

Role of Toll-like receptors, NOD-like receptors and RIG-I-like receptors in endothelial cells and systemic infections

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Summary

Bacteraemia and viraemia are characterised by pathogens entering the bloodstream. Endothelial cells are among the first cells coming into contact with the microbes and also some endogenous molecules which are released by tissue damage. As part of the innate immune system, endothelial cells respond to these contacts by producing inflammatory mediators and expressing surface molecules. The initial sensing of microbial and endogenous danger-associated molecules is mediated by so-called pattern recognition receptors (PRRs). PRRs can be classified in dif-

ferent protein families such as the Toll-like receptors, the NOD-like receptors and the RIG-I-like receptors. By activating inflammatory gene transcription and posttranslational processing, PRRs control the immediate innate immune reaction and also the subsequent adaptive immune response. Here we describe the current knowledge of extra- and intracellular PRRs in endothelial cells and their potential role in sepsis and vascular diseases.

Keywords

Toll-like receptor, NOD-like receptors, RIG-I-like receptor, endothelial cells

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Introduction

The endothelium is located at the interface between circulating blood and the surrounding tissue. The over 60 trillion endothelial cells of the human body cover an area of several square metres. They serve a multitude of functions that help to maintain organ homeostasis, including vasoregulation, vascular permeability and provision of an anticoagulant surface (1–3). Endothelial cells can be exposed to pathogens and bacterial products entering the bloodstream or to endogenous molecules which are released by tissue damage. As a part of the innate immune system, endothelial cells respond to these contacts with structural changes, and with production of inflammatory cytokines which contribute to leukocyte recruitment and activation. Moreover, endothelial cells react with expression of adhesion molecules which mediate platelet adhesion and leukocyte trafficking (1, 4). These innate immune reactions normally contribute to the containment and elimination of the invading microbes. During systemic infections such as sepsis, however, a generalised and overwhelming activation of endothelial cells by microbial and endogenous danger molecules as well as cytokines can be harmful. It con-

tributes to coagulopathy, increased vascular permeability, arterial hypotension and organ dysfunction (2, 5).

The initial sensing of microbes as well as endogenous molecules released by tissue damage is mediated by so-called pattern recognition receptors (PRRs) which are expressed in different host cells including endothelial cells (4). They sense pathogen-associated molecular patterns (PAMPs) derived from microbes such as the bacterial cell wall components lipopolysaccharide (LPS) and peptidoglycan or microbial nucleic acids. In addition, different PRRs are activated by endogenous danger-associated molecular patterns (DAMPs) including ATP or hyaluronan which are released by tissue damage. All PRRs share the following features: they are (I) germline-encoded, (II) expressed in a wide variety of host tissues and cells including endothelial cells, and (III) recognise a limited number of conserved microbial and endogenous molecules. PRRs differ, however, in the signalling cascade and the host response activated by their engagement. PRRs include the well-known Toll-like receptors (TLRs), as well as the cytosolic NOD-like receptors (NLRs) and RIG-I-like receptors (RLRs) (4, 6–10). The TLRs, RLRs and some NLRs activate transcription of inflammatory genes depending

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on transcription factors including nuclear factor- κ B (NF- κ B). In contrast, other NLR members do not regulate gene transcription but regulate proteolytic processing of cytokines and activate inflammatory cell death pathways. For example, production of the master cytokine interleukin (IL)- β can be regulated by a TLR-mediated NF- κ B activation which drives proIL-1 β expression, and by NLR-mediated caspase-1 activation which drives cleavage of proIL-1 β into the mature cytokine. This review aims to summarise the recent findings about different PRRs and their role in diseases affecting endothelial cells.

