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Review Article

Pattern recognition receptors in equine endotoxaemia and sepsis

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Summary

Pattern recognition receptors (PRRs) on host cells detect pathogens to activate innate immunity which, in turn, initiates inflammatory and adaptive immune responses. Successful activation of PRRs is, therefore, critical to controlling infections and driving pathogen-specific adaptive immunity, but overactivity of PRRs causes systemic inflammation, which is detrimental to the host. Here we review the PRR literature as it relates to horses and speculate on the role PRRs may play in sepsis and endotoxaemia.

Keywords: horse; endotoxaemia; sepsis; innate immunity; pathogen recognition receptor; pathogen-associated molecular pattern

Introduction

Sepsis and/or endotoxic shock commonly accompanies conditions such as neonatal bacterial sepsis, infectious or proximal enteritis, metritis, retained placenta, colitis, strangulating gastrointestinal lesions and bacterial pneumonia [1]. Sepsis is a systemic illness caused by microbial invasion, whereas endotoxaemia occurs when endotoxin, such as lipopolysaccharide (LPS) from Gram-negative bacteria, is present in the systemic circulation [2]. Sepsis presents more challenges than endotoxaemia because bacteria express many cell surface molecules or pathogen-associated molecular patterns (PAMPs), including LPS, bacterial lipoproteins, lipoteichoic acid, peptidoglycan and bacterial DNA, many of which may be present in the circulation at one time. Traditionally, Gram-negative bacteria have been associated with sepsis; however, in humans, Gram-positive bacteria may be equally important in disease pathogenesis [3–5]. In equine neonatal sepsis, both Gram-negative and Gram-positive bacterial isolates have been identified as causative agents [6–9], and Gram-positive bacteria are increasingly being isolated from neonatal and adult animals [8–11].

Pathogens and PAMPs are recognised by an extensive group of pattern recognition receptors (PRRs), each detecting specific ligands (Table 1). Activation of PRRs by PAMPs triggers the production of pro- and anti-inflammatory mediators, as well as initiating adaptive immune responses. Pattern recognition receptors include Toll-like receptors (TLRs), lectin receptors, Retinoic acid inducible gene I-like receptors and nucleotide-binding oligomerisation domain (NOD)-like receptors (NLRs) and may reside on the cell surface, in the endoplasmic reticulum, in endosomes, lysosomes, endolysosomes or the cytosol [12]. Successful activation of PRRs is critical in order for bacterial infections to be cleared successfully by the host, but overactivation of these receptors can lead to systemic inflammation and shock-like syndromes. Antagonism of PRRs therefore represents an exciting new therapeutic target for clinical syndromes such as sepsis and endotoxaemia [13].

Toll-like receptors

Toll-like receptors are the best characterised of the PRRs. The extracellular domain of all TLRs is constructed of 19–25 leucine-rich repeats that contain hydrophobic residues at distinctive intervals to form a horseshoe structure [14,15]. The exact structure and alignment of the different components of the leucine-rich repeats determines how ligands bind. The shapes of the binding pockets vary between species, which results in differential responses to PAMPs [16]. There are at least 10 TLRs, but in this review we will focus on only the TLRs that recognise bacteria.

Toll-like receptor 4

Toll-like receptor 4 (TLR4), the first fully characterised mammalian PRR, recognises the lipid A component of LPS and is the receptor activated during endotoxaemia [17]. Mice without TLR4 are more susceptible to Gram-negative bacterial infections [18]. The structure of LPS bound to TLR4 and its co-receptor myeloid differentiation protein-2 (MD2) has been solved [19]. First LPS is extracted from plasma by lipopolysaccharide binding protein [20]; the lipid A is then transferred to CD14 [21], which then transfers it to TLR4 and MD2 [22]. Each bacterial species produces a structurally unique lipid A, which affects its efficacy at TLR4 [15]. Variant lipid A structures are recognised in a mammalian species-specific manner. Lipid As from *Salmonella entericia* serovar Typhimurium (*S.* Typhimurium) or *Escherichia coli* are agonists in all species; the *E. coli* partial structure lipid IVa is an antagonist in humans [23], a partial agonist in horses [24] and a full agonist in mice [25]. *Rhodobacter sphaeroides* lipid A is an antagonist in humans and mice [26], but is an agonist in horse cells [18]. The species specificity in TLR4 ligand recognition, particularly in the horse, is becoming increasingly well understood and is dependent on subtle structural differences in MD2 and TLR4 [24]. Also, TLR4 recognises a number of other ligands, including respiratory syncytial virus fusions proteins, mouse mammary tumour virus envelope proteins, *Streptococcus pneumoniae* pneumolysin and the plant-derived cytostatic drug paclitaxel [27], although precisely how these PAMPs bind to the receptor is unclear. The importance of LPS in equine diseases such as acute abdominal disease [28,29], adynamic post operative ileus [30], laminitis [31], exertion [32], neonatal sepsis [33,34] and recurrent airway obstruction [35–38] has been the subject of extensive research efforts, particularly with respect to colic. This makes the TLR4–MD2 receptor complex an attractive therapeutic target for a number of equine diseases.

