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Review Article

PEPTIDOGLYCAN AND LIPOTEICHOIC ACID IN GRAM-POSITIVE BACTERIAL SEPSIS: RECEPTORS, SIGNAL TRANSDUCTION, BIOLOGICAL EFFECTS, AND SYNERGISM

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ABSTRACT—In sepsis and multiple organ dysfunction syndrome (MODS) caused by gram-negative bacteria, lipopolysaccharide (LPS) initiates the early signaling events leading to the deleterious inflammatory response. However, it has become clear that LPS can not reproduce all of the clinical features of sepsis, which emphasize the roles of other contributing factors. Gram-positive bacteria, which lack LPS, are today responsible for a substantial part of the incidents of sepsis with MODS. The major wall components of gram-positive bacteria, peptidoglycan and lipoteichoic acid, are thought to contribute to the development of sepsis and MODS. In this review, the literature underlying our current understanding of how peptidoglycan and lipoteichoic acid activate inflammatory responses will be presented, with a focus on recent advances in this field.

KEYWORDS—Gram-positive bacteria, sepsis, MODS, cytokines, peptidoglycan, lipoteichoic acid

INTRODUCTION

There are approximately 500,000 cases of sepsis with multiple organ dysfunction syndrome (MODS) annually in the United States (1). With a crude mortality of 35%, it is the number one killer in surgical intensive care units (2–13). Since the introduction of antibiotics 50 years ago, there has been no decline in the mortality of patients with sepsis. In fact, despite advances in pharmacology, as well as in fluid replacement and organ supportive therapy, the death rate of sepsis increased during the years 1980 to 1992 almost 2-fold from 4.2 per 100,000 in 1980 to 7.7 in 1992 (13). The resurgence of gram-positive bacterial infections has marked a dramatic change in the prevalence pattern of nosocomial infections. Today, 50% of all cases of sepsis are caused by gram-positive bacteria (11, 13–15), and it is likely that this proportion will continue to rise in the years to come.

The gram-positive bacterium *Staphylococcus aureus* is one of the bacteria most commonly isolated from patients with sepsis (15–18). The treatment of patients with sepsis caused by *S. aureus* has been further complicated in recent years because of the alarming outburst of methicillin-resistant *S. aureus* (MRSA) strains (19, 20). Today, around 50% of nosocomial *S. aureus* isolates are methicillin-resistant (20–22). In surgical ICUs, MRSA strains cause increased risk of postoperative complications, increased length-of-stay at the ICU, increased treatment costs, and increased mortality.

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Peptidoglycan (PepG) and lipoteichoic acid (LTA) are two of the major cell wall components in gram-positive bacteria (Fig. 1). Both PepG and LTA have been shown to stimulate inflammatory responses in a number of *in vivo* and *in vitro* experimental models. This review focuses on these effects of PepG and LTA. Gram-positive bacteria also produce the membrane bound lipopeptides and some secrete exotoxins, such as staphylococcal enterotoxin B (SEB) and toxic shock syndrome toxin (TSST-1). These components are important in the pathophysiological conditions associated with specific infections. However, this will not be extensively discussed in this paper.

The pathophysiological mechanisms leading to hemodynamic disturbances and organ injury in patients with sepsis are still not fully understood. In gram-negative infections, the cell wall component lipopolysaccharide (LPS, endotoxin) is the main initiator of the cascade of cellular reactions that may lead to circulatory failure and organ injury. From several studies, it is known that the release of the proinflammatory cytokines tumor necrosis factor alpha (TNF-α), interleukin 1 beta (IL-1β), and IL-6 is implicated in the hemodynamic disturbances and organ injury of sepsis (6, 23–34). Concurrent with the formation of inflammatory cytokines, generation of the anti-inflammatory cytokine IL-10 is induced in macrophages in response to LPS (35, 36). Increased amounts of IL-10 are found in plasma from septic patients (37–39), and this cytokine is thought to be the functional repressor of monocyte activation in blood from these patients (40). Gram-positive bacteria do not contain LPS, and the cellular mechanisms by which these organisms trigger the deleterious cytokine response leading to organ failure are not clear. It is, thus, important to identify the

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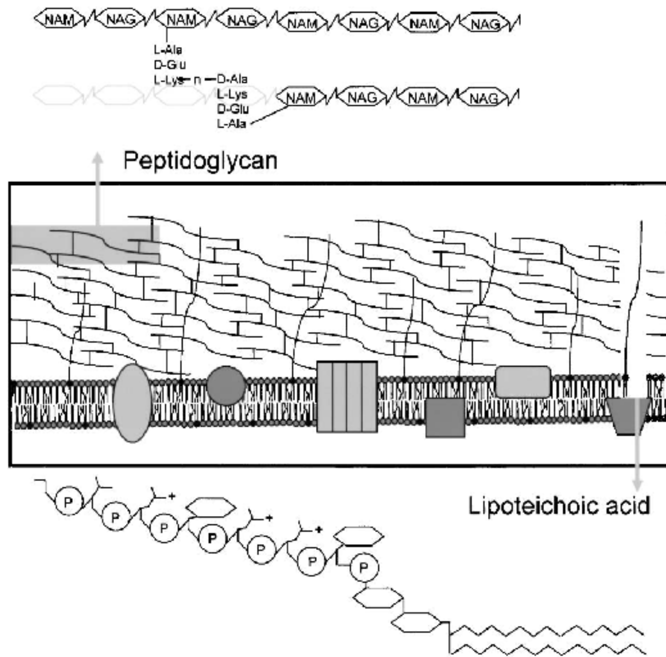

FIG. 1. **Illustration of the general structure of the cell wall of gram-positive bacteria**. NAM, N-acetyl muramic acid; NAG, *N*-acetyl glucoseamine; L-Ala, L alanine; D-Glu, D-glutamate; L-Lys, L Lysine.

chain of events that cause cytokine release in infections caused by gram-positive bacteria. This includes the identification of 1) components in gram-positive bacteria that initiates cytokine production, 2) receptors on host immune cells responsible for recognition of such components, and 3) signaling pathways leading to cytokine production.

