Within-host Envelope Remodelling and its Impact in Bacterial Pathogen Recognition

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Abstract

Following colonization of host tissues, bacterial pathogens encounter new niches in which they must gain access to nutrients and cope with stresses and defence signals generated by the host. For some pathogens, the adaptation to a new 'within-host' lifestyle involves modifications of envelope components that bear molecular patterns normally recognized by the host innate immune system. These new modified patterns limit host recognition, therefore promoting immune evasion and pathogenicity. In this review, we describe how envelope components like the peptidoglycan or lipopolysaccharide can be altered within the host to impair responses triggered by pattern recognition receptors (PRRs). We also discuss the few cases reported to date of chemical modifications that occur in the envelope of some intracellular bacterial pathogens when they reside inside eukaryotic cells. These envelope alterations may have evolved due to the sentinel role performed by PRRs over pathogen-specific molecular patterns. The available data indicate that only selected pathogens seem to evade recognition due to 'within-host' envelope changes, with most of them displaying such patterns also in non-host environments. Given the importance of these alterations, future studies should focus in the responsible pathogen regulators – most yet unknown – that could be targeted to prevent immune evasion.

Introduction

Bacterial pathogens confront a plethora of adverse conditions and diverse defences imposed by the host after their initial encounter. Both partners continuously evolve based in this confrontation and this fact is illustrated by the multiple mechanisms of immune evasion

characterized to date. Immune evasion encompasses strategies directed to target key elements of the host defence machinery ('direct attack' strategy) or to interfere with host recognition ('masking' strategy). In this review, we mostly discuss cases of impaired recognition, including those examples derived from changes in cell envelope components that occur 'within the host'. We emphasize changes in molecular patterns of these components that are normally detected by dedicated host receptors and, therefore, understood as changes that has consequences such as immune evasion.

Due to the bulk of data accumulated in the recent years, the review describes immune evasion exclusively in bacterial pathogens. We nevertheless recommend to the reader other recent reviews that address immune evasion in viral (Beachboard and Horner, 2016; Messaoudi *et al*., 2015); parasitic (Flávia Nardy *et al*., 2015; Nakada-Tsukui and Nozaki, 2016; Radolf *et al*., 2016; Stijlemans *et al*., 2016; Wright and Rayner, 2014); and fungal infections (Jiménez-López and Lorenz, 2013). Of hallmark importance is also the immune evasion associated to tumour development in various types of cancer (van der Burg *et al*., 2016).

Immune evasion strategies based on a 'direct attack' to innate host defences

Mammalian hosts recognize intruder organisms using innate defences that act as primary barrier to control infections. These defences include the complement cascade and the phagocytosis by activated macrophages and dendritic cells. Many pathogen proteins alter in one or another way diverse activities in the complement and can interfere with the phagocytic process. These strategies are mostly exploited by some Gram-positive pathogens, which use numerous immune evasion proteins either located in the pathogen surface or actively secreted to the milieu (Koymans *et al*., 2016). The literature provides many examples of blockage of immune receptors, proteases or antimicrobial peptides; active degradation of immune receptors, antimicrobial peptides or DNA (e.g. neutrophil extracellular traps, NET); host cell lysis triggered by a variety of toxins; interference in the coagulation process and overstimulation of host defences with proteins acting as super-antigens (Foster *et al*., 2014; Koymans *et al*., 2016). In the specific case of *Staphylococcus aureus*, it has been estimated that the pathogen could use up to 35 distinct secreted proteins to evade host immunity. A few examples include inhibitors of neutrophil serine proteases (Stapels *et al*., 2016); surface proteins that impede opsono-phagocytosis and, in addition, act as super-antigens (Pauli *et al*., 2014); proteins that induce tumour-necrosis factor 1 receptor (TNFR1) in neutrophils, which neutralizes circulating TNF-α (Hong *et al*., 2016); proteins promoting apoptosis in B cells (Hong *et al*., 2016); and, proteins that assemble into 'sequestering' complexes with factor H (FH) and the complement protein C3b (Amdahl *et al*., 2013). A similar 'trapping' mechanism on complement proteins has been shown for the surface protein Fhb of *Streptococcus suis* (Li *et al*., 2016). Group B streptococci have also been shown to use an ectonucleotidase, CdnP, to degrade cyclic nucleotides such as cyclic-di-AMP that stimulate the cGAS-STING cascade involved in production of interferon beta (INF-β) (Andrade *et al*., 2016). For a more complete analysis of these evasion strategies falling in the category of 'direct attack' we recommend excellent recent reviews (Foster *et al*., 2014; Geoghegan and Foster, 2015).

The masking strategy: immune evasion based on the interference with host receptors recognizing microbe-associated molecular patterns

The Toll-like receptor family

The innate immune system also relies in receptors that recognize microbe-associated molecular patterns (MAMP). These 'pattern recognition receptors' (PRR) differentiate based on the specific molecular pattern they recognize and their subcellular location (Caruso *et al.*, 2014; Strober *et al.*, 2006). An important class of PRR are the membrane-embedded Toll-like receptor (TLR) family, which bind pathogen's molecules either at the plasma membrane or in membranes of endocytic compartments (Strober *et al.*, 2006).

