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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.

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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

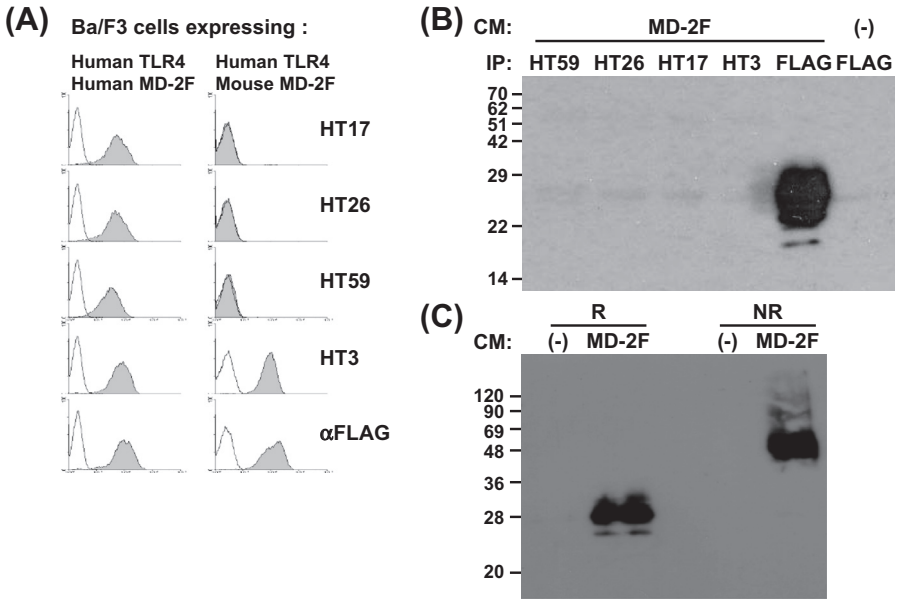

Fig. 1. Characterization of anti-TLR4/MD-2 mAbs. (A) Ba/F3 cells expressing human TLR4/MD-2F or human/mouse TLR4/MD-2F were stained using the indicated Bio-mAbs or FLAG M2 mAb followed by PE-conjugated secondary reagents. MAb reactivity was analyzed using flow cytometry. The open histogram represents staining with secondary reagents alone. (B) CM from CHO cells secreting sMD-2F or parental cells was immunoprecipitated using the indicated mAbs and subjected to western blotting using Bio-FLAG mAb. HT3 and FLAG M2 mAbs were used as negative and positive controls, respectively. (C) Immunoprecipitants of control and sMD-2F CM using FLAG-M2 agarose were resolved by 12% SDS–PAGE under reducing (R) non-reducing (NR) conditions and subjected to western blotting using Bio-FLAG mAb as in (B). Dimer or polymer complexes were detectable under non-reducing conditions.

our mAbs in more detail, we stimulated TLR4/MD-2/CD14-expressing Ba/F3-transfectant cells with LPS, and mAb reactivity was analyzed using flow cytometry. Staining intensity for HT17 and HT26 was dose-dependently decreased by short-term LPS stimulation in CD14-expressing cells (Fig. 2A and B). This was inhibited by pre-incubation with anti-CD14 mAb (Fig. 2B). In contrast, the other complex-dependent mAb, HT59, and the complex-independent TLR4 mAb, HT3, remained reactive to surface TLR4 even after LPS stimulation. This excluded TLR4/MD-2 loss from the cell surface by internalization or shedding. We performed similar experiments with CD14-negative transfectant cells to define membrane-anchored CD14 as a molecular requirement (Fig. 2C). Decreased staining intensity was not observed with either HT17 or HT26 mAbs, which was consistent with the finding of the anti-CD14 mAb blocking study. Based on a previous study [7], we examined the contribution of LBP as a soluble determinant. We stimulated TLR4/MD-2/CD14-expressing cells in serum-free medium, which was replenished with sCD14, LBP, LBP-N or fetal calf serum (FCS) (Fig. 2D). Staining intensity was clearly decreased when these cells were stimulated in the presence of LBP or FCS. In contrast, sCD14 and LBP-N lacking LPS-transfer activity to CD14 were ineffective. Therefore, the HT17 and HT26 epitopes may be disrupted by LPS stimulation in a CD14- and LBP-dependent manner. The three mAbs were classified in at least two groups: HT17/HT26 vs. HT59, based on their distinct reactivity to LPS-stimulated TLR4/MD-2. HT17 and HT26 recognized LPS-induced conformational change of TLR4/MD-2.