Toll-like receptors

The 10 human TLRs are located at either the cell surface (TLR1) (2, 4–6, 10) or lysosomal/endosomal membranes (TLR3) (7–9) (Fig. 1). They are characterised by a cytoplasmic Toll/IL-1 receptor homology (TIR) domain responsible for downstream signalling, and an extracellular leucine-rich repeat (LRR) domain which most likely mediates ligand binding. TLR2 in cooperation with TLR1 or TLR6 recognises bacterial tri- or diacylated lipopeptides, respectively, as well as lipoteichoic acids, related glycolipids and yeast molecules (11–16) (Table 1). TLR3 and TLR7/8 detect double-stranded (ds)RNA which is an intermediate in viral replication, and (viral) single-stranded (ss)RNA, respectively (17, 18). TLR4 is the receptor for LPS of Gram-negative bacteria and might also be activated by viral proteins, endogenous heat shock protein HSP60, oxidised low-density lipoprotein (LDL), and fibrinogen (19–21). TLR5 recognises bacterial flagellin on the cell surface (22), and TLR9 viral and bacterial Cytosin-phosphatidyl-Guanosin (CpG) DNA in endosomes (23).

The downstream signalling of the TLRs is mediated by the four TIR domain-containing adaptor molecules myeloid differentiation primary response protein 88 (MyD88), MyD88 adaptor-like (Mal), TIR domain-containing adaptor-inducing interferon β TRIF) and TRIF-related adaptor molecule (TRAM) which are differentially engaged by different TLRs (24, 25). All TLRs except TLR3 initiate a MyD88-dependent signalling pathway

leading to NF- κ B-dependent expression of inflammatory mediators. TLR7–9 are additionally capable of stimulating a different MyD88-dependent signalling pathway leading to activation of the transcription factor interferon regulatory factor 7 (IRF7) and subsequent type I interferon (IFN) responses. TRIF is recruited to TLR3 and TLR4 and stimulates a late NF- κ B activation as well as IRF3 and IRF7 activation leading to type I IFN responses (24, 25).

Different endothelial cells express TLR1, TLR3, TLR4, TLR5, TLR6 and TLR9, but not TLR7 and TLR8 (26–31). The expression of TLR2 is low in some endothelial cells under steady state conditions (32). Recent data, however, showed that at least HUVEC and HCAEC respond reasonably to TLR2 agonists (4, 33). TLR2 expression can be up-regulated by stimulation of endothelial TLR4 (32). Moreover, crosstalk between neutrophils and endothelial cells has been shown to lead to up-regulation of TLR2 in endothelial cells in mice intraperitoneally injected with LPS (34). Whereas the TLR signalling pathway is basically preserved in different host cells, slight differences appear to exist between classical immune cells and endothelial cells. For example, mouse endothelial cells appear to lack TRAM and fail to initiate a TRIF/TRAM-dependent signalling (35).

During infection, endothelial cells are activated directly through recognition of microbial molecules (PAMPs) by endothelial TLRs and indirectly by host-derived mediators produced by immune cells after TLR activation of these cells. Endothelial cells respond to these signals with structural changes resulting in increased vascular permeability, and production of inflammatory cytokines as well as chemokines (2, 4). Moreover, endothelial cells up-regulate expression of adhesion molecules and coagulatory proteins leading to increased attachment of platelets as well as leukocytes and stimulation of the coagulation cascade (2, 36). Whereas these mechanisms contribute to defense against invading pathogens during local infections, they can lead to oedema, hypotension and coagulopathy during systemic infections and sepsis (2, 36).

Transgenic mice with endothelial cell specific blockage of NF- κ B activation showed repressed expression of endothelial

