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Review

Endogenous ligands of TLR2 and TLR4: agonists or assistants?

Clett Erridge[1]

Department of Cardiovascular Sciences, Glenfield General Hospital, University of Leicester, Leicester, United Kingdom

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ABSTRACT

The mammalian TLRs serve as key sensors of PAMPs, such as bacterial LPS, lipopeptides, and flagellins, which are present in microbial cells but not host cells. TLRs have therefore been considered to play a central role in the discrimination between "self" and "non-self". However, since the discovery of their microbial ligands, many studies have provided evidence that host-derived molecules may also stimulate TLR2- or TLR4-dependent signaling. To date, more than 20 of these endogenous TLR ligands have been proposed, which have tended to fall into the categories of released intracellular proteins, ECM components, oxidatively modified lipids, and other soluble mediators. This review aims to summarize the evidence supporting the intrinsic TLR-stimulating capacity of each of these proposed endogenous ligands with a particular emphasis on the measures taken to exclude contaminating LPS and lipopeptides from experimental systems. The emerging evidence that many of these molecules may be more accurately described as PAMP-binding molecules (PBMs) or PAMP-sensitizing molecules (PSMs), rather than genuine ligands of TLR2 or TLR4, is also summarized. The relevance of this possibility to the pathogenesis of chronic inflammatory diseases, tumor surveillance, and autoimmunity is discussed. *J. Leukoc. Biol.* 87: 989–999; 2010.

Introduction

The ability to discriminate between "self" and "non-self" lies at the heart of mammalian innate immunity. This essential defensive process is mediated largely by the detection of a small number of key "danger-associated" molecules by germline-encoded receptors of the innate immune system, of which the TLRs are a major family [1]. Traditionally, it has been considered that the danger-associated molecules sensed by TLRs are highly conserved PAMPs, which are expressed by bacteria, viruses, or other pathogens, but are not present in mammalian cells [2]. For example, BLPs, LPS, and flagellins are recognized by TLR2, TLR4, and TLR5, respectively [2]. In this model, the engagement of PAMP with its corresponding TLR results in the rapid induction of proinflammatory intracellular signaling cascades, including MyD88-dependent phosphorylation of MAPKs, activation of NF-κB signaling, and the resulting up-regulation of diverse inflammatory mediators, such as cytokines, chemokines, and adhesion molecules, which together, serve essential functions in promoting antimicrobial responses [2].

More recently, however, an additional role for TLRs has been proposed. Specifically, a number of reports have emerged to suggest that diverse molecules of host-cell origin may also serve as endogenous ligands of TLR2 or TLR4 [3]. These molecules represent members of a recently identified family of molecules, termed "alarmins", which have been proposed to serve as mediators of inflammation that may be expressed or released in response to tissue damage and therefore, have also been described as DAMPs [4]. In view of this notion, a revised model of TLR function has been put forward, suggesting that the expression of certain alarmins or DAMPs may also serve to stimulate TLR signaling directly, thereby pre-emptively inducing an inflammatory response in the absence of microbial challenge [4, 5].

To date, there have been at least 23 reports of distinct endogenous ligands of TLR2 or TLR4, representing molecules of diverse source and structure, ranging from those associated with cell damage and ECM turnover to inflammatory mediators and oxidatively modified lipids. However, it should be noted that of these 23 reported endogenous TLR ligands, eight have been reported in subsequent studies to have no intrinsic TLR-stimulating potential when highly pu-

Abbreviations: AGE=advanced glycation end-product, BLP=bacterial lipopeptide, DAMP=damage-associated molecular pattern, ECM=extracellular matrix, F-EDA=fibronectin extra domain A, h=human, HEK-293=human embryo kidney 293, HMGB1=high-mobility group box 1, HSP=heat shock protein, LAL=limulus assay, LBP=LPS-binding protein, LDL=low-density lipoprotein, LRR=leucine-rich repeat, m=murine, MD2=myeloid differentiation protein 2, mmLDL=minimally modified LDL, MRP-8/14=myeloid-related protein-8/14, OxLDL=oxidized low-density lipoprotein, OxPAPC=oxidized palmitoyl-arachidonyl-phosphatidyl-choline, Pam_3CSK_4=palmitoyl-3-cysteine-serine-lysine-4, PAMP=pathogen-associated molecular pattern, PBM-PAMP-binding molecule, PMB-Polymyxin B, PSM=PAMP-sensitizing molecule, SAA=serum amyloid A, sCD14=soluble CD14, SFA=saturated fatty acid, TEV=tobacco etch virus

1. Correspondence: Department of Cardiovascular Sciences, Glenfield General Hospital, University of Leicester, Leicester, UK, LE3 9QP. E-mail: ce55@le.ac.uk

rified reagents were examined [6–14]. Thus, a key question that remains to be addressed in this emerging field is whether each of the proposed endogenous TLR ligands may possess genuine TLR-stimulating potential or whether these reports instead reflect contamination of the reagents used to perform these studies with the established ligands of these receptors, namely BLP and LPS.