The discovery a decade ago that the lipid-shuttle LPS-binding protein and the CD14-receptors mediate cellular responses to LPS (41–49), represented an important step forward in our understanding of how LPS stimulates cells. However, it soon became evident that CD14 was unable to transduce signals across the cell membrane because it is restricted to the outer cell membrane and has no transmembrane domain (50, 51). A few years later, several novel LPS receptors were identified, for example, the β2-integrins (52–54). However, none of these were able to transduce the LPS signal. Hence, intense efforts have been made to identify receptor(s) that could communicate the presence of bacterial infection to the interior of the phagocyte.

Not until a mammalian homologue of the *Drosophila* Toll protein was cloned in 1997, a new breakthrough was made. In *Drosophila*, the Toll protein had shortly before been demonstrated to be involved in antifungal defense (55, 56). It was then discovered that a mutation in the mammalian Toll-like receptor 4 (TLR4) subtype was responsible for the failure of C3H/HeJ mice to respond to LPS (57). This established TLR4 as the LPS signaling receptor. Today, ten TLRs have been cloned (58–63), of which seven have identified ligands. Because TLR2 has been shown to be essential for efficient host defense against gram-positive bacterial infections (64, 65) and recognition of their cell wall components, the literature relating to this receptor as well as its ligands and signaling pathways will be covered in some detail in this paper.

PEPG IN THE SEPTIC RESPONSE

PepG, the main component of the gram-positive bacterial cell wall, is a heteropolymer consisting of a glycan backbone of alternating units of *N*-acetyl-glucosamine and *N*-acetyl-muramic acid, with short peptides linked to the lactyl groups of the muramic acid moieties (Fig. 1). During gram-positive bacterial infections, PepG is released from the bacteria and can reach the systemic circulation (66). PepG can be detected in human plasma using the silkworm larvae plasma test (66, 67). Some antibiotics may enhance the release of PepG (60- to 85-fold) and LTA (4- to 9-fold) from *S. aureus* in culture (68). Recent data suggest that during ethanol- or hemorrhage-induced intestinal injury, PepG can translocate from the intestine to the systemic circulation (69, 70).

Over the last 30 years, a vast number of cellular activities have been assigned to PepG. During the 1970s and 1980s, the research was focused on the ability of PepG to cause polyclonal activation of T and B lymphocytes, a function that today is associated with superantigens. The ability of PepG to activate complement and monocytes/macrophages was demonstrated 20 years ago (71–75). Subsequent studies demonstrated a role of PepG in initiating the deleterious host cytokine response associated with sepsis and organ injury. For instance, several studies examined the release of TNF from human monocytes cultured with *staphylococci* and their PepG (76, 77). It was found that PepG induced the release of TNF with similar kinetics to that induced by LPS, and 1 μg of PepG per milliliter of culture medium was required to induce the release of immuno-detectable amounts of TNF. Most importantly, it was noted that PepG substructures, such as the stem peptide, the pentaglycin bridge, or the soluble synthetic PepG monomer muramyl dipeptide (MDP), all were unable to induce TNF release from human monocytes (77). In a subsequent report, it was demonstrated that PepG and teichoic acid both stimulate human monocytes to release TNF-α, IL-1β, and IL-6 (76). The LPS inhibitor polymyxin B did not influence these responses, indicating that the effect was not caused by potential contamination with LPS. Together these findings showed that PepG, like LPS, induces the production of several inflammatory cytokines associated with sepsis, and that the macroscopic structure of the PepG polymer is an intrinsic feature of its inflammatory properties.

In another study on human monocytes, serum components were found to potentiate the PepG-induced release of TNF-α (78). In the presence of 10% serum, low concentrations of PepG (0.01 to 0.1 μg/mL) induced the formation of TNF-α with potency similar to the one of LPS. The priming effect of serum on these PepG responses was abrogated by heat-treatment (56°C, 1 h) and by depleting serum of the complement components C1 and C3/C4; Ref. 78), suggesting that activation of complement by PepG is central for the cytokine response in human monocytes. Cellular responses to high concentrations of PepG were however found to depend on immunoglobulin G (78). A few years later, production of IL-12 in response to *S. aureus* PepG was shown in the murine macrophage cell line J774 (79). This was an important finding, as IL-12 is required for the induction of interferon (INF)γ genera-

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tion in T cells. PepG is also reported to stimulate endothelial cells directly (80). When a mixed suspension of PepG and LTA was added to endothelial cells *in vitro*, enhanced adhesiveness for granulocytes were noted after 24 h. This corresponded with increased expression of intracellular adhesion molecule 1 on the cell surface, and release of the chemokine IL-8 (80).

In a human whole blood model, we found that *S. aureus* PepG induced the release of TNF-α, IL-6, and IL-10, which coincided with accumulation of mRNAs for these cytokines in both monocytes and T cells (81). The significance of this cytokine induction in T cells is unknown, but the observation reinforced the contention that PepG has immediate effects on T cells, noted two decades earlier (82–84). Whether the induction of cytokine mRNA in T cells results from the direct interaction of PepG and T cell receptors (as superantigens) or from paracrine mediators, such as IL-12 produced by monocytes, remains to be elucidated. Using a gene array approach, a different study examined the regulation of 600 genes in human monocytes by *S. aureus*, PepG, LPS, and INFγ (85). The genes most strongly activated by these compounds were chemokines (IL-8 and MIP-1α), whereas the genes of proinflammatory cytokines (TNF-α, IL-1, IL-6) were induced to a lesser extent (85).