The most extensively characterized member of the TLR family is TLR4, which, together with its co-receptor MD2, recognizes the lipid A component of the lipopolysaccharide, LPS (Maeshima and Fernandez, 2013). It has been proposed that individual LPS molecules are those that are recognized by TLR4, which implies previous extraction from supramolecular lamellar membrane aggregates released from the outer membrane (Gioannini *et al.*, 2004). Other studies point to a contribution of the aggregate structure to TLR4 stimulation (Zughaier *et al.*, 2007). LPS is extracted from bacterial membranes by LPS-binding protein (LPB), a serum protein that transfers the cargo to the surface protein CD14. It is at this stage when CD14 subsequently deliver LPS to facilitate assembly of the TLR–LPS–MD2 tripartite complex (Maeshima and Fernandez, 2013). Following LPS binding at the host cells surface, the complex dimerizes to stimulate downstream signalling by interaction with the adaptor proteins TIRAP and MyD88. The TLR–LPS–MD2 complex is also know to signal once it has been internalized in endosomes, a process involving the adaptor proteins TRAM and TRIF (Maeshima and Fernandez, 2013). The stimulation derived from the plasma membrane leads to 'early' activation of the host pro-inflammatory regulator nuclear factor kappa B (NFκB) whereas the activation of the TRAM-TRIF pathway results in type I interferon production and a 'delayed' NFκB response. In both pathways, the cytosolic region of TLR4 binds to the distinct adaptor proteins once the TLR4–MD2-LPS complex is formed. Structural studies with distinct lipid A variants have demonstrated distinct signalling fluxes depending on the stability of this tripartite complex (Maeshima and Fernandez, 2013). Changes in lipid A structure are, in fact, a common strategy used by some Gram-negative pathogens to dampen first lines of host innate immune response (see Maeshima and Fernandez, 2013; Needham and Trent, 2013).

Besides TLR4, other important cell surface TLR involved in recognizing MAMP include those targeting bacterial lipoproteins (TLR1, TLR2 and TLR6) and flagellin (TLR5). Intracellular TLR located in the membrane of endosomal compartments include TLR3, TLR7, TLR8 and TLR9, which recognize nucleic acids from virus or bacteria (Kawasaki *et al.*, 2011).

The NLR receptor family

A second major line of innate immune defence involving PRR are the members of the nucleotide-binding oligomerization domain (NOD)-like receptor (NLR) family. NLR locate in intracellular locations where they recognize diverse molecules of invasive pathogens (Caruso *et al.*, 2014; Franchi *et al.*, 2009). NLR have three well defined regions consisting

of a C-terminal leucine rich repeat (LRR) domain, a central nucleotide-binding domain (NACTH) and a variable N-terminal domain. This latter domain differentiates the distinct NLR subfamilies, named NLRA, NLRB, NLRC and NLRP (Feerick and McKernan, 2017). The respective N-terminal domains bear an acidic transactivation domain (NLRA subfamily); baculovirus inhibitor repeat (NLRB subfamily); caspase recruitment domain, CARD (NLRC subfamily); or a pyrin domain (NLRP subfamily).

This review will focus mainly in the NLRC receptors NOD1 and NOD2, which bear CARD domains and recognize peptidoglycan (PG)-derived fragments, triggering production of inflammatory cytokines and antimicrobial molecules (Caruso *et al*., 2014; Kaparakis-Liaskos, 2015; Strober *et al*., 2006). A large number of data have confirmed a discriminating ligand specificity for these two receptors (Chamaillard *et al*., 2003; Girardin *et al*., 2003; Hasegawa *et al*., 2006). Thus, NOD1 recognizes PG fragments bearing the γ-D-glutamy l-meso-diaminopimelic (DAP) (iE-DAP) motif present in stem peptides of PG from many Gram-negative bacteria and some Gram-positive bacteria such as *Listeria monocytogenes* and *Bacillus subtilis*. NOD2 recognizes N-acetyl-muramyl-L-alanyl-γ–D-glutamic acid (muramyl-di-peptide, MDP), which is widely conserved in PG of many Gram-positive and Gram-negative bacteria. Whereas NOD1 is expressed ubiquitously in all cell types, NOD2 expression is restricted to immune cells, including macrophages, dendritic cells, neutrophils and T cells (Kaparakis-Liaskos, 2015; Moreira and Zamboni, 2012). NOD2 expression can increase in response to stimuli such as LPS, tumour necrosis factor (TNF) or interferon gamma (INF-γ).

NOD1 and NOD2 reside in an auto-inhibited monomeric state in the cytosol. Upon ligand binding, they multimerize in a state that exposes their CARD domains for interacting with the receptor-interacting serine/threonine kinase 2 (RIPK2) (Caruso *et al*., 2014). Despite their cytosolic location, recent studies have shown that recognition of PG fragments mediated by NOD1/NOD2 and their interaction with RIPK2 occurs in association to endomembranes (Irving *et al*., 2014; Nakamura *et al*., 2014). Thus, NOD1 binds PG fragments internalized via bacterial outer membrane vesicles (OMV) and interacts with RIPK2 in early endosomes (Irving *et al*., 2014). PG recognition mediated by NOD1 can also promote the formation of autophagosomes in a RIPK2-dependent manner (Irving *et al*., 2014). Intriguingly, stimulation of autophagy was observed exclusively in non-immune cells, underscoring the importance of this innate defence mechanism in non-phagocytic cells invaded by intracellular bacterial pathogens. Unlike NOD1, NOD2 recognizes PG and interacts with RIPK2 in endosomal platforms enriched in the late endosome-lysosomal markers LAMP1 and LAMP2 (Nakamura *et al*., 2014). Of interest, this latter study also demonstrated the presence in these endosomes of transporters such as SLC15A3 and SL15A4, capable of transporting the NOD2 ligand MDP from *Salmonella*-containing phagosomes to the cytosol (Nakamura *et al*., 2014). Therefore, PG fragments can reach the host cell cytosol in infections caused by invasive intracellular pathogens directly, as it is the case of pathogens adapted to a cytosolic lifestyle; or, indirectly, following transport from the phagosomal compartment by host transporters such as SLC15A3 and SL15A4. These transporters were reported also to contribute to release of PG fragments to the cytosol following uptake of OMVs or endocytosis of soluble PG fragments (Lee *et al*., 2009). An alternative route for delivery of PG fragments to the cytosol is supposed to exist due to the connection demonstrated between the activity of specialized type III and IV secretion systems and NOD1-dependent host responses. This association was reported in infections caused by