Addressing this question is of particular relevance to current research in the fields of chronic inflammatory diseases, such as atherosclerosis, insulin resistance, and arthritis, which have been shown to be potentiated by as-yet-unidentified ligands of TLR2 and/or TLR4 [15–17]. Specifically, it remains to be determined whether the TLR ligands responsible for promoting these conditions are of endogenous or microbial origin. In light of these uncertainties, this review will therefore aim to summarize the current evidence supporting each of the reported endogenous ligands of TLR2 and TLR4 and also to highlight the potential factors that may confound the interpretation of current findings.

DIVERSE ENDOGENOUS MOLECULES HAVE BEEN PROPOSED AS TLR LIGANDS

Table 1 summarizes the results of 64 studies that have reported the ability of endogenous molecules to stimulate signaling via TLR2 or TLR4. Of the 23 agents listed, five are intracellular, six are ECM components, four are modified lipids or lipoproteins, and eight fall into other categories. Almost one-quarter (13) of these reports suggest that TLR2 and TLR4 are stimulated by proposed molecules. However, it should also be noted that the TLR-stimulating properties of approximately one-third (8) of the proposed molecules were questioned in subsequent studies, which failed to demonstrate intrinsic TLR-stimulating properties of these molecules when highly purified reagents or rigorous anticontamination procedures were used [6–14]. For example, the TLR-stimulating capacities of HSP60 and HSP70 were shown to be absent in LPS-free HSP preparations [6, 7]. Likewise, the use of low endotoxin rHMGB1, derived from mammalian expression systems, revealed no capacity of this molecule to stimulate TLR signaling [9, 10]. The synthetic β-amyloid peptide was also shown subsequently to be incapable of stimulating TLR signaling when presented to cells in a purified form [12, 13], and highly purified pharmacological-grade, low m.w. hyaluronan did not promote NF-κB activation or cytokine secretion in murine macrophages [14]. A focus of our laboratory has been to investigate the possibility that modified lipids or lipoproteins may stimulate TLR-dependent signaling in the context of atherosclerosis. Against expectation, however, we have found no TLR-stimulating capacity of oxidized phospholipids or of moderately or heavily OxLDL when using low endotoxin reagents and protocols [11]. Moreover, we could not detect a TLR-stimulating capacity of SFAs, finding instead that most previous studies in this area had used commercially sourced fatty acid-free albumin to complex lipids, a reagent that can often be contaminated with endotoxin and BLP [8]. Findings such as these have led some researchers to reconsider the evidence supporting the TLR-stimulating capacity of proposed endogenous ligands [80].

RECENTLY IDENTIFIED SOURCES OF POTENTIAL ENDOTOXIN AND LIPOPEPTIDE CONTAMINATION

The possibility that experimental reagents may be contaminated with bacterial endotoxin (LPS) has been a consideration for researchers of immune mediators for decades [3, 80]. Indeed, the perceived immunological properties of several molecules have been found subsequently to be a result of endotoxin contamination [6, 7, 80, 81]. Most frequently, this has been a result of the use of recombinant proteins expressed in *E. coli* [3, 18–21, 77, 80], as used by 15 of the 64 studies summarized in Table 1. In view of these concerns, however, most studies of potential endogenous TLR ligands have used alternative preparative techniques. For example, 26 studies used commercially sourced reagents or gifts from other researchers, 12 used reagents prepared from primary tissues (including blood), and five examined recombinant proteins expressed in mammalian systems. Thus, the established view is that most of the studies listed in Table 1 are likely to have used reagents with little opportunity for microbial contamination.

However, more recent evidence has revealed that endotoxin and lipopeptide contamination may be more prevalent in commercially sourced reagents than appreciated previously [8, 80]. For example, we have found that the eluates of commonly used protein purification columns can be contaminated with BLP, despite the use of ethanol as a preservative in such columns (**Fig. 1**). Moreover, enzymes used routinely for protein tag removal in mammalian recombinant protein expression systems, such as TEV protease, or other preparative enzymes may be commonly contaminated with LPS and lipopeptide, presumably reflecting their recombinant origin (Fig. 1). A further concern regarding the examination of recombinant proteins derived from mammalian expression systems is that some surveys have suggested that as many as 25% of mammalian cell lines may harbor ongoing mycoplasma contamination [82]. Findings such as these suggest that significant potential for LPS or BLP contamination may exist for reagents used in any of the studies listed in Table 1, including those that have not used bacterial expression systems.

