Anti-inflammatory potential of probiotics: lipoteichoic acid makes a difference

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Lipoteichoic acid (LTA) mutants of lactobacilli suppress inflammation in animal models of experimental colitis. The fact that a single mutation of an administered *Lactobacillus* strain can result in enhanced probiotic efficacy is surprising given the genetic diversity and complexity of the intestinal niche, but at the same time exciting from a microbiological, immunological and gastroenterological point of view. In this Opinion article, we discuss the possible impacts of LTA modification in probiotic bacteria in the context of the current knowledge regarding the proinflammatory capacity of LTA, structure–activity relationships of LTA, intestinal LTA recognition in healthy and colitis conditions and anti-inflammatory molecules of lactobacilli.

Probiotics and immunomodulation

The immunomodulatory potential of probiotic bacteria is increasingly of interest for prophylactic and therapeutic options in various complex disorders, ranging from diarrhea to allergy [1]. However, the clinical studies are not unambiguously positive. In addition, many hypotheses on the molecular mechanisms of probiotics are poorly supported by experimental data. A better understanding of these mechanisms will help to interpret disparate clinical data and assist the selection of the best probiotic strains for new clinical studies targeting particular applications [1]. Recent studies with probiotic mutants lacking or modifying putative effector molecules [2–4], as well as studies with purified putative effector molecules [5], are important steps forward.

Many beneficial effects of probiotics originate in the gastrointestinal tract (GIT). Cells that are in direct contact with GIT microbes, such as dendritic cells (DCs) and intestinal epithelial cells (IECs), have various pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs) [6]. PRRs recognize conserved microbe-associated molecular patterns (MAMPs), which can occur in bacterial surface molecules such as lipopolysaccharides (LPS), flagellin, lipoteichoic acid (LTA), peptidoglycan, but also in bacterial DNA. The interaction between a MAMP and PRR results in a signaling cascade that ultimately mounts a molecular response such as cytokine expression, mucin production in IECs, or molecules for antigen presentation

in DCs. Cytokine expression by IECs and DCs skew T cell polarization, differentiation and proliferation and thus govern the overall mucosal immune response to specific MAMPs, both at the time and in future encounters. The response of a specific host cell against encountered microbes is not determined by a single MAMP–PRR interaction, but by multiple MAMP–PRR interactions [6]. Because probiotic bacteria contain various MAMPs, their use may modulate and tailor these MAMP–PRR interactions [2–4,7,8].

Exciting results with *Lactobacillus* LTA mutants in experimental colitis

An attractive application of probiotics is in the treatment of patients with inflammatory bowel diseases (IBD), such as Crohn's disease and ulcerative colitis. The pathogenesis of these diseases is not fully understood, but involves an aberrant mucosal immune response against the GIT microbiota in genetically susceptible hosts [9]. Recently, Mohamadadeh *et al.* showed that modification of the cell surface of the probiotic *Lactobacillus acidophilus* NCFM by a single gene mutation confers improved anti-inflammatory properties to this strain in mouse models for IBD [2]. These findings strengthen previous observations in *Lactobacillus plantarum* [3] and *Lactobacillus rhamnosus* [4]. All three studies have targeted LTA, a key surface molecule of Gram-positive bacteria [10]. Complete removal of LTA in *L. acidophilus* NCFM [2], removal of the D-alanyl (D-ala) substitutions in *L. rhamnosus* GG [4] or a shift from mainly D-ala to glucosyl substitutions in *L. plantarum* NCIMB8826 [3] resulted in strains capable of more efficiently alleviating inflammation. In *L. acidophilus*, the LTA-negative mutant downregulated the expression of the proinflammatory cytokines interleukin-12 (IL-12) and tumor necrosis factor (TNF) and enhanced IL-10 in DCs compared to wild type. Moreover, treatment of mice with the mutant compared to wild type prevented and alleviated dextran sulfate sodium (DSS)-induced colitis and prevented CD4⁺CD45RB^{high}T cell mediated colitis via induction of IL-10 and regulatory T cells [2]. The *dlt*– mutant of *L. plantarum*, showing a replacement of D-ala with glucose substitutions, was also shown to reduce the secretion of IL-12, TNF and interferon- γ (IFN- γ) and enhance IL-10 in monocytes compared to its corresponding wild type. *In vivo*, the *dlt*– mutant was significantly more

