Exploring the immunomodulatory potential ofmicrobial-associated molecular patterns derivedfrom the enteric bacterial microbiota

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*Abstract*

The human intestinal lumen represents one of the most densely populated microbial niches in the biological world and, as a result, the intestinal innate immune system exists in a constant state of stimulation. A key component in the innate defence system is the intestinal epithelial layer, which not only acts as a physical barrier, but also as an immune sensor. The expression of pattern recognition receptors, such as Toll-like receptors, in epithelial cells allows innate recognition of a wide range of highly conserved bacterial moieties, termed microbial-associated molecular patterns (MAMPs), from both pathogenic and non-pathogenic bacteria. To date, studies of epithelial immunity have largely concentrated on the inflammatory properties of pathogenic antigens; however, this review discusses the major types of MAMPs likely to be produced by the enteric bacterial microbiota and, using data from *in vitro* and animal model systems, speculates on their immunomodulatory potential.

*Introduction*

The intestine represents the body’s largest mucosal surface, with the adult human intestine estimated to cover an area of ~250 m2 ([Artis, 2008](#_ENREF_5)). The highly-folded luminal surface of the intestinal wall significantly increases absorption efficiency and, as such, represents the largest surface area of the body exposed to the environment and its high microbial load ([DeSesso & Jacobson, 2001](#_ENREF_28)). Humans have co-evolved with indigenous microbial populations, termed *microbiota*, which inhabit various niches of the body ([Hooper *et al.*, 2012](#_ENREF_57)) and the adult intestine represents one of the most densely populated microbial habitations in the biological world ([Artis, 2008](#_ENREF_5)). The enteric microbiota predominantly consists of bacteria, with estimated populations of ~1014 bacteria, with up to 500 species represented ([Gill et al., 2006](#_ENREF_41), [Guarner & Malagelada, 2003](#_ENREF_46)), however, methanogenic archaea, eukaryotes (yeasts) and viruses (mainly bacteriophages) are also present ([Lozupone *et al.*, 2012](#_ENREF_83)). Due to the vast expanse of intestinal tissue exposed to the microbiota, the local innate immune system is in a constant state of stimulation, and a chronic, low-level proinflammatory response is characteristic of enteric immune homeostasis ([Macpherson & Harris, 2004](#_ENREF_84), [Artis, 2008](#_ENREF_5)).

The intestinal epithelium plays an active role in the innate immunity and pattern recognition receptors (PRRs) are utilised to detect the presence of bacteria and their associated antigens. PRRs are germline-encoded, sensory molecules which recognise a range of highly conserved bacterial motifs, termed ‘pathogen-associated molecular patterns’ (PAMPs) ([Medzhitov, 2001](#_ENREF_90)). However, the ability of PRRs to recognise these bacterial moieties is not limited to just pathogens, and so the term ‘*microbial*-associated molecular patterns’ (MAMPs) may be more accurate ([Medzhitov, 2001](#_ENREF_90), [Sanderson & Walker, 2006](#_ENREF_105)) and will be used throughout this review. Epithelium-associated, enteric immune cells, such as macrophages, dendritic cells, T-cells and B-cells, differentially express two major groups of PRRs, the cell surface Toll-like receptors (TLRs) and the intracellular nucleotide-binding oligomerisation domain (NOD) receptors ([Hornung et al., 2002](#_ENREF_58), [Iwasaki & Medzhitov, 2004](#_ENREF_65), [Akira et al., 2006](#_ENREF_3)). Non-professional immune cells of the intestinal epithelium, such as enterocyte cells, also constitutively express the two groups of PRRs ([Furrie et al., 2005](#_ENREF_38), [Gribar et al., 2008](#_ENREF_45)), thus vastly enhancing the recognition of MAMPs.

TLRs are type I integral membrane glycoproteins found within the plasma and endosomal membranes of mammalian cells ([Takeda & Akira, 2005](#_ENREF_118)). TLRs consist of 3 distinct domains ([Botos *et al.*, 2011](#_ENREF_15)); a MAMP-binding extracellular domain, which contains a variable number of leucine-rich repeats (LRRs) ([Bell *et al.*, 2003](#_ENREF_10)); a transmembrane domain, which spans the host cell membrane, thus holding the receptor in place; and a cytoplasmic signalling domain, the Toll/IL-1R homology (TIR) domain, which is responsible for the intracellular transmission of the stimulatory signal ([Akira *et al.*, 2006](#_ENREF_3)). TLRs recognise a wide range of microbial moieties (see Table 1) and engagement by their respective ligand(s) triggers activation of intracellular signalling cascades leading to the induction of genes involved in anti-microbial host defence, such as those encoding proinflammatory cytokines and chemokines ([Aderem & Ulevitch, 2000](#_ENREF_1)).

