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Septic Shock by Gram-Negative Infections: Role of Outer Membrane Proteins

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1. Introduction

The magnitude of septic shock as a clinical problem is often understated. Despite advances in our ability to diagnose and treat infectious diseases, severe sepsis leading to shock due to gram-negative infections remains one of the leading causes of mortality worldwide. Septic shock develops because of a dysregulation in the host response, and the mechanisms initially recruited to fight infection produce life-threatening tissue damage and death. Recent research has witnessed a significant increase in our understanding of host-pathogen interactions, particularly in the area of innate immunity and the molecular recognition of gram-positive and gram-negative bacteria. Important new mediators of sepsis and novel mechanisms of host-cell toxicity have been identified and, together with clinical trials targeting pathways considered central to sepsis pathogenesis, provide new insight into the molecular and cellular basis of sepsis for the formulation of new strategies of intervention.

Research on septic shock pathogenesis by gram-negative bacteria is mainly focused on the understanding of the molecular and cellular role played by lipopolysaccharide (LPS). Strong experimental evidence and clinical observations suggest that the release of proinflammatory cytokine mediators by LPS-responsive cells (mainly macrophages, endothelial cells and neutrophils) in response to toxic products sets in motion the genetic and physiologic program that manifests as shock. The best characterized of these toxic components is LPS, which is considered as a paradigm for other less well-characterized toxic microbial molecules. The immune protection stimulated by highly purified LPS in animals does not resolve the symptomatology of septic shock, while LPS mixed to outer membrane proteins shows a better protective activity. Several studies evidence the major role played by outer membrane proteins in the molecular interaction between the host cell and the gram-negative bacteria. Endotoxin-associated proteins consist of a complex of several major proteins that are intimately associated with the LPS. Very little is known about release of non-LPS gram-negative outer membrane components such as OMPs in sepsis. Among the OMPs, porins have been shown to play an important role in pathogenesis of bacterial infections. Porins were pyrogenic in rabbits and elicited a localized reaction when used as the sensitizing and eliciting agent. Porins were also shown to kill D-galactosamine sensitized LPS-responsive and LPS-unresponsive mice. Treatment of Human Umbilical Vein Endothelial Cells: (HUVEC) with porins increased the transmigration of different leukocyte populations, in

particular of neutrophils. Porins by several gram-negative bacteria induce cytokine release by human leukocytes as well as enhancement of cytokine gene expression. Also, other components of the bacterial envelope are important in the induction and pathogenesis of septic shock such as bacterial lipoproteins (LP). As anti-LPS therapies does not seem to improve by the addition of proteins from the outer membrane or small fragments of these proteins, a great alternative to existing strategies will involve the blockage of signal transduction pathways, cytokine and inflammatory mechanisms.

2. The outer membrane of gram-negative bacteria

Bacteria in order to face unpredictable and often hostile environment have evolved a sophisticated and complex cell envelope that protects them while allowing selective passage of nutrients from the outside and waste products from the inside. There are three principal layers in the envelope: the outer membrane (OM), the peptidoglycan cell wall, and the inner membrane (IM). The two membrane layers delimit an aqueous cellular compartment called periplasm. The OM is a characteristic feature of Gram-negative bacteria, and in fact Gram-positive bacteria lack this structure. The OM is a lipid bilayer intercalated with proteins, superficially resembling the plasma membrane. The OM does contain phospholipids but they are confined to the inner leaflet of this membrane. The outer leaflet is composed of glycolipids, mainly lipopolysaccharide (LPS).

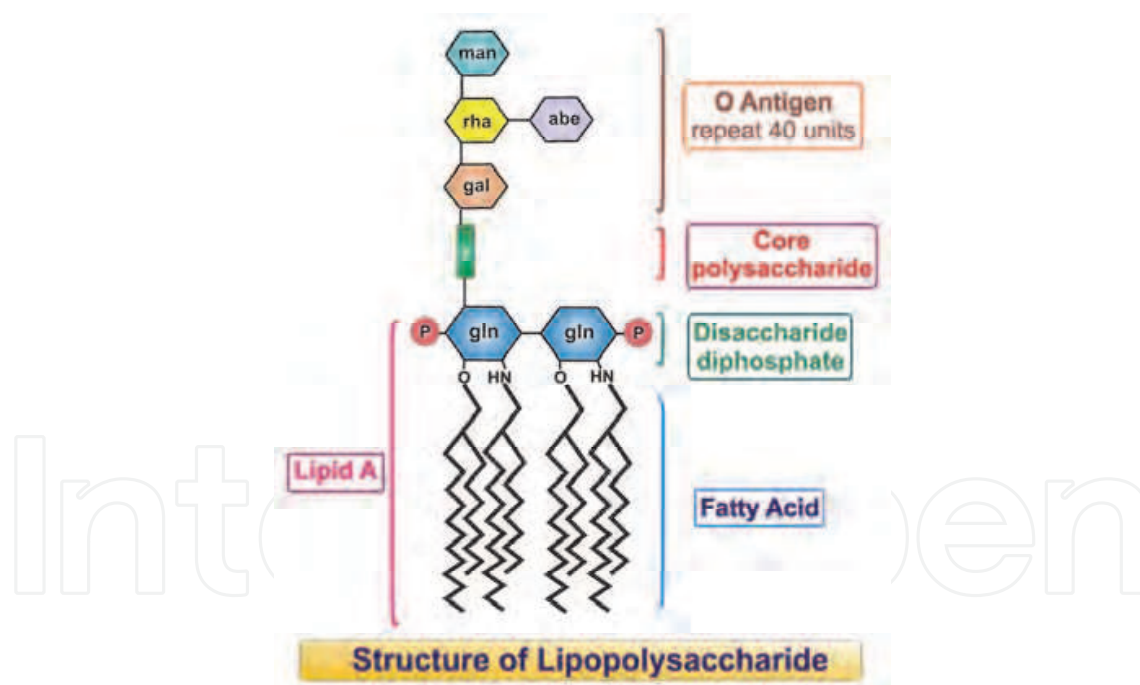


Fig. 1. Schematic representation of the structure of lipopolysaccharide (LPS).

