Visualization of the Molecular Dynamics of Lipopolysaccharide on the Plasma Membrane of Murine Macrophages by Total Internal Reflection Fluorescence Microscopy

Samia Shawkat1, Risuke Karima1, Tadashi Tojo1, Hisashi Tadakuma1, Shin-ichiroh Saitoh1, Sachiko Akashi-Takamura1, Kensuke Miyake1, Takashi Funatsu1, and Kouji Matsushima1†

From the 1Department of Molecular Preventive Medicine, Graduate School of Medicine, and the 6Environmental Science Center, University of Tokyo, Tokyo 113-0033, Japan, the 3Division of Infectious Genetics, Institute of Medical Science, University of Tokyo, Tokyo 108-8639, Japan, and the 6Department of Physics, School of Science and Engineering, Waseda University, Tokyo 169-8555, Japan

The molecular action of Alexa 594-labeled lipopolysaccharide (LPS) from Escherichia coli was examined on living peritoneal macrophages of C57BL/6 mice by total internal reflection fluorescence microscope (TIRFM), and the molecular kinetics of LPS was analyzed. TIRFM visualization of the action of fluorescence-labeled LPS revealed an increase in the mean fluorescence intensity of LPS on the plasma membrane of wild type macrophages at 60 min after administration, indicating the oligomerization of LPS after binding to the macrophages. Additionally, a time-dependent sharp decrease in the mean diffusion coefficient of LPS was observed. On the other hand, both mean fluorescence intensity and diffusion coefficient of LPS in cases of TLR4−/−, MD2−/−, Myd88−/−, and TRIF−/− macrophages were significantly different from the corresponding values of wild type macrophage, whereas differences were also noticed among these molecule-deficient macrophages. Furthermore, statistical analysis indicated the major role of receptors (TLR4 and MD2) and intracellular signaling molecules (MyD88 and TRIF) in oligomerization and lowering of the diffusion rate of LPS on the plasma membrane of murine macrophages, respectively.

Lipopolysaccharide (LPS), the major constituent of the Gram-negative bacterial outer membrane, induces intense inflammatory responses. The complex of LPS and LPS-binding protein (LBP) interacts with CD14, a receptor on macrophages/monocytes and neutrophils. Membrane-bound CD14 (mCD14) is a glycosylphosphatidylinositol-linked molecule anchored on the cell surface, but it can also remain in the circulation as soluble CD14 (1). The revelation of importance of Toll-like receptor 4 (TLR4) for the initiation of the intracellular signaling pathways provided insight into the LPS signaling cascade (2).

TLR4 is known to require the coexpression of MD2 in the optimal recognition of LPS (1, 2). According to the available reports, TLR4-mediated response to LPS can be divided into two different cascades: an early MyD88-dependent response through TLR4 and a delayed MyD88-independent response (3, 4). The early MyD88-dependent response induces an early activation of NF-κB, IRF3, and IFN-β (3). On the other hand, in the delayed MyD88-independent signaling pathway, TRIF has been established as a key adaptor molecule that is responsible for the later activation of NF-κB, IRF3, and is a more potent inducer of IFN-β compared with MyD88 (3).

Although a few studies have alluded to the direct association of LPS with the TLR4-MD2 complex (5–7), the molecular action of LPS on the plasma membrane of living cells is yet to be fully clarified. Reports describing the involvement of LPS-signaling molecules in the dynamics of LPS molecule on the living cell surface by direct visualization are lacking. The elucidation of the molecular mechanism of the recognition of LPS by the TLR4-MD2 complex may contribute to the development of the intervention against LPS-induced inflammation.

TIRFM is an ideal means to study the molecular dynamics of biological interaction of cellular molecules in vitro. When an excitation light beam passes across a solid (e.g. glass coverslip or tissue culture plastic) incident at a high angle, greater than the critical angle of total internal reflection upon the solid/liquid surface at which the sample adheres, a thin layer of illumination is produced that is called “evanescent field.” This is the unique feature of TIRFM that enables excitation of the fluorophores near the portion of the plasma membrane adhered to coverslip while avoiding excitation of a much larger number of fluorophores farther out in the liquid. TIRFM typically involves very thin (less than 100 nm) optical sectioning, which means that the signal to noise ratio is much better than with confocal images, and cellular photodamage and photobleaching are also minimal. This is one of the most effective ways to reduce background of fluorescence microscopy to
roles of TLR4/MD2/MyD88/TRIF molecules in dynamics of LPS on the living cell.

EXPERIMENTAL PROCEDURES

Reagents and Antibodies—LPS of Escherichia coli, serotype 055:B5, RPMI 1640 medium (with L-glutamine), and penicillin-streptomycin were purchased from Sigma, and fetal bovine serum was purchased from Invitrogen. Alexa 594-labeled LPS of *E. coli*, serotype 055:B5, was purchased from Molecular Probes Inc. (Eugene, OR). Recombinant mouse LBP (rmLBP) was purchased from Cell Sciences (Canton, MA). Hanks’ balanced salt solution (HBSS) was purchased from Invitrogen. Sodium pyruvate was purchased from Cambrex. An ELISA kit to detect mouse tumor necrosis factor α was purchased from R & D Systems, Inc. (Minneapolis, MN).