NOD receptors are a group of cytoplasmic receptors which are important for the recognition of intracellular bacteria. The first NOD receptor identified, NOD-1, recognises a derivative of peptidoglycan, γ-D-glutamyl-meso-diaminopimelic acid (iE-DAP), found exclusively in Gram-negative bacteria ([Girardin *et al.*, 2003a](#_ENREF_42), [Chamaillard *et al.*, 2003](#_ENREF_23)). Subsequently, the structurally similar NOD-2 was identified and found to confer cell responsiveness to the minimal bioactive peptidoglycan motif, muramyl dipeptide (MDP), found in both Gram-positive and Gram-negative bacteria ([Girardin *et al.*, 2003b](#_ENREF_43), [Inohara *et al.*, 2003](#_ENREF_62)). Ligand binding to NOD-1 or NOD-2 leads to receptor oligomerisation, which induces the recruitment of the serine/threonine kinase Rip2/RICK ([Takeda & Akira, 2005](#_ENREF_118)). NOD-receptor-bound Rip2/RICK subsequently activates the NF-κB-mediated expression of proinflammatory cytokines ([Akira *et al.*, 2006](#_ENREF_3), [Masumoto *et al.*, 2006](#_ENREF_88)).

*Enteric-derived MAMPs and their immunomodulatory potential*

As mentioned previously, MAMPs constitute highly conserved microbial motifs and the following sections review those factors which are likely to be produced by the intestinal microbiota. Additionally, the potential immunomodulatory role of each MAMP is discussed.

### CpG-DNA

Bacterial DNA contains a ~20-fold greater frequency of unmethylated 2'–deoxyribo(cytidine-phosphate-guanine) (CpG) dinucleotides than vertebrate DNA ([Ewaschuk *et al.*, 2007](#_ENREF_35)), thus predisposing it to MAMP activity with mammalian host cells ([Bauer *et al.*, 2001](#_ENREF_8)). Methylated bacterial DNA loses its stimulatory potential ([Ewaschuk *et al.*, 2007](#_ENREF_35)), thus confirming that its MAMP activity is attributable the increased expression of unmethylated CpG motifs. Moreover, the stimulatory effects of bacterial DNA on mammalian immune cells, can be mimicked by CpG-containing synthetic oligodeoxynucleotides (CpG-ODNs) ([Dalpke *et al.*, 2006](#_ENREF_26)).

Hemmi *et al.* ([2000](#_ENREF_50)) demonstrated that Toll-like receptor (TLR)-9 confers responsiveness to bacterial DNA in host macrophages and B-cells, as their counterparts isolated from TLR-9-deficient mice were not susceptible to the physiological effects elicited by CpG-DNA. Human intestinal epithelial cell lines (HT29, Caco-2 and T84 cells) were subsequently shown to constitutively express TLR-9 mRNA, the up-regulation of which was stimulated by pathogenic CpG-DNA ([Akhtar *et al.*, 2003](#_ENREF_2)). Furthermore, Akhtar *et al.* ([2003](#_ENREF_2)) also showed an increased secretion of the proinflammatory IL-8, by intestinal epithelial cells, in response to CpG-DNA. Nevertheless, it was subsequently suggested by Dalpke *et al.* ([2006](#_ENREF_26)) that stimulation of TLR-9 would be difficult *in vivo*, thus limiting the physiological importance of TLR-9. However, their work was undertaken utilising the macrophage model, therefore, only intracellular TLR- 9 was considered. In stark contrast to this, Ewaschuk *et al.* ([2007](#_ENREF_35)) described an up-regulation of apical surface expression of TLR-9 protein in intestinal epithelial cells, in response to pathogenic *Salmonella enterica* DNA, thus suggesting sensitisation of the epithelial cells to further challenge by CpG-DNA. Intestinal epithelial cells constitutively express TLR-9 on their external surface and are responsive to CpG-DNA ([Akhtar et al., 2003](#_ENREF_2)), therefore, it is possible that commensal-derived DNA plays a role in homeostatic intestinal inflammation. This hypothesis is supported by the findings of a key study, by Rachmilewitz et al. ([2004](#_ENREF_97)), which demonstrated that the probiotic effects of the bacterial preparation, VSL#3, were mediated via a TLR-9 pathway. Additionally, a more recent study showed that TLR-9-deficient mice were more susceptible to experimental colitis (induced by administration of dextran sulfate sodium (DSS)), when compared to their wild-type counterparts ([Lee et al., 2006](#_ENREF_76)).