Toll-like receptor 2

Toll-like receptor 2 (TLR2) recognises mycobacterial products, Gram-positive bacteria and their associated PAMPs, including lipopeptides, peptidogylcan, lipoteichoic acid and lipoarabinomannan [13]. Ligands bind to heterodimers of TLR2 with either TLR1 or TLR6 [16,39]. The TLR1/2 heterodimer recognises triacylated lipopeptides, whereas the TLR2/6 complex recognises diacylated lipopeptides [13]. Toll-like receptor 2 knockout mice are hypersusceptible to Gram-positive bacterial infections, including sepsis and meningitis [40], and people with mutations in the *TLR2* gene have an increased susceptibility to infection with Gram-positive organisms [41]. TLR2 mRNA is present in the normal lung of horses and is increased after LPS challenge [38,42]. Currently, there is no evidence for TLR2 playing a role in equine sepsis, although if Gram-positive organisms

TABLE 1: Ligands for Toll-like receptors (TLRs) and NOD-like receptors (NLRs)

PRR	Ligands	Origin of the ligand	Reference (equine reference in bold)
TLR1/2	Triacyl lipopeptides (Pam_3CSK_4)	Gram-positive bacteria, mycobacteria	[112], **[113]**
	OspA	*Borrelia burgdorferi*	[114]
	Soluble factors	*Neisseria meningiditis*	[115]
	Porin porB	*Neisseria meningiditis*	[116]
TLR2	Lipoprotein/lipopeptides	Gram-positive bacteria, Mycoplasma, Mycobacteria, Spirochetes	[117], [118], **[113]**
	Lipoteichoic acid	Gram-positive bacteria	[118]
		Treponema maltophilum	[119]
	Peptidoglycan	Gram-positive bacteria	[120]
	Lipoarabinomannan	Mycobacteria	[121], **[122]**
	NapA	*Borrelia burgdorferi*	[123]
	Glycoinositolphospholipids	*Trypanosoma cruzi*	[124]
	Phenol-soluble modulin	*Staphylococcus epidermidis*	[125]
	Glycolipids	*Treponema maltophilum*	[119]
	Porins	*Neisseria meningitidis*	[116]
	Zymosan	*Saccharomyces cerevisiae*	[126]
	Phospholipomannan	*Candida albicans*	[127]
	Atypical LPS	*Leptospira interogans, Porphyromonas gingivalis*	[128], [129]
	Heat shock protein 60 (Hsp60)	Host, *Chlamydia*	[130]
	Hsp70	Host	[131]
	Hsp96	Host	[132]
	High-mobility group protein-1 (HMGP1)	Host	[133]
	Hyaluronic acid	Host	[134]
	Low-molecular weight hyaluronic acid	Host	[135]
	Haemagglutinin	Measles virus	[136]
	Structural viral proteins	Herpes simplex virus	[137]
		Cytomegalovirus	[138]
		Respiratory syncytial virus	[139]
		Lymphocytic choriomengitis virus	[140]
	Outer membrane protein A	*Klebsiella pneumoniae*	[141]
	Heat-killed bacteria	*Listeria monocytogenes*	[142]
	S-protein	SARS virus	[143]
TLR4	LPS	Gram-negative bacteria	[17]
	Mannan	*Saccharomyces cerevisiae, Candida albicans*	[144]
	Glucuronoxylomannan	*Cryptococcus neoformans*	[145]
	Hsp60	Host, *Chlamydia pneumonia*	[130]
	Hsp70	Host	[131]
	HMGP1	Host	[133]
	Low-molecular weight hyaluronic acid	Host	[135]
	Oligosaccharides of hyaluronic acid	Host	[146]
	Haemagglutinin B	*Porphyromonas gingivalis*	[147]
	Flavolipin	*Flavobacterium meningosepticum*	[148]
	ER-112022, E5531, E5564, E6020	Synthetic compounds	[96], [149]
	Taxol	Plant product	[150]
	Fusion protein	Respiratory syncytial virus	[151]
	Envelope proteins	Mouse mammary tumour virus, Moloney murine leukaemia virus	[152]
	Type III repeat extra domain A of fibronectin	Host	[153]
	Polysaccharide fragments of heparan sulfate	Host	[154]
	Fibrinogen	Host	[155]
	αA crystallin and HSPB8 (Hsp22)	Host (recombinant *Escherichia coli* produced)	[156]
	β-Defensin 2	Host	[157]
TLR5	Flagellin	Gram-positive and Gram-negative bacteria	[43], [158], **[52]**
TLR6/2	Zymosan	*Saccharomyces cerevisiae*	[126]
	Diacyl lipopeptides (mycoplasmal macrophage-activating lipopeptide-2)	Mycoplasma	[159]
	Heat-labile soluble factor (GBS-F)	Group B streptococcus	[160]