LPS is a complex glycolipid exclusively present in the outer leaflet of the OM of gram-negative bacteria. LPS is one of the molecules responsible for the endotoxic shock associated with the septicemia, and is a sure indicator of infection as the human innate system is sensitized to this molecule. LPS molecules consist of a bisphosphorylated lipid (lipid A) forming the hydrophilic region of the outermost membrane leaflet which is stabilized by divalent cations and a hydrophilic polysaccharide (PS), extending outward from the

bacterium. A schematic structure for LPS from *Escherichia coli* together with the chemical structure of lipid A (Figure 1) reveals its key features. The LPS consists generally of two distinct regions, a core oligosaccharide chain of repeating units, the O-specific chain, which constitutes the major anti-LPS immune response. The core is covalently bound to the lipid A through an acidic sugar, the 3-deoxy-D-manno-oct-2-ulopyranosonic acid (Kdo). The outer core region consists of neutral or amino hexoses such as D-glucose, D-galactose, D-glucosamine, D-galactosamine or N-acetyl derivatives, while the inner core also contains heptose residues which are often substituted by phosphate, pyrophosphate or diphosphoethanolamine. Kdo represent a covalent bridge between lipid A and heptose units joined by diester phosphate linkages. The general pattern of the lipid A from diverse gram-negative bacteria is highly conserved. The lipid A from *E. coli* has a β -1,6-linked D-glucosamine disaccharide phosphorylated in positions 1 and 4'. Lipid A often contains up to four moles of (R)-3-hydroxytetradecanoic acids symmetrically distributed on the two glucosamine residues of the backbone. The hydroxyl in position 6' is linked to Kdo (Figure 1). The core oligosaccharide is very variable among bacterial species; so different species can express uniquely modified types of LPS. The O-antigen, if present, is the most variable part of LPS and shows even a high degree of variability between different strains of the same species.

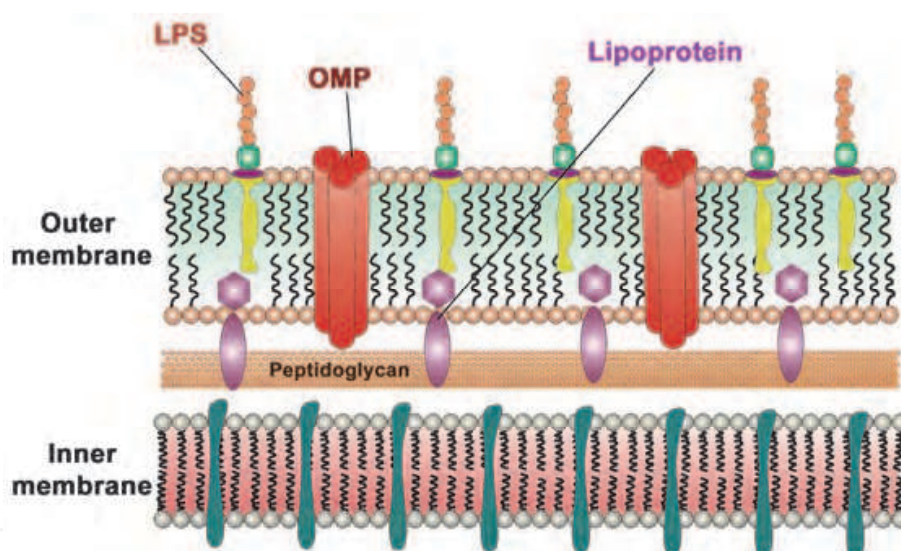


Fig. 2. Schematic representation of the inner and outer bacterial membrane.

With few exceptions, the proteins intercalated in the OM can be divided into two classes, proteins that traverse the membrane and assume a β -barrel structure and lipoproteins, anchoring the outer membrane to the underlying peptidoglycan stratum (Figure 2). Lipoproteins contain lipid moieties that are attached to an amino-terminal cysteine residue. It is generally thought that these lipid moieties embed lipoproteins in the inner leaflet of the OM, and are thus not supposed to be transmembrane proteins. Lipoproteins are low molecular weight proteins and are considered to be the most abundant proteins in the *E. coli* cell on the basis of molecular members. Lipoproteins are generally covalently linked to the peptidoglycan, but may also be present without covalent bonds.

The outer membrane proteins (OMPs) of gram-negative bacteria have been well characterized; they assume a β -barrel conformation. The OMPs serve as a molecular filter for

hydrophilic substances, and mediate the transport of nutrients and ions across the membrane into the periplasm. The OMPs can be divided into three classes (Nikaido, 2003): porins, substrate specific transporters and active transporters. Porins are a group of trimeric proteins that form pores of a fixed diameter through the lipid bilayer of the membrane. They constitute the major component of the OM and are thus indicated as “major outer membrane proteins” of high molecular weight.

Porins form passive pores that do not bind their substrates; they form trimeric, water-filled pores, through which relatively small (<600 Da) solutes diffuse, driven by their concentration gradient. For nutrients that are present at low concentrations in the extracellular environment, passive diffusion is no longer efficient and transport occurs via substrate-specific (substrate specific porins and transporters) and active transporters (Galdiero, 2007). The active transporters (FepA and FhuA) bind their substrates with high affinity and transport them against a concentration gradient. This process requires energy, which is provided by the inner membrane protein Ton B. The substrate-specific porins and transporters contain low affinity substrate saturable binding sites that allow efficient diffusion of substrates at very low concentration gradients. Among the substrate specific porins are LamB (maltose and maltodextrins) and SerY (sucrose); among the substrate specific transporters are Tsx and FadL, while among auto-transporters are NaLP and Hia. Whereas the composition, structure and function of the OM are well known, its assembly in the absence of energy sources has remained largely enigmatic. All the components of the OM are synthesized in the cytoplasm or at the cytoplasmic face of the IM, and they have to be transported across the IM and through the periplasm to reach their destination and to assemble into the OM.

3. The porins

The most abundant proteins of the bacterial outer membrane are porins which form channels with various degrees of selectivity (Schulz, 2002). Porins form β -barrels and their structures typically contain 14, 16 or 18 β -sheets. The majority of porins studied so far belong to the 16 or 18 stranded bacterial porins; and the general motif of their structural architecture is the closure of the barrel by pairing of the first and last β -strand in an antiparallel way. All strands are connected by eight or nine long loops, facing the extracellular side, with seven or eight small turns in the periplasmic space. In all porins, the constriction at the barrel center is formed by an inserted long loop L3, which is not exposed to the cell surface but folds back into the barrel, forming a constriction zone at half the height of the channel and contributing significantly to the permeability of the pore. Another feature is the presence of aromatic girdles with tyrosine and phenylalanine residues located at the outer and inner membrane boundaries. Residues located between these girdles and facing the hydrophobic lipid environment are mainly leucine, valine and isoleucine residues. At the very C-terminus almost all porins have a phenylalanine residue that is fundamental for proper import and folding in the outer membrane.

Porins made of 16 strands are called general or non-specific porins and form pores allowing the diffusion of hydrophilic molecules, showing no particular substrate specificity, despite some selectivity for either cations or anions; while 18 strands porins are substrate specific porins. Porins are passive diffusion channels with a pore diameter ranging from 15 Å for the general porins to 6 Å for the highly selective porins. Larger pores usually contain charged residues at opposite sides that form a local transversal electric field at the pore eyelet. This

field constitutes an energy barrier for low-polarity solutes so that the bacterium can exclude unwanted nonpolar molecules such as antibiotics while presenting a spacious eyelet for collecting large polar molecules such as sugars. A systematic study changing the pore properties by point mutations showed a strong correlation between the eyelet cross section and diffusion rate. Charge reversals affect selectivity and voltage gating. Interesting results were obtained with mutations at loop L3, for example the specificity of the sucrose porin was changed toward that of the maltoporin.

