, transforming growth factor (TGF)-β and thymic stromal lymphopoietin (TSLP), are expressed in intestinal epithelial cells, as a result of their interactions. Stimulation of cell TLR mediated by bacteria leads to up-regulation of TGF-β and IL-10, which in turn may limit the responsiveness of intestinal DCs resulting in the expansion and/or survival of T-cells with regulatory capacities, and limiting the ability of driving Th1, Th2 and Th17-cell responses. (b) Pathogenic bacteria have virulence factors that interact with intestinal epithelial-cell barrier and DCs resident in the intestine. Invasion of epithelium and direct interaction with DCs lead to activation of TLR and enhanced production of pro-inflammatory cytokines including interferon (INF)-γ and IL-12, which are capable of driving Th1, Th2 and Th17 response. RA, retinoic acid; sIgA, secreted Ig A; Th, T helper cell; Treg, T regulatory cell.

family of evolutionary conserved PRR that recognise a wide range of microbial components[1].

TLR were first identified in *Drosophila* in association with dorsal–ventral embryonic polarity[18], followed by a recognition of sequence similarity between the cytoplasmatic portion of Toll and that of signalling IL-1 receptor (IL-1R) components (TIR module)[19]. In mammals, the TLR family includes 11 proteins (TLR1–TLR11), although there is a stop codon in the human TLR11 gene, which results in lack of production of human TLR11[20]. All are single-spanning membrane proteins: the extracellular domain is composed of leucine-rich repeats, and the cytoplasmatic domain is defined by a TIR motif[21]. The TLR differ from one another in the cell types on which they are expressed, their ligand specificity, the signalling adaptors they utilise and the cellular responses they induce[22].

Activation of TLR occurs after binding of the ligand to extracellular leucine-rich repeats. In human subjects, TLR1, 2, 4, 5, 6 and 10 are outer-membrane associated and primarily respond to bacterial surface-associated PAMP. TLR3, 7, 8 and 9 are found on the surface of endosomes, where they respond primarily to nucleic acid-based PAMP from viruses and bacteria (Table 1)[1,21]. TLR expression has been detected in many types of immune cells. Importantly, TLR expression is related to the functional states of different subtypes of T-cells[23].

Interaction of a TLR (all TLR except TLR3) with its specific ligand leads to the recruitment of intracellular toll-IL-1 receptor (TIR) domain-containing adaptors such as MyD88 (myeloid differentiation primary response gene (88)), TIRAP (TIR domain-containing adaptor protein), TRIF (toll receptor – IL-1 receptor factor) or TICAM 1

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Table 1. Expression pattern of Toll-like receptors (TLR) in different immune cells and their main pathogen derived activators

TLR	PAMP	Immune cells*
TLR 1/TLR 2	Triacyl lipopeptides	Most cell types including DCs and C-cells
TLR 2	Lipoproteins, PGN, zymosan Lipoarabinomannan, porins	Peripheral mononuclear leucocytes, DCs, monocytes and T-cells
TLR 3	DsRNA	DCs, NK cells and T-cells
TLR 4	LPS, Hsp70	Macrophages, DCs and T-cells
TLR 5	Flagellin	Monocytes, DCs, NK cells and T-cells
TLR 6/TLR 2	Diacyl lipopeptides	High expression in B-cells and DCs, low in monocytes and NK cells
TLR 7	ssRNA	B-cells, DCs, monocytes and T-cells
TLR 8	ssRNA	Monocytes, DCs, NK cells and T-cells
TLR 9	CpG DNA	DCs, B-cells, peripheral mononuclear leucocytes, macrophages, NK and microglial cells
TLR 10	Unknown	B-cells, DCs, monocytes and T-cells

*Based on Liu & Zhao[23].
PAMP, pathogen-associated molecular patterns; CpG DNA, unmethylated CpG DNA sequences; LPS, lipopolysaccharide; PGN, peptidoglycans; NK, natural killer.