TABLE 1: Cont.

PRR	Ligands	Origin of the ligand	Reference (equine reference in bold)
TLR9	Purified HSV-2 DNA	HSV-2	[161]
	Unmethylated CpG DNA	Bacteria, virus, yeast, insects	[162]
	Chromatin–IgG complexes	Host	[163]
	Haemozoin	*Plasmodium falciparum*	[164]
NOD1	γ-D-Glu-DAP (iE-DAP) dipeptide structure in peptidoglycan	Gram-negative bacteria	[165]
	GM tripeptide	Gram-negative bacteria	[72]
	D-lactyl-L-Ala-γ-Glu-*meso*-DAP-Gly (FK156)	Gram-negative bacteria	[166]
	Heptanolyl-γ-Glu-*meso*-DAP-Ala (FK565)	Gram-negative bacteria	[166]
NOD2	Muramyl dipeptide (MDP) structure in peptidoglycan	Gram-positive and Gram-negative bacteria	[165]
	MurNAc-L-Ala-g-G-Glu-L-Lys (M-TRILys)	Gram-positive bacteria	[165]
NLRP3		Encephalomyocarditis virus	[167]
		Vesicular stomatitis virus	[167]
		Influenza virus	[168]
	Hyphae	*Candida albicans*	[169]
	Hyphae	*Aspergillus fumigatus*	[170]
		Saccharomyces cerevisiae	[171]
	β-Glucan	Fungi	[171]
	Muramyl dipeptide (MDP)	Gram-positive and Gram-negative bacteria	[172]
	Nigericin	*Streptomyces hygroscopicus*	[173]
	Maitotoxin	*Marine dinoflagellates dinoflagellates*	[173]
	Gramicidin	*Bacillus brevis*	[174]
	Aerolysin	*Aeromonas hydrophilia*	[175]
	α-Toxin	*Staphylococcus aureus*	[174]
	Haemozoin	*Plasmodium falciparum*	[176]
		Listeria monocytogenes	[177]
		Sendai virus	[178]
	ATP	Host	[173]
	Uric acid crystals	Host (e.g. gout associated)	[179]
	Silica	Airborne pollutants	[180]
	Asbestos	Airborne pollutants	[180]
	Alum	Vaccine adjuvant	[181]
	β-Amyloid	Host	[182]
NLRC4	Flagellin	*Salmonella, Legionella, Listeria, Pseudomonas*	[84], [177]
		Candida albicans	[183]
		Shigella flexneri	[184]
AIM2	dsDNA	Bacterial, viral and host	[91]

OspA, outer surface protein A; NapA, neutrophil activating protein A; HSV-2, Herpes Simplex Virus-2; γ-D-Glu-DAP, γ-D-glutamyl-meso-diaminopimelic acid; MurNAc-L-Ala-g-G-Glu-L-Lys, N-acetylmuramic acid-L-Alanine-g-Glutamyl-L-Lysine.

are isolated from a clinical case, it is highly likely that this PRR will be at least partly responsible for driving any inflammatory response.