### Peptidoglycan

Peptidoglycan (PGN) is an essential cell wall component in virtually all bacteria and is particularly abundant in Gram-positives, where it accounts for 30-70 % of their cell wall mass ([Schleifer & Kandler, 1972](#_ENREF_106)). It is a mesh-like polymer consisting of *β*(1–4)-linked *N*-acetylglucosamine (NAG) and *N*-acetylmuramicacid (NAM), crosslinked by short peptides and is responsible for the maintenance of cell morphology and the resistance of osmotic pressure of bacterial cells ([Dziarski, 2003](#_ENREF_31)). As a consequence of its presence in virtually all bacterial, substantial abundance in Gram-positive bacteria and absence from eukaryotic cells, PGN presents a perfect target for the host innate immune system ([Dziarski, 2003](#_ENREF_31)). PGN is only released in relatively low amounts during mitotic division, however, it demonstrates potent immunological activity in mouse and human macrophages, subsequently stimulating the significant release of proinflammatory cytokines ([Schwandner *et al.*, 1999](#_ENREF_109), [Takeuchi *et al.*, 1999](#_ENREF_121), [Wang *et al.*, 2001](#_ENREF_130)). Consequently, Gram-positive pathogens demonstrate significantly increased release during infection ([Dziarski & Gupta, 2005](#_ENREF_32)).

Initially, it was commonly accepted that TLR-2 mediated cellular sensitivity to PGN in human macrophages ([Schwandner *et al.*, 1999](#_ENREF_109), [Takeuchi *et al.*, 1999](#_ENREF_121), [Wang *et al.*, 2001](#_ENREF_130)), and that responsiveness was enhanced by the co-receptor CD14 ([Schwandner *et al.*, 1999](#_ENREF_109), [Iwaki *et al.*, 2002](#_ENREF_64)), however, this proposed stimulatory pathway was subsequently challenged by Trovassos *et al.*, who claimed TLR-4, not TLR-2, conferred cellular responsiveness to purified PGN ([Trovassos *et al.*, 2004](#_ENREF_124)). Nevertheless, a re-evaluation of the phenomenon, by Dziarski and Gupta ([2005](#_ENREF_32)), conclusively demonstrated that TLR-2 *was* essential for the stimulation of macrophages by PGN, and suggested the results observed by Trovassos and colleagueswere due to the destructive and incomplete nature of the purification methods they used. Constitutive expression of TLR-2 mRNA has previously been observed in both *ex-vivo* colonic epithelial tissue and *in vitro* colonic epithelial cells lines (HT29, Caco-2 and T84 cells) ([Melmed *et al.*, 2003](#_ENREF_91), [Furrie *et al.*, 2005](#_ENREF_38)), thus suggesting the potential for intestinal immune modulation by microbiota-derived PGN. In addition, mutations in the *NOD-2* gene, the product of which confers host cell responsiveness to the PGN derivative, muramyl dipeptide (MDP), is strongly associated with the pathogenesis of Crohn's disease ([Hugot et al., 2001](#_ENREF_61), [Ogura et al., 2001](#_ENREF_92)), thus further strengthening the notion that peptidoglycan could potentially play a role in the intestinal homeostasis. More recently, a study by Macho Fernandez *et al*. (2011) indicated that PGN and its derived muropeptides are active in the probiotic functionality of *Lactobacillus salivarius* Ls33 and, therefore, might represent a useful therapeutic strategy in the treatment of IBD.

### Lipopolysaccharide (LPS)

LPS is an amphiphilic membrane phospholipid ([Fenton & Golenbeck, 1998](#_ENREF_36)) which is essential for cell viability and outer membrane permeability of Gram-negative bacteria ([Rietschel *et al.*, 1994](#_ENREF_101)). It also plays a key role in protection of the bacterium against host immune defences, enzymatic degradation and antibiotic attack ([Holst *et al.*, 1996](#_ENREF_55)). Since only the *Sphingomonas* genus is found to lack LPS ([Alexander & Rietschel, 2001](#_ENREF_4)), its ubiquitous expression in other Gram-negative bacteria presents the mammalian innate immune system with a major target ([Erridge *et al.*, 2002](#_ENREF_34)).

LPS is a glycolipid macromolecule consisting of three domains; the distal hydrophobic O-specific chains, or O-antigens, which extend from the bacterial surface; the interconnecting core region; and the hydrophobic lipid A region which acts as the membrane anchor ([Bishop, 2005](#_ENREF_13)). O-antigens present a major target for the host’s antibody response of the adaptive immune system as they represent the extreme outer limits of the bacterial cell ([Erridge *et al.*, 2002](#_ENREF_34)). Nevertheless, it is the glycolipid membrane anchor, lipid A, which represents the biologically active moiety of LPS, with both free and synthetic lipid A molecules shown to reproduce the effects of whole LPS ([Galanos *et al.*, 1985](#_ENREF_39)).