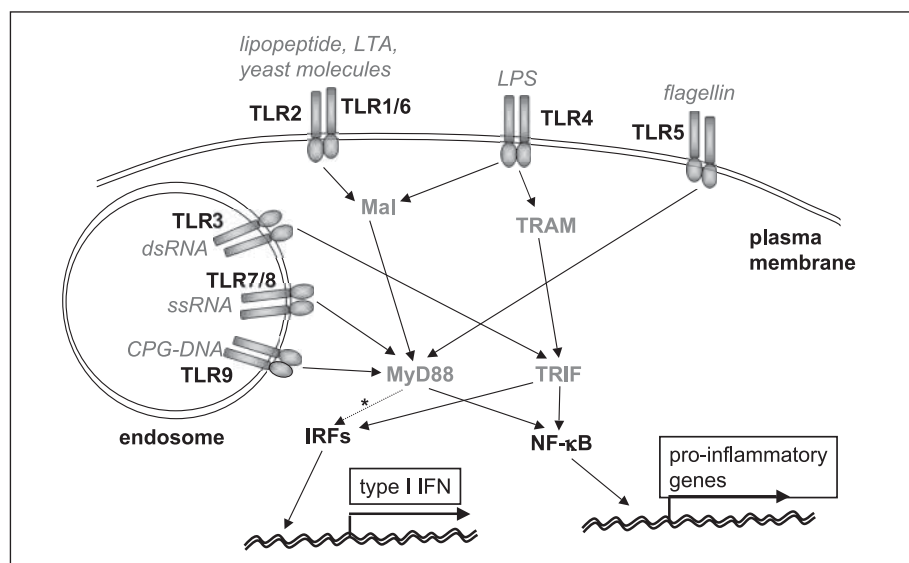


Figure 1: Overview of the human TLRs as discussed in the text. TLRs recruit different adapter molecules and initiate signalling pathways leading to activation of NF- κ B and/or IRFs. TLR7 and TLR8 are not expressed in endothelial cells. *, this pathway is only activated by TLR7–9.

Table 1: The human TLRs.

Name	PAMPs/activators	Adapter	Function/activation of	Expression in endothelial cells
TLR1	triacylated lipopeptide (in cooperation with TLR2)	MyD88	NF- κ B-dependent gene expression	+
TLR2	tri- or diacylated lipopeptides (in cooperation with TLR1 or TLR6), lipoteichoic acids, glycolipids, yeast molecules	MyD88, Mal	NF- κ B-dependent gene expression	+
TLR3	dsRNA	TRIF	NF- κ B-dependent gene expression, IRF-dependent expression of type I IFNs	+
TLR4	LPS, viral proteins, endogenous heat shock protein, oxidised LDL, fibrinogen	MyD88, Mal, TRIF, TRAM	NF- κ B-dependent gene expression, IRF-dependent expression of type I IFNs	+
TLR5	flagellin	MyD88	NF- κ B-dependent gene expression	+
TLR6	diacylated lipopeptide (in cooperation with TLR2)	MyD88	NF- κ B-dependent gene expression	+
TLR7	ssRNA	MyD88	NF- κ B-dependent gene expression, IRF-dependent expression of type I IFNs	-
TLR8	ssRNA	MyD88	NF- κ B-dependent gene expression, IRF-dependent expression of type I IFNs	-
TLR9	CpG-DNA	MyD88	NF- κ B-dependent gene expression, IRF-dependent expression of type I IFNs	+
TLR10	?	MyD88		?

cell adhesion molecules, reduced neutrophil infiltration into multiple organs, decreased endothelial permeability, ameliorated multiple organ injury, reduced hypotension, and abrogated intravascular coagulation when subjected to endotoxaemia, as well as improved survival compared to wild-type mice in a bacterial sepsis model (37). Moreover, sepsis-related pulmonary failure appears to be dependent on activation of endothelial TLR4 which regulates neutrophil sequestration into the lung (38). In addition, TLR3 in endothelial cells might contribute to dysfunctional coagulation and fibrinolysis during viral haemorrhagic fever (39). Taken together, TLRs in endothelial cells are critically involved in protective host defense against infections but can also be involved in harmful microvascular dysfunction and organ failure during systemic infections.

NOD-like receptors

In humans, the NLR family comprises 22 members with eight of them functionally characterised (7–9, 40) (Table 2). Most NLRs are located in the cytosol. They consist of a central nucleotide-

binding oligomerisation (NOD) domain, and of C-terminal LRRs which possibly mediate ligand binding. In addition, they contain N-terminal effector binding domains such as caspase recruitment domains (CARD), pyrin domains (PYD), or baculovirus inhibitor of apoptosis repeat (BIR) domains which mediate activation of different signalling pathways (7–9, 40).