(TIR domain-containing adaptor molecule 1) and TICAM 2 (toll-like receptor adaptor molecule 2) through TIR–TIR interactions. Uniquely, TLR4 employs all four adaptors. Another adaptor, SARM (sterile alpha and TIR motif containing 1), which negatively regulates TRIF, was recently reported in human cells. These interactions result in the recruitment of the IL receptor-associated kinase (IRAK) family (IRAK1, IRAK2, IRAK4 and IRAK-M) and TNF receptor-associated factor 6 (TRAF6) to the receptor complex. This leads to the activation of mitogen-activated protein kinases (MAPK) (ERK-MAPK 1, JNK-MAPK 8 and p38-MAPK 14) and transcription factors (NF-κB and AP-1 (jun oncogene)) that are critical for the induction of various inflammatory and anti-inflammatory cytokines. Furthermore, TLR-mediated signalling was shown to control DC maturation, inducing the up-regulation of various maturation markers such as CD80, CD83 and CD86 and the chemokine receptor CCR7[1,24].

Toll-like receptors 2, 1 and 6

As a general rule, TLR2 recognises PAMP from Gram-positive bacteria (e.g. lipoproteins, lipopeptides, peptidoglycans and lipoteichoic acid) as well as lipoarabinomannan from mycobacteria, phenol-soluble modulin from *Staphylococcus*, zymosan from fungi and glycosylphosphatidylinositol from *Trypanosoma cruzi* and complete pathogens, including *Chlamydia* and viruses such as herpes simplex virus[17,25]. The diversity of ligand recognition by TLR2 is possible because TLR2 forms a heterodimeric complex with TLR1 or TLR6. The TLR1–TLR2 complex recognises bacterial triacylated lipopeptides and is important in the response to *Neisseria meningitides*, while the TLR6–TLR2 complex recognises bacterial diacylated lipopeptides and is critical in the response to *Staphylococcus aureus*[26]. The importance of the cell surface expression of TLR2, 1 and 6 for their function is underscored by the association observed between the inability of TLR1 to traffic to the cell surface and impaired innate immune function[27].

Toll-like receptor 4

TLR4 is the main receptor for lipopolysaccharide (LPS). The first genetic evidence of the importance of TLR4 in innate immune response was reported in C3H/HeJ and C57BL/10SCr mice strains, which harbour a mutation in *Tlr4*[28]. Likewise, two mutations affecting the extracellular domain of TLR4 receptor (D299G and T399I) have been associated with differences in LPS responsiveness in human subjects[29].

TLR4 is the main and probably the only receptor for LPS. However, TLR4 recognises other molecules from different origins, including some agonists derived from plants. TLR4 also recognises taxol, a well-known anticancer drug and a respiratory syncytial virus fusion protein. Furthermore, TLR4 is activated by endogenous ligands, such as heat shock proteins 60 and 70, fibronectin, hyaluronic acid, fibrinogen and heparin sulphate[25]. Expression of human TLR4 is restricted to a small number of cell types, including endothelial cells, B-cells and predominantly myeloid cells (monocytes, macrophages, DCs and granulocytes)[30].

LPS-mediated TLR4 signalling involves four proteins. LPS first binds to serum LPS-binding protein, which transfers an LPS monomer to CD14, a glycosylphosphatidylinositol-anchored cell surface receptor that also exists as a serum protein. In turn, the CD14–LPS complex activates TLR4 and MD2 (a small cysteine-rich glycoprotein that binds to the ectodomain of TLR4 in the endoplasmic reticulum) and then transits to the cell surface in an active TLR4–MD2 complex. Both proteins (MD2 and TLR4) are required for normal responsiveness to LPS *in vitro* and *in vivo*[31,32]. It is noteworthy that the activity of TLR4 may be modulated by MD2 protein levels. Several studies have shown that exogenously added soluble MD2 can bind to TLR4 and enable LPS-dependent stimulation of epithelial cells that express TLR4 but not MD2[32,33].

