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Invited Minireview

Molecular Basis for Innate Immune Recognition of Microbial Components

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SUMMARY: Recognition of bacterial envelope constituents is one mechanism used by mammalian cells to initiate responses leading to bacterial killing, or, unfortunately, responses that also cause fatal septic shock. Many cell surface receptors by which these microbial components are recognized have been identified and characterized over the past a few years. In addition to CD14, which has been shown to be involved in the recognition of many microbial components, Toll-like receptors and MD-2 have been identified as factors playing a role in the receptor complexes of these components. Here we review the recent findings regarding the molecular basis for the recognition of microbial components.

Introduction

Mammalian defense responses to infectious pathogenic agents are comprised of innate and adaptive immune responses. Adaptive immune responses, which are also called acquired immune responses, are generated through clonal expansion of antigen-specific lymphocytes. The great diversity of antigen-specific lymphocytes is generated through somatic gene rearrangement. On the other hand, innate immune responses are not mediated through clonal expansion of antigen-specific lymphocytes, and the molecules involved in innate immune responses are encoded by separate genes. A major question regarding innate immunity is how the innate immune system acquires diverse defenses against various kinds of infectious agents.

The innate immune system is present in various kinds of organisms such as plants, invertebrates, and vertebrates. Therefore, it is believed that the innate immune system is evolutionally conserved. For example, the ability of elimination of foreign substances by phagocytes and the defense response involving antibacterial proteins (peptides) are conserved in mammals and insects. In 1996, *Drosophila* Toll was shown to be involved in immune responses such as the induction of antifungal peptides (1). In 1997 and 1998, human Toll-like receptors (TLRs), which are structurally related to *Drosophila* Toll, were identified (2,3), and subsequent research has shown that TLRs play a central role in mammalian innate immune recognition. In this paper, we summarize the recent findings concerning the mechanisms of ligand recognition by TLRs, especially TLR4.

Innate immunity and macrophage activation

The microorganisms that are encountered in the daily life of a normal healthy individual only occasionally cause a perceptible disease. Microbial invasions are initially countered by innate defense mechanisms that exist in all individuals and act within minutes of infection. Only when the innate host defenses are by-passed or evaded by microorganisms, is an adaptive immune response induced. Innate immunity not only constitutes an early defense response, but also facilitates the induction of an adaptive immune response through secretion of cytokines by macrophages.

The innate responses of macrophages are very important for host defense. In addition to engulfment of opsonized particles coated with antibodies and/or complement, macrophages can recognize and ingest many pathogens directly. On their surface, macrophages have several different receptors that bind microbial components common to many pathogens, and thereby induce phagocytosis and the release of cytokines. The induced cytokines recruit new phagocytic cells and effector molecules to the sites of infection. Lipopolysaccharide (LPS) is one of the most potent inducers of cytokines among microbial components.

LPS is a constituent of the outer membrane of Gram-negative bacteria, and contains a polysaccharide and a lipid A portion (Fig. 1A), the latter of which mediates many LPS responses (4). The importance of LPS-induced immune responses in host defense was demonstrated by the analysis of LPS-hyporesponsive mice, C3H/HeJ. C3H/HeJ is a mutant mouse strain that is hyporesponsive to LPS, and macrophages derived from C3H/HeJ mice did not produce inflammatory cytokines on LPS-stimulation. It is noteworthy that C3H/HeJ mice were more susceptible to Gram-negative bacteria, such as *Salmonella typhimurium* (5) and *Escherichia coli* (6), than was its cognate wild type pair, C3H/HeN, and the susceptibility of C3H/HeJ mice to *S. typhimurium* is closely linked to the Lps^d allele, which governs the defective response to LPS in C3H/HeJ mice (5). Recent research has demonstrated that the Lps^d allele encodes TLR4 (see below).