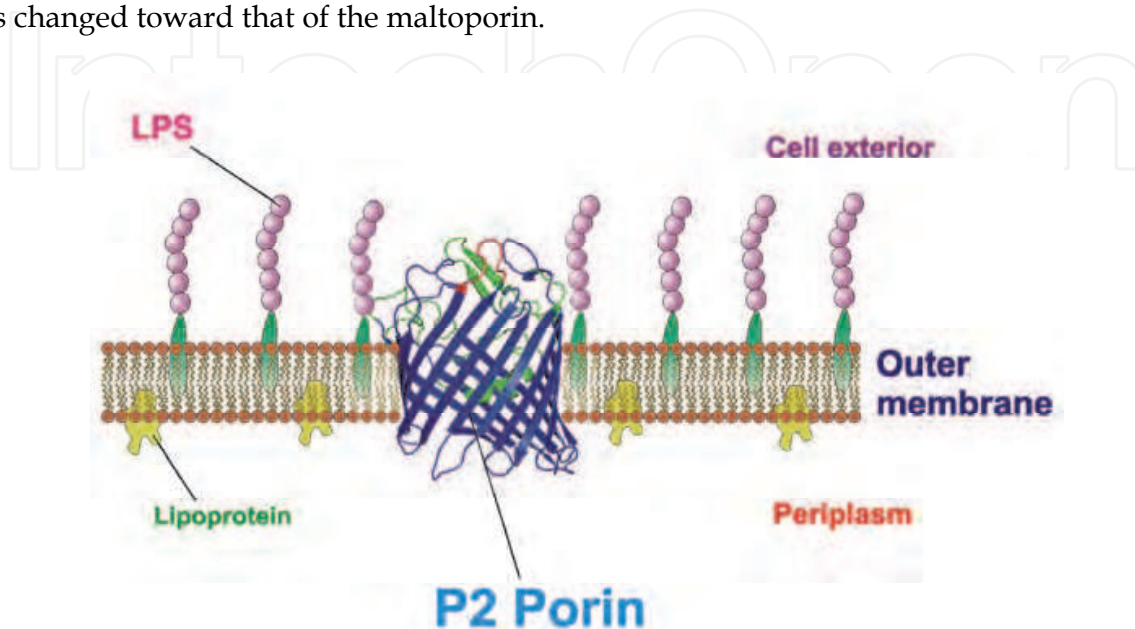


Fig. 3. Three dimensional model of the P2 monomer from *Haemophilus influenzae* type b. Surface loops are shown in green except L7 that is red. The extracellular space is located at the top of the figure and the periplasmic space is at the bottom. The position of the membrane bilayer is shown.

All porins form homotrimers in the OM; each subunit produces a channel and the trimer therefore contains three channels. For most porins, loops L1, L2 and L4 are important for monomer-monomer interactions within the porin trimer; loop L3 is internal; loops L5, L6 and L7 are superficial; loop L8 folds back into the barrel interior, contributing to the formation of the channel opening at the external side (Figure 3). Data from the literature indicate that peptide sequences corresponding to superficial loops are responsible for most of the biological activity of porins. In particular, loop L7 of porin OMPK36 from *Klebsiella pneumoniae* is involved in the interaction with C1q (Alberti, 1995); loops L5, L6 and L7 of porin P2 from *Haemophilus influenzae* activate JNK and p38 mitogen-activated protein kinase (MAPK) pathways (Galdiero, 2003) and induce the release of TNF- α and IL-6 (Galdiero, 2006); most functional antibodies raised to NTHI are directed to loop L5, which is thought to contain strain-specific and immunodominant epitopes (Yi, 1997); antibodies to loop L6 of NTHI showed complement-dependent bactericidal activity (Haase, 1994); the surface exposed loop regions are immunodominant as shown by immunizing mice with whole bacterial cells (Neary, 2001); synthetic peptides representing epitopes of outer membrane protein F of *Pseudomonas aeruginosa* elicit antibodies reactive with whole cells of heterologous immunotype strains of *Pseudomonas aeruginosa* (Hughes, 1992); major immunogenic epitopes of PorA and FetA of *meningococci* correspond to contiguous peptide

sequences located in putative surface-exposed loops of those proteins (Maiden, 1991; Thompson, 2003).

4. Lipoproteins

Lipoproteins are a major component of the outer membrane of bacteria, with low molecular weights (about 7000 Da). Lipid modification of bacterial proteins enables the anchoring of hydrophilic proteins to hydrophobic surfaces through the hydrophobic interaction of the attached acyl groups to the cell wall phospholipids, allowing the protein to function effectively in the aqueous environment (Kamalakkannan, 2004). Lipoproteins can localize in various places of the cell. *E. coli* has more than 90 lipoproteins, the majority of which is located at the periplasmic face of the OM, with some present at the periplasmic face of the IM (Narita, 2004). Although all the known lipoproteins in *E. coli* face the periplasm, in some gram-negative bacteria, lipoproteins are also present on the outer leaflet of the OM. However, little is known about the exact mechanism of how they translocate across the OM whether they are exposed or not to the outside surface of the outer membrane. Moreover, in those *E. coli* strains that have defects in LPS structure, the lipoproteins seem to react with antilipoprotein serum.

Lipoproteins are low molecular weight proteins lacking histidine, tryptophan, glycine, proline and phenylalanine. They are linked by the ϵ -amino group of their C-terminal lysine to the carboxyl group of every tenth to twelfth meso-diaminopimelic acid residue of the peptidoglycan. The N-terminal portion of the lipoprotein consists of glycercylcysteine to which two fatty acids are linked by two ether linkages and one fatty acid by an amide linkage. The amide-linked fatty acid consists of 65% palmitate, with the rest being mainly monosaturated fatty acids. The fatty acid bound as esters are similar to the fatty acids found in the phospholipids of the inner layer of the membrane. Lipoproteins exist in the membrane also as free form without covalent bonds to the peptidoglycan. There are about 2.4×10^5 molecules of the bound form per cell, and about twice as much of the free form. The total free and bound lipoprotein molecules 7.2×10^5 make lipoproteins the numerically most abundant protein in the membrane. Lipoproteins are required for virulence and play a variety of roles in host-pathogen interactions, from surface adhesion to initiation of inflammatory processes (Kovacs-Simon, 2011).