LIMITATIONS OF CURRENT ANTICONTAMINATION METHODOLOGIES

To address the possibility that test reagents may be contaminated with molecules of microbial origin, most studies of potential endogenous TLR ligands have used key experiments to control for potential LPS contamination (with 13 exceptions [24, 39, 51, 59, 61–65, 78, 79, 83, 84]). The most common approach has been the use of the LAL assay to detect contaminating LPS, and 38 studies have used this

TABLE 1. Diversity of Proposed Endogenous Ligands of TLR2 and TLR4

Refs.	TLRs	Cell types	Preparation	LAL	PMB	AC
HSPs						
[18]	TLR2/4	RAW, 293-TLRs, DC	CS, ER	—	—	a
[19]	TLR2/4	THP-1, 293-TLRs	CS, ER	<5 pg/ml	—	
[20]	TLR2/4	293-TLRs, VSMC	CS, ER	<0.2 pg/ml	—	a
[21]	TLR4	TLR4*d*MAC	CS, ER	<0.1 EU/mg	—	
Necrotic or dying cells						
[22]	TLR2	293-TLR2	FT	—	—	g
[23]	TLR2/3	TLR2/3*d* Schwann cells	FT	Low	—	
[24]	TLR2	TLR2, TRIF, MyD88*d* GMC	FT	—	—	
[25]	TLR4	TLR*d* mouse *in vivo* T cell response	ODX	—	—	l
[26]	TLR2	MyD88*d,* TLR2*d* MAC, 293-TLR2	UV	—	Yes	b
[27]	TLR2	293-TLR2/4, TLR2d DC, *in vivo*	FT	Low	—	
HMGB1						
[28]	TLR2/4	TLR2,4*d* neutrophils, RAW	PT	<10 pg/ml	Yes	
[29]	TLR2/4	293-TLRs, RAW	PT	<10 pg/ml	—	
[30]	TLR2/4	hMAC, 293-TLRs, TLR2/4*d* mMAC	MR, CP	0.6 pg/μg	Yes	
MRP-8/14						
[31]	TLR4	293-TLR4, TLR4*d* MAC, MON	ER	<1.2 pg/mg	Yes	a
Cardiac myosin						
[32]	TLR2/8	293-TLRs, MON	PT	—	—	g
F-EDA						
[33]	TLR4	293-TLR4, TLR4*d* SPL, THP-1	ER, CP, LC	<1 ng/ml	Yes	a, c, d
[34]	TLR4	MAC, *in vivo*	ER, LC	<0.6 pg/μg	—	c, h, i
Biglycan						
[35]	TLR2/4	293-TLRs, TLR2/4*d* MAC, *in vivo*	MR, CP, LC	<32 EU/mg	Yes	a, b
[36]	TLR2/4	TLR2/4*d* MAC	MR, CP	Low	—	
Hyaluronan						
[37]	TLR4	hDC, TLR4*d* mDC	CS, ET	<0.1 ng/ml	Yes	
[38]	TLR2/4	HK, TLR4*d* skin	CS	Low	—	a
[39]	TLR2/4	COC	PT, RG	—	—	
[40]	TLR4	MH-S, TLR4*d* skin, 293-TLRs	CS, LC	—	—	i
[41]	TLR2	293-TLRs, MAC, DC	CS	<10 ng/ml	Yes	
[42]	TLR2/4	*In vivo* in mice	PB, PT	<40 pg/mg	Yes	
[43]	TLR4	Endothelial cells	RG, LC	<6.3 EU/mg	Yes	
Heparan sulfate						
[44]	TLR4	TLR4*d* DC	CS	—	—	a, e
[45]	TLR4	TLR4*d* in vivo	CS	Low	—	e
Tenascin-C						
[46]	TLR4	TLR2/4*d* MEFs, MAC, *in vivo*	ER, MR, CP	<10 pg/ml	Yes	a, f
Versican						
[47]	TLR2	TLR2,3,4,9*d* MAC, *in vivo*	LM, CP	—	—	j
mmLDL						
[48]	TLR4	J774, LR9, MAC, CHO-TLRs	PT, LO	<2 ng/mg	Yes	
[49]	TLR4	J774, CHO-TLRs, TLR4*d* MAC	PT, LO	<50 pg/mg	—	
[50]	TLR4	MyD88*d* MAC, J774	PT, LO	<50 pg/mg	—	
OxPAPC						
[51]	TLR4	HeLa	AE	—	—	
[52]	TLR4	MAC	CS	<0.1 EU/ml	Yes	
OxLDL						
[53]	TLR4	Granulosa cells	PT, CS	—	—	
AGE-LDL						
[54]	TLR4	TLR4*d* MAC, 293-TLR4	PT, GL	<5 pg/ml	—	a
Surfactant protein A						
[55]	TLR4	TLR4*d* MAC, CHO-TLR4	PT, CP	<140 ng/mg	Yes	a
β-defensin						
[56]	TLR4	DC	ER, LC	<0.5 EU/μg	—	a, b
[57]	TLR2/1	DC, MON, 293-TLRs, CHO-TLRs	ER	<0.5 ng/mg	—	a, c
[58]	TLR4	DC, MAC, XS52	ER, LC	<0.5 EU/μg	Yes	
SFAs						
[16]	TLR4	293-TLR4, MAC, 3T3-L1, SKMC	BSA	<40 pg/ml	—	