Toll-like receptor 5

Toll-like receptor 5 (TLR5) recognises flagellin, which forms the protein backbone of bacterial flagella [43]. Flagella are important for bacterial motility and for cellular invasion [44]. A wide variety of flagellated bacteria, such as *E. coli* and *Salmonella* spp., cause disease in the horse [45]. An evolutionarily conserved region of flagellin, D1, interacts with the leucine-rich repeats of TLR5 on the cell surface of diverse cell types, including neutrophils, monocytes, macrophages and epithelial cells [46–52]. Human peripheral blood monocytes express moderate amounts of TLR5, and activation by flagellin results in a strong expression of proinflammatory cytokines [50,53]. In mice, TLR5 mRNA expression is found in dendritic cells, but is not detected in neutrophils [49]. In the horse, both monocytes and neutrophils express TLR5 mRNA, but show differential expression of TLR5 proteins on the cell surface [52]. Toll-like receptor 5 agonists activate equine neutrophils, but not monocytes, alveolar macrophages or peritoneal macrophages despite the fact that these cell types contain TLR5 mRNA transcripts. Cytokine gene expression induced by flagellin in neutrophils was comparable with that stimulated by LPS or Pam_3CSK_4 (a synthetic TLR2 agonist) [52]. What role, if any, TLR5 may play in infectious diseases in the horse is unclear, but it may be important in shock, sepsis, acute respiratory diseases and gastrointestinal infection [54].

Toll-like receptor 9

Toll-like receptor 9 (TLR9), unlike TLRs 1, 2, 4, 5 and 6, which are all present in the cell membrane, primarily resides in the endoplasmic reticulum. It recognises unmethylated CpG containing DNA motifs from both bacteria and viruses [55]. The Cytosine-phosphate-Guanine (CpG) DNA activates dendritic cells and is important in initiating adaptive immune responses [56]. Toll-like receptor 9 forms homodimers before ligand binding [57], then undergoes multiple cleavage steps on or after ligand binding prior to signalling [58,59]. The precise order and timing of dimerisation and cleavage/activation remain to be established. Toll-like receptor 9 shows differential expression among normal and inflamed tissues [60–63]. Equine TLR9 is found in lymphocytes, polymorphonuclear cells, bronchial epithelial cells, *type II* cells in the equine lung [64,65], and the cornea, limbus and the conjunctiva of the equine eye [66]. Age has little influence on TLR9 expression in neutrophils [67], macrophages or dentritic cells [68]. The role of TLR9 in equine disease remains to be elucidated.

NOD-like receptors

Nucleotide-binding oligomerisation domains (NODs) form a specific family of cytosolic receptors (NLRs), which consists of over 20 structurally related proteins [69]. There are 2 distinct families of NLRs; the NLRP proteins contain a pyrin domain, and the NLRC proteins, such as NOD1, NOD2, NLRC3 and NLRC4 contain a caspase recruitment domain [70]. NLRP3, NLRP1 and NLRC4 form protein complexes called inflammasomes such that upon ligand binding a change in NLR confirmation leads to recruitment of an adaptor molecule (apoptosis-related speck-like protein [ASC]) and an effector molecule (pro-caspase-1) in an oligomeric complex. This complex activates a proteolytic cascade resulting in the maturation and release of, amongst others, proinflammatory cytokines of the interleukin-1 family [71]. The NLRs are emerging as very important therapeutic targets in many inflammatory diseases in humans.

Both NOD1 and NOD2 recognise bacterial ligands [72,73]. Whereas NOD1 is ubiquitously expressed, NOD2 is expressed only in monocytes, macrophages, dendritic cells and intestinal epithelial cells [74]. A peptidoglycan derivative (L-Ala-D-Glu-meso-DAP [diaminopimelic acid]), present in almost all Gram-negative bacteria, is recognised by NOD1 [75,76]. However, NOD2 detects M-dipeptide and GM-dipeptide, both of which are degradation products of peptidoglycans. GM-dipeptide is found in all bacteria, hence NOD2 can be regarded as a general sensor of peptidoglycan degradation products [72]. There are limited data on equine NOD1 and NOD2, but horses with Recurrent Airway Obstruction show upregulation of NOD2-induced nuclear factor-κB activation [77].