Cell wall components of *S. aureus*, as well as exotoxins such as staphylococcal TSST-1 and staphylococcal SEB, has also been shown to induce the production of INFγ, studied in an *ex vivo* whole blood model (86). Interestingly, it was observed that production of IFNγ induced by heat-killed *S. aureus* depended on the presence of endogenous TNF-α, IL-12, and IL-18. In contrast, SEB-induced IFNγ production seemed only to depend on IL-12, whereas IFNγ production induced by TSST-1 was not influenced by blockade of these paracrine factors (86). The production of IFNγ may have a number of biological effects, e.g., in the priming of epithelial cells to secrete cytokines in response to LPS, LTA, and PepG (87). In a recent report, *S. aureus* PepG was also found to induce procoagulant activity and tissue factor expression in human monocytes (88). These studies show that PepG may induce the expression of several cytokines and factors inducing coagulation associated with the pathophysiology of sepsis. The abovementioned studies have focused on elucidating the effects of PepG on monocytes and cell lines, whereas the responses to PepG *in vivo* have so far been scarcely studied. There is still little information about the effects of PepG on organ macrophages and the role of such interactions in septic organ injury.

It has been argued that PepG is not an important initiator of inflammatory responses because cellular responses to this wall component typically require concentrations of 1–10 μg/mL, which is several logs higher than the concentrations of LPS required for activating macrophages. This may either mean that PepG is not an important initiator of inflammation or that only a part of the PepG structure is responsible for its proinflammatory properties. Although there seems to be some support for the latter (89), it should be noted that the structure–activity relationship of the interactions of PepG with pathogen recognition receptors is not yet fully understood. PepG is insoluble in its native form (90), but may be enzymatically cleaved into smaller components *in vivo* and *in vitro*. In one study, the walls of *S. pneumoniae* was digested by *N*-acetylmuramoyl-L-alanine amidase, which hydrolyzes the bond between the sugar backbone and the stem peptides, and/or muramidase, which hydrolyzes the sugar backbone leaving disaccharide moieties (89). Solubilized wall fractions were separated by high-pressure chromatography and tested for their ability to stimulate leukocytes. Results from these experiments demonstrate that cross-linked sugar chains with at least three stem peptides were 100-fold more potent that undigested PepG, whereas simple stem peptides were largely inactive. In fact, such branched peptides representing less than 2% of the naïve PepG induced the release of TNF-α with similar potency to LPS (89). The synthetic PepG monomer muramyl dipeptide, which consists of the two sugar units linked to two amino acids (alanine and glutamine), as well as stem peptides, were alone unable to induce inflammatory responses (77).

In a recent *in vivo* study, we demonstrated that PepG of *S. aureus* causes organ injury and inflammation (local and systemic) in the rat (91). Intravenous injection of 10 μg/kg PepG was associated with increased serum values of aminotransferases, indicative of liver injury. The response to PepG *in vivo* was also characterized by increased plasma values of TNF-α and IL-10 and cytokine gene activation in the liver, lung, and kidney. These findings support the contention that PepG is a factor that may contribute to the deleterious host response in sepsis.

Recently, the production of synthetic PepG polymers was reported (92). Fragments containing two or four repeating units of *N*-acetylglucosamine-*N*-acetylmuramic acid with or without a two amino acid (L-Ala-D-isoGln) branch on each *N*-acetylmuramic acid were prepared. Only the peptide conjugates had the ability to stimulate human monocytes and induced the expression of inducible nitric oxide synthase (iNOS) in rat macrophages. Important differences in signaling mechanisms were observed between the native PepG and the synthetic partial structures. Most notably, none of the synthetic PepG structures could activate TLR2-transfected Chinese hamster ovary (CHO) or human embryonic kidney (HEK) 293 cells, and mouse macrophages only produced TNF-α in response to native PepG. These synthetic analogues of bacterial components may prove useful for studying the precise functional requirements of PepG for interacting with pathogen recognition receptors, and the distinct signaling pathways elicited.

IS LIPOTEICHOIC ACID AN IMPORTANT ENDOTOXIN?

LTA is a cell wall component unique to gram-positive bacteria, and belongs to a diverse class of sugar phosphate polymers, which contain an acyl group anchored to the cell membrane (Fig. 1). A nonacylated form of LTA, teichoic acid (TA), is covalently linked to PepG in the gram-positive bacterial cell wall. Since 1993, the standard procedure for isolation of LTA involved hot phenol extraction followed by hydrophobic interaction chromatography (93), a method adapted from a LPS purification scheme.

In the early 1990s, it was demonstrated that LTA activates immune cells of the host to produce cytokines *in vitro*. In one study, the ability of LTA isolated from a number of different gram-positive bacteria to induce the release of IL-1β, IL-6, and TNFα from adherent human monocytes were examined (94).

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The addition of LTA from *Bacillus subtilis*, *Listeria monocytogenes*, *Streptococcus pyogenes*, and a number of *Enterococci* strains induced the release of all three inflammatory cytokines. In contrast, LTA isolated from *Staphylococcus aureus* and *Streptococcus pneumoniae* failed to induce cytokine release (94). In the same study, it was shown that induction occurred both in the presence and absence of autologous serum, suggesting that stimulation was not dependent on complement. Moreover, after de-acylation the LTA lost its ability to stimulate these cells, showing that the lipid is pivotal for the ability of LTA to initiate cytokine production. In rat macrophages *in vitro*, it was later demonstrated that LTA from several species induced 1) reductive capacity in a MTT-assay, 2) secretion of TNFα and nitrite, and 3) tumoricidal activity, whereas *S. aureus* LTA again was inactive (95). These studies suggested that the ability of LTA to influence immune responses is species dependent, and that *S. aureus* LTA is not potent in this respect.

During the 1990s, commercial preparations of LTA were frequently used to study the functions of this bacterial component. Such preparations are isolated by hot phenol extraction without further purification. LTA generated by this method may stimulate cells only when used in higher doses. For instance, *S. aureus* LTA induced 1) the release of IL-12 in a monocytic cell line (1.0 to 10 μg/mL; Ref. 96); 2) the expression of iNOS in vascular smooth muscle cells (97) and in the mouse macrophage cell line J774 (98, 99); 3) the accumulation of TNF-α, IL-6, and IL-10 mRNA in both monocytes and T-cells in a whole human blood assay (10 to 100 μg/mL; Ref. 81); and 4) the expression of cyclooxygenase-2 in human pulmonary epithelial cells (100). LTA was also found to activate both classical and alternative complement pathways (101, 102). *In vivo*, phenol extracted LTA has been shown 1) to act in synergy with PepG to cause shock and organ injury in rats (99, 103) and 2) to protect against ischemia/reperfusion injury of the heart and kidney (104, 105). In a recent study, intranasal instillation of LTA to mice caused infiltration of neutrophils into the lungs (106). These studies suggest that LTA shares many properties with LPS. However, LPS is the most potent microbial structure that exists, and several logs higher concentrations of LTA are required to obtain the same TNF-α response.