Preparation of Murine Peritoneal Macrophages—TLR4−/−, MD2−/−, MyD88−/−, and TRIF−/− mice prepared by Dr. Shizuo Akira (Osaka University, Osaka, Japan) were used. CD14−/− mice were purchased from the Jackson Laboratory (Bar Harbor, ME). Wild type C57BL/6 mice were purchased from SLC. All of the mice were C57BL/6 strain, male, and 6–8 weeks of age. The mice were intraperitoneally injected with 1 ml of 4% thioglycollate. After 3 days, the mice were sacrificed, and peritoneal macrophages were harvested using 10 ml of phosphate-buffered saline. The cells were then centrifuged at 2000 rpm for 5 min and resuspended with RPMI 1640 medium supplemented with 10% fetal bovine serum. 1% sodium pyruvate was added to reduce auto fluorescence of the cells. The cells were spread on an 8-well cover glass chamber (Lab-Tek, Naperville, IL) and incubated at 37 °C for 48 h. Nonadherent cells were washed out with HBSS three times just before TIRFM observation. Care of all the animals used in this study was in accordance with our institutional guidelines.

Fluorescence Observation on the Plasma Membrane of Cells by TIRFM—For the observation of the fluorescent spot of Alexa 594-labeled LPS on the plasma membrane of mouse peritoneal macrophages, an objective type TIRFM (IX71; Olympus, Japan) was used. The incident light for evanescent illumination was introduced from the objective lens (PlanApo 100×; NA = 1.4) installed on an inverted microscope. For the evanescent wave excitation of the fluorescence image, a 532-nm laser (Coherent Inc.) and a long pass filter (Asahi Spectra Co.) were used.
The laser beam was passed through an electromagnetically driven shutter (Hamamatsu Photonics), and fluorescence images were captured with a monochromatic ICCD camera (Hamamatsu Photonics) combined with an image intensifier (Hamamatsu Photonics). The video images were contrast-enhanced with a digital image processor (AquaCosmos, version 2.6; Hamamatsu Photonics) and were recorded and stored in a computer hard disk.

After washing three times with HBSS, the cover glass chamber spread with mice peritoneal macrophages containing 100 μl of HBSS was set on the objective lens. A drop of mineral oil (Olympus) was added to the lens. Then additional an 100 μl of HBSS containing Alexa 594-labeled LPS and rmLBP was put into the well. The final concentrations of Alexa 594-labeled LPS and rmLBP were 20 and 1000 ng/ml, respectively. The cells were then observed by TIRFM with 4000 microwatts of 532-nm laser, and real time fluorescent images were recorded during specified intervals, namely, 5–15, 25–35, and 55–65 min following LPS administration. However, for simplicity, while plotting the graphs, the aforementioned intervals were denoted as 10, 30, and 60 min, after LPS administration, respectively.

ELISA—Peritoneal macrophages from 6–8-week-old, male, C57BL/6 strain mice were collected and prepared in the same way as described earlier. The cells were resuspended with RPMI 1640 medium supplemented with 10% fetal bovine serum and spread on 48-well plates. Then incubated in 37 °C for 2 h. Non-adherent cells were washed out with phosphate-buffered saline. Then RPMI 1640 medium supplemented with 10% fetal bovine serum was added. The cells were stimulated with 0, 1, 10, 100, and 1000 ng/ml of Alexa 594-labeled and unlabeled E. coli LPS of same strain and then incubated for 6 h at 37 °C. The supernatant was harvested, and tumor necrosis factor-α (TNF-α) production was analyzed using an ELISA kit.

Analysis—All of the images were analyzed using the automatic fluorescent spot analyzer (AquaCosmos, version 2.6; Hamamatsu Photonics). Higher FI and the typical movement of the murine peritoneal macrophages on the plasma membrane distinguished the fluorescence-labeled LPS molecules from the background noise. Controls devoid of LPS facilitated further the proper detection of the LPS molecule. DC was estimated using AquaCosmos software (version 2.6; Hamamatsu Photonics). FI versus DC plots were prepared using Microsoft Excel. Diagrams showing FI versus the number of LPS spots and DC versus the number of LPS spots were prepared using Origin6 software and Microsoft Excel, respectively. A graph of spots/μm² of cells versus time was prepared using Microsoft Excel. The number
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The labeled LPS administration are showing the gradual increase of number of higher fluorescent spots with time. Here, spots of Alexa 594-labeled LPS spots was counted from the video observation. In the case of the diameter of 100 fluorescence-labeled LPS spots using the following equation.

\[
MSD = (x - x_0)^2 + (y - y_0)^2 \quad \text{(Eq. 1)}
\]

Here, \(x_0\) and \(y_0\) are the initial coordinates, and \(x\) and \(y\) are the coordinates at any given time. The data between each group were compared by unpaired \(t\) test, and all of the data were considered to be significant at \(p\) value < 0.01.