In a healthy individual, the basal systemic concentration of LPS in the human body can be in the range of 3-10 pg/ml ([Alexander & Rietschel, 2001](#_ENREF_4)). Accordingly, the innate immune system can detect and, indeed, degrade such low concentrations of LPS in a phenomenon known as ‘LPS tolerance’ ([Hoffman & Natanson, 1997](#_ENREF_54), [Ulevitch & Tobias, 1999](#_ENREF_125)), which has been shown to aid in the defence against subsequent bacterial invasion by the parent strain (Hoffman and Natanson, 1997). However, larger quantities of LPS, often released by cell lysis during infection ([Caroff & Karibian, 2003](#_ENREF_20)), can have a highly detrimental effect on the host, resulting in fever, increased heart rate, septic shock and, ultimately, death from multiple organ failure and systemic inflammatory response ([Hoffman & Natanson, 1997](#_ENREF_54), [Caroff & Karibian, 2003](#_ENREF_20)). It is noteworthy that LPSs do *not* elicit their toxic effect by the killing of host cells, or even by the inhibition of host cellular function, but they are wholly dependent on the active inflammatory responses of the host cells ([Rietschel *et al.*, 1994](#_ENREF_101)).

 The first stage in host recognition of LPS is the binding of the acute phase reactant, LPS-binding protein (LBP) ([Hailman *et al.*, 1994](#_ENREF_48)), which predominantly originates from the liver and freely circulates in the blood ([Fenton & Golenbeck, 1998](#_ENREF_36)). The main function of LBP is to opsonise and deliver LPS to CD14, with each LBP molecule chaperoning 10 LPS molecules to the receptor ([Hailman *et al.*, 1994](#_ENREF_48)). CD14 is a member of the toll-like receptor (TLR) family ([Triantafilou & Triantafilou, 2002](#_ENREF_123)), however, it does not possess a cytoplasmic domain, and therefore lacks the ability to activate a transmembrane activation signal ([Triantafilou & Triantafilou, 2002](#_ENREF_123)). This implied that another receptor conferred responsiveness to LPS. Poltorak *et al.* ([1998](#_ENREF_95)) suggested that Toll-like receptor (TLR-4) was responsible for LPS sensitivity, as they found the human *TLR-4* gene was homologous to the murine *Lps* gene, which was shown to control leukocyte response to LPS ([Linder *et al.*, 1988](#_ENREF_78)). This work was followed with a prominent study undertaken by Hoshino *et al.* ([1999](#_ENREF_59)) which demonstrated TLR-4 to be the translational product of the *Lps* gene. They also generated TLR-4-deficient mice which, consequently, lacked responsiveness to LPS ([Hoshino et al., 1999](#_ENREF_59)), thus going some way to confirming TLR-4 as the LPS receptor. Subsequently, there was some speculation that TLR-2 could also play a role in LPS responsiveness ([Kirschning et al., 1998](#_ENREF_71), [Yang et al., 1998](#_ENREF_133)), however, this was soon nullified when meticulous repurification of LPS, removing any lipoprotein contaminants, showed TLR-4 alone was responsible ([Hirschfield *et al.*, 2000](#_ENREF_53)). Nevertheless, it was found that TLR-4 does not work alone in LPS recognition. A co-factor, MD-2, was seen to associate with TLR-4, forming a receptor complex, which proceeds to induce a proinflammatory intracellular signal transduction cascade once the CD14-bound LPS is transmitted ([Shimazu et al., 1999](#_ENREF_112), [Heumann & Roger, 2002](#_ENREF_51)).

 The innate immune response to LPS is generally orchestrated by CD14-expressing immune cells such as macrophages, which react to the presence of LPS by producing proinflammatory cytokines such as TNF-α, IL-6 and IL-8 ([Guha & Mackman, 2001](#_ENREF_47)). However, CD14-deficient cells are also able to respond to LPS in the presence of serum ([Hailman *et al.*, 1994](#_ENREF_48)), and the intestinal epithelial cell lines HT29 and Caco-2 are sensitive (monitored via IL-8 expression) to pathogen-derived LPS in serum-containing media ([Schuerer-Maly et al., 1994](#_ENREF_108), [Smirnova et al., 2003](#_ENREF_113), [Huang et al., 2003](#_ENREF_60)), thus suggesting the potential for epithelial sensitivity to commensal-derived LPS moieties. In addition, mutations of the *TLR-4* gene have been implicated in the pathogenesis of IBD ([Franchimont et al., 2004](#_ENREF_37), [Oostenburg et al., 2005](#_ENREF_93)), further demonstrating a possible role for commensal-derived LPSs in intestinal homeostasis. A recent study suggested a proinflammatory role for commensal-derived LPSs, inducing cytokine (IL-1 β) and chemokine (IL-18) release during chronic stress in rats (induced by electric shock) ([Maslanik et al., 2012](#_ENREF_87)); however, there is currently no information (to the authors’ knowledge) on the homeostatic immunomodulatory potential of LPS’s derived from the enteric microbiota.