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protective compared to wild type in a trinitrobenzenesulfonic acid (TNBS)-induced model of colitis [3]. Importantly, this *dlt*[−] mutant also inhibited visceral pain perception in healthy non-inflamed rats in contrast to wild type [11]. For *L. rhamnosus* GG, the *dltD* mutant, lacking D-ala substitutions on LTA, improved colitis compared to wild type in DSS-induced colitis, which correlated with a downregulation of the expression of the IL-12, IFN- γ and TLR2. Remarkably, the wild type appeared even detrimental upon severe inflammation [4], in agreement with previous observations [12]. Taken together, these studies highlight a crucial role for LTA modification in the probiotic efficacy of lactobacilli in experimental colitis.

Proinflammatory capacity of LTA

LTA is an important proinflammatory molecule of Gram-positive bacteria and, as such, LTA mutants should be investigated for probiotic therapy. The presence of such a proinflammatory molecule is suggested to be an undesirable characteristic for a probiotic bacterium, especially for applications in IBD. LTA is considered as the equivalent of Gram-negative LPS because both can trigger the production of proinflammatory cytokines, nitric oxide (NO), the activation of nuclear transcription factor NF- κ B and other proinflammatory mediators in a variety of cell types [13,10]. However, whereas the bio-activity of LPS is well recognized and observed in the nanomolar range, the activity of LTA frequently requires micromolar concentrations and is the subject of intense debate, partly due to the differences in isolation protocols resulting in different contaminants and loss of bio-active residues [13]. Although LPS and LTA show structural relatedness (macroamphiphile, presence of phosphate groups and acyl chains), they are structurally quite different from each other. These structural differences result in different PRR interactions. Although the lipid A fraction of most LPS molecules is recognized by TLR4 and MD2, with LPS-binding protein and CD14 as important coreceptors [14], most LTA molecules are ligands for TLR2/TLR6, with CD14 and CD36 as coreceptors [15]. Other important PRRs for LTA are the macrophage type I scavenger receptor [16] and the platelet-activating factor (PAF) receptor, which detects LTA and signals via transactivation of the epidermal growth factor receptor, resulting, for example, in mucin production in epithelial cells [17]. Furthermore, DC-SIGN and other lectin PRRs can detect glycan substituents in LTA [18], but they are less studied.

Structure–activity relationships of LTA

Teichoic acids are generally classified into either wall teichoic acids (WTA), covalently attached to peptidoglycan, and LTAs, attached to the cell membrane. Because WTAs lack a lipid part, they seem to be much less immunostimulatory than LTA, although detailed structure–activity relationship (SAR) studies have not yet been performed [18]. Here, we will focus on the current SAR knowledge of LTA. Although most LTA molecules have a similar basic structure, important strain-specific differences can occur. LTAs in the low-G+C subdivision of Gram-positive bacteria, such as the Firmicutes, consists of two fatty acid tails hydrophobically anchored in the cell membrane and a long

Box 1. Lipoglycans in Actinobacteria

In the high-G+C subdivision of Gram-positive bacteria, such as the Actinobacteria, LTAs are largely replaced by lipoglycans, such as the well characterized lipomannans and lipoarabinomannans of mycobacteria. They are also anchored to the membrane by a diacylglycerol moiety, but contain a linear or branched polysaccharide as the hydrophilic moiety, which may become charged by substitution with monoglycerophosphate branches or succinyl esters [16]. In contrast to LTA, structural information on the lipoglycans is limited. Interestingly, some structural information exists for bifidobacterial strains, which are commonly used as probiotics. *Bifidobacterium bifidum* contains a macroamphiphile with single Gro-P units attached as monomeric side chains to the glycan backbone by phosphodiester linkages (Figure 1c). These single Gro-P units are substituted with L-ala instead of D-ala. The lipid anchor is most likely a galactosyldiacylglycerol, part of which carries a third fatty acid [52]. To our knowledge, their interaction with PRR such as TLR2 has not yet been investigated. Greenberg *et al.* showed that lipoglycans of *B. bifidum* show a similar strong binding capacity to macrophage scavenger receptor compared to LTA [16], but their proinflammatory cytokine-inducing capacities remain largely unexplored. Interestingly, probiotics of *Bifidobacterium* seem to be generally less proinflammatory than lactobacilli in their capacity to modulate host responses [53]. Bifidobacteria also occur in higher numbers in the human GIT as compared with lactobacilli (3% vs. <0.1%) [32], which is interesting in view of the 'stealth' hypothesis (Box 2).