extracellular pathogens such as *Citrobacter rodentium* (LeBlanc *et al.*, 2008) and *Helicobacter pylori* (Viala *et al.*, 2004).

A third line of innate defence involved in recognition of pathogen molecules: the inflammasome

In addition to the important role played by TLR and NLR receptors in the control of bacterial infections, mammals have developed additional innate host defences to monitor intracellular infections. This defence mechanism relies in supramacromolecular platforms, named inflammasomes, which upon recognition of specific pathogen molecules activate the pro-inflammatory caspases caspase-1 and caspase-11 (this latter, identified in mouse, with orthologue caspases-4 and -5 in humans). Activation of these caspases, which takes place following processing of the respective pro-caspase precursors, leads to production of the inflammatory cytokines IL-1β and IL-18 (Storek and Monack, 2015; Stowe *et al.*, 2015). Importantly, lysis of the infected cell in which inflammasome undergoes activation results in release of these pro-inflammatory molecules, an event known as pyroptosis. Two alternative pathways of inflammasome activation are known: (i) the canonical, which relies in the consecutive action of a PRR that binds the pathogen 'danger' molecule to further interact with one bridge adaptor protein that ultimately activates pro-caspase-1 (Crowley *et al.*, 2016; Storek and Monack, 2015); and, (ii) the non-canonical, involving in the last stage the contribution of caspase-11 or its human caspases-4/5 counterparts (Crowley *et al.*, 2016). Inflammasome activation has been extensively studied in *Salmonella* infections, involving receptors such as NAIP1 and NAIP2, which bind pathogen proteins of the type III secretion machinery (PrgI, PrgJ); and NAIP5/NAIP6, which recognize flagellin (FliC/FljB). These receptors converge in the PRR protein NLRC4, which via its CARD domain activate subsequently pro-caspase-1, which processes itself auto catalytically. Processed caspase-1 then contributes to the production of IL-18 and 1L-1β by proteolysis of their precursor forms. Pro-caspase-1 can also be activated by the adaptor protein ASC (apoptosis-associated speck-like protein) after stimulation of NLRP3, which responds to increased levels of mitochondrial reactive oxidative species (mROS), often a signal associated to intracellular bacterial infections (Crowley *et al.*, 2016). Unlike the canonical pathway of inflammasome activation, much less is known regarding the non-canonical activation route involving caspase-11 and its human counterpart caspase-4/5. Interestingly, a recent study provided evidence for direct binding of LPS by these 'non-canonical' caspases (Shi *et al.*, 2014), which makes apparently unnecessary the intervention of upstream receptors. Another matter for further studies involves the modes of inflammasome activation in different host cell types. Data obtained in mouse infection *in vivo* model using *Salmonella* support the requirement of both caspase-1 and caspase-11 to control pathogen proliferation in intestinal epithelial cells (IEC) (Knodler *et al.*, 2014; Sellin *et al.*, 2014). Of interest is also the fact that inflammasome activation in neutrophils is not accompanied by pyroptosis (Crowley *et al.*, 2016).

Immune evasion and the alteration of envelope components

Previous sections summarized mechanisms by which innate immunity controls bacterial infections based on recognition of distinct MAMPs. Receptors such as TLR4, NOD1/NOD2, and inflammasome-related receptors target distinct envelope molecules. This 'multifactorial' defence increases the probability of an effective control of the infection. Despite

this arsenal of host receptors, successful pathogens have evolved mechanisms to evade them. A common strategy is the alteration of the envelope chemistry, rendering it invisible to host PRR. This review mostly focuses in this 'masking' strategy, differentiating modifications known to occur in LPS and peptidoglycan that different studies have shown to impair host recognition. We will emphasize those advances in the field attempting to characterize envelope changes that occur 'within the host'.

Evasion due to impaired LPS recognition by the TLR4–MD2 complex

The LPS has a complex chemistry in which three regions are clearly differentiated. One of these is the lipid-A component, recognized by the TLR4–MD2 complex. Due to this property, the lipid A is the LPS region with immunomodulatory activity (Maeshima and Fernandez, 2013; Maldonado *et al*., 2016; Neyen and Lemaitre, 2016). The other LPS regions include the central core oligosaccharide and the O-antigen, this latter harbouring a variable number of repeating oligosaccharide units in contact with the external milieu (Whitfield and Trent, 2014).