5. Outer membrane blebbing

Extracellular secretion is the major mechanism by which gram-negative pathogens communicate with and damage host cells. Vesicles released from the envelope of the growing bacteria serve as secretory vehicles for protein and lipids of gram-negative bacteria. Vesicles production occurs in infected tissues and is influenced by environmental factors. Vesicles play an important role in colonization, carrying and transmitting virulence factors into host cells and modulating host defense and immune response. Gram-negative bacteria release membrane vesicles of average diameter 10-300 nm into the environment during all stages of normal growth as well as in a variety of growth environments such as infected tissues. The amount of released vesicles is increased several folds during periods of bacterial stress such as exposure of microorganisms to antibiotics or human serum. The vesicles are formed by protrusions of the bacterial outer membrane that are released into the environment (Ellis, 2010). Outer membrane vesicles (OMVs) are formed by blebbing and

pinching off segments of the bacterial outer membrane (Kulp, 2010). These vesicles contain the main components of the outer membrane such as LPS, OMPs and fractions of the underlying bacterial periplasm. Importantly, OMVs are not a product of cell death since they are produced without concomitant bacterial lysis and newly synthesized proteins are present. Active concentrations of both LPS and porins are often accumulated at infection sites from either gram-negative bacteria outer membrane blebbing or bacterial lysis. Gram-negative bacteria contain about 10^5 molecules of porin per cell (molecular mass, about 36kDa) and about 3.4×10^6 molecules of LPS (molecular mass, about 4.5kDa), therefore, 10^6 to 10^9 bacterial cells are enough to reach a concentration of 500 ng/ml to 20 µg/ml (about 0.02 to 0.8 µM) for porin or a concentration of 100 ng/ml to 10 µg/ml (about 0.05 to 5 µM) for LPS.

OMVs from pathogenic bacteria contribute to the pathogenicity in vivo (Ellis, 2010). Thus, OMVs are likely a key factor in effecting an inflammatory response to pathogens, being immunogenic and capable of eliciting proinflammatory responses. Immunization with *Vibrio cholera* OMVs induces protection in mice (Schild, 2008); the OMVs immunized mice were protected against *Salmonella* infections (Alaniz, 2007). Furthermore, OMVs influence inflammation and disease in vivo; it was shown that, in response to *Helicobacter pylori* and *Pseudomonas aeruginosa* OMVs (Bauman, 2006), epithelial cells produce interleukin-8, a cytokine that plays a fundamental role in neutrophil and monocyte recruitment.

Septic shock has been associated with an early excessive inflammatory response to LPS and other bacterial components, among which OMPs and lipoproteins. During sepsis and septic shock large quantities of OMVs are released into serum and tissues. In particular, fragments containing LPS, OMPA and a protein of 17kDa, were affinity purified from filtrate of human serum incubated with *Salmonella enterica* serovar *Abortus equi* using O-chain-specific anti-LPS IgG (Freudenberg, 1992); similarly, complexes containing LPS and at least three OMPs, with molecular masses of 35, 18 and 5-9 kDa were affinity purified from filtrates of normal human serum incubated with *Escherichia coli* cells, using O-chain-specific anti-LPS IgG (Hellman, 2000). These molecules or macromolecular complexes have been shown to derive from the OMVs formed by the blebbing of bacterial cells.

6. OMPs and endothelial cells

Bacteria or bacterial products may constitute important inducers of surface molecule expression on endothelial cells (Rawadi, 1996). The microvascular endothelium plays an important role in regulating the exchange of fluids, macromolecules and cells between the blood and the extravascular tissues. The endothelium is a pervasive organ covering a surface area of 4000-7000 m². Endothelial cells are highly active, constantly responding to alteration in the local extracellular environment, as might occur in the setting of transient bacteremia or other important stress such as septic invasion. Endothelial cell activation occurs as a normal adaptative response, the nature and duration of which depends on the type of stimulus. Endothelial cell injury contributes significantly to the pathophysiology of bacterial sepsis and endotoxic shock. Components of the bacterial surface activate pattern recognition receptors on the surface of the endothelium. Gram-negative bacteria contain several surface molecules interacting with endothelial cells. The role of LPS is well known while the roles of other surface molecules of gram-negative bacteria are less understood. Several studies have recently shown the activity of major outer membrane proteins on endothelial cells. The bacterial surface contains a wide assortment of molecules that interfere

with the complex network regulating the leucocyte traffic. The initial adhesion of circulating leukocyte to vascular endothelium is induced by interaction of constitutively functional leukocyte homing receptors with regulated endothelial cell ligands or counter receptors. Leukocyte-endothelial cell interactions both in vivo and in vitro are active multistep processes, as clearly demonstrated in studies of neutrophil interactions with inflamed sites (Von Andrian, 1991). During sepsis a dramatic increase of endothelial cell surface molecules expression occurs that facilitate adhesion of blood leukocytes. These kinds of interactions have been mainly studied in brain microvascular endothelial cells (BMEC).

The crossing of the blood-barrier by circulating bacteria is a complex process, requiring several bacterial and host factors and their interactions, such as a high degree of bacteremia, binding to and invasion of BMEC, BMEC actin cytoskeleton rearrangements and related signaling pathways. Among *E. coli* structures necessary for crossing of the blood-brain barrier in vitro and in vivo, outer membrane protein A (OmpA) contributes to *E. coli* K1 invasion of BMEC (Kim, 2002). OmpA has been implicated as an important virulence factor in several gram-negative bacterial infections such as *E. coli* K1, a leading cause of neonatal meningitis associated with significant mortality and morbidity (Mittal, 2011). *E. coli* K1 OMPA interacts with a gp96 protein on human BMEC. Purified OMPA as well as gp96 and gp96 antibody inhibited *E. coli* K1 invasion of human BMEC in a dose dependent fashion. OMPA is a major outer membrane protein of *E. coli*; it is present as an 8-stranded and anti-parallel β -barrel structure in the membrane, connected by large hydrophilic surface exposed loops and short periplasmic turns (Smith, 2007). Although OMPA's role in pathogenesis has been demonstrated, the exact role of individual loops is still to be determined (Maruvada, 2011). In particular, the synthetic peptides representing a part of the first loop and the tip of the second loop of OMPA have been shown to inhibit *E. coli* adhesion to BMEC (Prasadarao, 1996). The first and second loops are shown to be the sites for the interaction with the carbohydrate epitope of the BMEC receptor glycoprotein. OmpA extracellular loops play a fundamental role in the pathogenesis of meningitis and may help in designing effective preventive strategies against this deadly disease (Mittal, 2011). Loop regions 1 and 2 play an important role in the survival of *E. coli* K1 inside neutrophils and dendritic cells, and loop regions 1 and 3 are needed for survival in macrophages. Mutations in loop 4 of OmpA enhance the severity of the pathogenesis by allowing the pathogen to survive better in circulation and to produce high bacteremia levels. Loop 2 appears to be involved in the majority of the interactions and represents an interesting target for immunization.