Among the best studied NLRs are the CARD-containing NOD1 and NOD2 which serve as cytoplasmic PRRs. Both NOD1 and NOD2 detect degradation products of bacterial cell wall components. These bacterial products get access to the host cell cytosol via endocytosis, host cell transporter proteins including peptide transporters PepT1 and PepT2, bacterial secretion systems, bacterial pore forming toxins, or are released by intracellular bacteria (41–45). Whereas NOD1 detects peptidoglycan fragments containing *meso*-diaminopimelic acid found mainly in Gram-negative bacteria, NOD2 detects muramyl dipeptides (MDP), the largest motif common to Gram-negative and Gram-positive bacteria (46–49).

Both NOD1 and NOD2 recruit the Rip2 kinase and perhaps CARD9 which activates a NF- κ B and mitogen-activated protein

Table 2: The human NLRs. Abbreviations: iE-DAP, gamma-D-glutamyl-meso-diaminopimelic acid (peptidoglycan fragment); MDP, muramyl dipeptide (peptidoglycan fragment); ROS, reactive oxygen species.

Family	Name	Synonym	Activator	Function/activation of	Expression in endothelial cells
NLRC	NOD1	CARD4	iE-DAP*	NF- κ B-dependent gene expression	+
	NOD2	CARD15	MDP	NF- κ B-dependent gene expression	low
	NLRC	NOD3	?	?	?
	NLRC4	IPAF, CARD12	flagellin	caspase-1-dependent IL-1 β /IL-18 processing, pyroptosis	+
	NLRC5	NOD27, CLR16.1	?	?	?
NLRP	NLRP1	NALP1	MDP, anthrax lethal toxin	caspase-1-dependent IL-1 β /IL-18 processing	+
	NLRP2	NALP2	?	caspase-1-dependent IL-1 β /IL-18 processing	?
	NLRP3	NALP3, CRYOPYRIN, CIAS1	RNA, uric acid crystals, ATP, bacterial toxins, MDP, hyaluronan, silica crystals and aluminium salts	caspase-1-dependent IL-1 β /IL-18 processing, pyroptosis	?
	NLRP4	NALP4	?	?	?
	NLRP5	NALP5	?	?	?
	NLRP6	NALP6	?	?	?
	NLRP7	NALP7	?	?	?
	NLRP8	NALP8	?	?	?
	NLRP9	NALP9	?	?	?
	NLRP10	NALP10	?	?	?
	NLRP11	NALP11	?	?	?
	NLRP12	NALP12	?	?	?
	NLRP13	NALP13	?	?	?
	NLRP14	NALP14	?	?	?
NLRB	NAIP	BIRC1	flagellin	caspase-1-dependent IL-1 β /IL-18 processing, pyroptosis	?
NLRA	CIITA	MHC2TA, C2TA	?	MHCII induction in APC	-
NLRX	NLRX1	NOD9	?	RLR negative regulation, ROS production	?

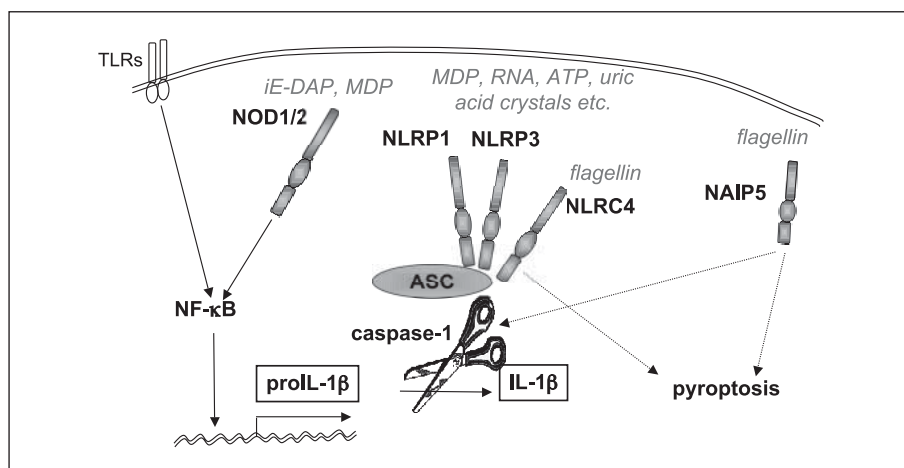
kinase (MAPK)-dependent inflammatory gene expression (50–52) (Fig. 2). NOD1 and NOD2 have been demonstrated to recruit from the cytosol to the plasma membrane, a process which might be involved in sensing bacterial products at the bacterial entry site but also in negative regulation of the receptor molecule (53–55). We and others recently showed that the membrane recruitment of NOD2 is mediated by PAK (p21-activated kinase)-interacting exchange factor = (β -PIX), Rac1 (Ras-related C3 botulinum toxin substrate 1) and the actin cytoskeleton (54, 56).

