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Regulatory Roles for CD14 and Phosphatidylinositol in the Signaling via Toll-like Receptor 4-MD-2[1]

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The complex consisting of Toll-like receptor 4 (TLR4) and associated MD-2 signals the presence of lipopolysaccharide (LPS) when it is expressed in cell lines. We here show that normal human mononuclear cells express TLR4 and signal LPS via TLR4. CD14 is a molecule that binds to LPS and facilitates its signaling. Little is known, however, about the relationship of CD14 with TLR4-MD-2. We show that CD14 helps TLR4-MD-2 to sense and signal the presence of LPS. CD14 has also been implicated in recognition of apoptotic cells, which leads to phagocytosis without activation. Membrane phospholipids such as phosphatidylserine (PS) or phosphatidylinositol (PtdIns) are thought to serve as the ligands for CD14 in apoptotic cells. We find that PtdIns acts as an LPS antagonist in the signaling via TLR4-MD-2. TLR4-MD-2 seems to discriminate LPS from phospholipids. The signaling via TLR4-MD-2 is thus regulated by CD14 and phospholipid such as PtdIns.

Bacterial infection is still a major threat to humans. Invasion of Gram-negative bacteria elicits immune responses. A major mediator of the responses is endotoxin/lipopolysaccharide (LPS), a component of the cell wall of Gram-negative bacteria. LPS-induced activation of monocytes/macrophages leads to secretion of a number of pro-inflammatory cytokines such as TNFα, IL-1, and IL-6. The defense programs are then activated and invading bacteria are eliminated. Excessive amount of the cytokines however may result in fatal endotoxin shock (1). LPS signaling has to be kept under the control during immune responses. It is therefore of particular importance to understand the molecular mechanism underlying LPS recognition/signaling for the control of immune responses and endotoxin shock.

LPS binding protein (LBP) is the plasma protein that firstly interacts with and recruits LPS from bacterial membrane to another protein CD14 (2, 3). CD14, which is present in plasma as well as on the monocyte cell surface, binds to LPS and facilitates its signaling (4, 5). CD14 is a glycosylphosphatidylinositol (GPI)-anchored protein and does not have a cytoplasmic signaling domain. Another molecule has been expected to transmit the LPS signal across the plasma membrane (3, 5). Toll-like receptors (TLR) are now identified as the signaling molecules (6,7). TLRs are transmembrane molecules consisting of extracellular leucine-rich repeats (LRRs) and an intracellular signaling domain, which is similar to the type I IL-1 receptor. TLR2 was firstly reported to signal the presence of LPS (8, 9). TLR2 was followed by TLR4, the gene of which is mutated in LPS low responder strains, C3H/HeJ and C57BL/10 mice (10–12). TLR4 alone however is not capable of sensing and signaling the presence of LPS (9, 13). Another molecule MD-2, which is physically associated with TLR4, is required for LPS recognition (13). The TLR4-MD-2 complex thus serves as the LPS recognition molecule.

In contrast to LBP or CD14, plasma lipoproteins and serum phospholipids have been shown to have a negative effect on LPS signaling. Lipoproteins promote the release of LPS from leukocytes and therefore attenuate

Abbreviations used: LPS, lipopolysaccharide; mCD14, membrane CD14; sCD14, soluble CD14; LBP, LPS-binding protein; PtdIns, phosphatidylinositol; PS, phosphatidylserine; PC, phosphatidylcholine; TNF, tumor necrosis factor; TLR, toll-like receptor; glycosylphosphatidylinositol, GPI; leucine-rich repeats, LRR; PBMC, peripheral blood mononuclear cells.

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LPS responses (14). Serum phospholipids, phosphatidylinositol (PtdIns) and phosphatidylserine (PS), bind to CD14 and inhibit LPS-induced activation of monocytes (15). Serum components thus seem to regulate host responses to LPS in both positive and negative ways. Despite these results, it is not clear whether these serum components, CD14 and phospholipids, influence the specific LPS signaling pathway via TLR4-MD-2. The present study addressed the issue.

METHODS

Cells and reagents. TNF-sensitive L929 cells were kindly provided by Dr. T. Suzuki (University of Kansas Medical Center). Stable transfectants used were described previously (13). Briefly, the IL-3-dependent line Ba/F3 were transfected with expression vectors encoding human TLR4 or human MD-2 as well as the p55κB luciferase reporter construct for measuring NF-κB activity. Neither the stable transfectant nor the original line Ba/F3 did not express mouse CD14. Another transfectant was established in the present study by transfecting an expression vector encoding human CD14 into the transfectant expressing TLR4-MD-2 (Fig. 4). The human CD14 cDNA was kindly provided by Dr. R. J. Ulevitch (San Diego, CA). These transfectants were maintained in 10% FCS RPMI1640 supplemented with 50 μM 2ME and IL-3.