charged hydrophilic chain with various spatial distributions and charges depending on the strain [19], whereas LTAs are largely replaced by lipoglycans in the Actinobacteria (Box 1). The hydrophilic region of many LTAs is composed of a 1,3-phosphodiester-linked polymer of glycerol-phosphate (Gro-P) variously substituted with D-ala or sugars (Figure 1a). The hydrophobic region of LTA is generally a glycolipid, often Glc(β 1-6)Glc(β 1-3)diacylglycerol [10].

Most SAR studies have been performed on LTA of *Staphylococcus aureus* and *Streptococcus* species because they can cause sepsis. Significant breakthroughs came with the chemical synthesis of these molecules because this minimizes the loss of bio-active substituents due to purification and the copurification of contaminants and allows SAR studies of different parts of the molecules [20]. *S. aureus* LTA is composed of poly-Gro-P, on average 30–50 residues, with D-ala and N-acetylglucosamine (GlcNAc) as major substituents. A synthetic analogue of this LTA, (D-ala)₄(GlcNAc)₁(Gro-P)₆gentiobiosyl-*sn*-dimyristoylglycerol, was shown to induce cytokine release with the same potency as native LTA [21,22]. For maximal activity, both the glycolipid and D-ala residues were required, whereas GlcNAc substituents could be omitted. Replacement of D-ala with L-ala resulted in a 100-fold decrease of activity [22].

Some SAR studies are also available on LTA molecules of lactobacilli. They generally possess a D-alanyl-poly(Gro-P) LTA in which the glycolipid anchor is composed of trihexosyl-diacylglycerol [10,23] (Figure 1b). LTAs of some lactobacilli have proinflammatory capacities. For example, LTA from *Lactobacillus casei* YIT9029 and *Lactobacillus fermentum* YIT0159 induce TNF expression in macrophages via TLR2 [24]. Although LTA of *L. plantarum* NCIMB8826 is a weaker inducer than LTA from *S. aureus*, it is also able to stimulate proinflammatory cytokines via TLR2 [3]. LTA isolated from the corresponding *dlt*[−] mutant only induced

Figure 1. Lipoteichoic acid (LTA) structure in comparison to bifidobacterial lipoglycocalactans. **(a)** Prototypical polyglycerophosphate LTA. In Type 1 LTA, the hydrophilic backbone is composed of repetitive glycerolphosphate (Gro-P) units and the lipophilic glycolipid is generally composed of Glc(β1-6)Glc(β1-3)diacylglycerol. Variations in the degree of substitution and poly-Gro-P chain length convey a microheterogeneous character to LTA [10]. Abbreviations: GlcNAc, N-acetylglucosamine; Glc, glucose; Gal, galactose. **(b)** Schematic representation of the structure of LTA from *L. rhamnosus* GG according to [45]. X = 74% D-ala, $n = 50$ (on average). The glycolipid anchor is composed of trihexosyl-diacylglycerol instead of typical diglucosyl-diacylglycerol. **(c)** Structure of the lipoglycans from various *Bifidobacterium* strains [52]. Values of n and m vary between 7 and 10 and between 8 and 15, respectively. Twenty to 50% of the mono-Gro-P residues are substituted with L-ala in an ester linkage. The lipid anchor is most likely a galactosyldiacylglycerol (with R_1 and R_2 as acyl chains), part of which contains a third fatty acid on the galactopyranosyl residue.