Molecules involved in LPS recognition by macrophages

CD14: CD14 is expressed on the surface of macrophages. Evidence supporting a central role of CD14 in LPS signaling includes the following: (i) LPS binds stoichiometrically to CD14 (7); (ii) transfection of CD14 confers LPS-responsiveness on

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the 70Z/3 pre-B cell line (8) and CHO-K1 cell line (9); and (iii) CD14-deficient knockout mice show dramatically reduced sensitivity to LPS (10). Furthermore, it has been demonstrated that CD14 is involved in the signaling induced by other microbial components, such as lipoarabinomannan, peptidoglycan, and lipoteichoic acid (Fig. 1) (11). Intracellular signaling induced by LPS in macrophages cannot be mediated directly by CD14, because CD14 is a GPI-anchored protein and does not possess an intracellular signaling region. Therefore a second receptor that mediates LPS and other microbial component signaling has been sought for a long time (12).

TLRs: Toll was first identified genetically as an essential molecule for embryonic patterning in *Drosophila* (13). Because the Toll signaling cascade showed a striking similarity with the mammalian IL-1-induced activation cascade of NF-κB, the involvement of the Toll-signaling cascade in *Drosophila* immunity was hypothesized. Also, the signaling cascade was subsequently shown to be essential for the induction of antifungal peptides in *Drosophila* (1). Toll is a transmembrane protein that has leucine-rich repeats in its extracellular portion and a cytoplasmic portion homologous to the intracellular signaling domain of the type I IL-1 receptor (designated as the TIR domain). A mammalian family, called the TLR family, which is structurally similar to *Drosophila* Toll has been reported. To date, ten members (TLR1-10) have been reported (2,3,14).

TLR4: Human TLR4 was the first reported TLR (2). Expression of constitutively active TLR4 in a human cell line induces NF-κB activation, and the expression of NF-κB-controlled genes for inflammatory cytokines as well as the expression of the co-stimulatory molecule B7.1, which is required for the activation of naive T cells (2). Subsequently, LPS-