### Lipoprotein

Lipoproteins (LPs) are proteins which contain lipid moieties covalently bound to an N-terminal cysteine residue ([Braun & Wu, 1994](#_ENREF_16)). They represent a key component in the outer membrane of Gram-negative bacteria, particularly in members of the *Enterbacteriaceae* family, such as *E. coli*, which naturally secrete them, in low levels, into the surrounding media ([Zhang *et al.*, 1998](#_ENREF_135)). LPs are also present, albeit it in much more limited quantities, in the cell wall of Gram-positives ([Sutcliffe & Russell, 1995](#_ENREF_117)).

Brightbill *et al.* ([1999](#_ENREF_17)) elucidated that host cellular responsiveness to bacterial lipoproteins in human macrophages is mediated via TLR-2. This was later confirmed by Wang *et al.* ([2002](#_ENREF_129)) who demonstrated that pre-treatment of human monocytes with low concentrations of LP imparts a TLR-2 ‘tolerance’ that protects against subsequent treatment with higher concentrations of LPs. However, it was later discovered that TLR-2 actually forms a heterodimer with TLR-1 to confer cell responsiveness to bacterial LPs in murine macrophages ([Takeuchi *et al.*, 2002](#_ENREF_120)). Spirochetal LPs, from *Treponema pallidum* and *Borrelia burgdorferi*, have been implicated in the pathogeneses of syphilis and Lyme disease, respectively ([Sellati *et al.*, 1998](#_ENREF_110)). Additionally, LPs elicit proinflammatory cytokine release in a range of human systems, such as whole blood ([Karched *et al.*, 2008](#_ENREF_68)), macrophages ([Zhang *et al.*, 1998](#_ENREF_135)) and neutrophils ([Soler-Rodriguez *et al.*, 2000](#_ENREF_114)); however, the immunomodulatory potential of either pathogen- or commensal-derived lipoproteins with intestinal epithelial cells has not (to the authors’ knowledge) yet been explored. This could be an area of particular interest in future studies, given that *E. coli* are among the first bacteria to colonise the neonatal intestinal ([Hooper, 2004](#_ENREF_56)), therefore, the elevated presence of lipoproteins in these bacteria could potentially have significant effects in the development of intestinal immunity.

### Lipoteichoic acid

Lipoteichoic acid (LTA) is a membrane-associated, amphiphilic polymer which extends from the cytoplasmic membrane, through the cell wall, to the outer surface of Gram-positive bacteria ([Buckley *et al.*, 2006](#_ENREF_18)). LTA is thought to aid in bacterial attachment to host cells ([Granato *et al.*, 1999](#_ENREF_44)), and is also immunologically active, having previously been demonstrated to elicit proinflammatory cytokine secretion from macrophage cells ([Standiford *et al.*, 1994](#_ENREF_115)). In contrast to this, LTAs from strains of potentially probiotic lactobacilli were unable to stimulate a proinflammatory response in the HT29 intestinal epithelial cell line, but actively inhibited *E*. *coli*- and LPS-induced IL-8 release in these cells ([Vidal *et al.*, 2002](#_ENREF_126)). Additionally, oral ingestion of LTA (isolated from *Staphylococcus aureus*), prior to induction of experimental colitis via dextran sulfate sodium (DSS), conferred protection in mice with colons depleted of commensal microbiota, subsequently reducing mortality, morbidity, and severe colonic bleeding ([Rakoff-Nahoum et al., 2004](#_ENREF_98)). From these contrasting studies we are unable to speculate what function LTA potentially plays in intestinal homeostasis, therefore it is evident that more research is required in this field. Also, there is some debate as to which of the Toll-like receptors (TLRs) confers host cell responsiveness to LTA. Schwandner *et al.* ([1999](#_ENREF_109)) demonstrated that human embryonic kidney cells were activated via TLR-2, however, [Takeuchi and colleagues](#_ENREF_111) disputed this, as their results showed that TLR-2-deficient mice were still responsive to LTA, whereas TLR-4-deficient mice were not ([Takeuchi et al., 1999](#_ENREF_121)), thus suggesting TLR-4 confers responsiveness.

### Flagellin

Flagellin is the highly antigenic, monomeric subunit of bacterial flagella ([Ramos *et al.*, 2004](#_ENREF_99)). Flagella are rotary motor-like structures, which are expressed by the majority of motile bacteria in the intestine ([Berg, 2003](#_ENREF_11)). Hayashi *et al.* ([2001](#_ENREF_49)) determined that bacterial flagella possess TLR-5 stimulatory ability, and it was confirmed shortly afterwards that TLR-5 exclusively confers cellular responsiveness to extracellular flagellin ([Gewirtz *et al.*, 2001](#_ENREF_40)). Monomeric flagellin is naturally released by bacteria, either by leakage due to uncapping or by active depolymerisation; however, it can also be sheared from the bacterial surface by host proteases or detergents ([Ramos *et al.*, 2004](#_ENREF_99)), as would be present in the intestine.