(*Continued on next page*)

TABLE 1. (Continued)

Refs.	TLRs	Cell types	Preparation	LAL	PMB	AC
HSPs						
[59]	TLR2	C2C12 myotubes	BSA	—	—	
[60]	TLR2/4	RAW, DC	BSA	<0.4 EU/ml	—	
[61]	TLR4	3T3-L1 adipocytes	BSA	—	—	
[62]	TLR2/4	RAW, 293-CD4-TLR4	Na^+	—	—	
[63]	TLR4	293-TLR4, RAW	Na^+	—	—	
[64]	TLR2	TLR4*d* MAC, 293/SW620-TLRs	Na^+	—	—	
[65]	TLR4	RAW	BSA	—	—	
[66]	TLR4	RAW	Na^+	—	Yes	
[67]	TLR4	3T3-L1/TLR4*d*MAC	BSA	—	Yes	
[68]	TLR4	L6 myotubes	BSA	—	Yes	
Fibrinogen						
[69]	TLR4	TLR4d MAC	CS	<1 EU/ml	Yes	a
[24]	TLR4	Cardiomyocytes	CS	—	—	
[70]	TLR4	293-TLR4	Not stated	<0.5 pg/ml	—	
[71]	TLR4	Human monocytes	CS, LC	<0.1 pg/mg	—	
[72]	TLR4	Podocytes	CS	—	Yes	a
SAA						
[73]	TLR2	TLR2*d* MAC, *in vivo*, HeLa	ER, CS	<0.1 ng/μg	Yes	a, b, k
[74]	TLR2	Hela, TLR2*d* MAC	ER, MR, CP	<0.54 EU/μg	—	a, b
[75]	TLR4	TLR4*d* MAC	ER, CS	<0.1 ng/μg	Yes	b
Antiphospholipid antibodies						
[76]	TLR2	TLR1,2,4,6*d* MEF	PT, CP	<0.5 EU/mg	—	j
Amyloids						
[77]	TLR2	293-TLR2, THP-1, TLR2*d* MAC	ER and SP	—	—	c
Angiotensin II						
[78]	TLR4	Rat VSMC	CS	—	—	
[79]	TLR4	Rat VSMC	CS	—	—	

This table summarizes the results of 64 studies that have reported TLR2- or TLR4-dependent signaling of 23 endogenous molecules. Listed are which TLRs were reported to be stimulated, the cell types used to show TLR2- or TLR4-dependent signaling, the method used for preparation of each proposed ligand, the amount of LPS quantified in each proposed TLR stimulant as measured by the LAL assay, whether PMB was used in any experiments and additional comments (AC) relating to other control methodologies. Abbreviations for methods of preparation of proposed TLR stimulants: AC = other anticontamination procedures applied to test reagents; AE = prepared by exposing PAPC to air for 72 h; BSA = SFAs were complexed to BSA; CP = column-purified; CS = commercially sourced; ER = *Escherichia coli*-recombinant; ET = enzyme-treated; FT = freeze thaw of cells; GL = glycated LDL prepared by incubating LDL with 50 mM glucose for 14 days; LAL = the amount of LPS quantified in proposed TLR stimulants as measured by the LAL assay; LC = LPS-removal column was used; LM = prepared by His-tag purification from Lewis lung carcinoma-conditioned medium; LO = prepared by incubating LDL with 15-lipoxygenase-O-expressing fibroblasts; MR = mammalian-recombinant; Na^+ = SFAs were presented as sodiated forms; ODX = necrotic cells were prepared by treatment with oxaliplatin, doxorubicin, or X-rays; PB = prepared from bacteria (Streptococcus); PMB = PMB was used in some experiments; PT = prepared from tissues; RG = gift from other researcher; SP = synthetic peptide; UV = primary and secondary necrosis of cells induced by treatment with ultraviolet radiation, streptozotocin, etoposide, or IFN-γ/TNF-α. Cell types: 293-TLRs = HEK-293 cells transfected with TLRs; 3T3-L1 = adipocyte cell line; CHO = chinese hamster ovary; COC = cumulus-oocyte complexes; DC = dendritic cells; GMC = glomerular mesangial cell; HK = human keratinocyte; MAC = macrophages; MEF = mouse embryonic fibroblasts; MH-S = mouse alveolar macrophage cell line; MON = human monocytes; RAW = RAW 264.7 macrophage cell line; SKMC = skeletal muscle cells; SPL = splenocyte; TLR2*d* = cells from or mice deficient in TLR2; TRIF = Toll/IL-IR domain-containing adaptor-inducing iFN-β; VSMC = vascular smooth muscle cells. AC: a = heat treatment of proposed ligand-reduced TLR signaling; b = protease treatment of proposed ligand-reduced TLR signaling; c = related proteins or peptides were without activity; d = treatment with LPS antagonist E5564 did not reduce activity; e = cotreatment with LPS-binding protein Limulus anti-LPS factor did not reduce activity; f = cytokine secretion profile different from LPS; g = potential microbial contamination discounted, as no response observed in 293-TLR4 cells; h = serum not required for responsiveness to proposed ligand; i = CD14 not required for responsiveness to proposed ligand; j = Mycoplasma testing was performed; k = lipoprotein-lipase did not reduce activity of proposed TLR2 agonist; l = dendritic cell incubated with HMGB1 alone did not release proinflammatory cytokines.