Furthermore, *in vitro* and *in vivo* experiments have shown that exposure of cells to LPS induces tolerance toward a second exposure to LPS and induces cross-tolerance to some other TLR ligands. In this context, de Vos *et al.* investigated whether *in vivo* exposure of human subjects to

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LPS induces tolerance in circulating leucocytes to other TLR agonists that rely on MyD88-dependent or MyD88-independent signalling. Analysis of TNFα, IL-1β, IL-6 and IL-10 levels in whole blood demonstrated that leucocytes were hyporesponsive to *ex vivo* LPS stimulation. Reduced cytokine release was also observed in whole blood further stimulated with MyD88-dependent ligands for TLR2, TLR5 and TLR7 or with whole bacteria. These data indicate that systemic LPS challenge of human volunteers induces cross-tolerance to multiple TLR ligands that signal in a MyD88-dependent or MyD88-independent manner, suggesting that LPS exposure of human blood leucocytes may hamper the inflammatory response to various microbial components[34].

Toll-like receptor 9

In 2000, Hemmi *et al*. showed that unmethylated CpG dinucleotide sequences in the flanking regions of bacterial DNA and their flanking regions activate mouse immune cells via TLR9, and that TLR9 is required for CpG sequences to induce monocytes and DCs to produce the IL-12 involved in Th1-cell activation[35].

TLR9 is widely reported to be a receptor for bacterial DNA but has also been implicated in the recognition of viral DNA[36], and it is now evident that mammalian DNA can be an effective TLR9 ligand. However, the DNA sequence required for TLR9 activation is controversial, since studies have published conflicting results on the nature of the DNA backbone and the route of DNA uptake[37]. TLR9 expression was preferentially detected in immune cell-rich tissue, including spleen, lymph nodes, bone marrow and peripheral blood leucocytes[38,39]. TLR9 is localised to the endoplasmic reticulum of DCs and macrophages. CpG DNA binds directly to TLR9 in ligand-binding studies; CpG DNA enters DCs in vesicular structures and moves into a tubular lysosomal compartment. Concurrent with the movement of CpG DNA in cells, TLR9 redistributes from the endoplasmic reticulum to CpG DNA-containing structures, which consequently also accumulate MyD88[40]. Thus, while ligand recognition occurs in endolysosomes, it has been reported that most if not all TLR9 resides in the endoplasmic reticulum of resting cells[41]. However, the specific details of how TLR9 transport is regulated between these compartments are not fully understood.

Likewise, TLR9 activation through apical and basolateral surfaces activates different intracellular signalling pathways in polarised ECs. Whereas basolateral TLR9 triggers IκBα (NF-κB inhibitor alpha) degradation and NF-κB pathway activation, apical TLR9 induces cytoplasmatic accumulation of ubiquitinated IκB and inhibition of NF-κB activation. The finding that apical TLR9 stimulation appears to confer tolerance to subsequent TLR challenge suggests that TLR9 plays an important role in maintaining intestinal homeostasis[42].

Furthermore, it has been shown that the ectodomain of TLR9 is cleaved in the endolysosome, such that no full-length protein is detectable in the compartment in which ligands are recognised. Notably, although the full-length and cleaved forms of TLR9 are capable of binding ligand, only the processed form recruits MyD88 upon activation, indicating that this truncated receptor, rather than the full-length form, is functional. This proteolytic regulatory step is consistent with a model in which TLR involved in nucleic acid sensing are translated as 'pro-receptors' in the endoplasmic reticulum and only function after being processed in the endolysosomal compartment. Moreover, it has been proposed that ectodomain cleavage represents a strategy to ensure proper self-/non-self-discrimination based on nucleic acid recognition[41].

Other Toll-like receptors (5, 3, 7 and 8)

TLR5 recognises bacterial flagellin, a principal component of Gram-positive and Gram-negative bacterial flagella, and the expression of TLR5 induces NF-κB activation and TNFα production[43].

Although it is well established that TLR5 recognises bacterial flagellin, it has been identified as an essential sensor for cytoplasmic flagellin. TLR5 activates NF-κB and MAPK, leading to the secretion of multiple cytokines (e.g. IL-6, IL-12 and TNFα), whereas cytoplasmic flagellin permits the activation of caspase-1 and the secretion of mature IL-1β[44,45].