3.3. All three TLR4/MD-2 mAbs recognize extracellularly formed TLR4/MD-2 with comparable affinity to intracellularly formed TLR4/MD-2

It was thought that TLR4-expressing cells could extracellularly form a complex with sMD-2 even when not expressing endogenous MD-2 [10–13,16]. However, an alternative extracellular association to intracellular association could not be excluded because of aggregation of sMD-2 in solution [9,10,12,14]. To address this issue, the reactivity of complex-specific mAbs to TLR4-expressing Ba/F3 cells after co-cultivation with sMD-2F-secreting CHO cells was investigated. Clear extracellular TLR4/MD-2 complex formation was observed using all three mAbs (Fig. 3A). In contrast, this specific complex was undetectable on co-cultivation with parent CHO cells or parent Ba/F3 cells. Furthermore, we compared mAb affinity for extracellularly formed TLR4/MD-2 with that for the intracellularly formed complex to determine whether the association modes were equivalent between extracellular and intracellular complex formation. The affinities of HT17, HT26 and HT59 for the extracellularly formed complex were comparable to those for the intracellularly formed complex as observed in a titration study (Fig. 3B).

3.4. Cell–cell interaction is not required for extracellular complex formation of TLR4 with sMD-2

We investigated whether cell–cell interaction is required for extracellular complex formation by incubating TLR4-expressing Ba/F3 cells with sMD-2F-containing CM, and stained them with complex-specific mAbs. As shown in Fig. 3C, the TLR4/MD-2 complex was formed on incubation with sMD-2 CM but not with control CM. This result indicates that cell–cell interaction between TLR4-expressing cells and sMD-2-secreting cells is not essential for extracellular TLR4/MD-2 formation.

3.5. Extracellularly formed complex changes its conformation in response to LPS stimulation similarly to intracellularly formed complex

Using the mAbs HT17 and HT26, which are sensitive to LPS-induced conformation change, we investigated whether the extracellularly formed TLR4/MD-2 complex is equivalent to the intracellularly formed complex in terms of function. TLR4/CD14-expressing Ba/F3 cells were co-cultured with sMD-2F-secreting CHO cells and the resultant extracellularly formed TLR4/MD-2 was stimulated with varying amounts of LPS (Fig. 4). The staining