method to confirm that tested reagents contained low levels of LPS. Next, most widely used is cotreatment of cells with PMB, an antibiotic that binds and sequesters LPS from the receptors of the innate immune system (22 studies). The TLR-stimulating capacities of proposed ligands were also shown to be sensitive to heat in 16 studies and protease in six studies, with the assumption that the biological activity of endotoxin is not lost during heat or protease treatment. As these experiments are considered to constitute rigorous exclusion of potential microbial contaminants, it has been

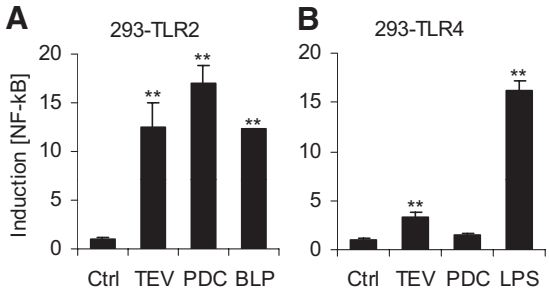

Figure 1. Potential sources of contaminants in nonprokaryote recombinant systems. HEK-293 cells transfected with a NF-κB-sensitive reporter, CD14 and TLR2 or TLR4/MD2, were challenged with a 1:100 dilution of commonly used tag removal enzyme TEV protease, saline eluate from an unused protein-desalting column (PDC), 100 ng/ml Pam_3CSK_4 (BLP), or 10 ng/ml LPS. **, $P < 0.01$, versus medium alone [control (Ctrl)].

widely accepted that the results of most of the listed studies represent discoveries of genuine endogenous ligands of TLR2 or TLR4.

However, recent evidence suggests that some caution may be required in the interpretation of the results of experiments using these control methodologies. For example, it is well established that the LAL assay is easily confounded by the presence of molecules that binds LPS. Indeed, LBP and bactericidal/permeability-inducing protein, were each shown to completely prevent the ability of the LAL assay to detect LPS [85]. Thus, it is possible that the LPS content of many of the proposed ligands could have been greatly underestimated if these ligands have any capacity to bind LPS. Moreover, the LAL assay is not able to detect lipopeptide contaminants [8], a notable consideration when almost half (28) of the reports suggest that their proposed agents stimulate TLR2 signaling.

A similar concern arises regarding the use of PMB. Although this molecule binds and sequesters enterobacterial LPS, it does not inhibit the signaling induced by LPSs of several nonenterobacterial organisms [86] or the capacity of BLP to stimulate TLR2 signaling [8]. Moreover, studies have shown that although PMB can entirely block the capacity of LPS to stimulate cellular cytokine release, it does not completely block signaling of LPS-contaminated HSPs, suggesting that agents that bind LPS may prevent LPS inactivation by PMB [6, 7]. Supportive of this notion is the observation that PMB does not completely block LPS signaling in the presence of sCD14 (**Fig. 2A**).

With respect to heat treatment, it should be noted that contrary to popular belief, the biological activity of endotoxin is in fact reduced significantly by heating, as highlighted recently by Tsan and Gao [80]. Moreover, substances that serve to enhance cellular sensitivity to low levels of LPS contamination may lose their capacity to do so following heat or protease treatment. For example, sCD14, which is a molecule that binds and enhances cellular responsiveness to LPS, appears to display the properties of a heat- and protease-sensitive endogenous ligand of TLR4 when in the presence of low-grade endotoxin contamination (Fig. 2B). Likewise, in the presence of low levels of lipopeptide contamination, sCD14 shows apparent endogenous TLR2-stimulating properties that are sensitive to boiling and protease (Fig. 2C).

Taken together, these findings suggest that if proposed endogenous TLR ligands have any capacity to bind or enhance cellular sensitivity to LPS or lipopeptide, it may be difficult to discount potential contamination of such molecules by the use of LAL assays, PMB, protease, or heat treatment alone. Future studies of candidate endogenous TLR ligands will therefore require the use of mammalian expression systems, mycoplasma testing of expression cell lines, avoidance of the use of recombinant enzymes, and rigorous

