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MD-2-dependent human Toll-like receptor 4 monoclonal antibodies detect extracellular association of Toll-like receptor 4 with extrinsic soluble MD-2 on the cell surface

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ABSTRACT

MD-2 is essential for lipopolysaccharide (LPS) recognition of Toll-like receptor 4 (TLR4) but not for cell surface expression. The TLR4/MD-2 complex is formed intracellularly through co-expression. Extracellular complex formation remains a matter for debate because of the aggregative nature of secreted MD-2 in the absence of TLR4 co-expression. We demonstrated extracellular complex formation using three independent monoclonal antibodies (mAbs), all of which are specific for complexed TLR4 but unreactive with free TLR4 and MD-2. These mAbs bound to TLR4-expressing Ba/F3 cells only when co-cultured with MD-2-secreting Chinese hamster ovary cells or incubated with conditioned medium from these cells. All three mAbs bound the extracellularly formed complex indistinguishably from the intracellularly formed complex in titration studies. In addition, we demonstrated that two mAbs lost their affinity for TLR4/MD-2 on LPS stimulation, suggesting that these mAbs bound to conformation-sensitive epitopes. This was also found when the extracellularly formed complex was stimulated with LPS. Additionally, we showed that cell surface TLR4 and extrinsically secreted MD-2 are capable of forming the functional complex extracellularly, indicating an additional or alternative pathway for the complex formation.

1. Introduction

The Toll-like receptor (TLR) family, which recognizes pathogen-associated molecular patterns, is essential for innate immunity against the invasion of pathogenic microorganisms [1]. TLR4 must be associated with MD-2 to sense lipopolysaccharide (LPS), a major cell-wall component of gram-negative bacteria [2]. Genetic deficiency in *TLR4* or *MD-2* causes complete loss of LPS responsiveness, resulting in increased vulnerability to bacterial infection [2,3]. MD-2 is a secretory glycoprotein; however, it remains on the cell surface in association with TLR4 when they are co-expressed in the endoplasmic reticulum [4]. MD-2 binds LPS directly and determines LPS pathogenesis in concert with the extracellular domain of TLR4 [5,6]. LPS binding evokes dynamic conformational change in the cell-surface TLR4/MD-2 complex, subsequently triggering signal transduction initiation [6]. In addition to MD-2, LPS-binding protein (LBP), glycosylphosphatidylinositol-anchored CD14 and its soluble form (sCD14) participate in LPS recognition, enhancing LPS sensitivity [7,8].

The TLR4/MD-2 complex is assembled within cells that express both molecules [4]. It was indicated that the complex can also form extracellularly when MD-2 is supplied as a secreted molecule [9–13]. However, soluble MD-2 (sMD-2) readily aggregates in solution when expressed and secreted in the absence of TLR4 by heterogeneous polymerization through intra- and/or inter-molecular disulfide bond formation [9,10,12,14]. However, a small fraction of the sMD-2 may remain monomeric, enabling constitutive complex formation on the cell surface, which responds to LPS stimulation [10,11,15]. Several studies suggested that human serum sMD-2 may act together with TLR4 as a part of the LPS receptor [11–13,16]. Serum sMD-2 was shown to confer LPS responsiveness indirectly by serological activity [11,13,16] and directly by immunological techniques using anti-MD-2 monoclonal antibodies

Abbreviations: Bio-, biotinylated; CM, conditioned medium; FCS, fetal calf serum; LPS, lipopolysaccharide; mAb, monoclonal antibody; MD-2F, C-terminally FLAG-tagged MD-2; LBP, LPS-binding protein; LBP-N, truncated N-terminal form of LBP; PE, phycoerythrin; sCD14, soluble form of CD14; sMD-2, soluble MD-2; stv, streptavidin; TLR, Toll-like receptor.

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(mAbs) [12,16]. However, it remains unclear whether the complex formed by the association of TLR4 with sMD-2 on the cell surface is essentially the same as that assembled in the endoplasmic reticulum. An alternative association is not excluded because of the lack of efficient probes to detect this specific complex.

In general, a mAb has the advantage of targeting a specific structure at the molecular or cellular level because of its high specificity. It is also useful in monitoring a molecular structure related to the activation state of a molecule. For example, some integrin mAbs discriminate between conformations depending on the activation state [17]. It was reported that the active state of murine TLR4 could be distinguished from its inactive state using a mAb [7,18].

In the present study, we used three mAbs specific for distinct combinatorial epitopes shared by TLR4 and MD-2 to determine whether the cell surface TLR4 and sMD-2 form a functional complex which is equivalent to the intracellularly formed complex. The mAbs recognized TLR4 only in the presence of sMD-2, suggesting that TLR4 could be assembled with sMD-2 on the cell surface. In addition, the extracellularly formed complex changed its conformation in response to LPS stimulation, which was detected by two of the three mAbs, as was also observed for the intracellularly formed complex. Our mAbs are novel probes for the detection of extracellular complex formation and monitoring of activation status of the complex. Our findings suggest the existence of an additional or alternative pathway for TLR4/MD-2 complex formation.