Different endothelial cells including HUVEC, HAEC and microvascular endothelial cells express NOD1 (57, 58). In contrast, basal expression of NOD2 in these cells is low but can be up-regulated by inflammatory mediators (58–60). We recently demonstrated that the NF- κ B and p38 MAPK activation as well as IL-8 secretion in endothelial cells infected with *C. pneumoniae* or *L. monocytogenes* were dependent on NOD1 (57, 58). Non-invasive *Listeria* or inactivated *Chlamydia*, however, failed to activate endothelial cells. Subsequent studies further demonstrated that NOD1 was also critical for the innate immune response to *L. monocytogenes* and *C. pneumoniae* *in vivo* (61,

62). In the *L. monocytogenes* infection, NOD1 in non-haematopoietic cells, which include epithelial and endothelial cells, was most important for controlling the outcome (61). Collectively, these studies showed that NOD1 is a critical component of the innate immune system of endothelial cells and other host cells.

NLRC4 (NLR protein containing a CARD 4, also known as IL-1 β -converting enzyme protease-activating factor = IPAF) is another member of the CARD-containing NLRs which has been indicated to be expressed in endothelial cells (63). Its function, however, has only been examined in macrophages and other leukocytes so far. NLRC4 together with caspase-1 and in some cases with the neuronal apoptosis-inhibitory protein 5 (Naip5/NAIP [Birc1e/BIRC1 = baculovirus inhibitor of apoptosis repeat containing protein 1]) and apoptosis-associated speck-like protein (ASC) forms multiprotein complexes called inflammasomes (40). Inflammasomes are multiprotein complexes which regulate proteolytic processing of cytokines such as proIL-1 β and proIL-18 into IL-1 β and IL-18, as well as an inflammatory cell death called pyroptosis. The NLRC4 inflammasome appears to sense bacteria expressing flagellin and/or type III/IV secretion systems (64–69).

Figure 2: Schematic representation of the function of some NLRs and of the two-step model for production of mature IL-1 β . After sensing their specific agonists, NOD1 and NOD2 as well as the TLRs activate a NF- κ B-dependent inflammatory gene expression which includes proIL-1 β . A second signal mediated by NLRs, NLRC4 or NAIP5 inflammasomes is necessary to cleave proIL-1 β to the active form IL-1 β . Some NLRs additionally activate an inflammatory cell death called pyroptosis. iE-DAP, gamma-D-glutamyl-mesodiaminopimelic acid (peptidoglycan fragment); MDP, muramyl dipeptide (peptidoglycan fragment).



Bacteria such as *Bartonella* or *L. monocytogenes*, however, which are capable of replicating in different host cells including endothelial cells down-regulate flagellin expression during the infection process (70, 71). This flagellin down-regulation might represent an important escape mechanism from NLRC4/Naip5-related defense in different host cells. Nonetheless, the functional role of NLRC4 and Naip5/NAIP in endothelial cells clearly needs further investigations.