It was recently demonstrated that commercial preparations of LTA isolated from *S. aureus*, *B. subtilis*, and *S. sanguis* contain large and variable amounts of LPS, and other non-LTA components with the ability to induce inflammatory responses (107, 108). For example, it was shown that the ability of these preparations to stimulate production of NO in RAW 264.7 mouse macrophages is strongly attenuated by the LPS-inhibitor polymyxin B (107). Further purification of these preparations of LTA by hydrophobic interaction chromatography resulted in two well-separated fractions. One fraction was highly enriched for LTA and a second highly enriched for LPS. Most notably, the LTA-enriched fraction alone did not induce production of NO in RAW 264.7 macrophages (107). In a different study, commercial preparations of LTA were subjected to hydrophobic interaction chromatography and nuclear magnetic resonance spectroscopy (108). The content was very heterogeneous and was characterized by decomposition of the LTA structure. LTA content averaged 61% for *S. pyogenes*, 16% for *B. subtilis*, and 75% for *S. aureus* (108). The decomposition was characterized by a loss of glycerol-phosphate units as well as of alanine and N-acetylglucosamine substituents. The LTA-enriched fraction from *B. subtilis* and *S. pyogenes*, but not LTA from *S. aureus*, induced the release of TNF-α, IL-1β, IL-6, and IL-10 in human blood. It seems that the inability of *S. aureus* LTA to trigger inflammatory responses, noted by several investigators (94, 95, 108), may be caused by decomposition during the hot phenol purification procedure, rather than reflect species specific differences in the inflammatory features of LTA. LPS equivalents of more than 10 ng/mg of LTA were found in the commercial preparations (108).

A novel method for purification of pure and biologically active *S. aureus* LTA based on extraction with n-butanol at room temperature was recently reported (109). Structural analyses by nuclear magnetic resonance and mass spectroscopy showed that D-alanine substituents important for the inflammatory properties of LTA are preserved by the novel purification method (109). Comparison in the ability to induce the release of TNF-α between LTA prepared with butanol and LTA extracted with phenol showed that several logs less butanol-LTA were required to induce cytokine production. Indeed, 10 ng/mL butanol-LTA from *S. aureus* or *B. subtilis* were sufficient to induce the release of TNFα in a diluted whole blood model (109). These studies re-established *S. aureus* LTA as an important inflammatory principle in gram-positive bacteria.

In a subsequent study, however, using an undiluted whole blood model, 10 to 30 μg of *S. aureus* butanol-LTA per milliliter of blood was required to induce the release of TNF-α or IL-6 (110), a concentration that was 3 logs higher than previously reported (109). It was found that this discrepancy in the response to LTA was reflected by the difference between undiluted vs. diluted whole blood, inasmuch as the cytokine induction by butanol-LTA was inhibited by serum (110). In line with this notion, it was demonstrated that soluble CD14 inhibited the cytokine induction caused by LTA of different origin (111), which contrasts with the earlier findings that cytokine responses in adherent monocytes are independent of serum (94). This effect of sCD14 is important, because sCD14 is present in normal serum at concentrations of 5 to 10 μg/mL. LTA also induces the formation of TNF-α and IL-6 in primary cultures of rat and human Kupffer cells *in vitro* (112), and this response is also inhibited by serum [Øverland and Wang, unpublished data].

The ability of LTA purified with butanol to initiate inflammatory responses has so far been scarcely studied *in vivo*. A recent study showed that intramuscular injection of butanol-extracted LTA to mice (0.05 to 5.0 μg/kg) had no effect on leukocyte rolling, leukocyte adhesion, or leukocyte emigration in the microcirculation, as observed by intra-vital microscopy (113). Furthermore, systemic injection of LTA (0.5 to 50 mg/kg) did not elicit a drop in the circulating leukocyte counts or an increase in neutrophil sequestration to the lung, as could be obtained by LPS. This failure of LTA to influence leukocyte endothelium interactions were confirmed in a surrogate human blood vessel assay *in vitro* (113). Together these findings suggest that the inflammatory properties of LTA are strongly

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suppressed by normal serum, and that LTA alone is probably not an important initiator of inflammation in blood.

Most recently, the preparation of synthetic LTA has been described (114). Synthetic LTA is expected to be a useful tool in delineating the structural requirements in the interactions between LTA and pathogen recognition receptors on host cells, free of confounding contaminants. However, to get a better understanding of the role of this cell wall component for the deleterious immune activation in sepsis leading to circulatory failure and organ injury, genetically modified bacteria with alterations in the biosynthetic machinery of LTA would be a better approach to accomplish this task. Alternatively, specific antibodies that neutralize the effects of LTA during gram-positive bacteremia would be a useful tool to investigate the specific role of LTA in this condition.

PATTERN RECOGNITION RECEPTORS IN HOST RESPONSES TO PEPG AND LTA

Bacterial components such as PepG and LTA activate the host defense by engaging TLRs and other pattern recognition receptors of the innate immune system. A complete overview of the literature on the different TLRs and their various microbial ligands are beyond the scope of the present paper, and has recently been published elsewhere (115, 116). This review will focus on receptors involved in mediating inflammatory responses to PepG and LTA, and hence the gram-positive septic response.