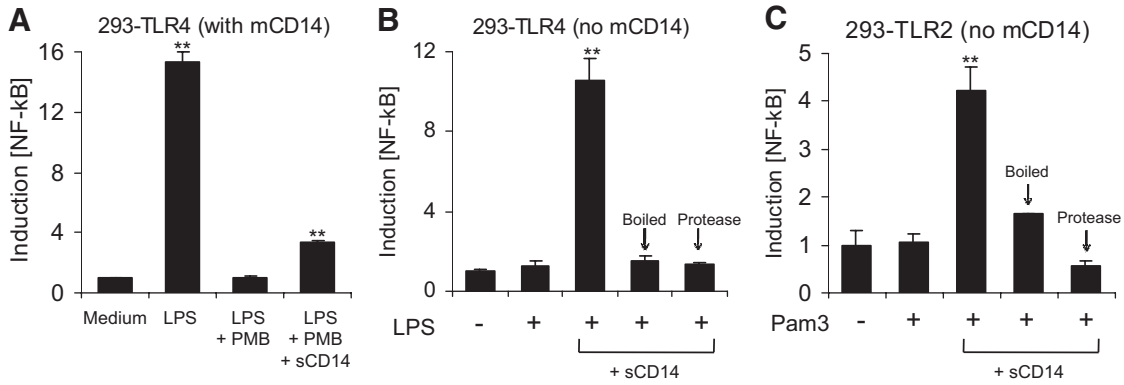

Figure 2. Effect of heat, protease, and PMB on the biological activity of sCD14/LPS complexes. (A) HEK-293 cells transfected with a NF-κB reporter, mCD14 and TLR4/MD2, were challenged with medium alone or 10 ng/ml LPS and 10 μg/ml PMB, with or without 1 μg/ml sCD14. (B) HEK-293 cells transfected with TLR4/MD2 but without mCD14 were challenged with 10 ng/ml LPS in the presence or absence of 1 μg/ml sCD14. LPS/sCD14 complexes were untreated, boiled for 10 min, or treated with proteinase K for 30 min before addition to cells. (C) HEK-293 cells transfected with TLR2 were challenged with 0.1 ng/ml Pam_3CSK_4 (Pam3), with or without sCD14, which had been untreated, boiled, or treated with proteinase K. **, $P < 0.01$, versus medium alone.

testing of all reagents and experimental accessories involved in the preparation and treatment of test compounds. In particular, assays that do not rely on the limulus enzyme system, such as the THP-1 cell or whole blood IL-1β release assays, may need to be used to detect non-LPS-based contaminants in preparatory reagents [87, 88].

EVIDENCE THAT DAMPs MAY SERVE AS PBMs

In view of the concerns raised above, it is interesting to note that many of the molecules listed in Table 1 have been shown to have the capacity to bind LPS or other PAMPs. For example, BSA and LDL have long been understood to bind and transport LPS [89, 90], and direct evidence of LPS binding has also been shown for HSPs [91], HMGB1 [9, 10], surfactant protein A [92], and β-defensins [93, 94].

Most of the other agents listed in Table 1 have not yet been tested for their capacity to bind LPS or lipopeptide, but some evidence already exists to suggest that they may contain lipid-binding domains capable of complexing LPS or lipopeptide. For example, the type III repeat domains of fibronectin have been shown to consist of seven antiparallel β-strands, which enclose a core of highly conserved hydrophobic residues [95]. It is tempting to speculate that the specialized type III EDA of alternatively spliced fibronectin may be adapted specifically to promote LPS binding or presentation. Tenascin-C also contains similar fibronectin type III domains [46]. The LRR architecture of the ligand-binding domain of TLRs has been shown to be involved in the binding of PAMPs [96], and this motif is also present in biglycan [35] and the related ECM component lumican, which has been shown to bind and sensitize cells to LPS [97]. Bacterial lipoteichoic acid was shown to bind to the fatty acid-binding sites on human plasma fibronectin [98], and more general lipid or lipoprotein binding was shown for versican and MRP-8/14 [99, 100].

Beyond lipid-binding domains, it is possible that other types of molecular motif may also enhance the binding of LPS or other PAMPs. For example, bacteria have been shown to bind to SAA and heparan sulfate [101, 102], and the diglucosamine motif of the lipid-A moiety of LPS may promote interaction with similar repeating disaccharide motifs present in the glycosaminoglycan hyaluronan (which is a polymer of D-glucuronic acid and D-N-acetylglucosamine) or heparan sulfate (most commonly, glucuronic acid and N-acetyl-glucosamine). Thus, of the 23 listed, proposed TLR ligands, nine have already been shown to bind LPS or to contain agents that bind LPS, a further five have been shown to bind other types of lipid or lipoprotein, and at least four of the remainder have putative or established bacteria or PAMP-binding properties (**Table 2**).