low levels of these proinflammatory cytokines. Because LTA from this mutant differs not only in D-ala residues from wild type, but also shows an increased number of glucose substituents and an increased length of the Gro-P backbone [25], the exact SAR of the modified LTA structure in the *dlt*-mutant remains to be unraveled. Recently, the LTA structure of *Lactococcus lactis* G121 was determined to be composed of a short Gro-P backbone (14–18 repeats), randomly substituted by D-ala and galactose, and a lipid anchor containing a heterogeneous mixture of different fatty acid compositions. This LTA induced IL-6 release from mononuclear cells, but did not activate TLR2 or TLR4 [26].

In contrast to proinflammatory activities, others have shown that LTA molecules of some lactobacilli are less immunostimulatory [27]. Compared to LTA from *S. aureus* and *Bacillus subtilis*, LTA from *L. plantarum* KCTC10887BP only scarcely induced NO in macrophages, showed a weaker activation of TLR2/CD14, and lower in-

duction of TNF in macrophages [27]. LTA from *L. plantarum* KCTC10887BP even acted as an antagonist of *S. aureus* LTA-induced TNF production in monocytic cells in a TLR2/CD14-dependent manner [28], of LPS-induced TNF production in monocytic cells and endotoxin shock in mice [29] and of *Shigella flexneri* peptidoglycan-induced inflammation in monocytic cells [30]. Recently, multi-spectrometric analyses of this *L. plantarum* LTA were compared with those of *S. aureus* LTA [31]. These analyses revealed that the major differences lie in the numbers (third acyl chain in *L. plantarum* LTA) and degree of saturation of the acyl chain in the glycolipid moiety (unsaturated fatty acids in *L. plantarum*), and the sugar substituents on the Gro-P chains (glucose and galactose sugars in *L. plantarum* versus GlcNAc in *S. aureus*), whereas they showed similar levels of D-ala.

Thus, SAR studies of LTA highlight that the lipid chain (number of acyl chains, degree of saturation) and substitutions on the Gro-P chains (D-ala or sugars) play a key role

in its pro-/anti-inflammatory activity, but many details remain to be unraveled on the SARs, including on the PRRs and associated coreceptors involved.

LTA recognition in the GIT

An important question arising from studies with *Lactobacillus* LTA mutants in experimental colitis is why LTA modification of probiotic microbes has such a major impact on their proinflammatory capacity, considering that the mammalian GIT is naturally exposed to a high number of Gram-positive microbes that also contain LTA and apparently do not cause inflammation. A possible explanation is that the LTA structures of the major Gram-positive gut members differ significantly from *Lactobacillus* LTA (Box 2). Most human and murine Gram-positive gut bacteria belong to *Firmicutes* followed by *Actinobacteria* [32]. Important genera are *Faecalibacterium*, *Clostridium*, *Eu-bacterium* (Firmicutes) and *Bifidobacterium* (Actinobacteria) [32]. *Lactobacilli* are not present in high numbers in the human GIT (less than 1%) [33]. Only very limited structural data on LTA or lipoglycans of the major gut bacteria are available. Therefore, studies on the structural diversity of LTA in the dominant microbiota species, and by extension GIT pathogens, will be highly relevant in the future.

Besides structural changes in the major intestinal MAMPs, other mechanisms are possibly involved in controlling the proinflammatory capacity of intestinal bacteria and their MAMPs [7,34]. IECs seem to have developed special mechanisms to tolerate the continuous presence of high numbers of MAMPs originating from commensals in the gut lumen. Cell culture experiments have shown that IECs express TLR2/6 and coreceptors at a limited level [35] and upregulate the expression of specific inhibitors of TLR signaling [36]. Whether IECs also acquire *in vivo* LTA tolerance immediately after birth as documented for LPS [37] remains to be substantiated. In addition, as the lipid chains of LTA are crucial for TLR2 interaction [38], these acyl chains must become exposed by shedding or lysis to induce signaling, thereby limiting the accessibility of these ligands for proinflammatory activities. The amount of free LTA in the GIT is not known, but it is hypothesized that LTA shed from bacteria is rapidly