The receptor NLRC4 (also known as IPAF) is expressed in myeloid cells [78,79]. It recognises a variety of pathogens, including *S*. Typhimurium [80], *Pseudomonas aeruginosa* [81], *Shigella* [82], *Legionella pneomophila* [83], bacterial flagellin [84,85] and a basal rod component of some bacterial *type III* secretion systems [86]. This receptor is present in the equine genome, but it is unclear what role it might play in equine bacterial diseases.

Many 'danger signals' are recognised by NLRP3 (cryopyrin or NALP3), both infectious (for example recognising *Staphylococcus aureus* [87], *Staphylococcus pneumoniae* [88] and *S*. Typhmuium [89]) as well as noninfectious, endogenous or exogenous molecules. The wide variety and diverse nature of these ligands suggest it is unlikely that they interact directly with NLRP3, but trigger inflammasome formation indirectly [90]. It is important in many chronic inflammatory syndromes in humans, and it is present in the equine genome.

A fourth inflammasome complex is formed by association of a pyrin and HIN200 domain containing protein family member (absent in melanoma 2 [AIM2]) with ASC and caspase-1 [90]. A cytosolic receptor, AIM2, recognises double-stranded DNA [91–93] and is an important sensor for bacterial double-stranded DNA from both *Listeria monocytogenes* and *Francisella tularensis* [94,95]. It is present in a limited number of mammalian species, of which the horse is one, and therefore this PRR is also potentially important in horses.

Antagonists of PRRs

Pattern recognition receptor agonists (for adjuvants) and antagonists are under development. Some antagonists at other PRRs have been described, but antagonists of TLR4 and TLR2 are likely to be most useful in equine endotoxaemia and sepsis.

Toll-like receptor 4 antagonists

Antagonism at TLR4 is the most obvious therapeutic target for equine endotoxaemia and sepsis. Development of TLR4 antagonists is challenging because many of the drugs developed are derived from bacterial lipid As that are antagonists in humans and mice, but this does not mean they will necessarily be antagonists in the horse. Lipopolysaccharide from *Rhodobacter sphaeroides*, for example, is a TLR4 antagonist in humans and mice, but it is an agonist in the horse and hamster [96,97]. E5531, a synthetic compound based on the lipid A structure of *Rhodobacter capsulatus*, is an antagonist in mice and humans, an antagonist in equine cell models, but an agonist in an equine whole-blood model [98–100]. A second-generation compound based on E5531, eritoran (E5564), is a potent antagonist of LPS in humans [101,102] and in horses [103], but in phase III clinical trials [104] it did not meet its primary end-point in humans with severe sepsis [105]. Several other TLR4 antagonists are currently being investigated in humans and mice for the treatment of different acute and chronic inflammatory diseases [13,106].

Toll-like receptor 2 antagonists

Antagonistic phospolipids for TLR2 have been synthesised, but currently there is little information available beyond their initial description [107]. Toll-like receptor 2 antibodies protect mice from lethal septic shock syndrome [108], and anti-TLR2 antibodies that prevent trafficking of the receptor from the endoplasmic reticulum to the cell surface were shown to inhibit *in vitro* and *ex vivo* ligand-driven cell activation [109]. Anti-TLR2 antibodies also show beneficial effects in arthritis and ischaemia-reperfusion injury models [110,111], but it is unlikely that commercial equine-specific TLR-blocking antibodies will be developed for horses. It is likely, however, that TLR2 antagonists may be useful for a range of equine conditions, for example, neonatal diarrhoea-associated sepsis, should these compounds become available for use in horses.

Conclusions

In conclusion, PRRs that recognise bacteria are likely to be useful therapeutic targets for treating equine sepsis and endotoxaemia. However, PRR antagonists will need careful clinical evaluation because of the controversial results emerging from human clinical trials due to the complex, multifactorial pathogenesis of these diseases. Use of TLR4 antagonists in endotoxic horses is likely to be successful, but whether PRR antagonists will be useful in septic foals is less clear. Infections with mixed bacterial species will potentially involve multiple PRRs, suggesting that combination therapy simultaneously inhibiting several PRRs may be necessary. Complete inhibition of PRRs is potentially detrimental, particularly in sepsis, because TLR4 and TLR2 knockout mice show increased mortality in response to Gram-negative or Gram-positive bacteria, respectively. Specific equine drugs will need to be developed to achieve a safe treatment that blocks systemic inflammation whilst retaining the protective immune responses against bacterial infection.

Authors' declaration of interests

No conflicts of interest have been declared.

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