TLR3, TLR7 and TLR8 are located in the intracellular endosomal compartment, where they sense microbial nucleic acids such as RNA and DNA[20]. TLR3 recognises the viral dsRNA and dsRNA produced during the replication of ssRNA viruses. TLR3 is expressed in the endosomes of immune cells (e.g. DCs, B-cells, natural killer cells and non-immune cells) and epithelial cells. However, TLR3 is not expressed on plasmacytoid DCs. Therefore, the role of TLR3 in viral infection is unclear[17].

TLR7 and TLR8 recognise viral and non-viral ssRNA and activate cytokine production through IFN regulatory factor 3 and 7. TLR7 is highly expressed on plasmacytoid DCs and TLR8 on myeloid DCs. TLR7 responds to ssRNA by producing INF type I and pro-inflammatory cytokines. TLR7 and TLR8 also respond to synthetic antiviral imidazoquinoline compounds such as R848, loxoribine and imiquimod and to ssRNA rich in virus-derived guanosine or uridine[1,17].

Toll-like receptors, probiotics and immunotolerance

Pre-industrialised areas and rural populations appear relatively protected from allergic disease. The hygiene hypothesis ascribes this protection to the effects of microbes and microbial products. Building on this concept, substantial research efforts are concentrated on probiotics[46].

The concept that intestinal microbiota can be positively modulated by the administration of bacteria was proposed by Metchnikoff in 1901. Probiotics are live commensal micro-organisms of the intestinal tract and are defined as living bacteria preparations with clinically documented health effects in human subjects[47].

The vast majority of probiotic bacteria are Gram-positive strains, mainly species of the *Lactobacillus* and *Bifidobacterium* genera, although *Lactococcus*, *Streptococcus*

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and *Enterococcus* species, as well as some non-pathogenic strains of *Escherichia coli* and certain yeast strains, are also qualified as probiotic[14,48]. They are known to affect the gastrointestinal tract and the gut-associated lymphoid tissue and have numerous effects on intestinal function and immune responses, including effects on DC function; skewing of T-cells towards Th1 polarization; competitive exclusion of pathogens; and suppression of intestinal inflammation by down-regulation of TLR expression and secretion of metabolites that may inhibit TNFα from blood mononuclear cells and by inhibition of NF-κB signalling in ECs. However, the mechanisms of probiotic activity remain poorly understood[47,48].

There is evidence that probiotic micro-organisms preferentially elicit Th3/Treg cells and appear to induce an anti-inflammatory response, mainly via interaction with TL9. Rachmilewits *et al*. showed that intragastric and subcutaneous administration of probiotic *E. coli* (strain DH5-α) DNA ameliorated the severity of colitis in a murine experimental colitis model (dextran sodium sulphate-induced colitis), whereas methylated probiotic DNA, calf thymus DNA and DNase-treated probiotics had no effect. They also found that the intragastric administration of γ-irradiated probiotics significantly decreased the severity of dextran sodium sulphate-induced colitis in TLR2- and TLR4-deficient mice, but had no effect in TLR9-deficient mice. Hence, they concluded that the protective effects of probiotics are mediated by their own DNA rather than by their metabolites or ability to colonise the colon, and that TLR9 signalling is essential to mediate the anti-inflammatory effect of probiotics[49].

The binding of natural commensal-origin DNA to apical TLR9 initiates an intracellular signalling cascade that is subsequently associated with attenuation of TNFα-induced NF-κB activation and NF-κB-mediated IL-8 expression. Ghadimi *et al*., using polarised HT-29 and T84 cell monolayers, demonstrated that apically applied DNA of *Lactobacillus rhamnosus* GG (a human commensal and probiotic bacteria) attenuated TNFα-enhanced NF-κB activity by reducing IκBα degradation and p38-MAPK phosphorylation[50].