degraded or inactivated by binding with soluble factors such as cationic peptides and immunoglobulin A (IgA) [39]. Furthermore, specific enzymes such as PAF-acetylhydrolase (PAF-AH) can be involved [40]. Because LTA and PAF both have a glycerol backbone with two lipid chains, Seo and Nahm [40] found that PAF-AH can also remove an acyl chain from LTA and inactivate it. The *in vivo* role of PAF-AH in the GIT remains to be demonstrated, but intestinally active PAF-AH reduced the incidence of necrotizing enterocolitis in an animal model [41]. The function of PAF-AH could be similar to intestinal alkaline phosphatase, which appears to inactivate the proinflammatory capacity of intestinal LPS [42]. Furthermore, production of acid by other microbes in the GIT could play a role in inactivating LTA because lactic acid has been shown to degrade LTA of *Streptococcus pyogenes* [43].

Phagocytic cells such as macrophages and DCs seem to have an increased capacity to respond to MAMPs compared to IECs [44]. In healthy subjects, DCs seem to be the most important phagocytes. Intestinal DCs can sample bacteria by passing dendrites between the IECs or sample in the dome of intestinal lymphoid follicles [39]. In colitis, where the epithelial barrier is disturbed and the number of macrophages is increased, bacteria show increased interaction with these cells, which could lead to a more proinflammatory LTA-driven response. Furthermore, many IBD patients and colitic animals show increased expression of PRRs [9]. Together with possible polymorphisms in PRRs such as NOD2, a cytoplasmic receptor for peptidoglycan, this suggests that MAMP-PRR interactions are different in colitis and more readily induce inflammation.

Taken together, LTA recognition in the GIT generally does not result in proinflammatory responses in healthy subjects due to various microbiota- and host-associated mechanisms, but could occasionally, such as in the case of colitis, result in inflammation and unbalanced immune responses.

Pleiotropic effects of LTA mutations

To better understand the effects of the *Lactobacillus* LTA mutants in experimental colitis models, it is important to realize that LTA mutations have strong pleiotropic effects on growth, cell morphology, cell division and cell wall organization [19]. The *L. rhamnosus* GG *dltD* mutant, which lacks D-ala residues on its LTA, shows, for example, an increased cell length, defects in septum formation and reduced gastric acidity tolerance [45]. The *L. plantarum* *dlt*- mutant also exhibits an increased cell length, defects in cell separation, increased autolysis and altered expression of peptidoglycan hydrolases [25]. Surprisingly, although LTA is almost completely absent in the *L. acidophilus* mutant, this mutant does not show a growth or morphological defect [2]. This is in contrast to other knockout mutants in the LTA polymerizing enzyme LtaS, which all show major defects [19]. However, *L. acidophilus* contains WTA, which shows some functionally similar roles in bacterial physiology [18]. Yet, the *L. acidophilus* LTA mutant did show reduced survival in simulated gastric and small intestinal juice, which could also impact on its performance in animal colitis models [2].

Box 2. The 'stealth' hypothesis

Various hypotheses exist on how the host immune system is able to discriminate between resident symbionts and ingested foreign microbes that could be potential pathogens. One hypothesis is that MAMPs from symbiotic microbes have undergone biochemical modifications to render them 'stealth' to PRRs [34]. This hypothesis is mainly based on the fact that the lipid A molecules of important members of the major gut Gram-negative Bacteroidetes phylum are tetra- or pentacylated, a form that is not agonistic and even antagonistic to the TLR4/CD14/MD2 complex [54]. Conversely, the lipid A molecules of Enterobacteriaceae, such as *Escherichia coli*, which are far less abundant, are generally hexacylated, and agonistic to TLR4 [14]. Recently, the lipid A phosphate position was also suggested to be an important factor in determining differential host TLR4 responses to phylogenetically-related symbiotic and pathogenic bacteria [55]. In agreement with this, intestinal alkaline phosphatase, which dephosphorylates and inactivates LPS, has been shown to play an important role in promoting mucosal tolerance to resident gut bacteria [42].