Among the major surface proteins, the 34K and 36K porins from *Salmonella typhimurium* modulate leukocyte migration by acting on endothelial cells and leukocytes. The transmigration increase was dose-dependent and optimal endothelial activation occurred after 4-6 hours using porin as stimulus, after 2-4 hours using LPS. Stimulation of leukocytes with either porins or LPS slightly increased their transmigration through porin-non-activated endothelial cells. The simultaneous stimulation in vitro of HUVEC with IL-1 β and either porins or LPS causes overlapping effects leading to a very high migration index (Galdiero, 1999). In natural inflammatory process the combination of several stimuli induces high endothelial permeability of vessels to migrating cells. The main adhesion molecules of endothelial cells are activated by porins. Neutrophil transmigration through HUVEC cells treated with porins was partially inhibited by MoAbs binding to E-selectin; the transmigration of lymphocytes and monocytes was partially inhibited by MoAb anti-VCAM-1; the transmigration of neutrophils, lymphocytes and monocytes was partially inhibited by MoAb anti ICAM-1. Soluble E-selectin and ICAM-1 were found in the

supernatants from IL-1 and TNF- α activated endothelial cells. Also porins were able to stimulate the release of soluble E-selectin and soluble ICAM-1. Protein H from *Pasteurella multocida* in vitro induces neutrophil adhesion and transmigration through bovine endothelial cells (Galdiero, 2000). An increase of the expression of the vascular cell adhesion molecule 1 on the aortic endothelium has been reported in rabbit experimentally infected with *Pasteurella multocida* (Galdiero, 2000). These results evidence a local and systemic microcirculatory dysfunction that is considered central in the development of multiple organ dysfunction syndromes in septic shock.

7. OMPs and host-cells

Among surface components, porins and LPS may be important inducers of biological activity in host-interactions. Several studies have been carried out to dissect the immunobiological activities of *Salmonella enterica* or *typhimurium* porins, showing that these proteins have important effects on macrophage viability and functions; in particular, porins inhibit their phagocytic activity in a dose dependent fashion by activating the adenylate cyclase system (Di Donato, 1986). Porins induce the activation of the complement system by acting both on the classic pathway and on the alternative pathway (Galdiero, 1984), acting as mitogens for B lymphocytes. Furthermore, in rats they increase the toxicity of cardio-toxic molecules (Galdiero, 1986) and damage renal tubules (Tufano, 1987). Porins are clearly endowed with pro-inflammatory activity; when injected into the rat paw induce dose-dependent edema with long-lasting effects. The inflammation induced by porins is sensitive to both steroid (dexamethasone) and non-steroid (indomethacin) anti-inflammatory drugs. The in vitro studies carried out on peritoneal cells of the rat show that porins are able to induce the release of histamine and also of prostacyclin. Porin-induced inflammation may depend on the release of histamine, even though the arachidonic acid metabolites may also participate. In fact, in vitro results exclude an increase of 6-keto-prostaglandin and subsequent prostacyclin release, whereas in vivo results confirm both the prolonged duration of porin-induced edema and its marked inhibition by indomethacin. Porin-induced inflammation was also observed in decomplicated animals; therefore, it is unlikely that the activation of the complement system plays a major role in the inflammation induced by porins (Galdiero, 1984). Porins isolated from *S. typhimurium* are lethal at the dose of 100 ng to both LPS-responder (BALB/cByS) and non responder (C3H/HeJ) mice sensitized with D-galactosamine. The lethal action could be prevented by anti-TNF- α serum. Porins were also pyrogenic to rabbits and elicited a Shwartzman reaction when used as the sensitizing and eliciting agent (Galdiero, 1994). *Haemophilus influenza* type b (Hib) porin also induces the early release of cytokines in central nervous system cells, amplifying the inflammatory response. Hib porin inserted into the fourth ventricle of the brain elicited the appearance of serum proteins and the development of brain edema. These modifications were followed by increase in the number of neutrophils both in cerebrospinal fluid and in the tissue sections around the porin inoculation site. IL-1 α , TNF- α and MIP-2 mRNA appeared quickly in the tissue near the inoculation site (Galdiero, 2001a).

Activation of the coagulation and fibrinolytic systems is an important manifestation of the systemic inflammatory response of the host to infection. The in vivo effect of a synthetic peptide corresponding to loop L7 from *Haemophilus influenzae* type b (Hib) porin was compared with the effect of the entire protein to evaluate its role on the coagulative/fibrinolytic cascade and the circulating markers of endothelial injury (Vitiello,

2008). Plasma was obtained from rats injected intravenously with the peptide and tested for fragment 1+2 (F1+2), tissue-type plasminogen activator (tPA), plasminogen activator inhibitor type I (PAI-1) antigen, von Willebrand factor (vWF) and soluble E-selectin (sE-selectin). The coagulative/fibrinolytic cascade was impaired as determined by the increased level of PAI-1. Concomitantly, E-selectin, a marker of endothelial injury, was also significantly elevated. In addition either loop L7 or Hib porin injection induced hyperglycaemia and inflammatory cytokine production. The data were correlated with hemodynamic functions (significant reduction of blood pressure and increase of heart rate). The results indicated that, in that experimental model, the loop L7 plays an essential role in the pathophysiologic events observed during gram-negative infection.

OMPA from *E. coli* K1 plays a fundamental role in pathogenesis and great importance are correlated with the host signaling events underlying its entry into host cells. OMPA contributes to endothelial cells activation through a ligand- receptor interaction. OMPA activates PI3K but exhibited no effect on RhoA activation. The RhoA and PI3K host cell signaling pathways involvement in *E. coli* K1 invasion of human BMEC was further supported by the treatment of human BMEC with Rho kinase inhibitor (Y27632) and PI3K inhibitor (LY294002) which resulted in significant greater inhibition of *E. coli* K1 invasion compared to individual inhibitors alone.

The properties of Lipid-A associated proteins (LAP) have been extensively reviewed by Hitchcock and Morris (Hitchcock, 1984). Preparations of LAP from *S. typhimurium* have IL-1 like properties. LAP from *Actinobacillus actinomycetemcomitans*, an aquaporin associated with various forms of inflammatory periodontal disease, stimulate the release of IL-1 β and IL-6 from human monocytes or human gingival fibroblast. LAP from *Porphyromonas gingivalis*, one of the causative organisms of periodontitis, are potent stimulators of IL-6 release from human gingivalis fibroblasts (Reddi, 1995).