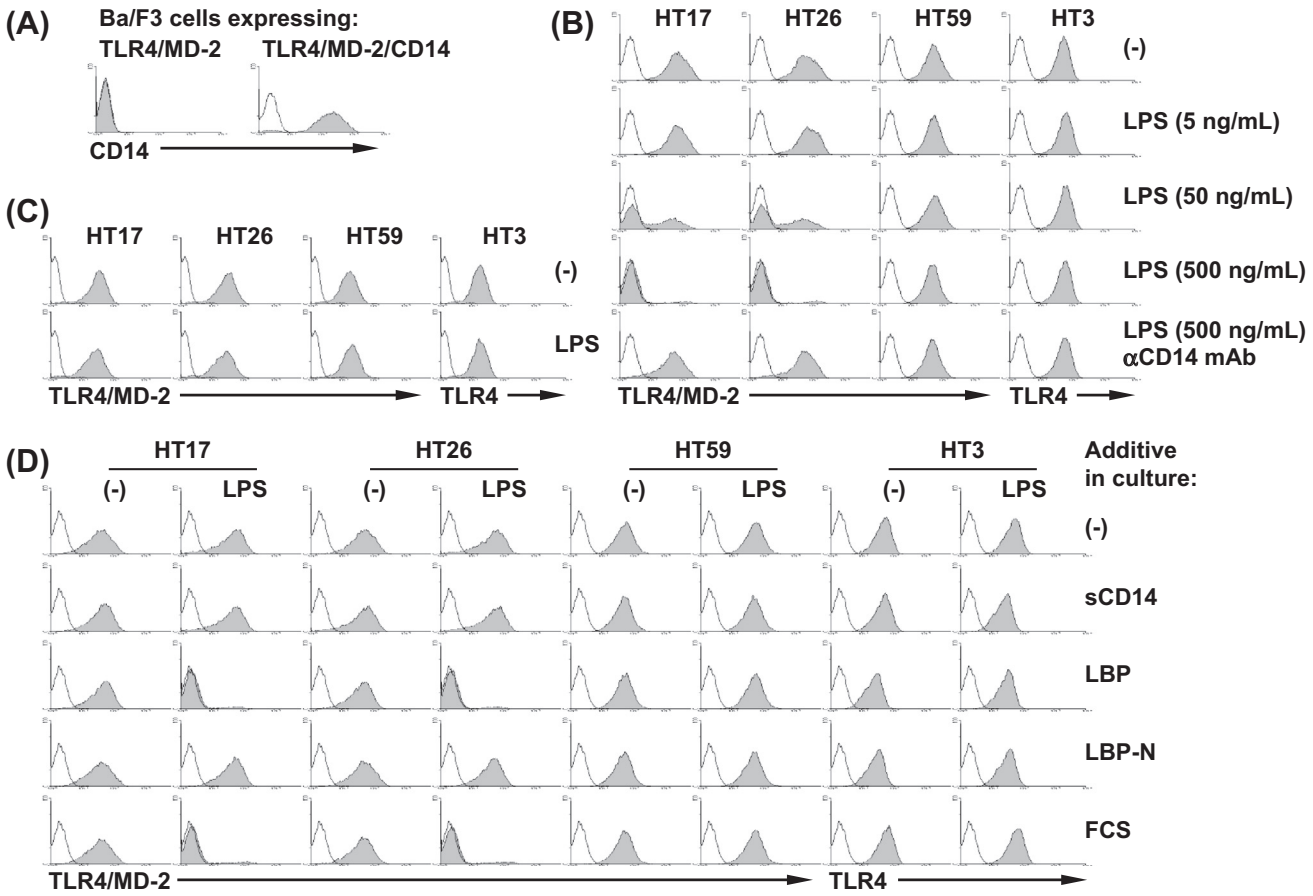

Fig. 2. HT17 and HT26 lose reactivity for TLR4/MD-2 on LPS stimulation in a membrane-bound CD14- and soluble LBP-dependent manner. (A) CD14-positive (right) and -negative (left) TLR4/MD-2F-expressing Ba/F3-transfected cells were stained with Bio-1B12 mAbs for CD14 and PE-conjugated stv, and analyzed using flow cytometry. The open histogram represents staining with PE-conjugated stv alone. (B, C), CD14-positive (B) and -negative (C) TLR4/MD-2F-expressing Ba/F3 cells were stimulated with LPS (C, 1 μg/mL) for 15 min in the presence or absence of anti-CD14 mAb (1B12, 10 μg/mL) prior to staining. Cells were stained and subjected to flow cytometry as in Fig. 1A. (D) TLR4/MD-2F/CD14-expressing Ba/F3-transfected cells were stimulated with 1 μg/mL LPS (right) for each mAb staining or only cultivated (left) for 15 min in serum-free medium containing either sCD14 (1 μg/mL), LBP (4 μg/mL), LBP-N (4 μg/mL) or FCS (5%) as indicated on the right side. After washing twice with phosphate-buffered saline, the cells were stained and analyzed using flow cytometry as in Fig. 1A.

intensities of HT17 and HT26 were dose-dependently decreased by LPS stimulation, as observed for the intracellularly formed complex (Fig. 2B). HT59 and HT3 intensities were unchanged. This result suggests that the extracellularly and intracellularly formed complexes are functionally equivalent.