Diphosphoryl lipid A derived from *Salmonella minnesota* Re 595 was purchased from Sigma (St. Louis, MO). The Re-chemotype LPS from *Salmonella minnesota* R595 was kindly provided by Dr. K. Hisatsune (Josai University, Japan). Human serum was purchased from BioWhittaker (Walkersville, MD). Phospholipids were purchased from Sigma (St. Louis, MO), dried, and resuspended in PBS by sonication.

Establishment of monoclonal antibodies against human TLR4 and CD14 and preparation of soluble CD14-depeleted serum. The anti-TLR4 mAb HTA125 was described previously (13). HTA1216 (IgG1/κ) another mAb to human TLR4 was established simultaneously. Specificity of HTA1216 was confirmed by staining and immunoprecipitation of the transfectant expressing TLR4-MD-2 (data not shown). We also established a mAb to human CD14, MA141. BALB/c mice (SLC, Shizuoka, Japan) were immunized with Ba/F3 expressing human CD14 + TLR4-MD-2 (see above), and immunized spleen cells were fused with the SP2/0 myeloma partner. Hybridoma supernatant was screened with staining of a transfectant expressing CD14 alone. Cross-blocking studies revealed that MA141 is similar in epitope recognition to commercially available mAbs to human CD14, MY4 and RMO52 (data not shown). Antibodies were purified from ascites derived from severe combined immunodeficient (SCID) mice. The purified anti-CD14 mAb was coupled to NHS-activated Sepharose 4 Fast Flow Media (Amersham Pharmacia Biotech AB, Uppsala, Sweden) according to the manufacturer's instruction. We also prepared the beads coupled with an irrelevant mAb for mock depletion. The coupled beads were used for depleting sCD14 in human serum. The binding of serum-derived CD14 to the beads was confirmed by biotinylation of the beads, electrophoresis, Western blotting, and probing with avidin. An ~50 kD signal was detected from the anti-CD14 mAb coupled beads but not from a control mAb-coupled beads (data not shown).

TNF assay with normal blood cells. Heparinized blood was obtained from healthy donors, diluted by 4-fold with serum-free RPMI 1640, and inoculated onto 96-well plate (270 μl/well). PBMCs were obtained by density gradient centrifugation of peripheral blood from healthy donors using Ficoll-Paque plus (Pharmacia Biotech, Uppsala, Sweden). PBMCs were resuspended in 2% FCS RPMI 1640, and inoculated onto 96-well plate at 5×10^6/ml. Whole blood cells or PBMCs were allowed to adhere for 2 h at 37°C, washed three times with RPMI 1640, and stimulated with 1 ng/ml LPS for 18 h in media containing 2% FCS. The mAbs were added 30 min earlier than LPS. The supernatants were collected and their TNF activity was determined by a functional cytotoxic assay using actinomycin D-treated L929 (16).

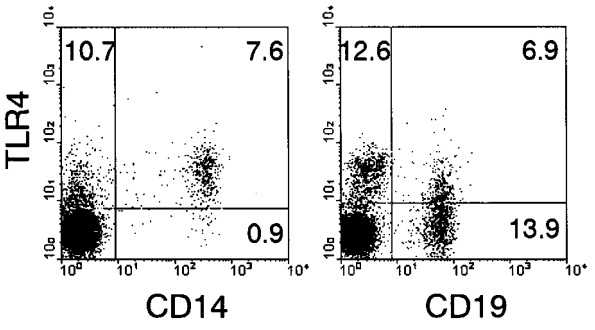

FIG. 1. Expression of TLR4 on peripheral blood mononuclear cells. PBMCs were stained with the anti-TLR4 mAb HTA1216 in combination with an Ab against CD14 or CD19. Percentages are shown in each quadrant.

Cell surface staining. Cell surface staining was conducted by using the biotinylated HTA1216 followed by streptavidin–phycoerythrin (PE) (Vector Laboratories, Inc., Burlingame, CA), FITC-labeled anti-CD14 mAb (RMO52, Beckman Coulter Inc., Fullerton, CA), and FITC-labeled anti-CD19 mAb (Leu-12, Becton Dickinson, Mountain View, CA). Cells were analyzed on a FACScan (Becton Dickinson).