In this context, Hall *et al*. found that gut flora DNA plays a major role in intestinal homeostasis through TLR9 engagement. *Tlr9*$^{-/-}$ mice displayed increased frequencies of CD4+Foxp3+ Treg cells at intestinal effector sites and reduced constitutive IL-17- and INF-γ-producing effector T (Teff) cells. In addition, gut flora DNA limited lamina propria DC-induced Treg cell conversion *in vitro*. Further, Treg/Teff cell disequilibrium in *Tlr9*-deficient mice led to impaired immune responses to oral infection and oral vaccination[51].

Nevertheless, *in vitro* assays by Vinderola *et al*. demonstrated that the interaction of the probiotic strain *Lactobacillus casei* CRL 431 with epithelial cells is mediated through TLR2[52]. As a consequence of these results, the authors studied the expression of two receptors (CD-206 and TLR2) present on the surface of macrophages and DCs and the effect of orally administered *L. casei* CRL 431 on this expression, using BALB/c mice as a model. They showed that the interaction between *L. casei* and gut-associated immune cells induced an increase in the number of CD-206 and TLR2 receptors, mainly in the cells involved in the innate immune response[53].

Grabig *et al*. studied TLR2 and TLR4 knockout mice and demonstrated that *E. coli* Nissle 1917 fails to improve colitis or modulate cytokine production in comparison with wild-type mice[54]. Likewise, Hoarau *et al*. reported that a fermentation product from *Bifidobacterium breve* C50 could induce maturation, high IL-10 production and prolonged survival of DCs via the TLR2 pathway[55]. Lee *et al*. demonstrated that the *Lactabacillus suntoryeus* HY7801 may be able to improve colitis via the inhibition of TLR4-linked NF-κB activation and harmful enzyme production of intestinal bacteria[56]. Likewise, the probiotic VSL-3 mixture reduces the severity of dextran sodium sulphate-induced colitis but not in the TLR9-deficient mouse. Therefore, different probiotic bacteria stimulate distinct TLR[57].

Additionally, Miettinen *et al*. characterised TLR gene expression in response to *L. rhamnosus* GG and *Streptococcus pyogenes* (an important human pathogen) in human primary macrophages. They observed that *L. rhamnosus* GG and *S. pyogenes* enhanced TLR2 expression in macrophages and also required TLR2 for NF-κB activation, but only *S. pyogenes* was able to up-regulate TLR3 and TLR7 gene expression. This up-regulation was dependent on INF-α/β. They therefore suggested that macrophages can discriminate between probiotic and pathogenic bacteria by INF-mediated TLR gene regulation[58].

TLR expressed by DCs (mainly TLR9, but also TLR4 and TLR2, among others) are engaged by commensal species following projection of DC dendrites across the epithelial-cell layer or following M-cell-mediated translocation of commensal bacteria into Peyer's patches and their subsequent uptake by DCs[59]. As a result, these DCs become conditioned and initiate appropriate responses upon contact with commensal microbiota, such as the differentiation of Treg, Th2 and IgA-secreting B-cells[12] (Fig. 1).

The functional properties of intestinal DCs are altered by factors present in the local environment. Activation of NF-κB expression in intestinal epithelial cells, perhaps as a result of commensal microbiota signalling via PRR, enhances the production of thymic stromal lymphopoietin. This and other epithelial cell-derived factors can act on DCs to down-regulate IL-12/23p40 production in response to bacterial stimulation. DCs conditioned in this manner preferentially drive classical Th2-type responses[60,61]. IL-10 and TGF-β may also have a role in limiting the responsiveness of intestinal DCs to bacterial or other activation signals (Fig. 1). These cytokines may derive from multiple sources, although an autocrine effect of TGF-β may be produced by DCs in response to epithelial cell-derived signals, including retinoic acid[12,62].