8. Activation of eukaryotic cell signaling and transcriptional activation induced by OMPs

The molecular mechanisms during the interaction of gram-negative bacteria with macrophages are well understood, but the mechanisms used by porins to activate cells is not well characterized. LPS, porins or other OMPs probably activate cells through similar but not identical mechanisms (Galdiero, 2003b). A variety of extracellular factors, such as growth factors or bacterial surface components, induce a complex cellular signaling by binding specific transmembrane receptors on the host cell membrane. The intracellular signaling pathways are complex networks of biochemical events that culminate in specific patterns of nuclear gene expression mediated by transcription factors. Signal transduction pathways and transcriptional activation known to occur during immune cell activation have been investigated by numerous authors and protein tyrosine phosphorylation plays a central role in transduction mediated by bacteria or LPS or toxins (Evans, 1998; Rosenshine, 1992; Weinstein, 1992). Cytoplasmic signal transduction is regulated by several enzymatic pathways among which the mitogen-activated protein kinase (MAPK) pathway is especially activated during the adhesion and penetration of bacteria into the host cell (Evans, 1998; Rosenshine, 1992) and when stimulating the cell with products of bacterial origin (Weinstein, 1992).

MAPK/extracellular signal-regulated kinases are serine/threonine protein kinase members of sequential protein phosphorylation pathways involving c-Jun N-terminal kinases (JNKs)

and ERKs (Davis, 2000). The MAPK pathway activates a number of transcription factors such as activating protein-1 (AP-1) and nuclear factor-kappa B (NF- κ B). The contribution of AP-1 family members to transcriptional regulation is controlled by a number of well characterized mechanisms (Karin, 1997). AP-1 is a ubiquitous class of gene regulatory factors and AP-1 proteins form either Jun-Jun homodimers comprised of members of the Jun family (c-Jun, JunD, and JunB) or Fos-Jun heterodimers derived from the various Fos family members. The AP-1 family members differ in their abilities to transactivate or repress transcription (Karin, 1997). NF- κ B is a dimeric transcription factor and has multiple functions in immunity and is also critical for development and cellular survival. Mammalian cells contain five NF- κ B subunits (p65, c-Rel, RelB, p50 and p52) which form various hetero- and homodimers. NF- κ B is present in the cytoplasm of resting cells bound to its inhibitor I κ B α . The activation of NF- κ B requires sequential phosphorylation, ubiquitination, and degradation of I κ B. Multiple kinases have been shown to phosphorylate I κ B at specific amino-terminal serine residues. In response to a large spectrum of chemically diverse agents and cellular stress conditions including LPS and porins, microbial and viral pathogens, cytokines and growth factors, NF- κ B translocates in the nucleus, activating expression of target genes mainly involved in inflammatory and immunological responses (Caamano, 2002).

Several studies have addressed the mechanism by which porins stimulate cells. *S. enterica* serovar typhimurium porins induce signal transduction in mouse macrophages (Gupta, 1999). Porin activation of macrophages results in increased inositol triphosphate and intracellular Ca²⁺ mobilization, translocation of protein kinase C (PKC) to the membrane, NO release within the macrophages and increased binding of infected macrophages resulting in macrophage activation and triggering of specific signaling pathways. *S. enterica* serovar typhimurium, *Mannheimia haemolytica*, and *Haemophilus influenzae* (Hib) porins induce tyrosine phosphorylation in THP-1 cells and in C3H/HeJ mouse macrophages (Galdiero, 2001), with Hib porin being the most powerful stimulator. Incubation of porins with either THP-1 or macrophages from C3H/HeJ mice resulted in tyrosine phosphorylation of specific host cell proteins with the appearance of tyrosine-phosphorylated proteins in the soluble cytoplasmic fraction, in the membrane fraction and in the insoluble protein fraction. The pattern of phosphorylation observed following LPS or porin stimulation is essentially similar, but a difference can be observed in the cytoplasmic fraction bands of 50-60 kDa, which are more evident after treatment with LPS, and in the insoluble fraction band of 80kDa and the cytoplasmic fraction band of 250kDa, which are more evident after porin treatment.

Among the most prominent tyrosine-phosphorylated bands in porin-stimulated cells, a number of proteins with a molecular mass that is similar to that of the family of tyrosine/serine/threonine protein kinases were observed. *S. enterica* serovar typhimurium porins induce tyrosine phosphorylation of ERK1-2. Porins of *S. enterica* serovar typhimurium were also able to stimulate protein kinase A (PKA), PKC and protein-tyrosine kinase (NT-PTKs) in U937 cells. In the cells pretreated with tyrphostin, a specific PTK inhibitor, or with H-89, a specific PKA inhibitor, or calphostin C, a specific PKC inhibitor, decrease of the relevant activity was observed (Galdiero, 2003a).

Neisserial porins induce protein tyrosine phosphorylation and alter the surface expression of the co-stimulatory molecule B7-2 (Massari, 2003). Recent evidence suggests that the Raf-1-MEK1/2-MAPK pathways are included among the proteins which are phosphorylated following porin stimulation (Galdiero, 2002). The use of some specific inhibitors of phosphorylation pathways such as SB-203580 (p38 inhibitor), PD-098059 (MEK/ERK kinase

inhibitor) and forskolin (Raf-1 inhibitor) demonstrated that they modulate in a different way cytokine mRNA expression in cells stimulated with porins. Neisserial porins induce nuclear translocation of the transcription factor NF- κ B in B cells and dendritic cells that was maximal by 3 h of stimulation (Massari, 2003). *S. enterica* serovar typhimurium porins also activate AP-1 and NF- κ B in U937 cells involving the Raf-1-MEK1/2-MAPK pathways (Galdiero, 2002); pretreatment with PD-098059 and with SB-203580 markedly affected the activation, indicating that the p38 signaling pathway is mainly involved in AP-1 and NF- κ B activation. In contrast, forskolin pretreatment did not block transcription factor activation by porins, suggesting that a Raf-1-independent pathway may also be involved following porin stimulation. Electrophoresis mobility shift assays, using antibodies to specific transcription factor protein subunits, showed that in U937 cells the AP-1 complex contains Jun-D and c-Fos heterodimers and probably no other homodimers or heterodimers. In U937 cells treated with LPS, AP-1 complexes containing Jun-D, c-Fos and c-Jun appeared, while stimulation by porins induces AP-1 complexes containing fra-2 in addition to the other subunits. The formation of a different complex represents a further difference between stimulation with LPS and stimulation with porins. This may be added to past observations where mRNA