Luciferase assay. Stable transfectants were inoculated onto 96 well plates at 1×10^5/well. Lipid A was also included. Culture medium (RPMI1640 with 2-ME and IL-3) contained 10% FCS or 10% human serum as indicated in each figure. Results under a serum-free condition were also shown in Fig. 3. After 4 h stimulation, cells were harvested, washed, and lysed in 100 μl lysis buffer and luciferase activity was measured using 10 μl lysate and 50 μl luciferase substrate (Nippon Gene, Toyama, Japan). The luminescence was quantitated as relative light unit (RLU) by a luminometer (Berthold Japan, Tokyo, Japan). The results in Fig. 3 were shown as relative luciferase activity by dividing each RLU value with that of cells without stimulation.

RESULTS

Expression of TLR4 on Normal Monocytes and B Cells

We first studied expression of TLR4 with the anti-TLR4 mAb HTA1216. The results are shown in Fig. 1. PBMCs were doubly stained with the anti-TLR4 mAb in combination with CD14 or CD19, markers for monocytes or B cells, respectively (Fig. 1). TLR4 was expressed on CD14-positive, CD11b/CD18-positive monocytes, as well as on a subpopulation of CD19-positive B cells. Some CD5-positive B cells were positive for TLR4 (data not shown).

TLR4 Mediates the LPS Signaling in Normal PBMCs

We next examined expression of TLR4 on PBMCs after stimulation with LPS, but no change was observed (data not shown). A mAb against TLR4,

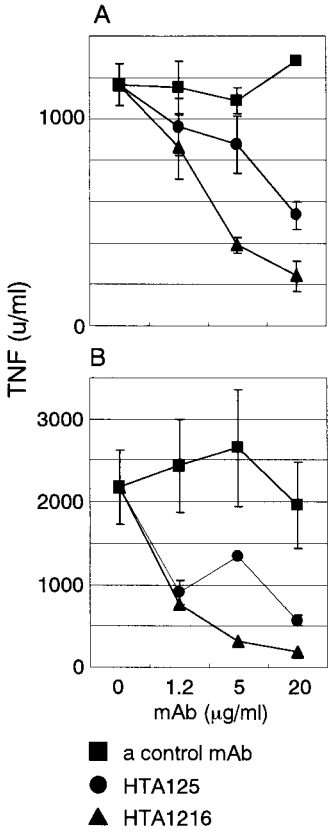

FIG. 2. Effects of anti-TLR4 mAbs on LPS-induced TNF production by human peripheral blood mononuclear cells. Human whole blood samples (A) or monocytes (B) were preincubated with graded concentrations of the indicated mAbs at 37°C for 30 min, and then stimulated with 1 ng/ml LPS at 37°C for 15 h. TNF activity in the supernatants was determined by the cytotoxic assay using L929 cells. The data shown are mean values ± standard errors from triplicate wells. Representative results were shown out of four (A) or two (B) experiments.

HTA125 or HTA1216, was then included in cultures of whole blood cells or adherent PBMCs with LPS, and TNF production was examined. Either mAb against TLR4 was antagonistic in LPS-induced TNF production by whole blood cells or adherent PBMCs (Fig. 2). HTA1216 was more effective in the inhibition. No agonistic activity was observed with either mAb when included in culture without LPS (data not shown). TLR4, probably with associated MD-2, serves to signal the presence of LPS on normal human monocytes.

CD14 Augments the LPS Signaling via TLR4-MD-2

The LPS signaling via TLR4-MD-2 was observed even under a serum-free condition (Fig. 3). Nevertheless, TLR4-MD-2 acquired about two orders of magnitude higher sensitivity in the presence of serum (Fig. 3). CD14 is present in serum, and has been implicated in LPS recognition (4, 5). To address a role for CD14 in the LPS recognition by TLR4-MD-2, we employed a

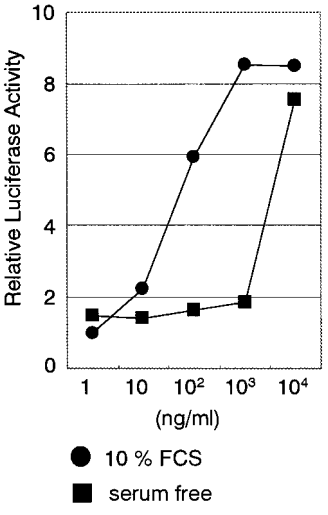

FIG. 3. Serum dependence of the LPS signaling via TLR4-MD-2. Ba/F3-derived transfectants expressing TLR4-MD-2 were stimulated with graded doses of lipid A in the presence (circle) or absence (square) of 10% fetal calf serum. After 4 h culture, cells were harvested, NF-kB activation was measured by luciferase assay, and data are expressed as relative luciferase activity (see Methods). Representative results are shown out of three experiments.