4. Discussion

We characterized extracellular TLR4/MD-2 complex formation using three mAbs with distinct epitopes. These mAbs reacted with only the human TLR4/MD-2 complex, and not with free sMD-2 or TLR4, indicating combinatorial epitopes formed through association of TLR4 with MD-2. The mAbs showed that cell-surface TLR4 can form an extracellular complex with sMD-2 similar to that assembled intracellularly. Direct intercellular interaction was not required for extracellular complex formation between TLR4-expressing cells and sMD-2-secreting cells. The extracellular and intracellular associations were demonstrated to be equivalent in a mAb titration study. Active conformational change of the extracellularly formed complex was confirmed using conformation-sensitive TLR4/MD-2 mAbs.

We characterized three anti-human TLR4/MD-2 mAbs that specifically recognize combinatorial epitopes produced by intracellular association of TLR4 with MD-2. These mAbs are classified into two groups: HT17 and HT26 vs. HT59, based on the reactivity to LPS-stimulated TLR4/MD-2. HT17 and HT26 lost such reactivity after stimulation with LPS whereas HT59 remained reactive. This finding is compelling because we recently demonstrated that HT17 and HT26 cross-blocked each other whereas HT59 was not cross-blocked by either HT17 or HT26 [19]. In addition, HT59 was cross-blocked by some TLR4 mAbs which had MD-2-independent epitopes, whereas HT17 and HT26 were not cross-blocked by any TLR4 mAbs [19]. The TLR4 chain may provide a more crucial antigenic determinant for HT59 than the MD-2 chain. Intriguingly, the HT17 and HT26 epitopes may partially overlap in the LPS-binding pocket or at the TLR4/MD-2 dimerization interface because they lost reactivity in LPS-stimulated cells. We demonstrated previously that LPS-induced TLR4/MD-2 clustering is enhanced in a CD14- and LBP-dependent manner in mice [7]. The molecular requirements for membrane-bound CD14 and soluble LBP were consistent with those for the loss of HT17 and HT26 reactivity by LPS. Therefore, it is possible that human TLR4/MD-2 dimerization may be paralleled by loss of HT17 and HT26 reactivity, although further investigation is required.

The mAb is a useful experimental and clinical tool in that it has high specificity for an antigen epitope. Therefore, the use of mAbs specific for the TLR4/MD-2 complex allows us to determine whether surface TLR4 can also form a functional complex with sMD-2 extracellularly. The detection of extracellular TLR4/MD-2