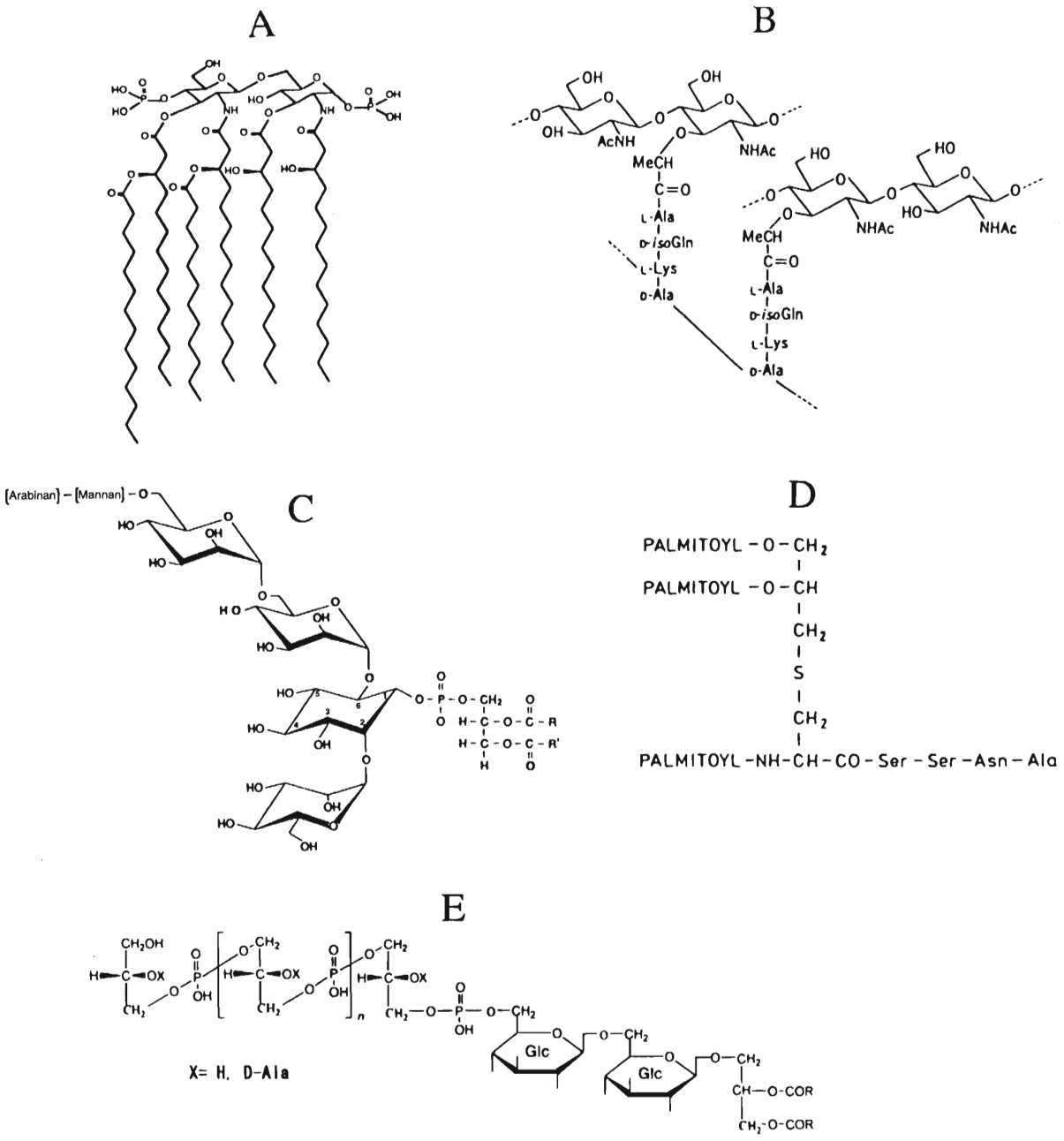

Fig. 1. Structures of microbial components that induce macrophage activation.
A) Lipid A, B) peptidoglycan, C) lipoarabinomannan, D) lipopeptide, E) lipoteicoic acid.

Table 1. Toll-like receptors (TLRs) and ligands

TLR	Cofactor	Ligand	Origin of ligand	References
TLR2	CD14	lipopeptide/lipoprotein	bacteria	19, 20
	TLR6, CD14	peptidoglycan	bacteria	18, 33, 35
	CD14	lipoarabinomannan	mycobacteria	17
	TLR6	MALP-2	mycoplasma	36, 37
		glycosylphosphatidylinositol	protozoan	38
TLR3		double-stranded RNA	virus	39
TLR4	CD14, MD-2	LPS	Gram-negative bacteria	15, 16, 22, 24
	CD14	lipoteichoic acid	Gram-positive bacteria	18
	MD-2	Taxol	*Taxus brevifolia* (plant)	28
	CD14	heat-shock protein 60	human	29
	glycosphingolipid	P fimbriae	*E. coli*	40
	CD14	teichuronic acids	*Micrococcus luteus*	41
TLR5		flagellin	bacteria	42
TLR6	TLR2, CD14	peptidoglycan	bacteria	18, 33, 35
	TLR2	MALP-2	mycoplasma	36, 37
TLR9		CpG DNA	bacteria	43

hyporesponsive mutant mice, C3H/HeJ, have been reported to possess a point mutation in TLR4 (15) that causes an inability to activate NF-κB (16). Unequivocally, TLR4 was demonstrated to be involved in LPS signaling, because macrophages derived from TLR4-deficient knockout mice did not respond to LPS (16).

Interestingly, it has been demonstrated that the signals induced by other CD14 ligands, such as lipoarabinomannan (17) and peptidoglycan (18), were mediated by TLR2 but not by TLR4. In addition to these CD14 ligands, recent research has shown that many other microbial components were also recognized by TLRs; these findings are summarized in Table 1.

TLR4 is involved in the recognition of LPS, a component of Gram-negative bacteria, whereas TLR2 is involved in the recognition of Gram-positive bacterial components such as peptidoglycan (18) and lipoprotein (19,20). C3H/HeJ mice, which are hyporesponsive to LPS, are known to be highly sensitive to infection by Gram-negative bacteria, as described above. TLR2-deficient mice are highly susceptible to Gram-positive *Staphylococcus aureus* infection (21). Reduction of cytokine production in response to bacterial infection is a cause of the susceptibility, because pretreatment with TNF-α and IL-1α protects C3H/HeJ mice from lethal *E. coli* infection (6).