The lipid A, which is embedded in the outer membrane, has a di-glucosamine backbone decorated with acyl chains (carbons 2, 3, 2′ and 3′) and phosphate groups (carbons 1 and 4′) (Whitfield and Trent, 2014). Lipid A chemistry has been extensively studied in *Escherichia coli*, in which it is normally hexa-acylated. Four of these acyl groups are bound to the 2, 3, 2′ and 3′ positions of the di-glucosamine whereas the other two acyl groups are secondary chains bound to the hydroxyl groups of the 2′-and 3′-linked chains. Importantly, the biosynthetic pathway of the lipid-A molecule, which involves acylation and phosphorylation reactions, is interconnected with the addition of the 3-deoxy-D-manno-oct-2-ulosonic acid (Kdo_2), the core oligosaccharide in the LPS of *E. coli*. Thus, addition of the secondary acyl chains to the tetra-acylated lipid-A occurs only after the two phosphate groups have been added to the 1 and 4′ positions and the Kdo_2 is incorporated (Whitfield and Trent, 2014). This hexa-acylated lipid A form of *E. coli* elicits a strong inflammatory response via the TLR4–MD2 complex (Maeshima and Fernandez, 2013). Variations in the chemistry of lipid A implying a reduced acylation state are normally associated to lesser degree of TLR4-MD2 activation (Maeshima and Fernandez, 2013; Needham and Trent, 2013). A recent study pointed to lower recognition of the lipid A by CD14 as a critical step to evade TLR4-MD2 signalling (Tan *et al*., 2015). Reduction in the number of lipid A acyl chains is a strategy widely used to evade TLR-MD2 signalling by diverse bacterial pathogens including, among others *S. enterica* serovar Typhimurium, *H. pylori*, *Burkholderia cenocepacia*, *Pseudomonas aeruginosa*, *Bordetella bronchispetica*, and *Yersinia* spp. (Needham and Trent, 2013). The reserve, increased lipid-A acylation, can also result in decreased TLR-MD2 activation. Representative cases are those of *Klebsiella pneumoniae* and *S. enterica* serovar Typhimurium (Dalebroux and Miller, 2014; Llobet *et al*., 2015). Other modifications in the lipid A chemistry are known to confer resistance to cationic antimicrobial peptides (CAMP) due to a reduction in the negative charge balance of the molecule. Changes altering the net charge of the molecule include the addition of phospho-ethanolamine, aminoarabinose, or galactosamine; dephosphorylation reactions in lipid A; the removal of outer Kdo region; and hydroxylation of lipid A acyl chains or the Kdo (reviewed by Needham and Trent, 2013).

Despite the extensive knowledge on lipid A chemical modifications, there is relatively

scarce information on the exact changes in the chemistry of this important envelope component when the pathogen colonizes host tissues. Most of the modifications reported for lipid A modifications have been characterized in bacteria grown in laboratory conditions (Needham and Trent, 2013). This remark also applies for the corresponding experiments involving stimulation of host receptors, which are generally based on material purified grown bacteria grown in axenic cultures. It is also important to note that while some modifications -compared to the 'reference' lipid-A from *E. coli*- are constitutive in other bacteria (e.g. phosphorylation of Kdo in *Haemophilus influenzae*, *Vibrio cholerae*, or *Bortedella pertussis*), other modifications are tightly regulated. In most instances, these modifications are orchestrated by regulators involved in controlling expression of virulence genes. Among this class of regulators are two-component systems such as PhoPQ, PmrAB, ParRA, CprRS, which control distinct modification such as lipid-A acylation (addition of extra acyl chains), deacylation, or the incorporation of phosphoryl-ethanolamine (PEA) or 4-amino-4-deoxy-L-arabinose in the sugars of the di-glucosamine backbone (Dalebroux and Miller, 2014; Whitfield and Trent, 2014). Besides these type of regulators, there is also evidence that small non-coding RNA (sRNA) are regulatory pieces that contribute to LPS modification in some enteric bacteria (Klein and Raina, 2015). This regulation has been reported to occur at the level of the enzyme that introduces the modification into the lipid A; for example, the sRNA MgrR that silences EptB, an enzyme that incorporates PEA to the Kdo (Moon *et al*., 2013). Other sRNA have been proposed to control indirectly LPS synthesis and modifications by modulating the availability of metabolic precursors (Klein and Raina, 2015). Recent studies have shown that these two-component regulatory systems could also be involved in controlling phospholipid content (Dalebroux *et al*., 2014), which agrees with the view of global changes in the envelope architecture ensuring survival following exposure to diverse host defences, including CAMP.

Most of the known modifications in lipid-A subjected to regulation have been demonstrated in envelope material isolated from wild-type and mutants lacking the corresponding regulators grown in axenic cultures. Conversely, there is scarce information of the exact changes occurring in lipid A or other regions of the LPS following colonization of host cells or tissues. Although some authors addressed this important issue using *in vitro* cultures of macrophages infected with *S.* Typhimurium (Gibbons *et al*., 2005), there is still no direct proof of the type of LPS produced by this pathogen *in vivo*. Nonetheless, many of the lipid-A modifications characterized in intracellular bacteria inside macrophages match those regulated by PhoPQ and PmrAB in bacteria grown in minimal media with acidic pH and low Mg^{2+} concentration (Dalebroux and Miller, 2014; Gibbons *et al*., 2005). Thus, remodelling of *S.* Typhimurium lipid A within the host may follow similar trends to those characterized in axenic media mimicking some host environmental conditions. To recall that the PhoPQ-PmrAB-mediated modifications culminate in a less inflammatory LPS, which could be beneficial to avoid TLR4-MD2 signalling.