mAb to human CD14 and a transfectant expressing CD14 as well as TLR4-MD-2 (Fig. 4). The original line Ba/F3 and derived lines do not express mouse CD14 (data not shown). Human serum was included in culture medium instead of fetal calf serum, and the anti-

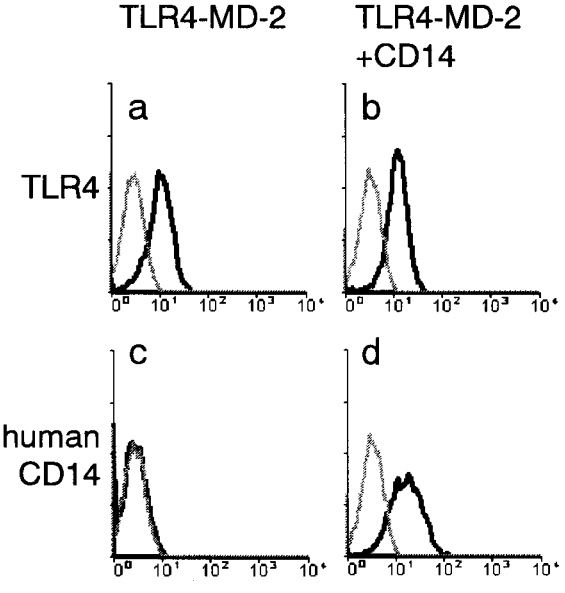

FIG. 4. Cell surface expression of TLR4 or CD14 on the transfectants. The transfectant expressing TLR4-MD-2 (a, c) or TLR4-MD-2 + CD14 (b, d) was stained with the mAb to TLR4 (HTA125: a, b) or to human CD14 (MA141: c, d), followed by goat-anti-mouse IgG-FITC (solid lines). Overlaid histograms (dotted lines) show staining with the second reagent alone.

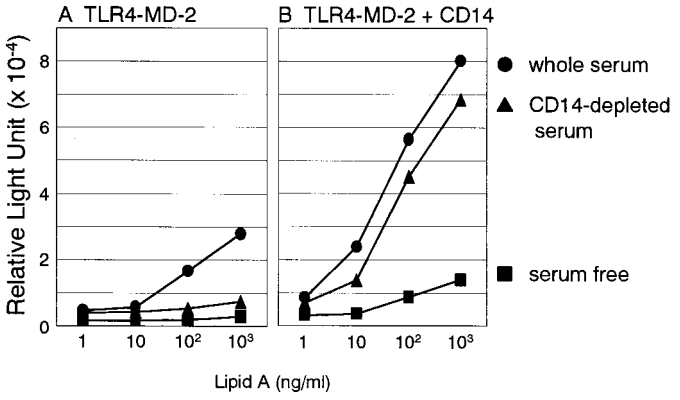

FIG. 5. The LPS signaling via TLR4-MD-2 is dependent on CD14. The transfectant expressing TLR4-MD-2 with or without CD14 was stimulated by lipid A in the culture medium containing human serum (circle), CD14-depleted serum (triangle), or no serum (square). After 4 h culture, cells were harvested and NF-kB activation was measured by luciferase assay. Representative results are shown out of three experiments.

CD14 mAb was used to deplete sCD14 (see Methods). The transfectant expressing TLR4-MD-2 was stimulated with LPS in the presence of intact or sCD14-depleted serum, and NF-κB activation was examined. The LPS responses were observed in the presence of human serum, although the responses were lower than those in the presence of fetal calf serum (Figs. 3 and 5). Comparable NF-κB activation was observed with the human serum depleted with the control beads that had been conjugated with an irrelevant mAb (data not shown). In contrast, the LPS responses with the CD14-depleted serum were as low as those under a serum-free condition (Fig. 5). Such reduction was not observed in the transfectant expressing mCD14 as well as TLR4-MD-2. However, serum is still required for the LPS responses. Another serum protein LBP is likely to contribute to the LPS responses. Interestingly, LPS-induced NF-κB activation was strengthened with cell surface CD14 as compared with sCD14 (see Discussion).