Of the ten TLRs known, only TLR2 has been clearly demonstrated to be involved in the host defense against gram-positive bacteria. TLR2-deficient (TLR2–/–) mice are susceptible to infection with *S. aureus* and *S. pneumoniae* (64, 65). Initial studies in cell lines aimed to elucidate which components in gram-positive bacteria interact with TLRs, showed that PepG and LTA are two likely candidates (117, 118). It was demonstrated that transfection of TLR2-receptors conferred upon CHO cells or HEK293 cells the ability to activate NF-κB in response to PepG and phenol extracted LTA. In both studies, co-transfection with CD14 enhanced this TLR2-mediated activation (117, 118). Subsequent studies in TLR2–/– and TLR4–/– mice were performed to elucidate the critical role of these receptors for cellular responses to microbial components (119). Peritoneal macrophages from TLR2–/– mice were unable to produce TNF-α in response to *S. aureus* PepG, whereas cells from TLR4–/– mice produced large amounts of TNFα. The only study that suggests that LTA utilizes TLR4 was performed with phenol (rather than butanol)-extracted LTA (119). Indeed, later studies using highly purified LTA strongly suggest that LTA signals via TLR2 in mouse macrophages (120).

TLR2 has been shown to respond to diverse bacterial products and also recognizes lipoproteins from a number of bacterial species (121), showing that TLR2 is not a specific receptor for PepG and LTA. In a different study, it was then demonstrated that TLR2-transfected CHO cells respond to heat-killed *Listeria monocytogenes* but not to Group B *Streptococci*, suggesting that TLR2 can distinguish between different gram-positive bacteria (122). Most notably, this study also showed that a monoclonal antibody to human TLR2 (clone TL2.1) attenuates the release of TNF-α from adherent human monocytes induced by gram-positive bacterial fragments (122). This was the first evidence that the response of human leukocytes to gram-positive bacteria involved the activation of TLR2. There is also some recent evidence that TLR4 confers resistance to pneumococcal infection by interacting with pneumolysin (123), and that Nod2 sense peptidoglycan by recognition of MDP (124).

The innate immune system must be able to recognize and respond to a vast number of microbial structures in the environment. To accomplish this task, different TLRs cooperate by forming homo-dimers and hetero-dimers (125). Most notably, TLR2 and TLR6 must act in concert to respond to gram-positive bacteria and the yeast cell wall particle, zymosan. TLR2 and TLR6 are recruited to the macrophage phagosome (126), where they recognize PepG (125). In contrast, TLR2 recognizes bacterial lipopeptides without TLR6. It appears that dimerization of cytoplasmic domains is required to induce the production of TNF-α, and that TLR2 may form functional partners with TLR6 and TLR1. This important study shows that the cytoplasmic domains of different TLRs are not functionally equivalents, as had been assumed, and that the ability of a specific cell type to respond to gram-positive bacteria is not solely defined by it's expression of TLR2. Differential expression and heterodimerization between TLRs increase the repertoire of cellular responses that may be mounted to an infectious stimulus, forming the basis for finely tuned and cell specific responses.

In human cells, the TLR2 protein is predominantly expressed on monocytes and neutrophils, and not on T cells, B cells, or NK cells (127). However, a number of other cell types may produce mRNA for TLR2 and other TLRs (115). The expression and activity of TLR signaling is also regulated by soluble accessory proteins known as MD-1 and MD-2 (128–130). The myeloid receptor CD14 acts in concert with TLR4/MD-2 signaling to mediate sensitive responses to LPS. Demonstrations that PepG binds to CD14 (131), and that blockade of this receptor inhibits signaling events induced by both PepG and LTA (96, 98, 132, 133), led to the contention that CD14 is involved in signaling of gram-positive infections. This hypothesis has been questioned by the surprising observation that CD14–/– mice produced more TNF-α in response to PepG than their normal littermates (134). In addition, a potent CD14 blocking antibody (18D11), which eradicates responses to LPS does not inhibit PepG-induced production of TNF-α in whole human blood (81). Together these studies indicate that CD14 may function primarily as a scavenger or decoy receptor for PepG. In line with this notion, the expression of CD14 on monocytes is up-regulated by PepG and down-regulated by LPS (135, 136), possibly reflecting the different functions of CD14 in responses to these two components.

Organ-specific mechanisms have been reported to modulate the innate host response to gram-positive bacteria. For example, surfactant protein A (SP-A) and SP-D, collectins of the C-type lectin superfamily along with mannan binding lectins, are known to exert innate immune defense of the lung. However, the specific function of these proteins has not been

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well understood. In a recent report, it was demonstrated by solid-phase binding assays that SP-D, but not SP-A, bind PepG and LTA in a saturable and specific manner (137). Binding to LTA and PepG was dependent on calcium, and was inhibited by addition of carbohydrates. The latter suggests that SP-D binds to PepG and LTA by its carbohydrate recognition domain (CRD). Although SP-A did not bind to PepG or LTA (137), this main protein constituent of pulmonary surfactant binds directly to the extra-cellular domain of TLR2 (138), thus inhibiting cellular responses to PepG in a concentration-dependent manner. These results indicate that binding of SP-A to TLR2 interferes with innate recognition of PepG, a mechanism that may reduce harmful TLR2-mediated immune activation in the lung early after exposure to gram-positive bacteria. This notion is supported by *in vivo* observations that SP-A−/− mice exhibit enhanced pulmonary inflammation and reduced bacteria clearance (139–141).

Even though we know today that TLRs play a pivotal roles in the signal transduction events leading to inflammation and host defense, the precise mechanisms by which pathogenic structures interact with TLRs are still not understood. For instance, it has been difficult to demonstrate binding of microbial ligands to TLRs. However, a recent publication reports that the extra-cellular domain of TLR2 directly binds *S. aureus* PepG in an *in vitro* system (142). A soluble form of recombinant TLR2 extra-cellular domain (sTLR2) generated by a baculovirus expression system, binds avidly to insoluble PepG precoated onto micro-titer wells, and an antibody against TLR2 could inhibit this binding. In contrast, sTLR2 binds very weakly to LPS. These authors also show that sTLR2 attenuates the inflammatory responses caused by PepG in certain cell lines, and that sCD14 increased the binding of sTLR2 to PepG (142). This study clearly shows that TLR2 has the capacity to bind PepG in-vitro.