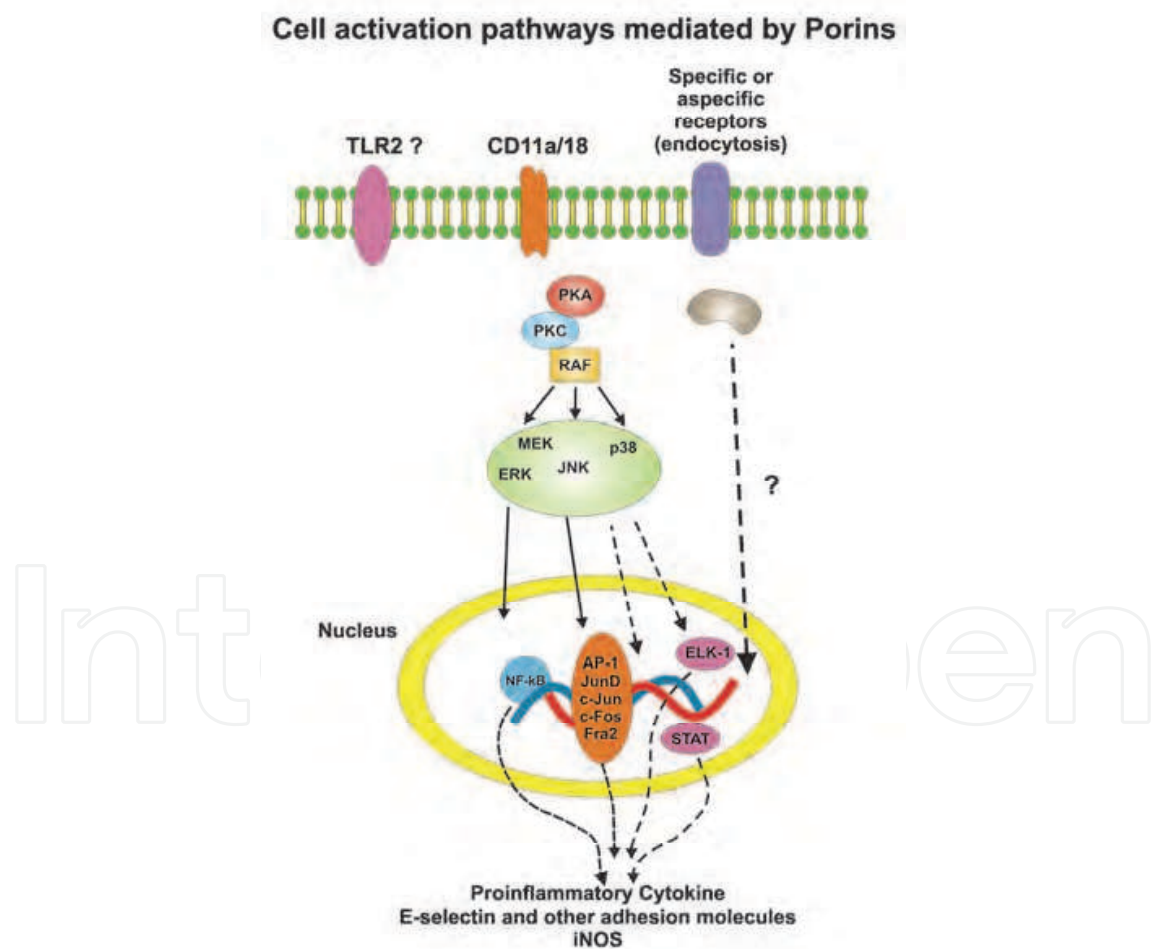


Fig. 4. Speculative scheme of porin signal transduction pathways. Putative porin-specific receptors are shown to be transmembrane. The solid arrows indicate the known association between superficial porin receptors and activation of several transcription factors; dotted arrows indicate hypothetical events.

cytokine expression after stimulation with porin begins after 120 min and continues for 5-6 h, while following LPS stimulation begins after 30 min and decreases at 120 min. Forskolin did not block NF- κ B translocation after porin stimulation. Raf-1 induces the dissociation of cytoplasmic NF- κ B-I κ B complexes (Li, 1993), suggesting that a Raf-1-dependent pathway may be involved in NF- κ B activation. However, it is known that PKC triggers the activation of several kinases suggesting that MEK/ERK pathways may also participate in NF- κ B activation by enhancing an AP-1-NF- κ B cross-coupling mechanism. The porin P2 from *Hib*, like porins from *S. enterica* serovar typhimurium, activates mainly but not exclusively the JNK and p38 pathways. Synthetic peptides, corresponding to the amino acid sequences of variable loop regions facing the cell exterior and thus more probably involved in the initial interaction with the host cell, proved to be able to activate the MEK1-MEK2/MAPK pathways similarly to the entire protein. In contrast, peptides modelled on internal β -strands were ineffective in inducing phosphorylation of such pathways (Galdiero, 2003c). A speculative scheme of signal transduction pathways involved in porin-mediated responses is depicted in Figure 4. Accumulating evidence has suggested that the regulation of transcriptional factors and the subunit composition by porin stimulation may affect the adaptive immune mechanism to modulate the production of biologically active proteins or peptides. The engagement of multiple pathways during signal transmission makes the possible use of molecular inhibitors as therapeutic agents very difficult; although recent findings show that peptides complementary to loop regions have a certain ability to block the activity of the porin (Cantisani, 2011).

9. OMPs and septic shock

Septic shock is a major cause of death in the world. Gram-negative infection frequently results in systemic manifestations of sepsis and septic shock. The systemic syndrome is caused by the host response to gram-negative surface components. This response may set in motion a cascade of pathophysiologic consequences that result in multiple organ systemic failure and death. The host response to gram-negative bacterial infection is complex and multifaceted. Strong experimental evidence and clinical observations suggest that the release of proinflammatory cytokine mediators in response to toxic bacterial products set in motion an uncontrolled pathophysiologic program that manifests as sepsis or septic shock. The best characterized and most important of these toxic products is gram-negative endotoxin. For the general scientific public, the terms endotoxin and LPS are interchangeable. The term lipopolysaccharide (LPS) obtained by using the Westphal extraction procedure (Westphal, 1952), should be reserved for purified bacterial LPS extracts which are free of detectable contaminants, particularly proteins. In contrast, the term endotoxin should be used to refer to macromolecular complexes of LPS, protein and phospholipid normally obtained by extraction of bacteria with trichloroacetic acid, butanol and EDTA. Endotoxin associated protein consist of complex of four or five major proteins that range in size from 10 to 35 kDa. Originally considered to be a superfluous carrier of LPS, endotoxin associated proteins are now recognized to have potent biological activities (Mangan, 1992). Endotoxin associated proteins are powerful mitogen for C3H/HeJ mice, which are hyporesponsive to LPS. Techniques previously used in the extraction of stable LPS from the endotoxin had greatly favored the study of this portion of the molecule, ignoring the denaturable protein fraction, allowing the identification of most of the effects of endotoxin with those of LPS. Subsequent extraction techniques for membrane proteins (in

their native form) then allowed the study of the protein fraction, which was extracted globally in the endotoxin (Hindennach, 1975). Although much is known about the role of LPS in septic shock, little is known about the role of the proteic components. Approximately 50% of the dry mass of the outer membrane of the gram-negative bacteria consists of proteins and more than 20 immunochemically distinct proteins (OMPs) have been identified in *E. coli*. Several OMPs have been shown to be potent inducers of cytokine synthesis. The most abundant of OMPs are porins. Porins isolated from *Salmonella enterica* serovar typhimurium, *Yersinia enterocolitica*, and *Mannheimia haemolytica* have been shown to stimulate the release of a range of proinflammatory and immunomodulating cytokines including IL-1, IL-4, IL-6, IL-8, TNF- α and INN- γ by monocytes and lymphocytes. Porins stimulate also the release of granulocyte-monocyte colony stimulating factor (GM-CSF), soluble intercellular adhesion molecule-1 (ICAM-1) and soluble E-selectin (SE-selectin) in a dose-dependent fashion by HUVEC cells (Donnarumma, 1996).