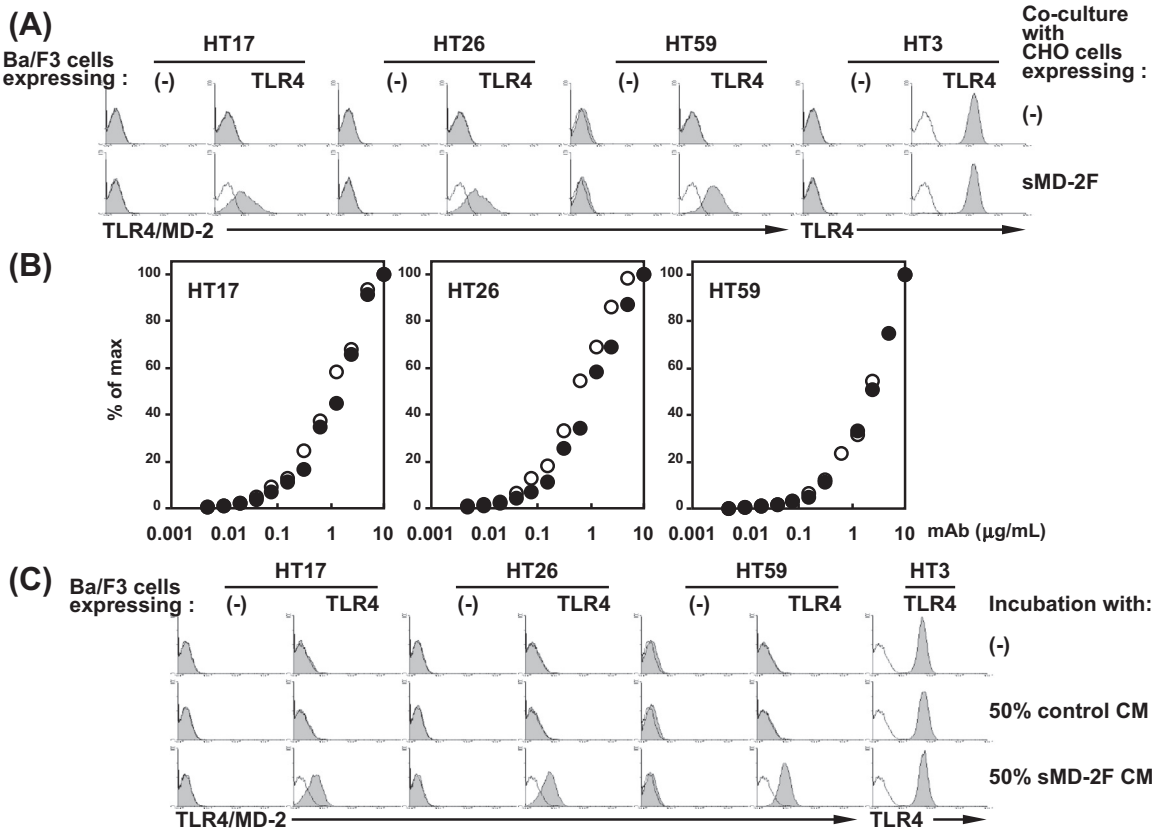

Fig. 3. All three TLR4/MD-2 mAbs detect extracellular TLR4/MD-2 complex formation with comparable affinity to the intracellularly formed complex. (A) TLR4-expressing or parent Ba/F3 cells were co-cultivated with either CHO cells expressing sMD-2F (lower) or parent CHO cells (upper) for 3–4 h. Ba/F3 cells were stained and analyzed using flow cytometry as in Fig. 1A. (B) The extracellular TLR4/MD-2F complex (open circles) was formed by 1-day co-cultivation of TLR4-expressing Ba/F3-transfected cells with CHO cells expressing sMD-2F and then stained with the indicated Bio-mAbs and PE-conjugated stv. TLR4/MD-2F-expressing stable Ba/F3-transfected cells were stained as in the identical experiments for the intracellularly formed TLR4/MD-2 complex (closed circles). Mean fluorescent intensity of TLR4/MD-2 staining by varying the concentration of the Bio-mAbs was determined using flow cytometry; the percentage of the maximal intensity is presented. (C) TLR4-expressing or parent Ba/F3 cells were co-incubated with either 50% CM from CHO cell expressing sMD-2F or from parent CHO cells for 3–4 h, as indicated on the right side. Cells were analyzed as in Fig. 1A.