Inhibition of the LPS Signaling via TLR4-MD-2 by PtdIns

It is interesting and important to know whether the phospholipids influence the signaling via TLR4-MD-2. Our system employing transfectants allowed us to address this issue. The transfectant expressing TLR4-MD-2 was incubated with PS, PC, or PtdIns, but no NF-κB activation was observed (data not shown). We next studied effects of these phospholipids on the LPS signaling via TLR4-MD-2. PtdIns showed pronounced inhibition, whereas PS or PC revealed a modest or little effect, respectively (Fig. 6). The inhibition by PtdIns is dose-dependent and maximal inhibition was achieved at 10 μg/ml. PtdIns is contained in serum at about 50–100 μg/ml (15). We included serum at 10%, which would contain 5–10 μg/ml endogenous PtdIns. Therefore a 2- to 3-fold increase in PtdIns seems to greatly inhibit the LPS signaling via TLR4-MD-2. PtdIns or PS was not toxic, since the antagonistic activity with PtdIns or PS was not apparent in the transfectant expressing CD14 as well as TLR4-MD-2 even at the highest concentration 100 μg/ml (Fig. 6). CD14 on the cell surface seemed to regulate the antagonistic activity of PtdIns or PS.

DISCUSSION

The present study showed for the first time that TLR4 is expressed on PBMCs, and the mAbs against TLR4 blocked the LPS-induced TNF production by PBMCs (Figs. 1 and 2). TLR4 therefore signals the presence of LPS in normal leucocytes. Considering broad expression of the MD-2 mRNA (13), it is likely to be expressed and associated with TLR4 in normal PBMCs. The LPS signaling via TLR4-MD-2 seems to be employed by normal human leukocytes.

CD14, which circulates as a soluble form and is present on the cell surface of monocytes, has been shown to bind to LPS (3–5). By using transfectants and the mAb to CD14, we showed that the LPS signaling via TLR4-MD-2 became about two orders of magnitude more sensitive in the presence of CD14. It seems easier for TLR4-MD-2 to recognize the complex consisting of

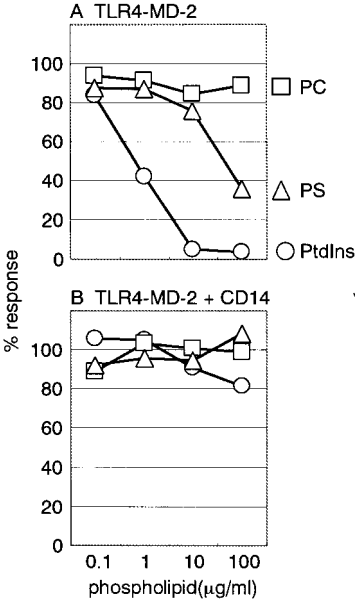

FIG. 6. The antagonistic activity of PtdIns to the LPS signaling via TLR4-MD-2. The transfectant expressing TLR4-MD-2 alone (A) or with CD14 (B) was stimulated by lipid A (100 ng/ml) in the culture medium that contained 10% FCS. Graded doses of PtdIns (circle), PS (triangle), PC (square) were included. After 4-h culture, cells were harvested and NF-κB activation was measured by luciferase assay. Representative results are shown out of five experiments.

LPS and CD14 than LPS alone. The direct binding of the CD14-LPS complex and TLR4-MD-2 would be the simplest mechanism explaining LPS recognition by TLR4-MD-2. However, the binding assay using radiolabeled LPS (17) failed to detect the direct binding of LPS to the transfectant expressing TLR4-MD-2 (our unpublished observation). The assay was conducted in the presence of serum, which contained LBP and CD14. LPS or the CD14-LPS complex may not directly bind to TLR4-MD-2. It is possible that additional molecules are working in between. This possibility is supported from the findings with drosophila Toll. The drosophila defense programs against pathogens require not only Toll but also the Toll ligand Spaetzle (18). Bacteria or fungi may be recognized by drosophila Toll indirectly with an aid of Spaetzle (19). Similarly, a ligand for TLR4-MD-2 if any could help LPS recognition in concert with CD14.

