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Cetacean Toll-like receptor 4 and myeloid differentiation factor 2, and possible cetacean-specific responses against Gram-negative bacteria

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ABSTRACT

Toll-like receptor 4 (TLR4) and myeloid differentiation factor 2 (MD-2) are essential for recognizing the lipopolysaccharides (LPS) of Gram-negative bacteria. We determined the sequences of cDNAs encoding TLR4 and MD-2 from cetaceans and generated three-dimensional (3D) models for a better understanding of their modes of interaction and LPS recognition. The 3D reconstructions showed that cetacean TLR4 and MD-2 formed a horseshoe-like structure comprised of parallel β-strands and a β-cup structure consisting of two anti-parallel β-sheets, respectively. The $(TLR4\text{-}MD\text{-}2)_2$ duplex-heterodimer was shown to form a symmetrical structure. Comparison with the interfaces of the complexes in other mammals revealed that cetacean TLR4s have some amino acid residue substitutions involved in duplex-heterodimer formation and in species variation for LPS recognition. These substitutions in the changed amino acid residues may alter the interaction among TLR4, MD-2, and LPS and modify the TLR4/MD-2 immunological responses.

1. Introduction

Recently, the incidence of infectious disease in marine mammals has been increasing. For example, infection by the Gram-negative bacteria *Brucella* spp. is now broadly distributed in a variety of marine mammal species [1,2]. A better understanding of the defense mechanisms against bacteria in marine mammals is becoming more important in efforts to conserve them. However, little is known about their immune systems.

Innate immunity is a first-line defense against invading microorganisms and important in both vertebrates and invertebrates [3,4]. It promotes immediate inflammatory responses and also plays a major role in inducing the adaptive immune responses [5]. Toll-like receptors (TLRs) are a family of type I transmembrane glycoproteins that play a critical role in innate immunity by recognizing broadly conserved pathogen-associated molecular patterns that are absent from the host molecules [6,7]. To date, 13 mammalian TLR family members have been identified based on their functions and sequences, which include leucine-rich repeats (LRRs) in the extracellular region and toll/interleukin I-receptor (TIR) domains in their intracellular region [8,9]. TLR4 forms a complex with myeloid

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differentiation factor 2 (MD-2) which directly binds to lipopolysaccharide (LPS), a major cell wall constituent of Gram-negative bacteria.

LPS is known to be a strong elicitor of innate immunity and is comprised of an amphipathic lipid A component, a hydrophilic polysaccharide core, and O-antigen. Among these, lipid A, which is composed of two phosphorylated glucosamines to which six lipid chains are attached, is the primary inducer of immunological responses to LPS. TLR4 and MD-2 form a heterodimer, TLR4-MD-2 within the endoplasmic reticulum (ER), move to the cell surface [10–12].

X-ray crystallographic analyses of human and mouse TLR4-MD-2 heterodimer complexes have shown that TLR4 and MD-2 form a horseshoe-like and β-cup structure, respectively, and that MD-2 binds to the N-terminal and central parts of TLR4 in the ER to form the heterodimer with LPS binding affinity [13,14]. Seven amino acid residues of TLR4 and nine of those of MD-2 are involved in their primary binding to form the heterodimer [13,14]. On the cell surface, lipid A binds directly to MD-2 within the complex, with five of its six lipid chains buried deep inside the β-cup pocket of MD-2. The remaining chain is exposed to the surface of MD-2. Two phosphate groups of lipid A are located at the entrance of the MD-2 pocket. The exposed lipid chain and negatively charged two phosphate groups of lipid A in the complex TLR4-MD-2-LPS trigger a formation of duplex-heterodimer $(TLR4\text{-}MD\text{-}2\text{-}LPS)_2$, which were completed by the complicated interactions with hydrophobic and hydrophilic bindings among TLR4, MD-2, and LPS [15]. The formation of duplex-heterodimer $(TLR4\text{-}MD\text{-}2\text{-}LPS)_2$, is essential to activate a signaling pathway mediating the defense against Gram-negative bacteria, which entails the production of cytokines such as interleukin-6, tumor necrosis factor-α, and/or interferons [6,7]. The difference in the response against LPS within animal species has also been a focus of research. For example, although lipid A behaves as an agonist for TLR4 immunological responses in nearly all mammal species, lipid IVa, a precursor of lipid A, acts as an antagonist in humans but as an agonist in the mouse and horse [16–19]. This suggests that specific variation in immune responses against pathogens has significant influence on the mammalian innate immunity.