Other indirect evidences supporting modification of lipid-A chemistry *in vivo* induced by host signals come from studies showing inactivating mutations in genes encoding for lipid A acylases, as *lpxL*, in clinical isolates of *Neisseria meningitidis* (Fransen *et al*., 2009). *H. pylori* is also known to express as sole LPS species a variant lacking both phosphate groups (one being replaced by PEA) and having four acyl chains. *H. pylori* LPS has low stimulatory capacity for TLR4-MD2 (Cullen *et al*., 2011). For *Y. pestis* it was shown that LpxP, the acyl transferase that adds additional acyl chains to the tetra-acylated lipid A, is

temperature sensitive not being functional at the host temperature, 37°C (Kawahara *et al.*, 2002). This tetra-acylated lipid A turned to be less stimulatory for TL4-MD2 (Kawahara *et al.*, 2002), explaining the ability of *Y. pestis* to evade this innate immune defence.

Direct analysis of lipid A produced by pathogens within the host

A recent study by Llobet *et al.* (2015) has established the first evidence of lipid A remodelling *in vivo* in response to host signals. These authors infected mice with the respiratory pathogen *K. pneumoniae* to subsequently recover bacteria from the colonized lungs. Using state-of-the-art chromatography techniques, a novel lipid A species bearing a 2-hydroxylated myristoyl chain not present in the bacteria used to infect the animals, was identified (Llobet *et al.*, 2015). The study also provided evidence for the enzyme responsible for this derivation, LpxO, and two important aspects that may explain the strong ability of *K. pneumoniae* to evade innate immune responses. First, the lipid A produced by this pathogen *in vivo* is less inflammatory. Second, it confers resistance to CAMP. Intriguingly, the authors reported that the lipid A produced by bacteria exposed to a CAMP named colistin resembles that found in bacteria obtained from lung homogenates. This observation prompts to reconsider the risk-benefit cost of using this antimicrobial compound in clinical settings giving the possibility of positive selection of bacteria producing a less inflammatory LPS and, as consequence, with higher probability of succeeding in the infection.

The peptidoglycan: a plastic molecule influencing host innate immune responses

The peptidoglycan (PG) is a giant macromolecule consisting in glycan chains of alternating N-acetyl-glucosamine (GlcNAc)-N-acetyl-muramic residues (MurNAc) cross-linked by stem peptides (Typas *et al.*, 2011). As abovementioned, innate defences directed by NOD1 and NOD2 rely on the recognition of specific PG fragments with defined motifs, such as iE-DAP and MDP, respectively. Experiments carried out *in vitro* with purified compounds showed that modification of these motifs has a strong effect in NOD1 and NOD2 sensing. Taking as reference the motif MurNAc-L-Ala-γ-D-Glu-*meso*-DAP-D-Ala (M-Tetra) and a large repertoire of derivate molecules, Girardin *et al.* (2003) showed that the preferred substrate for NOD2 is muramyl-dipeptide (M-Di) and that the presence of *meso*-DAP in third position interfers with signalling by this receptor. By contrast, preferred substrates for NOD1 included those having the γ-D-Glu-*meso*-DAP (iE-DAP) motif (Chamaillard *et al.*, 2003). Interestingly, while compounds as M-Tri (MurNAc-L-Ala-γ-D-Glu-*meso*-DAP), Tri (L-Ala-γ-D-Glu-*meso*-DAP) and Di (γ-D-Glu-*meso*-DAP) stimulated NOD1 signalling, that was not the case for M- Tetra (MurNAc-L-Ala-γ-D-Glu-*meso*-DAP-D-Ala) (Girardin *et al.*, 2003). This latter result underscores the importance of the *meso*-DAP residue for NOD1 signalling implying that residues linked to the L-chiral centre of this diamino acid may impose steric hindrance for NOD1 to properly bind the ligand. Although the most common strategy followed by pathogens to evade NOD1/NOD2 sensing relies in alterations of the PG structure (see below), PG recycling can also play an important role in evasion of innate immunity by limiting the amount of muropeptide shed from the pathogen to the extracellular environment. This was first shown with *Shigella flexneri* mutants partly defective in PG recycling, which triggered NOD1-dependent activation of NF-κB at higher extent than

wild-type bacteria (Nigro *et al.*, 2008). Differences in PG recycling between *N. meningitidis* and *N. gonorrhoeae* are also associated to distinct NOD1-dependent NF-κB activities, with meningococci having more efficient PG recycling and, as consequence, shedding fewer immunostimulatory PG fragments (Woodhams *et al.*, 2013).

Peptidoglycan structural modifications leading to 'intrinsic' immune evasion

The PG structure of certain bacteria varies in defined sites respect to the PG of model bacteria as *E. coli*, which bears canonical NOD1 and NOD2 motifs. Some of these differences can impact host recognition notoriously. A striking example is that of pathogenic spirochaetes of the *Borrellia* and *Treponema* genera, which are Gram-negative bacteria displaying in their PG L-ornithine as third residue of the stem peptide instead of *meso*-DAP (Beck *et al.*, 1990; Yanagihara *et al.*, 1984). The presence of L-ornithine in this position impairs NOD1 signalling (Girardin *et al.*, 2003), which is consistent with the low immunogenicity associated to the PG of spirochaetes. Intriguingly, *in vitro* studies with purified muropeptide showed that L-ornithine in the third position of a muramyl-tripeptide can modulate positively NOD2 sensing (Girardin *et al.*, 2003). Future studies should clarify these observations assessing PG spirochaetal immunogenicity in the context of the infection.