A new class of immune receptors involved in the innate recognition of PepG has recently been cloned in both insects and mammals (143–145). PepG recognition proteins (PGRP) bind PepG with high affinity and mediate antibacterial functions (146). In *Drosophila*, activation of Toll by gram-positive bacteria is dependent on a circulating PGRP (147). A *loss-of-function* mutation in PGRP (termed *semmelweis*) blocks activation of Toll by gram-positive bacteria and causes reduced resistance to infection (147). Recent data also demonstrate that PGRPs mediate activation of the NF-κB analogue relish (148) and an immune defense against gram-negative bacteria in *Drosophila* (149). Mouse PGRP inhibits growth of gram-positive bacteria (150). Moreover, mice deficient of the PGRP-S type have increased susceptibility to intraperitoneal infection with gram-positive bacteria of low pathogenicity, but not with pathogenic gram-positive or gram-negative bacteria (151). PGRP-$S^{-/-}$ mice exhibited normal inflammatory responses to bacteria, but their neutrophils were defective in intracellular killing and digestion of non-pathogenic gram-positive bacteria. Together these data strongly suggest that PGRPs mediate important host defense functions against bacterial infection. Four human PGRPs have been cloned so far (143, 152), each with unique expression patterns (152); PGRP-S is a soluble protein expressed in the bone marrow and to a lower degree in neutrophils, the trans-membrane PGRP-L is expressed in the liver and colon, and PGRP-Iα and PGRP-1β (trans-membrane) are highly expressed in esophagus with low expression also in tonsils and thymus. Functional data with respect to human PGRPs in induction of immunity in health and disease awaits further studies.

CELLULAR SIGNALING IN THE SEPTIC RESPONSE

An overview of some of the major signaling events downstream of TLR2 that lead to the production of inflammatory mediators in macrophages is shown in Figure 2. The evidence for implications of mitogen-activated protein kinase (MAPKs) and phosphatidide inositol 3-kinase (PI3-K) in the septic response and signaling events that intersect with MAPK and PI3-K signaling are subjected to some attention below.

In human sepsis, activation of MAPKs has been implicated in destructive granulocytosis, and manipulation of the MAPK system has been attempted to restore immune function in sepsis. Most notably, inhibition of MAPK activation in granulocytes isolated from patients with severe sepsis, caused restoration of LPS-mediated granulocyte apoptosis (153). In a murine model of polymicrobial sepsis, Song et al. recently demonstrated that p38 MAPK mediate splenic immune-suppression, and that inhibition of p38 MAPK can restore lymphocyte function and improve survival in this model (154, 155). Moreover, MAPK activation has been implicated in endotoxin tolerance (156) and inactivation of alveolar macrophages after trauma-hemorrhage and subsequent sepsis (157).

A central role of PI3-Kγ activated by G protein-coupled receptors (GPCR) has also been demonstrated in inflammation (158). Neutrophils from PI3-Kγ−/− mice displayed impaired respiratory burst, reduced migration towards a range of chemotactic stimuli and defective accumulation in a model of septic peritonitis. In further support of this contention, a study of murine endotoxemia showed that lung edema, neutrophil recruitment, NF-κB activation and pulmonary levels of IL-1β and TNFα were reduced in PI3-Kγ−/− mice compared with normal littermates (159). These studies show that PI3-Kγ plays an important role in signaling events leading to neutrophil activation and recruitment. Based on these observations, these signaling pathways may be a suitable target for manipulation aimed to reduce excessive organ inflammation and injury in sepsis.

The signaling system mediated by cAMP has been reported to repress activation of ERK, p38 MAPK and JNK in LPS-stimulated peritoneal macrophages (160) and to inhibit LPS-induced expression of TNF-α, NF-κB, and iNOS in Kupffer cells and monocytes (161–163). In contrast, cAMP up-regulates the production of cyclooxygenase 2 and PGE2 (164). Together this indicates that cAMP signaling events counteract the inflammatory response. Cyclic AMP is also able to inhibit LPS-mediated and IL-1-mediated iNOS production, indicating that the inhibitory effect of cAMP may work on TIR-family receptors in general (165, 166). Similarly, reduction

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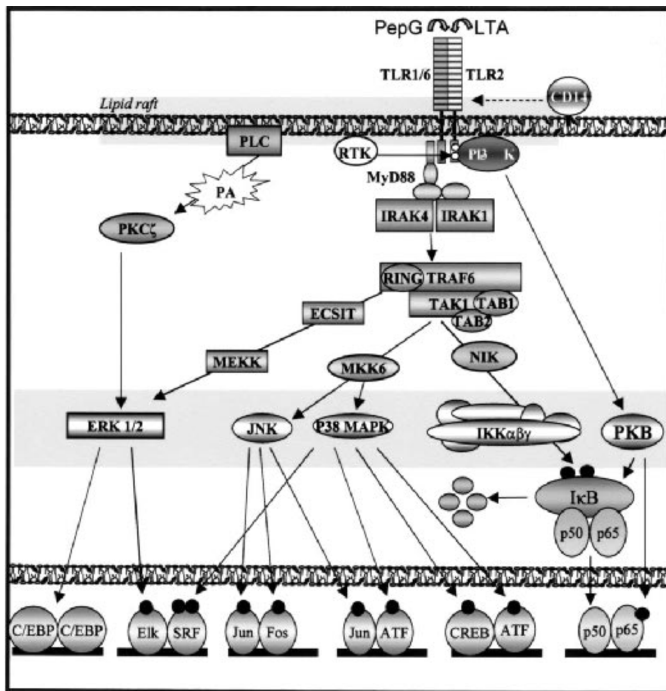