EVIDENCE THAT DAMPs MAY SERVE AS PSMs

It is well established that cellular sensitivity to LPS is critically regulated by soluble proteins that bind and present LPS, such as LBP and sCD14 [110, 111]. These molecules also markedly increase cellular sensitivity to BLPs [112–114]. Interestingly, recent evidence suggests that many of the proposed endogenous TLR ligands may also have potential to enhance the sensitivity of cells to LPS, lipopeptide, or indeed other PAMPs (as summarized in Table 2). For example, HMGB1 was shown to disaggregate LPS and transfer monomers to sCD14 and to hPBMCs, thereby enhancing their responsiveness to LPS [9, 10]. HMGB1 also enhanced PBMC responses to BLP and CpG DNA [9]. Likewise, β-amyloid was shown to increase cellular sensitivity to BLP and LPS [12, 13]. Cells cotreated with LPS and low m.w. hyaluronan secreted more cytokine than cells treated with LPS alone [106]. Fibrinogen and fibronectin were shown to prime neutrophils toward greater responsiveness to LPS [108, 104], and fibronectin was shown additionally to enhance microglial cell responses to lipopeptide and CpG DNA [105]. Mice genetically deficient in biglycan were shown to be considerably less responsive to LPS than wild-type animals [35] in a manner similar to that shown for the related LRR-containing ECM component lumican [97]. Finally, the macrophage cytokine response to Gram-negative bacteria was shown to be enhanced significantly in the presence of SAA [101]. Thus, of the 23 listed, proposed TLR

TABLE 2. PBM and PSM Properties of Proposed Endogenous TLR Ligands

	LPS-binding	Other lipid/PAMP-binding	PAMP-sensitizing
HSP	[91]	[91]	[103]
Necrotic cells[a]	[9, 91]	[9, 91]	[9, 91]
HMGB1	[9]	[9]	[9, 10]
MRP-8/14	?	[100]	?
Cardiac myosin	?	?	?
F-EDA	?	[98]	[104, 105]
Biglycan	(LRR?)	Putative	[35]
Hyaluronan	?	?	[106]
Heparan sulfate	?	[102]	?
Tenascin-C	?	(EIII?)	?
Versican	?	[99]	?
mmLDL	[89]	Yes	?
OxPAPC	?	Yes	?
OxLDL	[89]	Yes	?
AGE-LDL	[89]	Yes	?
Surfactant protein A	[92]	Yes	?
β-Defensin 2	[93, 94]	[93, 94]	?
SFAs (BSA[b])	[107]	Yes	?
Fibrinogen	?	?	[108]
SAA	?	[101]	[101]
Antiphospholipid antibodies	?	?	?
β-Amyloid	?	[109]	[12, 13]
Angiotensin II	?	?	?

[a]Necrotic cells are likely to release PBMs and PSMs, such as HMGB1 and HSPs. [b]Binding information shown for BSA, which was used to solubilize fatty acids in previous studies. EIII = Fibronectin extra domain IIIa motif present.

ligands, nine have already been shown to increase the sensitivity of cells to established PAMPs, such as LPS (Table 2).

Discoveries such as these have led several authors to suggest that certain alarmins may serve as enhancers of pathogen-sensing rather than de facto stimulants of TLRs [9, 10, 53, 97]. Thus, given the evidence that almost all of the proposed endogenous TLR ligands have been shown to bind or enhance responsiveness to particular PAMPs (Table 2), it is tempting to speculate that many of the currently labeled endogenous TLR ligands could in fact be better described as PBMs or PSMs.

POTENTIAL RELEVANCE OF PBMs AND PSMs TO CHRONIC INFLAMMATORY DISEASES

There is ample evidence to suggest that many of the molecules listed in Table 1 are expressed or accumulate at sites of tissue damage or excessive tissue remodeling, such as atherosclerotic or arthritic lesions, and it has been suggested that these molecules may play a direct role in promoting inflammation in these diseases [44, 46, 49, 99, 115–117]. However, in light of the evidence cited above, it is possible that these molecules mediate their proinflammatory effects, not by direct stimulation of TLRs as currently suggested but rather, by enhancing the sensitivity of local cells to PAMPs or other inflammatory mediators.

In this model, many of the proposed TLR ligands listed in Table 1 could be considered to function in a manner similar to that of sCD14 but unlike direct TLR stimulants such as LPS. For example, sCD14 itself is not sufficient to promote inflammatory signaling, but its presence can enhance the sensitivity of monocytes and macrophages to LPS or lipopeptide by several orders of magnitude [111–114]. Moreover, many other cell types, such as endothelial cells and epithelial cells, remain rather insensitive to LPS despite expression of TLR4, as they lack expression of CD14 [110, 111, 118]. Reversing this CD14 deficiency by the addition of sCD14 restores sensitivity of these cells to low concentrations of LPS [110, 111, 118]. Thus, it is possible that the transient appearance of PSMs in response to tissue injury may serve a purpose similar to that of CD14, in that cellular sensitivity to PAMPs may be increased. Such responses may be advantageous to the host, in that damaged tissues, which are likely to be at higher risk of infection, may subsequently become able to respond more quickly or more thoroughly to the presence of PAMPs in the eventuality of microbial infection.