Coombes *et al*. speculated that there may be constitutive low-level recruitment of DCs in the steady state from blood precursors that would be capable of driving Th1- or Th17-cell responses. These DCs may either act as sentinels for the presence of pathogenic species or constitutively initiate cell-mediated immune responses against the commensal microbiota to ensure that it is kept under control. In contrast to the commensal microbiota, some pathogenic

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species possess virulence factors that allow them to invade the intestinal epithelium and subvert immune responses in order to enhance their replication. Invasion of the epithelium leads to activation of cytosolic PRR and enhanced production of chemokines and pro-inflammatory cytokines. Neutrophils, macrophages and DC precursors are recruited to the site and become activated by a combination of signals from pathogens and pro-inflammatory cytokines and chemokines. Whether these DC precursors also give rise to the populations of DCs present in the steady state remains unclear. Although DCs resident in the tissues before infection may not take on pro-inflammatory functions, it is possible that their ability to promote Treg-cell differentiation may be impeded[12] (Fig. 1).

While intestinal DCs are clearly involved in the generation of active immune responses against pathogens/antigens, commensal micro-organisms are able to activate DC-dependent immune regulatory mechanisms, generating low-level immune responses aimed at controlling normal microbiota without causing disease[12] (Fig. 1).

Another possible mechanism that can induce tolerance is through the negative regulation of TLR. Many factors are known to have the ability to attenuate or abrogate TLR signalling, but the role of many of these has not yet been characterised in the intestine. Only six inhibitors of TLR have been identified in the gastrointestinal tract to date: peroxisome proliferator-activated receptor-γ, A20 (a cytoplasmic zinc finger protein), NOD2 (a cytoplasmic protein with a leucine-rich-repeat domain), IRAK-M, SIGIRR (single Ig and TIR domain) and Tollip (toll interacting protein). These molecules, which have been shown to regulate intestinal homeostasis, appear to exert their effect through TLR2, TLR3 and TLR4[63].

In this regard, repeated stimulation of TLR4 in human subjects has been postulated as a protective mechanism to limit excessive inflammation and prevent septic shock. Several *in vitro* studies have shown that repeated stimulation of TLR induces unresponsiveness to the same TLR ligand in cell lines, B-cells and plasmacytoid DCs[64]. It has therefore been suggested that the continuous presence of specific bacterial components results in a state of hyporesponsiveness in otherwise reactive ECs. Down-regulation of TLR surface expression and up-regulation of inhibitory Tollip with decreased phosphorylation of IRAK might all contribute to this hyporesponsiveness[65]. Likewise, transfection of Tollip in ECs resulted in decreased responsiveness to stimulation with LPS and lipoteichoic acid. These cells continued to show normal reactivity to TNF-stimulation, suggesting that Tollip is involved in endotoxin tolerance in a TLR-specific manner. However, it has been suggested that Tollip controls the magnitude of the inflammatory cytokine production response to IL-1β and LPS[63].

Conclusion

We are developing a better understanding of how commensal/probiotic micro-organisms can create an overall tolerant state mediated by the action of TLR on DCs. It is clear that TLR9 signalling is essential to mediate the anti-inflammatory effect of probiotics. However, different studies have implicated other TLR such as TLR3 and TLR7 in the tolerance induced by commensal and probiotic bacteria. After activation by commensal and probiotic micro-organisms, DCs initiate appropriate responses such as the differentiation of Th0 to Treg, which has an inhibitory effect on Th1, Th2 and Th17 inflammatory responses.

TLR on DCs are also implicated in the generation of protective immune responses against pathogens inducing pro-inflammatory cytokines such as IL-12. While there is substantial evidence from *in vitro* and animal studies that known and potential probiotics have strain-specific immunomodulatory capacities, the results of human intervention trials have been far less convincing. One potential explanation might be that the composition of intestinal microbiota is likely to vary to a much greater extent among individual human subjects than among individual mice kept in the same environment and fed the same diet. Genetic differences in the expression of PRR and other factors contributing to the response to bacterial signals are also likely to contribute to the variability in responses to probiotic treatment[14]. Hence, further research is required to study the effect that specific probiotics exert on the immune system in human DCs, animal models and, finally, in human intervention studies.

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