Fig. 2. **Signaling downstream of TLR2**. Upon recognition of a TLR2 ligand (e.g., PepG or LTA) a range of intracellular signaling events occurs, leading to the activation of signaling kinases of the MAPK family (ERK1/2, JNK, and p38; Refs. 191–195), PKB (196), and IKKβ (197–199). Further, several transcription factors, including nuclear factor κB (NF-κB, p50, p65; Ref. 197), Jun/Fos (Refs. 200, 201), ATF (200), the complex of serum response factor (SRF) and Ets-like protein (Elk; Ref. 202), C/EBP (195), and CREB (200) are activated by the kinase cascade, resulting in the expression of a wide range of proinflammatory mediator genes. The first event that initiates the signaling cascade is most likely the colocalization and clustering of receptors and signaling molecules at the plasma membrane. Clustering of TLR2, TLR6, and CD14 in the recognition of secreted microbial products from group B *Streptococcus* (GBS) have been shown (203), and the studies on TLR4 signaling have revealed that the cluster is localized to lipid rafts (detergent-insoluble membrane regions rich in cholesterol and glycolipids) (204). The first intracellular molecule recruited to the complex is the Toll/IL-1 receptor (TIR)-domain containing adapter molecule MyD88 (myeloid differentiation factor 88; Refs. 205, 206). MyD88 then recruits IL-1 receptor associated kinases/IRAKs (207). When multimers of IRAKs are formed, they gain the ability to catalyze auto- and cross-phosphorylations and recruit a larger complex consisting of TNF receptor-activated factor/TRAF6, TAK1, TAB1, and TAB2 (208, 209). Several subforms of IRAK exist, and whereas IRAK-4 has been demonstrated to phosphorylate IRAK-1 and be indispensable for further signaling events (210), IRAK-M was recently reported to be an inhibitory subform (211). Further on, TAK1 will be activated and released, and may activate IKKβ and MAP kinase kinase 6/MKK6 (212, 213). The NF-κB–inducing kinase (NIK) is required for the activation of IκB kinaseβ (120, 212), which initiates IκB degradation and NF-κB nuclear translocation (197). MKK6 is responsible for activation of the MAPKs p38 and JNK (199). In the presence of the adapter protein ECSIT and MEK kinase 1/MKK1, TRAF6 may also activate ERK1/2 (214), thus generating a multitude of downstream responses. Another pathway that may activate ERK1/2 independently of TRAF6, involves the atypical PKC isoform PKCζ, a pathway demonstrated to be activated by LPS but not by Toll-like receptors directly (215). PKCζ activation have been associated with an accumulation of phosphatidic acid (PA) (216). Interestingly, accumulation of PA (217) is linked to activation of ERK (218) and to endocytosis (219), and inhibitors of endocytosis block ERK activation (219). The activation of the PI3-kinase/PKB pathway have also been demonstrated for gram-positive mediators, and PI3-K was shown to form a complex with TLR-2 at the plasma membrane and initiate NF-κB activation in a PKB-dependent fashion (196). In this pathway, tyrosine phosphorylation of the TIR-domain of TLR2 by a RTK was shown to be essential. PepG, peptidoglycan; LTA, lipoteichoic acid; TLR, Toll-like receptor; PLC, phospholipase C; PKB, protein kinase B; PKC, protein kinase C; PI3-K, phosphatidide inositol 3 kinase; RTK, receptor tyrosine kinase; MyD88, myeloid differentiation factor; IRAK, IL-1 receptor associated kinase; TRAF, TNF receptor-activated factor; TAK1, transforming growth factor beta activated kinase 1; TAB, TAK1 binding protein; MAPK, mitogen-activated protein kinase; MKK, MAPK-kinase; MEKK, MAPK-kinase-kinase; ERK1/2, extracellular signal responsive kinase; JNK, Jun N-terminal kinase; IκB, inhibitory kappa B; IKK, IκB kinase; ECSIT, evolutionary conserved intermediate in Toll pathway; C/EBP, CCAAT enhancer binding protein; CREB, cyclic AMP responsive element binding protein; SRF, serum response factor; ATF, activating transcription factor.

of cAMP levels in Kupffer cells caused by the uncoupling of cAMP production by ethanol, show a related stimulatory effect on the production of TNF-α. (167). The implication of the cAMP signaling system in sepsis is supported by several studies showing that cAMP levels are regulated in experimental animal models of polymicrobial sepsis (168–170), trauma-hemorrhage and resuscitation (171), and endotoxic shock (172). Most notably, the Kupffer cells seem to be central in this respect (168, 169, 171–173). Aberrant cAMP signaling has also been demonstrated in blood from patients presenting with severe sepsis and septic shock (174). The ability of cAMP to regulate vasoactive mediators such as NO has prompted attempts to modulate this system. In one study, administration of cAMP caused improved systemic vasoconstriction caused by endotoxin in dogs (175). Studies have also implicated PGE2 mediated cAMP signaling in blunted T lymphocyte responses in sepsis by modulation of the T cell receptor regulator p59fyn (176). Altogether, these observations indicate that an inhibitory role of the cAMP pathway on TLR-mediated production of inflammatory mediators may exist and should be further examined. It should also be emphasized that even though TLR and TLR signal transduction is central in the inflammatory response, a number of other receptors also trigger signaling events that contribute to the integrated response.

INTERACTIONS OF PEPG AND LTA IN SEPSIS AND ORGAN INJURY

It is becoming increasingly clear that LPS cannot fully reproduce all clinical features of sepsis. This implies that other contributing factors co-operate with LPS to trigger the aberrant signaling events leading to sepsis and organ injury. It was noted more than 20 years ago that the synthetic PepG monomer MDP can act as an adjuvant and enhance immune responses (177–180). For example, Pabst and co-workers showed that pre-treatment of mouse peritoneal macrophages with MDP or LPS primed the cells for enhanced production of superoxide anions ($O2^-$) in response to phorbol myristate acetate (PMA). The priming effect was not observed with stereo-isomers of MDP (178). It was also demonstrated that intravenous injection of MDP enhanced lethal toxicity of LPS in mice (181–183). The priming effect of MDP *in vitro* is enhanced by IFN-γ (184). Parant and colleagues reported that MDP and LPS act synergistically to induce TNF-α in mice, and maximal effect was noted when MDP was given several hours before LPS (185). It should be stressed that there is good evidence that MDP alone is unable to induce inflammatory cytokine production in cells (76). Thus, MDP and LPS interact in a synergistic manner to enhance inflammatory responses in-vitro and in-vivo, and cytokines may contribute to the consequences of

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such interactions. However, the molecular basis for this phenomenon is still unknown.