Flagellin unquestionably plays an important and highly complex role in intestine homeostasis, as it has been implicated as a major antigen in Crohn’s disease ([Lodes et al., 2004](#_ENREF_81), [Targan et al., 2005](#_ENREF_122)) and, paradoxically, as a protective moiety against spontaneous colitis ([Vijay-Kumar *et al.*, 2007](#_ENREF_127)). Streiner *et al.* ([2000](#_ENREF_116)) first showed that flagellin has the potential to stimulate an immune response from intestinal epithelial cell lines. However, it was subsequently demonstrated that, *in vivo*, flagellin must be first be translocated from the mucosal to the serosal domain of the epithelial layer ([Gewirtz *et al.*, 2001](#_ENREF_40)), despite intestinal epithelial cell lines exhibiting both basolateral *and* apical TLR-5 expression ([Cario & Podolsky, 2000](#_ENREF_19)). A significant level of translocation is normally considered a trait of pathogenic bacteria ([Ljungdahl *et al.*, 2000](#_ENREF_80)); therefore it can be hypothesised that the intestinal epithelium is able to distinguish between commensal and pathogenic flagellins simply by the physical exclusion of commensal bacteria. Epithelial responses to commercially available flagellin (isolated from the enteric pathogen, *Salmonella typhimurium*) have been well characterised with HT29 and Caco-2 intestinal epithelial cell lines, as both were shown to secrete significantly increased levels of IL-8 in the presence of flagellin ([Bannon, 2008](#_ENREF_7)). However, to date, the epithelial responses to non-pathogenic flagellins have little been considered.

### Membrane vesicles (MVs)

Membrane vesicles (MVs) are small (50-250 nm diameter), spherical, bilayered membranous structures ([Beveridge, 1999](#_ENREF_12)) produced by Gram-negative bacteria. MVs are not MAMPs in their own right, but rather represent a collection of MAMPs, as their composition, conformation and surface chemistry are small scale reproductions of the intact outer membrane of Gram-negative bacteria ([Beveridge, 1999](#_ENREF_12), [Schooling & Beveridge, 2006](#_ENREF_107)). Lipopolysaccharides (LPSs), outer membrane proteins (OMPs), phospholipids and periplasmic proteins are all present in MVs ([Beveridge, 1999](#_ENREF_12), [Kesty & Kuehn, 2004](#_ENREF_69)) and proteins such as transmembrane porins, murein hydrolases, transporter proteins, flagelin, and other virulence factors have all been identified in MVs by proteomic studies ([Lee *et al.*, 2008](#_ENREF_75)). It is starting to become apparent that these small membranous structures have the potential to deliver bacterial products to eukaryotic cells ([Kaparakis *et al.*, 2010](#_ENREF_67)).

A number of roles and functions have been suggested for MVs, including periplasmic equilibrium maintenance ([McBroom & Kuehn, 2007](#_ENREF_89)), antibiotic protection ([Ciofu et al., 2000](#_ENREF_24), [Manning & Kuehn, 2011](#_ENREF_85)), quorum sensing ([Mashburn-Warren & Whitely, 2006](#_ENREF_86)), biofilm maintenance ([Schooling & Beveridge, 2006](#_ENREF_107)) and gene transfer ([Dorward *et al.*, 1989](#_ENREF_29), [Yaron *et al.*, 2000](#_ENREF_134), [Renelli *et al.*, 2004](#_ENREF_100)). However, a direct role in the virulence of Gram-negative bacteria is the most strongly supported. Kadurugamuwa and Beveridge ([1995](#_ENREF_66)) first suggested the virulent nature of MVs due to the enrichment of antigenic LPS molecules and the inclusion of host tissue-destructive enzymes in MVs isolated from the respiratory pathogen *Pseudomonas aeruginosa*. Enterotoxigenic *E. coli* (ETEC) MVs preferentially package heat-labile (LT) toxin in their luminal space, protecting it from extracellular enzymatic activity and delivers it directly to the cytoplasm of target cells ([Kesty *et al.*, 2004](#_ENREF_70)). A similar system was also seen in *Helicobacter pylori*, with its MVs encapsulating and transporting its major virulence factor, *H. pylori* vacuolating toxin ([Parker *et al.*, 2010](#_ENREF_94)). The immunomodulatory potential of MVs was recognised when MVs isolated from *H. pylori* were shown to elicit IL-8 release in human gastric epithelial cells ([Ismail *et al.*, 2003](#_ENREF_63)). More recently, MVs isolated from *H. pylori* have been shown to elicit IL-8 responses in human gastric epithelial cell lines through the novel delivery of peptidoglycan to the intracellular PAMP receptor, NOD1 ([Kaparakis *et al.*, 2010](#_ENREF_67)). Additionally, MVs from *P. aeruginosa* have been shown to be potent activators of the proinflammatory response, stimulating the secretion of IL-8 in human lung epithelial cells ([Bauman & Kuehn, 2006](#_ENREF_9)) and MIP-2 and IL-6 from murine macrophages ([Ellis et al., 2010](#_ENREF_33)). Nevertheless, the interaction of MVs with intestinal epithelial cells have, surprisingly, been little studied, with only a recent investigation demonstrating that MVs isolated from the enteropathogen, *Vibrio cholerae*, elicit IL-8 from Int407 intestinal epithelial cells, via a NOD-1-mediated pathway (Chatterjee and Chaudhuri, 2012). In addition, despite the large population of Gram-negative bacteria present in the intestinal lumen, the immunological role of MVs produced by non-pathogenic enteric bacteria is yet to be elucidated. However, a study by Shen *et al*., (2012) has recently suggested that capsular polysaccharide (PSA)-containing MVs, isolated from *Bacteroides fragilis*, can protect against a 2,4,6-trinitrobenzenesulfonic acid (TNBS)-induced experimental model of colitis in mice. In contrast, we have observed, *in vitro*, that MV’s isolated from the commensal bacterium, *E coli* strain C25, stimulates a concentration dependent increase in the secretion of IL-8 from the intestinal epithelial cell lines HT29 and Caco-2 (Patten and Collett, unpublished results).