Another important change in PG structure that can potentially affect host recognition involves amidation of the *meso*-DAP residue. This modification is prominent in Corynebacteriales (Levefaudes *et al.*, 2015). Structural analysis of the PG of *Corynebacterium glutamicum* revealed that 100% of the D-Glu residues (second position of stem peptide) were amidated whereas that chemical modification was also observed for ≈80% of the *meso*-DAP residues (Levefaudes *et al.*, 2015). A high percentage of *meso*-DAP residues present in the PG are also amidated in other Gram-positive bacteria such as the intracellular pathogen *L. monocytogenes* (Boneca *et al.*, 2007) and *B. subtilis* (Atrih *et al.*, 1999). This scenario of only a fraction of the respectively muropeptides harbouring chemical modifications in a defined position is recurrent for the PG of many bacteria (Cava and de Pedro, 2014). Of interest, a recent study showed that amidated *meso*-DAP is weakly recognized by NOD1 (Vijayrajratnam *et al.*, 2016), which could be compatible with the low immunogenicity shown for the PG of Corynebacteriales. As in the case of L-ornithine present in the PG of spricohetes, amidated *meso*-DAP increases slightly NOD2 sensing (Girardin *et al.*, 2003). Based on these data, it is tempting to postulate that evasion mechanisms directed to avoid NOD1 sensing might have opposite consequences on NOD2-based defences. Future studies should investigate this interesting scenario.

meso-DAP amidation has also been extensively analysed in pathogenic mycobacteria, especially in the case of *Mycobacterium tuberculosis*. The PG of this pathogen has unique structural modifications involving glycolylation (instead of acetylation) of a fraction of the muramic acid residues and amidation of some of the D-Glu and *meso*-DAP residues (Angala *et al.*, 2014). A recent study performed with synthetic muropeptides has shown that amidation of D-Glu and *meso*-DAP reduces NOD1 activation (Wang *et al.*, 2016). The same study provides evidence of partial impairment of NOD2 signalling associated to the glycolylation of the muramic acid residue (Wang *et al.*, 2016). This observation however contrasts with previous studies inferring a contribution of N-glycolylation of the muramyl residue to NOD2-dependent immunogenicity (Hansen *et al.*, 2014) and increased recognition of

N-glycolyl muramyl dipeptide by NOD2 (Coulombe *et al.*, 2009). Other recent studies with PG from *M. leprae* show that neither D-Glu amidation nor the presence of glycine instead of L-alanine as first residue of the stem peptide interfere with NOD2 signalling (Schenk *et al.*, 2016). Globally, it can be concluded that among those modifications reported for the mycobacterial PG, that involving *meso*-DAP amidation seems to affect at a higher degree recognition by innate immune defences.

Another widely characterized structural modification in the PG of some bacteria involves deacetylation and O-acetylation in specific sites of the muramic acid residue (Cava and de Pedro, 2014). These modifications confer lysozyme resistance (Rae *et al.*, 2011) and interfere with immune signalling. PgdA and OatA, the two enzymes involved in these modifications, play an important role in immune evasion by *L. monocytogenes* (Aubry *et al.*, 2011; Boneca *et al.*, 2007). This pathogen contains a fraction of the muramic acid residues either deaceylated or O-acetylated, and these modifications are tightly regulated by response regulators and dedicated sRNA that ensure up-regulation of these enzymes *in vivo* (Burke *et al.*, 2014).

The PG of *H. pylori* displays also features that could diminish its immunogenicity, including deacetylation of the N-acetyl-muramyl residue by PgdA (Wang *et al.*, 2010). An early study showed that when *H. pylori* remodels substantially the PG structure following entry into in stationary phase. In this condition, there is a marked increase in muropeptides bearing the L-Ala-γ-D-Glu dipeptide (Costa *et al.*, 1999). *H. pylori* has also other interesting features as a high percentage of muropeptides bearing a pentapeptide stem chain and the lack of L–D-type cross-links among stem peptides. *H. pylori* PG was postulated to have lower potential to stimulate NOD1 given the high percentage of muropeptides harbouring dipeptide stem peptides (up to 20% in growth cessation), which lack the essential *meso*-DAP residue. This conclusion agrees with the observation that a defect in the amidase AmiA, which results in decrease in the amount of muropeptides bearing dipeptide as stem peptide, correlated with increased immunogenicity (Chaput *et al.*, 2006). A further study in *H. pylori* supported the presence of additional peptidases acting in the stem peptide that could contribute to generate the characteristic helical rod shape of this pathogen and influence its capacity to colonize the stomach (Sycuro *et al.*, 2012). Noteworthy, this study showed that the absence of these peptidases has no effect in the pro-inflammatory response of the infected animal despite a 3-fold increase in tripeptides in the PG (Sycuro *et al.*, 2012). Further studies are needed to elucidate the exact contribution of the *H. pylori* PG to signalling and the relative contribution of the distinct fragments (bearing motifs with low- or high-immunogenicity) that are released during infection. Early studies also reported the capacity of *cag*$^+$ *H. pylori* to stimulate NOD1 via its specialized type IV secretion system (Viala *et al.*, 2004), which agrees with the higher susceptibility to *H. pylori* infection displayed by NOD1-deficient mice (Viala *et al.*, 2004).