In the present study, we determined the sequences of cDNA encoding cetacean TLR4 and MD-2 and constructed three-dimensional (3D) models to determine the possible cetacean-specific immune responses against Gram-negative bacteria.

2. Materials and methods

2.1. Leukocyte and tissue samples

Peripheral blood leukocytes were collected from captive cetaceans, a Pacific white-sided dolphin (*Lagenorhynchus obliquidens*) and a killer whale (*Orcinus orca*), which were kept at the Yokohama Hakkeijima Sea Paradise aquarium and the Kamogawa Sea World aquarium, respectively. The leukocytes were isolated from blood samples by centrifugation (2800 rpm, 25 min) using a Histopaque1083 (Sigma) and stimulated with 5 μg/ml LPS (*Salmonella Minnesota* R595, Alexis Biochemicals) for 24 h. They were then used for RNA extraction. For DNA extraction to examine the variation in the sequences of TLR4 among cetaceans, testis tissue samples from common minke whales (*Balaenoptera acutorostrata*) and sperm whales (*Physeter macrocephalus*) collected under the Japan Whaling Research Program II in 2001 were used.

2.2. Determination of sequences of cDNAs encoding cetacean TLR4 and MD-2

Total RNA was extracted from the stimulated leukocytes using an RNeasy Mini Kit (Qiagen). To determine the complete sequences of cDNAs encoding TLR4 and MD-2, the cDNAs were amplified using a OneStep RT-PCR Kit (Qiagen) with the appropriate primers designed in this study (Table 1). The RT-PCR protocol entailed reverse transcription to cDNA at 50 °C for 30 min and denaturation of the DNA at 95 °C for 15 min, followed by 35 cycles of amplifi-

Table 1
Primers used in this study to detect TLR4 and MD-2.

Primer name	Direction[a]	Sequence	Position[b]
TLR4			
TLR4 SiF3	F	5′-CAGAAAATGCCAGGATGATG-3′	−14–6
TLR4 F3	F	5′-CTGAGCTTTAACTACCTGAG-3′	191–211
TLR4 F4	F	5′-TCCAGCTTCCCAGAACTGCA-3′	239–259
TLR4 F1	F	5′-TATTCAAGGTCTGGCTGGTT-3′	753–773
TLR4 F2	F	5′-TCAAGGACCAGAGGCAGCTC-3′	1797–1817
TLR4 R3	R	5′-TATTGAACCAGGCACCTTTA-3′	753–773
TLR4 R1	R	5′-ATTTAGATCTGAGCTTCAAT-3′	1224–1244
TLR4 R2	R	5′-CAGACCTGGCAGTTTCTTAG-3′	2282–2302
TLR4 SiR3	R	5′-GTGCTTCTTTCCCAGATGGA-3′	2561–2581
MD-2			
MD-2 F3	F	5′-GAGTCTGATGATTAGTTAC-3′	−21–40
MD-2 F9	F	5′-CCCAGTGCGCAAACAAGTTA-3′	261–281
MD-2 R5	F	5′-GGATCTGTAACTCCTCTG-3′	68–86
MD-2 R6	F	5′-CTTCAGAGCTCGCTCTGAAGG-3′	308–329
MD-2 R14	R	5′-ACTGAAGAAAGGCTCCTTTGC-3′	424–445

[a] F: forward, R: reverse.
[b] The nucleotide numbers in TLR4 and MD-2 are based on sequence data for the Pacific white-sided dolphin determined in this study. A minus sign indicates the region upstream of the first ATG.