Exopolysaccharides (EPSs)

Although not typically recognised as MAMPs, there is growing evidence that exopolysaccharides (EPSs), which are long chain polysaccharides released into the surrounding media during bacterial growth, have an immunomodulatory function. EPS-producing bacteria are increasingly used in the food industry and, indeed, naturally reside within the intestine ([Badel *et al.*, 2010](#_ENREF_6)). EPSs form a highly viscous local environment ([Roller & Dea, 1992](#_ENREF_102)), thus enhancing bacterial nutrient and water entrapping abilities ([Poulsen, 1999](#_ENREF_96)). EPSs have also been suggested to play a major role in bacterial attachment ([Watnick & Kolter, 1999](#_ENREF_131)) and are thought to play a key role in bacterial protection against bacteriophages, antibiotics, lysozyme enzymes and metal ions ([Looijesteijn *et al.*, 2001](#_ENREF_82), [Durlu-Ozkaya *et al.*, 2007](#_ENREF_30)).

EPSs are separated into two categories; homosaccharides and heterosaccharides ([Laws *et al.*, 2001](#_ENREF_73)). Homosaccharides, such as cellulose, dextran and levan, are made up of only one type of monosaccharide ([Laws *et al.*, 2001](#_ENREF_73)), conversely, heterosaccharides consist of multiple repeats of oligosaccharides, which themselves are comprised of 3-7 sugar residues ([Laws *et al.*, 2001](#_ENREF_73)). These oligosaccharide precursors typically contain D-glucose, D-galactose and L-rhamnose sugars ([De Vuyst & Degeest, 1999](#_ENREF_27)) and occasionally include amino-sugars, such as *N*-acetyl-D-glucosamine and *N*-acetyl-D-galactosamine ([Badel *et al.*, 2010](#_ENREF_6)). Heterosaccharides are mainly produced by mesophilic and thermophilic bacteria, such as lactic acid bacteria (LAB) ([Cerning, 1990](#_ENREF_21), [De Vuyst & Degeest, 1999](#_ENREF_27)) and bifidobacteria ([Ruas-Madiedo *et al.*, 2006](#_ENREF_103), [Ruas-Madiedo *et al.*, 2010](#_ENREF_104)).

Kefiran, an EPS produced by a number of strains of lactobacilli in the fermented milk drink, Kefir, has been shown to possess a number of systemic physiological activities; these include wound-healing properties, reduction of blood pressure and cholesterol levels, and the retardation of tumour growth in experimental models ([Vinderola *et al.*, 2006](#_ENREF_128)). Kefiran also exhibits a potential role in intestinal homeostasis, with an increase in luminal IgA and of both pro- and anti-inflammatory cytokines, such as IFN-γ, TNF-α, IL-6 and IL-10, observed in the small and large intestine ([Vinderola *et al.*, 2006](#_ENREF_128)). In concordance with these homeostatic effects, a study by Sengül *et al.* ([2006](#_ENREF_111)) demonstrated that EPS-producing bacteria were able to significantly attenuate the inflammation of an experimental colitis model, induced via intracolonic administration of acetic acid, in rats. Additionally, this is supported further by evidence at the cellular level, as murine macrophages challenged with various EPSs, (isolated from strains of lactobacilli and bifidobacteria) demonstrate augmented release of both pro- and anti-inflammatory cytokines, such as TNF-α, IL-6 and IL-10 ([Chabot et al., 2001](#_ENREF_22), [Bleau et al., 2010](#_ENREF_14), [Wu et al., 2010](#_ENREF_132)). The mitogenic activity of EPSs isolated from strains of lactobacilli and bifidobacteria is also well characterised, with studies showing the promotion of human, murine, porcine and bovine macrophage proliferation ([Kitazawa *et al.*, 1998](#_ENREF_72), [Chabot *et al.*, 2001](#_ENREF_22), [Wu *et al.*, 2010](#_ENREF_132)).