H. pylori is also known to produce varied forms of co-existing lipid-A having distinct degree of acylation (Suda *et al.*, 2001) and an O-antigen that mimics Lewis (Le) blood group antigens (Lina *et al.*, 2014). Future studies, to be performed in bacteria extracted from animals, could provide insights into the relative enrichment of the molecular patterns having distinct immune-stimulatory features during the colonization of the gastric epithelium.

Modification of the peptidoglycan moiety 'within the host' can affect innate immune defences

To date, most studies focused in the elucidation of PG structure in bacterial pathogens have been carried out in laboratory conditions. Like the case of the lipid A, our knowledge of the exact chemistry of envelope components that are produced by pathogen within the host is still limited. This absence of data contrasts with the accumulating evidence supporting elaborated mechanisms involved in the regulation of enzymes that participate in the biosynthesis, hydrolysis or remodelling of the PG (Egan *et al.*, 2017). The PG structure can be remodelled in bacteria grown in axenic cultures and it is conceivable that similar alterations also occurs within the host (Fig. 2.1).

Some examples of responses affecting activity of PG enzymes are known for the case

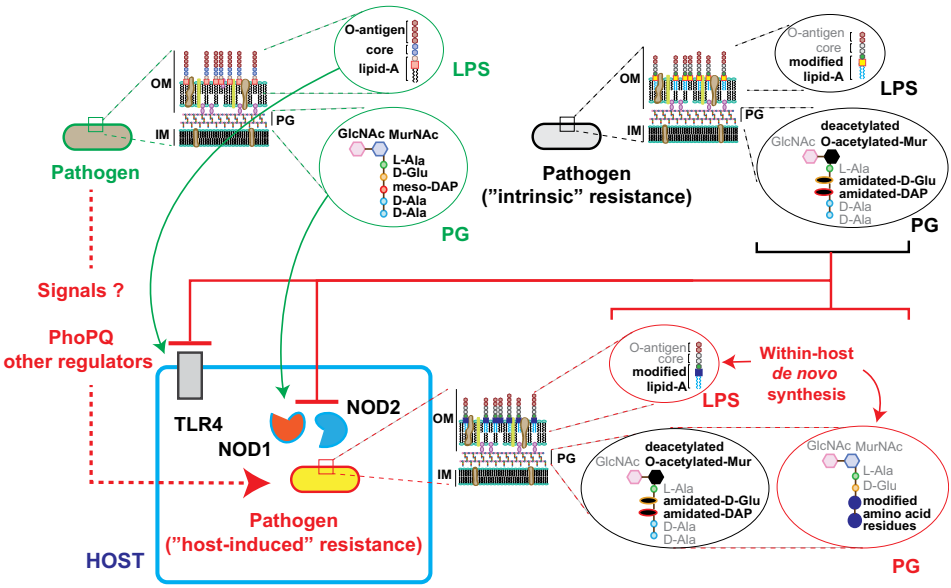

Figure 2.1 Evasion of TLR4 and NOD1/NOD2 signalling based on changes of lipid A and peptidoglycan (PG) structure. The scheme depicts a distinct chemistry of lipid A and PG present in some bacteria grown in non-host environments, which can affect recognition by TLR4 and/or NOD1/NOD2. This mode of evasion is referred as 'intrinsic resistance'. The scheme also denotes the synthesis *de novo* within the host of new molecular patterns in envelope components such as the lipid A component of lipopolysaccharide (LPS) and PG. This evasion mechanism, involving new molecular patterns can impair TLR4 and NOD1/NOD2 signalling, is referred as 'host-induced'. Note that the signals and regulators involved in the changes in these pathogen-associated molecular patterns occurring during the infection are mostly uncharacterized. One of the regulators known to be activated within the host and mediate changes in the LPS, membrane phospholipids and PG in several Gram-negative pathogens is the two-component system PhoP-PhoQ. Although the scheme refers to the envelope structure of Gram-negative, the concepts of 'intrinsic' and 'host-induced' capacity for evasion are equally applicable to Gram-positive bacterial pathogens. IM, inner membrane; OM, outer membrane; PG, peptidoglycan; LPS, lipopolysaccharide. See text for more details.

of the 'resuscitation-promoting factors' (Rpf) of mycobacteria (Kana and Mizrahi, 2010). These factors include glycosidases and endopeptidases that, in response to signals linked to nutrient availability, promote cultivability of dormant bacteria (Kana and Mizrahi, 2010).

In one of the few studies that accomplish the structural analysis of PG in intracellular bacteria, Quintela *et al.* showed that *S.* Typhimurium remodels substantially the PG when proliferates actively within cultured epithelial cells (Quintela *et al.*, 1997). These authors purified PG from intracellular bacteria and, when compared to that of bacteria used in the inoculum, they noticed a substantial increase in the relative proportion of cross-linked muropeptides lacking one of the GlcNAc-MurNAc disaccharide units. These muropeptides could be produced only by the action of an amidase acting in the MurNAc-L-Ala amide bond (Quintela *et al.*, 1997). Such an activity targeting un-cross-linked muropeptides could result in the release of stem peptides, with potential to target NOD1, ubiquitously expressed by all cell types. In this scenario, one could therefore assume a phenomenon favouring a pro-inflammatory response in the infected cell. Future investigations are also needed to clarify the source (either host or pathogen) of this yet uncharacterized amidase activity. Interestingly, a recent study has shown that intracellular *S.* Typhimurium up-regulates within the host cell an enzyme with D-L-endopeptidase activity against the γ-D-Glu-*meso*-DAP bond, which is regulated by the PhoP-PhoQ two component system (Rico-Pérez *et al.*, 2016). Such an activity could potentially attenuate the effect on NOD-1-mediated signalling by decreasing the number of peptides bearing the iE-DAP motif and shed by the pathogen inside the infected cell. These examples clearly manifest the ability of intracellular bacterial pathogens to remodel PG structure within the host.