2. Materials and methods

2.1. Cells

Ba/F3, CHO-DG44 and derived transfected cells were maintained as described previously [7]. Ba/F3-transfected cells expressing human TLR4 [19], TLR4/C-terminally FLAG-tagged MD-2 (MD-2F) [20] and TLR4/MD-2F/CD14 [19] were produced previously. Human/mouse TLR4/MD-2F-expressing Ba/F3-transfected cells were a gift from Dr. Miyake (Tokyo University, Tokyo, Japan) [5]. TLR4/CD14-expressing Ba/F3-transfected cells were produced by the electroporation of human CD14/pEFBOS [21] using human TLR4/pCAGGS1 [20].

2.2. Reagents and antibodies

LPS (*Escherichia coli* O:111) was purchased from Wako Pure Chemical Industries (Osaka, Japan). Mouse anti-human TLR4 mAb (HT3) [19], anti-human TLR4/MD-2 mAbs (HT17, HT26, HT59) [19] and rat anti-human CD14 mAb (1B12) [21] were generated previously. Biotinylated (Bio-)mAbs were prepared as described previously [7]. Other antibodies were purchased from the following companies: Bio-anti-FLAG-M2 mAb from Sigma–Aldrich Co. (St. Louis, MO); unlabeled and agarose-immobilized anti-DYKDDDDK mAb (1E6) from Wako Pure Chemical Industries; phycoerythrin (PE)-conjugated goat anti-mouse IgG polyclonal antibody from Southern Biotechnology Associates (Birmingham, AL); PE-conjugated streptavidin (stv) from BioLegend (San Diego, CA); horseradish peroxidase-conjugated stv from Pierce (Rockford, IL). Recombinant human sCD14, LBP and the truncated N-terminal form of LBP (LBP-N) were generated previously [7].

2.3. Preparation of sMD-2-containing conditioned medium (CM)

CHO-DG44 cells were transfected with a pCAGGS1 vector containing human MD-2F [20] using a Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA). Stable clones with high sMD-2-secreting ability were established as described previously [7]. CM was collected by centrifugation after established stable clones were cultured for more than 3 days.

2.4. Cell staining and flow cytometry

Cells were stained and subjected to flow cytometry using FACScan or FACScalibur (Becton Dickinson, Franklin Lakes, NJ) as described previously [7].

2.5. Analysis of extracellular formation of the TLR4/MD-2 complex

Parent or sMD-2F-secreting CHO-DG44-transfected cells (5×10^5) were cultivated for 1 day in 24-well plates, washed twice with phosphate-buffered saline and co-cultured with Ba/F3-transfected cells (2×10^6) for 3–4 h at 37 °C in a humidified CO_2 incubator. Alternatively, Ba/F3-tranfected cells (2×10^6) were incubated with sMD-2-containing or control CM for 3–4 h in 1 mL of culture medium in 24-well plates. Following co-cultivation or incubation, floating Ba/F3 cells were stained and subjected to flow cytometry. For titration experiments, CHO-transfected cells were co-cultured with Ba/F3-transfected cells (1×10^7) for 24 h in 10 mL of growth medium on a 10-cm dish.

2.6. Immunoprecipitation and western blotting

sMD-2F was immunoprecipitated from 5 mL of CM by a 2-h incubation with agarose-immobilized anti-DYKDDDDK mAb (50 μL 50% slurry) or indicated mAbs (20 μg) conjugated with protein G Sepharose 4 fast flow (50 μL 50% slurry, Amersham Biosciences, Piscataway, NJ) at 4 °C with gentle rotation. After washing three times with 20 mM Tris pH 8.0, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF and 0.1% Triton X-100, bound proteins were eluted by boiling in Laemmli buffer, resolved in a 12% SDS–PAGE gel, transferred to an Immobilon-P membrane (Millipore Co., Bedford, MA) and probed with Bio-FLAG M2 mAb and horseradish peroxidase-conjugated stv. Immunoreactive proteins were visualized on X-ray film using enhanced chemiluminescence detection reagents (GE healthcare, Buckinghamshire, UK).

3. Results

3.1. Characterization of anti-human TLR4/MD-2 mAbs

We recently generated a series of anti-human TLR4 mAbs by immunizing *TLR4*$^{-/-}$ mice [19]. Three mAbs in particular, HT17, HT26 and HT59, showed affinity for only human TLR4/MD-2, but not for TLR4, on the cell surface [19]. This suggests that their epitopes are composed of combinatorial structures generated by complex formation of TLR4 with MD-2. Further characterization revealed that these three mAbs did not bind mouse TLR4/MD-2 [19] or a chimeric complex consisting of human TLR4 and mouse MD-2F (Fig. 1A). In contrast, an FLAG mAb reacted with these complexes, which confirmed surface expression of TLR4/MD-2. The reactivity of HT17, HT26 and HT59 was found to depend on the association of TLR4 with MD-2; otherwise their epitopes would associate with human MD-2 irrespective of complex formation. To exclude this possibility, we tested the affinity of the mAbs for sMD-2F using immunoprecipitation. None of the three mAbs could precipitate sMD-2, in contrast to control FLAG mAb (Fig. 1B). Therefore, we concluded that these three mAbs recognize combinatorial epitopes produced uniquely by intracellular complex formation of TLR4 with MD-2.

3.2. HT17 and HT26, not HT59, loses reactivity to TLR4/MD-2 on LPS stimulation in a CD14- and LBP-dependent manner

Ligand binding and resultant conformational change of a receptor potentially influences mAb reactivity [7,17,18]. To characterize