With a large number of EPS-producing bacteria naturally residing in the intestine, it is surprising that very little research has been undertaken into the interaction of EPSs with the intestinal epithelial layer itself. Previous studies have investigated the potential of EPSs as antiproliferative or anticytoxicitic agents with intestinal epithelial cells (IECs) ([Ruas-Madiedo *et al.*, 2010](#_ENREF_104), [Liu *et al.*, 2011](#_ENREF_79)), but, the immunomodulatory effects of EPSs on IECs has largely been neglected in the literature. However, Lebeer *et al.* ([2012](#_ENREF_74)), as part of a much larger investigation, have reported that EPSs isolated from the known probiotic, *Lactobacillus rhamnosus* GG, had no significant effect on IL-8 mRNA expression in Caco-2 cells. Additionally, a recent review article presented preliminary data in which co-culture with EPS-producing strains of bifidobacteria differentially modulated the secretion of inflammatory cytokines, including IL-8 and IL-6, in the Caco-2 intestinal epithelial cell line ([Hidalgo-Cantabrana *et al.*, 2012](#_ENREF_52)).

The evidence presented above confirms that EPSs directly associate with host cells in the intestine, however, the molecular mechanisms by which they interact is not fully understood. Chabot *et al.* ([2001](#_ENREF_22)) suggested EPSs could exert their action via the mannose receptor and a more recent study by Ciszek-Lenda *et al.* ([2011](#_ENREF_25)) demonstrated a cross-tolerance between LPS and EPSs in macrophages, thus indicating the possible involvement of TLR-4-meidated pathway. Nevertheless, another study, undertaken by Lin *et al.* ([2011](#_ENREF_77)), on a novel EPS (TA-1) isolated from the thermophilic marine bacterium *Thermus aquaticus*, provides the strongest candidate for an EPS receptor. TA-1 was shown to stimulate the release of proinflammatory cytokines, TNF-α and IL-6, from murine macrophages via a TLR-2-mediated pathway ([Lin *et al.*, 2011](#_ENREF_77)). This is consistent with the fact that TLR-2 is a well characterised receptor for a range of microbial components ([Takeda *et al.*, 2003](#_ENREF_119), [Akira *et al.*, 2006](#_ENREF_3)).

### *Conclusions*

Like their pathogenic counterparts, commensal bacteria are able to stimulate an immune response from the intestinal epithelial layer; however, the mechanisms of this low level inflammatory reaction are, as yet, largely unknown, but are likely to involve pattern recognition receptors, such as TLRs. A number of possible contributory factors, and their receptor(s), have been discussed in this review. However, it is apparent from the small number of studies undertaken thus far, which consider the MAMPs of the enteric bacterial flora, that much more research is needed in this field, in order to further uncover the complex relationship between the commensal microbiota and the intestinal innate immunity.

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| --- | --- | --- | --- |
| Pattern recognition receptor (PRR) | Location(s) | Ligand(s) | Source(s) |
|
| TLR-2 | Plasma membrane | Peptidoglycan  | Bacteria |
|  |  | Phospholipomannan | Fungi |
|  |  | Haemagglutinin | Measles virus |
| TLR-2/TLR-1 | Plasma membrane | Lipoprotein | Bacteria |
|  |  | Triacyl lipopeptides | Gram-negative bacteria |
| TLR-2/ TLR-6 | Plasma membrane | Zymosan  | Fungi  |
|  |  | Diacyl lipopeptides | Mycobacteria  |
|  |  | Lipoteichoic acid | Gram-positive bacteria |
| TLR-3 | Endosomal membrane | dsRNA | Viruses |
| TLR-4 | Plasma membrane | Lipopolysaccharide | Gram-negative bacteria |
|  |  | Mannan | Fungi |
| TLR-5 | Plasma membrane | Flagellin | Bacteria |
| TLR-7 | Endosomal membrane | ssRNA | Viruses |
| TLR-8 | Endosomal membrane | ssRNA | Viruses |
| TLR-9 | Plasma/endosomal membrane | CpG-DNA | Bacteria |
|  |  |  | Viruses |
|  |  |  | Protozoa |
| TLR-10 | Endosomal membrane | Unknown | Unknown |

Table 1 – Human TLRs and their known MAMPs