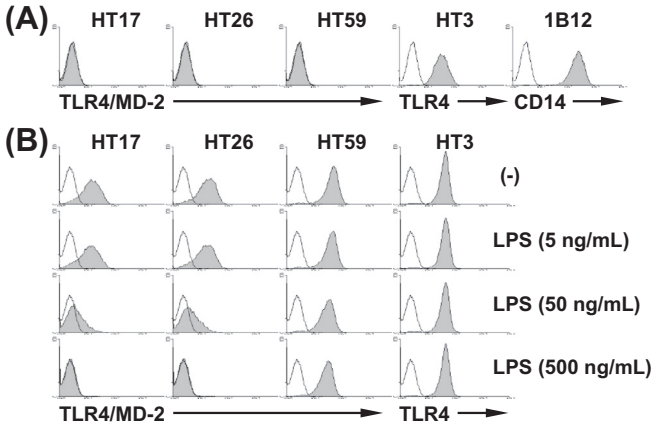

Fig. 4. The extracellularly formed complex changes its conformation similarly to the intracellularly formed complex in response to LPS stimulation. (A) TLR4 and CD14-expressing Ba/F3-transfected cells were stained with Bio-1B12 mAbs for CD14 and PE-conjugated stv, followed by flow cytometric analysis. The open histogram represents staining with PE-conjugated stv alone. (B) TLR4/CD14-expressing Ba/F3 cells were co-cultivated with sMD-2F-secreting CHO cells as in Fig. 3B. After washing twice with PBS, the cells were stimulated with LPS, stained and analyzed using flow cytometry as in Fig. 2B.

complex formation using a tag sequence fused to MD-2 or an MD-2-specific mAb was performed previously [10,12,16]. As a typical example, human TLR4/mouse MD-2F complex can be detected by FLAG mAb, as shown in Fig. 1A. However, this does not prove the presence of a functionally active TLR4/MD-2 complex, because these detection methodologies are independent of the mode of association between TLR4 and MD-2. Indeed, the human TLR4/mouse MD-2F chimeric complex is unresponsive to LPS stimulation despite the clear detection of MD-2F association [5] (Fig. 1A). In contrast, the reactivity of HT17, HT26 and HT59 rely largely on combinatorial epitopes generated by the intact TLR4/MD-2 complex. HT17, HT26 and HT59 mAbs were unreactive with human TLR4/mouse MD-2F despite positive staining with FLAG mAb for MD-2F. Even subtle conformational differences, dependent on the association state of complex, are believed to affect the sensitivity of these mAbs.

Because of the ready aggregation of sMD-2 and limited availability of efficient probes to detect the molecular structure generated by complex formation, it remains unclear whether the surface TLR4 complex formed with sMD-2 is indistinguishable from that formed intracellularly. It is a matter for debate whether sMD-2 in serum forms the complex, despite previous reports [12,13,16], because the active monomeric form of sMD-2 appears to be an extremely minor portion compared to the abundant inactive polymeric forms (Fig. 1C) [10,11,15]. Serum sMD-2 has been indirectly indicated from serological activity [11,13,16] and using anti-MD-2 mAbs [12,16]. However, sMD-2-like molecules with serological activity cannot be completely ruled out. MD-2 mAb does not necessarily prove that the extracellularly formed complex is equivalent to the intracellularly formed one in terms of association mode and functional activity to induce signals in response to LPS.

Because the mAb is also likely to display affinity for the inactive sMD-2 polymer, this may lead to false-positive detection of active sMD-2 in such assays. Here we demonstrated that all three mAbs were reactive for TLR4 when cells were co-cultured with sMD-2-secreting cells or incubated with sMD-2-containing CM. These results suggest that the equivalent complex is assembled with sMD-2 on the cell surface. Furthermore, we also demonstrated, using conformation-sensitive HT17 and HT26 mAbs, that LPS induced an active conformational change in the extracellularly formed complex. Therefore, the LPS-induced activation mechanism of the extracellularly formed complex appeared to be equivalent that of the intracellularly formed complex.

In conclusion, we demonstrated that cell-surface TLR4 and sMD-2 form a functional complex that is equivalent to that created intracellularly in terms of the association mode and LPS-induced conformation change. This finding supports an additional or alternative pathway of complex formation. MAbs for the TLR4/MD-2 complex and sensitive to LPS-induced conformation change could be novel probes for the effective detection and molecular characterization of extracellular complex formation. In addition, the combined use of these mAbs with standard anti-TLR4 mAbs would be useful to characterize the expression pattern of free and complexed TLR4, and their activation status under physiological conditions.

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