CD14 on the cell surface strengthened LPS-induced NF-κB activation about 2-fold as compared with sCD14 (Fig. 5). Efficient recruitment of LPS to TLR4-MD-2 by CD14 would explain higher sensitivity to LPS, but not the stronger signaling. Membrane CD14 may positively regulate the TLR4-MD-2 signaling besides recruiting LPS to the cell membrane. In keeping with this, Trapping *et al.* (20) showed that recruitment of LPS by membrane-anchored LBP did not augment LPS responses. It might be possible that membrane CD14 is associated with TLR4-MD-2 and augments the signaling, although our coprecipitation studies using mAbs to CD14 or TLR4 did not reveal physical association of these molecules (our unpublished results).

The present study demonstrated that PtdIns acts as an LPS antagonist on the signaling via TLR4-MD-2. Wang *et al.* (15) showed that PtdIns inhibits the uptake and signaling of LPS in mCD14 expressing cells, and that PtdIns is internalized and used as a source of arachidonate for leukotriene synthesis (21). It is possible that these products have an inhibitory effect on the TLR4-MD-2 signaling. However, our results failed to demonstrate clear inhibition by PtdIns in the Ba/F3 cells expressing mCD14 and TLR4-MD-2 (Fig. 6). Moreover, PtdIns alone did not inhibit the ligand-independent signaling via TLR4-MD-2, which was apparent in the absence of LPS (9, 13). We prefer another possibility that LPS and PtdIns compete for CD14 interaction. Two distinct complexes, CD14-LPS and CD14-PtdIns, are likely to be present simultaneously in serum. TLR4-MD-2 signals only the presence of the former, but not of the latter. The CD14-PtdIns is not recognized by TLR4-MD-2. TLR4-MD-2 seems to discriminate between these two complexes.

Our assay system included serum in which phospholipids are contained. Even in the presence of serum-derived phospholipids, exogenous PtdIns or PS was able to inhibit the LPS signaling via TLR4-MD-2, although the inhibition was evident only in mCD14-negative cells. Under a physiological condition, PtdIns is likely to act on mCD14-negative cells such as B cells or endothelial cells rather than mCD14-positive monocytes. TLR4 is expressed on a subpopulation of CD19-positive B cells (Fig. 1). B cells would be sensitive to the PtdIns antagonism, since they do not express mCD14. Anti-phospholipid Abs were present in normal serum as natural Abs (22). Moreover, B cells seem to produce anti-phospholipid Abs in response to LPS inoculation or bacterial infection (23, 24). Some of anti-phospholipid Abs react with LPS, clear it from the circulation, and thereby prevent endotoxin shock (22). However, excessive production of anti-phospholipid Abs would be harmful and lead to immune complex deposition and/or coagulation disorders. Indeed, autoantibodies to phospholipids have been described as anti-cardiolipin Abs or lupus anticoagulant in lupus-like autoimmune disorders (25, 26). The amount of anti-phospholipid Abs therefore has to be kept under the control. The present study raises a possibility that endogenous PtdIns has a role in keeping the amount of anti-phospholipid Abs developed in bacterial infection within a normal range by acting antagonistically against LPS.

Recent studies identified apoptotic cells as a source of autoantigens (27, 28). Surface blebs of apoptotic cells contain self antigens which are recognized by autoantibodies developed in autoimmune diseases such as lupus, scleroderma, and dermatomyositis. These self-antigens need to be cleared instantly; otherwise autoantibodies may be produced (29, 30). Molecules such as a complement component C1q (31), C-reactive protein (32), serum amyloid protein (SAP) (33), and CD14 (34) have a role in clearing surface blebs of apoptotic cells, small nuclear ribonucleoproteins, DNA/chromatins, and apoptotic cells, respectively. Mice lacking C1q or SAP suffer from lupus like autoimmune disorders (33, 35). Interestingly, these molecules also recognize and/or clear pathogens. C1q helps Abs to clear pathogens. CRP binds to the C carbohydrate of *Streptococcus pneumoniae* (36). Finally, CD14 binds to LPS (4). It is interesting and important to study how self-antigens are discriminated from pathogens. CD14 itself does not recognize the difference, since it binds to either LPS or PtdIns (4, 15). TLR4-MD-2 delivers an activation signal in the presence of LPS, but not in the presence of PtdIns. TLR4-MD-2 may distinguish LPS from PtdIns, with the aid of CD14. In this regard, it is particularly important to understand the molecular mechanism underlying LPS recognition by TLR4-MD-2. Aberrant recognition of PtdIns as LPS by TLR4-MD-2 or a defect in the PtdIns antagonism to LPS may lead to autoantibody development.

Further studies concerning the distinction by TLR4-MD-2 between self-antigens and pathogens would contribute to understanding of pathophysiologic mechanisms underlying lupus-like autoimmune disorders.

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