The implication of interactions between PepG and LTA on the pathophysiology of septic shock and multiple organ failure has been studied in our laboratory (99, 103). In one study it was demonstrated that infusion of PepG and phenol-extracted LTA cause shock and multiple organ failure in the anaesthetized rat, whereas each component alone was unable to do so. Shock and organ injury were associated with the induction of iNOS in the organs and enhanced plasma values of TNF-α and INFγ (103). MDP was later shown to be the minimal essential PepG structure required to induce shock and organ failure in the rat *in vivo* (99). In addition, there is evidence that PepG and LTA act synergistically to cause respiratory failure and sepsis in the pig (186). Even though interactions between PepG and LTA are suggested by these studies, the potential effect of contamination with LPS in the commercial preparations of LTA (107, 108) has not been accounted for. This opens the possibility that the observed priming could be due to contaminating LPS in the LTA preparation, and not caused by LTA itself. In keeping with this notion, we have recently demonstrated that LPS and PepG act synergistically to cause organ failure and shock in the rat (187). In addition to LPS, commercial preparations of LTA contain various non-LTA molecules with the ability to induce the production of TNF-α (108, 188). Hence, immunostimulatory components in the LTA preparations different from LPS (or LTA), may act in synergy with PepG and be responsible for induction of organ injury and shock in rats. The conclusions with respect to potential interactions between PepG and LTA and their implications for development of shock and organ injury await further studies using highly purified butanol-extracted LTA.

Despite the large body of evidence demonstrating the potent synergistic interactions between LPS and PepG/MDP on induction of immune responses, the impact of such interactions in generation of septic shock and organ failure are still not well understood. In our laboratory, we used a whole human blood model to study synergistic interactions between naïve PepG structures and LPS on production of both pro- and anti-inflammatory cytokines (135). Together PepG or LPS induced the release of high amounts of inflammatory cytokines TNF-α and IL-6 that could not be obtained by several-fold higher concentrations of each stimulus alone. This phenomenon was observed with different concentrations of each stimulus and after different incubation periods. In contrast, PepG and LPS did not seem to enhance the production of the anti-inflammatory cytokine IL-10 (136). We also demonstrated that, in clear opposition to LPS, PepG causes increased expression of the CD14 receptor on the surface of monocytes (135, 136). Increased expression of CD14 induced by PepG may sensitize cellular responses to LPS, and thus be implicated in the increased sensitivity to LPS caused by PepG. The synergistic effect of MDP and LPS on the release of IL-8 was studied in the human monocytic cell line THP-1 (189). MDP, which alone failed to potently induce IL-8 release, enhanced the production of this cytokine in response to both LPS and crude phenol LTA (189). Together these studies show that synergistic responses of PepG and LPS are operative in human leukocytes, and suggest that the inflammatory balance is shifted in favor of a pro-inflammatory response by such interactions. In should be noted that the synergistic interactions between LPS and PepG to induce inflammatory responses in macrophages seem to be cell specific, and such synergy was not observed in rat or human Kupffer cells [Øverland and Wang, unpublished observation].

The mechanistic basis for the increased inflammatory response triggered in cells upon simultaneous exposure to LPS and PepG/MDP is not clear. Increased expression of CD14 induced by PepG may be involved (136), as noted above. On the other hand, since both LPS and PepG bind to the CD14 receptor (47, 131), they would be expected to compete for CD14 occupancy and act as reciprocally inhibitors, rather than to act synergistically. TLR2 is known to activate cells via TLR2, whereas TLR4 transduce signaling by LPS. On this basis, the hypothesis was put forward that PepG-LPS synergy occurs as a consequence of simultaneous TLR2-TLR4 activation (187). However, another TLR2 agonist, LTA, does not interact with LPS in the induction of cytokines in human blood (Wang et al., unpublished data) or in primary cultures of rat Kupffer cells (112). In a recent publication by Wolfert et al. (190), MDP alone was shown to induce TNF-α mRNA in human Mono Mac 6 cells, but the transcript was not translated into protein. In combination with either LPS or PepG, MDP caused additive accumulation of TNF-α mRNA. The authors suggest that LPS and PepG remove a block in translation of the mRNA produced in response to MDP. This study demonstrates that MDP suffices to activate gene transcription, but is unable to drive translation into TNF-α protein. Subsequently, this suggests that the signaling pathways for transcription and translation of TNF-α are distinct, which may reflect a "security-valve" in the induction of inflammation. The data also, at least in part, indicated the mechanisms by which MDP cause enhanced immune responses to LPS. However, the author failed to observe synergy between naïve PepG and LPS (190).

FUTURE PERSPECTIVES

Sepsis with multiple organ failure still carries unacceptable high mortality and morbidity. Whereas we know that LPS is important in initiating many of the early events involved in the devastating inflammatory response in sepsis, it has become clear that other microbial components such as PepG and LTA also may play a role. To date, the roles of PepG and LTA in the development of sepsis and organ injury are still unclear, but a large number of publications showing that these components can trigger or amplify inflammatory responses suggest that they may contribute. The use of specific inhibitors or antagonists of PepG and LTA during infections may be helpful to clarify their roles in this respect. Both PepG and LTA use TLR2 for signaling, but different biological responses are induced by them. This demonstrates that the cell wall components such as PepG or LPS cannot be merely reduced to a ligand of a TLR2 or TLR4, probably because a multitude of different phagocyte receptors contribute to forming the biological response. Increasing amount of literature moreover suggest that different microbial components such as PepG and LPS

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may act synergistically to cause organ injury in sepsis. The molecular basis for such synergistic interactions is not yet understood, however, the differential use of TLRs and other innate phagocyte receptors may be involved. Future studies should continue to unravel the involvement of different microbial components such as PepG, LTA, LPS and CpG DNA in the septic response, and potential synergistic interactions between them. By doing this, the aberrant signaling events leading to the devastating condition of sepsis and organ failure and new ways to intersect them will emerge.

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