The NLRP (NLR protein containing a PYD, also known as NACHT-LRR-PYD-containing proteins = NALP) proteins express PYD domains and can be classified as another NLR subgroup. Some NLRPs including NLRP1 (NALP1) and NLRP3 (NALP3) form inflammasomes upon interaction with ASC and the caspase-1. These protein complexes regulate processing of proIL-1 β and proIL-18 into functionally active cytokines as well as pyroptosis (72–75), similarly to the NLRC4 inflammasome discussed above. At least in some cases, the NLRP1 inflammasome appears to also contain NOD2 (76). Whereas the NLRP1 inflammasome is activated by anthrax lethal toxin and muramyl dipeptide (MDP) (77, 76), the NLRP3 inflammasome is stimulated by a variety of molecules including microbial RNA, bacterial toxins, gout-associated uric acid crystals, ATP, hyaluronan, silica crystals and aluminium salts (78–86).

Several studies suggest that inflammasomes play important roles in systemic infections and sepsis. Caspase-1-deficient mice are resistant to endotoxin shock and *E. coli* sepsis (28, 87), and mice lacking ASC or NLRP3 were fully or partially, respectively, protected from lethal effects of LPS (85, 88). Moreover, mice deficient in caspase-12, which negatively regulates caspase-1, are resistant to polymicrobial sepsis (89). Polymorphisms in human caspase-12 have been shown to drastically impact the inflammatory response of the individuals to endotoxin, and the susceptibility to severe sepsis (90). A recent study further found that expression of ASC, caspase-1, NLRP1, and NLRP12 were significantly lower in monocytes of patients with septic shock compared with critically ill control subjects, and that NLRP1 mRNA levels were linked to survival in patients with sepsis (91).

NLRP1, ASC and caspase-1 are also expressed in endothelial cells (92, and B. Opitz, unpublished data). In addition, the IL-1 family cytokine IL-33 is highly expressed in endothelial cells (93, 94). In contrast to IL-1 β or IL-18, IL-33 appears to be inac-

tivated by processing mediated by caspase-1 inflammasomes (95). It was speculated that IL-33 represents an endogenous danger signal (DAMP) which is released by tissue damage during trauma as well as infection and activates immune responses (94, 95). In addition, IL-33 appears to play important roles in cardiovascular diseases (96).

Overall, it appears that NLRs in haematopoietic and endothelial cells are critically involved in local and systemic infectious diseases affecting the vascular system although further investigation is necessary.

RIG-I-like receptors

The RNA helicases retinoic acid inducible gene-I (RIG-I) and melanoma differentiation-associated gene 5 (MDA5) constitute a further PRR family called RIG-I-like receptors (RLRs) (97, 98). Both proteins are localised in the host cell cytosol and consist of a DexD/H box RNA helicase domain as well as two CARDs. RIG-I was demonstrated to recognise ssRNA bearing a 5'-triphosphate moiety and mediate immune responses to various ssRNA viruses, whereas MDA5 is activated by other RNA viruses and the synthetic dsRNA poly I:C (99, 100, 101). Both, RIG-I and MDA5 engage the CARD-containing adapter molecule IFN regulator factor-1 (IPS-1, also called MAVS, VISA or Cardif) (102–105). IPS-1 in turn activates signalling pathways leading to IRF3/7-dependent type I IFN responses, and to NF- κ B-dependent inflammatory gene expression.

RIG-I and MDA5 are expressed in endothelial cells including HUVEC and expression of RIG-I is up-regulated by pro-inflammatory stimulation (106, 107). The function of these molecules during infections with Cytomegalovirus, Dengue virus, Hantaan virus and other clinically important viruses which replicate inside the endothelium needs to be determined.

Concluding remarks

The immune response to local and systemic infections is dependent on extracellular and intracellular PRRs. The role of the TLRs in endothelial cells in these processes has been well acknowledged. In addition, recent studies indicate that the NLRs in endothelial cells and other cells are also critically involved in pro-

tective immune responses to infections as well as in overwhelming immune reactions as seen in sepsis. Further analysis of the endothelial PRRs is, however, necessary. In the long run, interference with extra- and intracellular PRRs as well as with downstream signalling molecules may lead to novel intervention strategies to treat local and systemic inflammatory conditions.

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