Another possibility that may need to be considered is that the up-regulation of PBMs could lead to an accumulation of PAMPs in damaged tissues. Although it is generally considered that PAMPs are absent from the sterile tissues of the body, work from this laboratory and others [119–121] has shown that LPS derived from the intestinal microflora can reach concentrations of 1–10 pg/ml in the blood of healthy subjects under certain conditions. Asymptomatic transient bacteremia are also commonly observed in some healthy subjects [122, 123], suggesting that other types of PAMP may also be present, if transiently, in the blood. Evidence to support the notion that circulating PAMPs could accumulate at sites of inflammation or tissue damage is available in the frequent observations of bacterial DNA and peptidoglycan in arthritic joints [124, 125] and bacterial DNA, LPS, and peptidoglycan in atherosclerotic lesions [126–129]. This problem could also be exacerbated by the presence of PSMs, as resident cells, which would otherwise remain unresponsive to low concentrations of PAMPs, could become activated in response to local PAMP accumulation. This model therefore predicts that a chronic cycle of PAMP-mediated sensitization of tissue to further PAMP exposure could be generated, whereby the induction of inflammation maintains the level of local PBMs and PSMs, which in turn promotes further inflammatory signaling (as illustrated in **Fig. 3**). In this scenario, the chronic inflammatory cycle would depend largely on the continuing presence of circulating PAMPs, suggesting that lowering of systemic PAMP concentrations below the level required to sustain PSM generation may be of use in dampening potential chronic inflammatory cycles.

POTENTIAL RELEVANCE OF PBMs AND PSMs TO AUTOIMMUNITY AND TUMOR SURVEILLANCE

There may also be a role for PBMs and PSMs in the regulation of other disease processes, particularly those involving

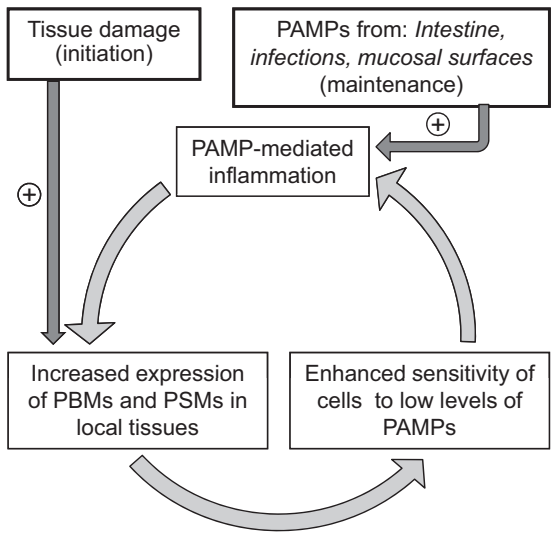

Figure 3. Potential role of PBMs and PSMs in the maintenance of chronic inflammation. In this proposed model, cycles of chronic inflammation may be initiated by tissue damage or inflammation leading to localized up-regulation of PBMs and PSMs, such as biglycan, HMGB1, lumican, HSPs, F-EDA, and tenascin-C. This leads to a local accumulation of PAMPs and lowers the threshold of cellular responsiveness to PAMPs. Both of these processes may contribute to further inflammation, which in turn maintains elevated, local PBM and PSM expression. In this model, the cycle is maintained largely by ongoing exposure to PAMPs rather than by the inherent inflammatory potential of DAMPs or alarmins.

tumor surveillance or the induction of autoimmunity. For example, TLR2 signaling was shown to enhance metastases of certain forms of pulmonary tumor, and it has been suggested that tumor cells of this type could exploit the expression of endogenous TLR2 ligands to promote their growth or metastases [130]. By contrast, it has also been shown that beneficial T cell-dependent immunity to some forms of tumor can depend on the stimulation of TLRs, particularly TLR2 and TLR4, expressed by macrophages or dendritic cells [25, 27]. Specifically, it has been proposed that TLR stimulants may be released from necrotic tumor cells, such as those responding to chemotherapeutic agents or radiotherapy, and that the success of such therapies may depend on the release of endogenous TLR ligands from such cells [25, 27]. Autoimmune processes may also be modulated by the expression of PBMs or PSMs, as it has been suggested that secondary necrosis of pancreatic β cells could represent a key trigger in the development of T cell responses to β cell-derived autoantigens and that this breakdown in tolerance could be promoted by the release of endogenous TLR2 ligands from necrotic cells [26]. Whether the substances released from necrotic cells in these conditions promote stimulation of TLRs directly or indirectly by acting as PBMs and PSMs remains to be determined in future studies.

CONCLUDING REMARKS

Recent evidence suggests that many of the currently reported endogenous ligands of TLR2 and TLR4 may in fact be better described as PBMs or PSMs. It is possible that the expression of these molecules may serve a beneficial purpose initially by enhancing the sensitivity of compromised tissues to potential microbial challenge, although it is proposed that this process may also play a role in the maintenance of chronic inflammatory diseases such as arthritis and atherosclerosis. Further work using rigorous control methodologies will be required to establish which of the currently proposed endogenous TLR ligands possess intrinsic TLR stimulatory capacity and which may be better described as PBMs or PSMs.

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KEY WORDS:
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