In vitro and in vivo experiments supported the involvement of porins in the septic shock pathogenesis. The administration of porin to animals affects their hemodynamics, body temperature, blood clotting, cellular and humoral immunities proliferation of B lymphocytes and macrophages, and release of various endogenous mediators. The role played by porins and in general by OMPs in sepsis has been further supported by conflicting results obtained with the immunotherapy. The notion that the core regions of most strains of gram-negative bacterial LPS were quite similar, supported the development of a broadly effective immunotherapy for gram-negative sepsis using antibodies raised against LPS, through the use of a bacterial strain with an outer membrane that features no side chains, while bearing only the conserved core elements of LPS. The strain selected was the J5 mutant of *E. coli* O111:B4, whose LPS contains only the core determinants, primarily lipid A. Although it has generally been assumed that immunoglobulins to rough mutant *E. coli* J5 protect by binding to LPS, it has been demonstrated that IgG in those antisera bind only weakly to LPS from heterologous gram-negative strains. Also anti-lipid A monoclonal antibodies did not induce the expected results (Siber, 1985). Recently, it has been demonstrated that IgG in polyclonal antiserum raised to heat-killed *E. coli* J5 binds to three conserved gram-negative bacterial outer-membrane proteins. (5-9, 18, and 35 kDa). These OMPs are exposed on the surface of bacterial cells and are released into human serum in complexes that also contain LPS (Binkley, 1945). The role of porins in pathogenesis is also confirmed by studies on the development of an effective vaccine against serogroup B *Neisseria meningitidis*. The nonimmunogenicity of serogroup B capsular polysaccharide has led to the development of outer membrane vesicle (OMV) vaccines, based on the presence of PorB (Jolley, 2001; Wright, 2002). The porin proteins adopt a β -sheet structure within the outer membrane with surface exposed loops (Van der Ley, 1991). OMPs epitopes mainly involved in the interaction with the host cells are those on the surface. The wide antigenic variability of gram-negative bacteria is due also to the great sequence amino acid variability of surface exposed loops. Although, cross-reactivity of the major OMPs of *Enterobacteriaceae* has been reported by several investigators (Hofstra 1979, Hofstra, 1980), their role in the pathogenesis of sepsis and shock has not been fully dissected.

Bacterial lipoproteins are important in the induction and pathogenesis of septic shock; in fact, they induce proinflammatory cytokine production in macrophages and lethal shock in LPS-responsive and nonresponsive mice. Lipoproteins are released from growing bacteria and released lipoproteins may play an important role in the induction of cytokine production and pathologic changes associated with gram-negative bacterial infections; treatment of bacteria with antibiotics significantly enhances lipoprotein release.

Lipoproteins activate macrophages; induce lethal shock in mice, and act synergistically with LPS to induce these responses (Zhang, 1997). Some gram-negative microorganisms have the ability to secrete lipoproteins to the extracellular environment; among those peptidoglycan-associated lipoproteins, Pal is released into the bloodstream during infection, and this process contributes to the development of septic shock (Hellman, 2002; Liang, 2005). Lipoproteins play an important role in septic shock induced by bacteria; moreover, they act synergistically with LPS to induce lethal shock which suggest that they activate cells through different mechanisms. Bacterial lipoproteins have been shown to affect both the innate and acquired immune system via TLR2 signaling and generation of cytotoxic T lymphocytes and bactericidal antibodies (Masignani, 2003).

10. Novel perspectives for therapies

Gram-negative sepsis remains a significant cause of morbidity and mortality in site of the ongoing development of new antimicrobial agents (Lazaron, 1999); the reason may be attributed to the failure of antimicrobial therapy to address the described pathogenetic mechanism involved in the systemic inflammatory response due to gram-negative bacteria. The systemic syndrome is caused by the host response to gram-negative infection; which sets in motion a cascade of pathophysiologic consequences that result in dysregulation of hemodynamics, oxygen use, and intermediate metabolism, and often results in multiple organ failure with further increased morbidity and mortality; these may happen also after apparent eradication of the original infection. The immunotherapy in the treatment of sepsis and shock did not produce the expected results. In fact, polyclonal *E. coli* J5 antiserum is not suitable for commercial development, especially for the viability of antiserum activity. The mass production of IgM monoclonal antibodies allowed the obtainment of an antibody E5 binding more specifically to Lipid A. E5 monoclonal antiserum, tested in two randomized placebo-controlled clinical trials demonstrated no clinical benefit to patients with gram-negative sepsis (Greenman, 1991). Also a human hybrid monoclonal antibody, HA-1A has been problematic. Anti-LPS core directed antibodies have not shown a survival benefit in clinical trials. The sometimes protective results observed using polyclonal *E. coli* J5 antiserum, may be attributed to the presence of antibodies against surface epitopes of OMPs. The great variability of surface loops of OMPs makes it rather difficult the preparation of specific antiserum that could be used for all gram-negative infections. As death by septic shock has been derived by an early excessive inflammatory response, therapeutic strategies have been designed to block the cytokines and other mediators involved into pathogenesis. However, the sepsis and septic shock are not restricted only to the activation of the inflammatory response, but also to compensatory anti-inflammatory mechanism usually leading to immunosuppression. Patients in this state have a poor prognosis; in fact, the majority of deaths occur in patients with sepsis who are immunosuppressed (Adib-Conquy, 2009).

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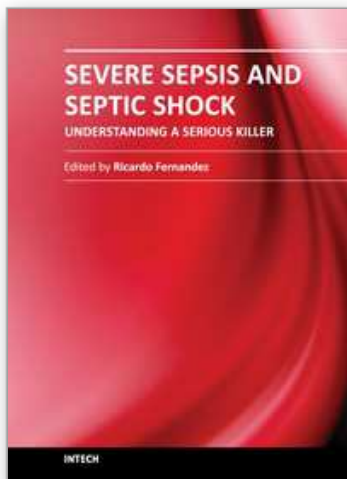
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Despite recent advances in the management of severe sepsis and septic shock, this condition continues to be the leading cause of death worldwide. Some experts usually consider sepsis as one of the most challenging syndromes because of its multiple presentations and the variety of its complications. Various investigators from all over the world got their chance in this book to provide important information regarding this deadly disease . We hope that the efforts of these investigators will result in a useful way to continue with intense work and interest for the care of our patients.

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