Other recent studies have provided evidence of responses to 'host signals' that influence PG structure. When exposed to bile salts, *S.* Typhimurium alters PG structure by lowering the amount of lipoprotein bound covalently to the PG and decreasing the percentage of peptide bridges of the L-D-(*meso*-DAP-*meso*-DAP) type (Hernández *et al.*, 2015). Interestingly, mutants lacking the enzymes responsible for these modifications were shown to be more resistant to bile salts (Hernández *et al.*, 2015). These observations highlight the importance for the pathogen of negative regulation acting to remove enzymes probably unnecessary in the host environment.

Evasion of the inflammasome defence

The inflammasome has emerged as a highly elaborated mechanism to cope with invasive pathogen that colonize the intracellular niche of both phagocytic and non-phagocytic cells (Crowley *et al.*, 2016). Several forms of evading this important innate host defence have been postulated. One of them involves down-regulation of components recognized by the distinct receptors forming part of the canonical inflammasome. A relevant case is that of flagellin, which is strongly down-regulated by intracellular *S.* Typhimurium in persistent infections (Nuñez-Hernández *et al.*, 2013). This pathogen also switches the type III secretion system (T3SS) when colonizes the phagosomal compartment, changing from that encoded by the *Salmonella*-pathogenicity island 1 (SPI-1) to that encoded by SPI-2. Such reduction in the expression of structural proteins of the SPI-1 T3SS is proposed to lower the capability of the pathogen to stimulate the inflammasome at late infection times (Crowley *et al.*, 2016).

A recent report provided a new mechanism of inflammasome evasion based on maintaining envelope integrity and, in this way, avoiding release of potential stimulatory molecules recognized by the inflammasome receptors. In this work, Sampson *et al.* implicated Cas9, a component of the CRISPR-Cas system, in envelope homeostasis of *Francisella novicida* (Sampson *et al.*, 2014), an intracellular pathogen causing diseases in a variety of mammalian species, including humans. Infection with mutants defective in Cas9 resulted in overstimulation of the inflammasome system, a phenomenon that could be abrogated by a defect in ASC, a key adaptor required for activation of pro-caspase-1 or TLR2 (Sampson *et al.*, 2014). These data point to the importance that the control of envelope integrity has for the pathogen in intracellular infections. In this line, it is important to recall on the massive release of outer membrane vesicles (OMVs) that some intracellular pathogens experience, probably as result of confronting stresses imposed by the host cell in the cytosol or phagosomal locations. Pathogens such as *Legionella pneumophila* and *S.* Typhimurium release OMV within the infected cell and this process has been linked to processes such as pathogen intracellular replication or the delivery of protein effectors (García-del Portillo *et al.*, 1997; Jager *et al.*, 2015; Jung *et al.*, 2016). Of interest, production of OMV in *S.* Typhimurium is tightly linked to LPS remodelling that is controlled by the PhoP-PhoQ system (Elhenawy *et al.*, 2016). This correlation supports direct implication of virulence regulators in OMV formation. Indeed, OMV have been shown in a variety of pathogens to be important pieces in the infection process (Laughlin and Alaniz, 2016; O'Donoghue and Krachler, 2016; Pathirana and Kaparakis-Liaskos, 2016). Like in the case of lipid A and PG, the future challenge will be to characterize the content of OMV produced and released by the pathogen in extracellular or intracellular locations. This information is vital to understand the role played by OMV in immune evasion. Currently, there are on-going technical efforts in the proteomics field to reach this goal (Lee *et al.*, 2016).

Concluding remarks

Evolution has shaped host–pathogen interactions in many ways. A key step is the initial first encounter between pathogen and the host, in which molecular patterns recognized as 'foreign' by the host innate defences play a fundamental role. Despite the exhaustive analysis of envelope components in a myriad of bacterial pathogens, we are still far from knowing whether those components undergo significant changes inside the host and, as consequence, elicit one or another type of response in the host. We should be aware of this limitation since, to date, most of the studies rely in stress conditions artificially mounted in the laboratory and supposed to mimic the environment faced by the pathogen during host colonization. Another aspect to be evaluated in future studies is the known heterogeneity occurring in the multiple pathogen–host cell encounters as the infection proceeds. These can differ either stochastically or a result of specific host cell types invaded by the pathogen with unique molecular cross-talks (Bumann, 2015). Current technical advances should also allow to define at the molecular level those changes occurring in LPS, PG and other envelope components in extra- and intracellular infections. This should pave the way to identify the involved regulators and design new antimicrobial molecules. The work by Llobet *et al.*, which describes changes in the lipid A of *K. pneumoniae* LPS when the pathogen colonizes the lung and the low immunogenicity accompanying that modification (Llobet *et al.*, 2015),

will certainly encourage investigators to focus on the characterization of pathogen envelope components within the host.

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