MD-2: Expression of TLR4 in the Ba/F3 pro-B cell line (22) and human embryonic kidney 293 cell lines (23) did not confer LPS responsiveness on these cells. In addition to that of TLR4, expression of MD-2, a molecule that physically associates with TLR4, in Ba/F3 confers LPS responsiveness (24). The expression of CD14 enhances LPS-induced NF-κB activation via the TLR4•MD-2 complex (24). A point mutation of MD-2 abolishes the LPS-induced signaling in the CD14-transfected CHO cell line (25). MD-2 associates with the extracellular portion of TLR4 and the existence of the TLR4•MD-2 complex on macrophages has been demonstrated (26). These findings demonstrate that MD-2, as well as TLR4, is involved in LPS signal transduction. A model of the LPS receptor complex is shown in Fig. 2. However, the function of MD-2 in ligand recognition by TLR4 has not been characterized well. It is possible that MD-2 is required for cell surface expression of TLR4, because MD-1, which is structurally similar to MD-2, is essential for cell surface expression of RP105, which is structurally similar to TLR4 (27).

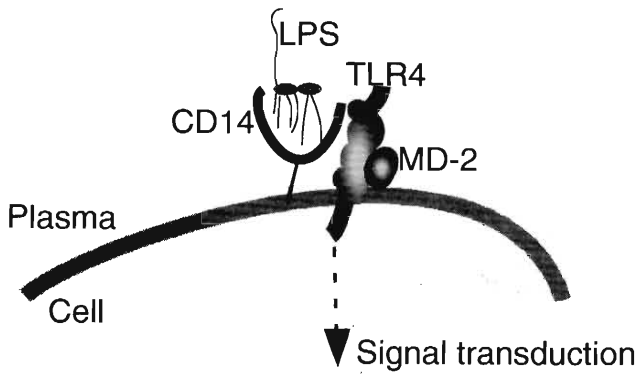

Fig. 2. Molecules involved in LPS-recognition by macrophages.

Molecular events involved in the recognition of foreign substances by TLRs

As shown in Table 1, TLRs mediate signals induced by various kinds of microbial components. It has been shown that TLR4 is involved in the signal transduction induced by LPS, Taxol (28), lipoteicoic acid (18), and heat shock protein 60 (29). However, the molecular mechanism by which TLR4 recognizes these stimulants has not been characterized well. In our experiments, ^{125}I-labelled LPS bound well to CD14, but not to the TLR4•MD-2 complex. LPS agonist Taxol, which induces the cellular signal mediated by the TLR4•MD-2 complex, also did not significantly bind to the complex (28). These results show that the TLR4•MD-2 complex is not a receptor that shows high affinity to its ligand. Interestingly, Taxol has an LPS-mimetic action on mouse macrophages, but not on human macrophages. We have shown that MD-2 is involved in this species-specific LPS-mimetic action, suggesting that MD-2 is involved in ligand discrimination (28,30). Furthermore, the involvement of TLR4 in the discrimination of lipid A has been demonstrated (31,32). These findings lead us to the speculation that the TLR4•MD-2 complex directly recognizes its ligand, but that the association is weak and undetectable.

To date, ten TLRs have been reported, but it is not clear how a restricted family of such receptors has the capacity to recognize the wide spectrum of TLR stimuli known to exist. Interestingly, the recognition of some TLR stimuli, such as peptidoglycan and zymosan, is explained by the cooperation of TLR2 and TLR6. In contrast, for the recognition of another TLR2 ligand, lipopeptide, the cooperation of TLR2 and TLR6 was not required (33). The repertoire for pattern recognition of pathogens by the innate immune system may be increased by cooperation between TLRs.

Finally, the discovery of TLRs is a breakthrough for the understanding of innate immunity. It is hoped that these findings will enable the development of new immuno-stimulatory adjuvant and anti-inflammatory drugs. In addition to TLRs, Nod1 and 2 have been reported to be intracellular LPS receptors and to confer LPS responsiveness on a transfected cell line (34). Nod1 and 2 are cytosolic proteins which possess leucine-rich repeats, and more than 20 proteins exhibiting structural similarity to Nod1 have been registered in public databases (34). The study of this protein family may provide new insights into innate immunity.

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