Anti-inflammatory *Lactobacillus* molecules

Taking only LTA into account does not fully explain the enhanced probiotic efficacy of the three *Lactobacillus* LTA mutants in experimental colitis. These mutants are not only less proinflammatory but also more anti-inflammatory, which suggests that they also exhibit enhanced expression of anti-inflammatory molecules. Given the pleiotropic effects of LTA mutations, this is not unlikely. The surface layer proteins of *L. acidophilus* are potential mediators of the anti-inflammatory effect [46]. The SlpA protein has been previously shown to regulate immature DC and T cell functions and have anti-inflammatory properties by interaction with the PRR DC-SIGN [8]. The *dltD* mutant of *L. rhamnosus* GG shows enhanced secretion of a soluble protein p40 [4], which can ameliorate inflammation in experimental colitis [5]. Whether this is indeed an important factor in the enhanced anti-inflammatory of the *dltD* mutant remains to be determined. Also, other *L. rhamnosus* GG molecules appear to have anti-inflammatory capacities. The exopolysaccharides play an immunomodulatory role by forming a shield against innate immune factors in the intestine [47], and might similarly be anti-inflammatory as reported for the cell wall polysaccharides of *L. casei* Shirota [48]. Moreover, because the *L. rhamnosus dltD* mutant [45] and *L. acidophilus ltaS* mutant [2] are more susceptible to the harsh conditions of the GIT, subsequent enhanced lysis could liberate bacterial DNA, which in the case of LGG has documented anti-inflammatory properties in IECs via interaction with TLR9 [49]. In addition, DNA of other probiotics was sufficient to mediate anti-inflammatory effects in murine colitis models via TLR9 [50].

Concluding remarks

Although various natural *Lactobacillus* strains show anti-inflammatory effects [51], three independent studies with different *Lactobacillus* strains have shown that LTA mutants can be superior in alleviating experimental colitis compared to wild type [2–4]. Given the microbial diversity and complexity of the intestinal niche, the fact that a single mutation of an administered *Lactobacillus* strain can result in enhanced probiotic efficacy is surprising and exciting from a microbiological, immunological and gastroenterological point of view. However, further questions need to be answered (Box 3). More SAR studies need to be established for LTA molecules of pathogens, commensals and probiotics. For instance, because different LTA mutants were used in

the different *Lactobacillus* strains, ranging from complete removal of LTA [2] over removal of D-ala [4] to replacement of D-ala with glucosyl substitutions [3], it is not yet clear what determines the exact proinflammatory nature of *Lactobacillus* LTA. In addition, the fact that LTA mutants show alleviation of colitis symptoms clearly indicates the existence of *Lactobacillus* molecules that can confer anti-inflammatory effects. The nature and specific importance of different molecules in the anti-colitis effects of these *Lactobacillus* strains requires further research. Nevertheless, recent studies highlight that the field of probiotics has entered an interesting era. The use of probiotic mutants carrying dedicated mutations helps our molecular understanding of how ingested probiotics interact with the host immune system. Mutants such as these enable studies on the role of probiotic molecules presented on the surface of the administered live bacterial cells. This study is an important step towards insights in the mode of action of probiotics with focus on the specificity of their cell surface properties. Hopefully, this can ultimately result in more efficient medical practices with well-selected probiotic strains.

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Box 3. Outstanding questions

- Is the biochemical composition of LTA a key determinant in the proinflammatory/anti-inflammatory potential of probiotics? In other words, is it possible to establish a SAR that will help to screen and select probiotics for specific use?
- Are bifidobacteria that lack LTA less proinflammatory and thus better suited as probiotics against IBD?
- Can the immunostimulatory capacity of *Lactobacillus* LTA also have beneficial applications, e.g. in allergy or as an adjuvant in vaccines?
- Does the GIT become tolerant to LTA from gut microbes early in the life of the individual? What happens when this tolerance is not properly induced? Does this play a role in the pathogenesis of IBD?
- Do tailored probiotic strains have clinical potential or are they only useful to validate proofs of concept?

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