RESULTS

Immunological Activity of Alexa 594-labeled LPS—We stimulated mouse peritoneal macrophages with Alexa 594-labeled LPS and unlabeled LPS of same strain. Our data showed that both fluorescence-labeled and unlabeled LPS triggered almost the same amount of tumor necrosis factor \(\alpha\) (Fig. 2). It proves that the fluorescence-labeled LPS that we used in our experiment is immunologically functional.

Visualization of the Molecular Dynamics of LPS on the Peritoneal Macrophages of Wild Type Mouse—We first investigated the molecular action of Alexa 594-labeled LPS on the plasma membrane of peritoneal macrophages of 6–8-week-old male C57BL/6 wild type mice. The cells were observed under TIRFM at 4000 microwatts of green laser at 532 nm at different time intervals.

The fluorescence as well as the number of LPS spots gradually increased with time after administration of LPS, and accordingly they were easily distinguishable from the background noise (supplemental Movies S1–S3). Fig. 3A shows a typical movement pattern of a fluorescence-labeled single LPS spot on the cell surface, and Fig. 3B demonstrates the gradual decay of FI because of photobleaching a certain period after the initiation of the observation. In the case of the diagram of the MSD of fluorescence-labeled LPS spots versus time (Fig. 3C), when we analyzed MSD of only two Alexa 594-labeled LPS spots against time, our graph showed a linear pattern, but when we calculated the MSD of 100 LPS spots, the graph was not linear. It is likely that an individual LPS spot can move freely on the surface of the cell, when the spots bind with receptors and adaptor molecule diffusion becomes restricted.

Fig. 4A shows a time-dependent change of distribution of fluorescence-labeled LPS spots. Compared with 5–15 min after LPS administration, the number of spots in the higher fluorescence range progressively increased at 25–35 and 55–65 min (Fig. 4B) after LPS administration. At 5–15 min the range of FI was 40–70 AU. The range extended up to 75...
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![Graphs and images showing molecular dynamics of LPS on the living cell.](image)

FIGURE 5. Time-dependent changes of molecular dynamics of Alexa 594-labeled LPS on wild type murine peritoneal macrophages. Log values of DC expressed in μm²/s were plotted. A, the change in mean FI on murine peritoneal macrophage after 5–15, 25–35, and 55–65 min of Alexa 594-labeled LPS administration. The values are expressed as the means ± S.E. B, the change in mean DC on murine peritoneal macrophage after 5–15, 25–35, and 55–65 min of Alexa 594-labeled LPS administration. Log values of DC expressed in μm²/s were plotted, and the values are expressed as the means ± S.E. C, number of fluorescence-labeled LPS spots/μm² was calculated from the video images of wild type macrophages at 10-, 30-, and 60-min intervals of LPS administration and plotted in a graph. *, p < 0.01 versus 10 min; **, p < 0.01 versus 30 min; ***, p < 0.01 versus 10 and 30 min.

at 25–35 min along with an apparent decrease in the number of spots with FI levels of ~60 AU in comparison with the result at 5–15 min after LPS administration, whereas many spots with FI below 55 AU still could be found at 25–35 min. In contrast, at 55–65 min after LPS administration, a striking reduction in the number of spots with FI below 55 AU in addition to a remarkable increase in the number of spots above 70 AU could be found. Compared with the case of 5–15 min after LPS administration, the diagram of number of spots versus FI at 55–65 min of LPS administration (Fig. 4B) showed a more diffused pattern with several distributed peaks, which indicates the formation of larger oligomers at 55–65 min. This change was accompanied by a decrease in the number of spots in the lower fluorescence range and an increase in the number of spots with the lower DC. The statistical analysis of the data at different time intervals confirmed the apparent time-dependent change of both FI and DC (Fig. 5, A and B). The mean FI of 55.73 ± 0.31 AU (n = 520) after 10 min increased to 59.53 ± 0.35 AU (n = 524) after 60 min of LPS administration, whereas the corresponding mean logarithms of DC values expressed in μm²/s were −0.156 ± 0.026 (n = 520) and −0.467 ± 0.036 (n = 524), respectively. It is important to point out here that, Fig. 5A (plotting mean FI) at first glance may give an impression that there was no change in FI up to 30 min. However, although the statistical mean FI was almost the same at 5–15 and 25–35 min, as demonstrated in Fig. 4B, the appearance of spots with FI of 70–75 AU and the apparent reduction of the spots with FI levels of ~60 AU at 25–35 min compared with the result at 5–15 min show the time-dependent change in FI at 25–35 min. In Fig. 5C the number of fluorescence-labeled LPS spots/μm² of wild type macrophages showed a gradual increase with time, indicating higher binding of LPS spots with its concerning receptors and adaptor molecules.

The Role of LBP and CD14 in the Dynamics of LPS on the Plasma Membrane of the Living Cell—Next, we examined the involvement of LBP and CD14 in the molecular movements of LPS on the plasma membrane of wild type mouse peritoneal macrophages. In our experiments, consistent with the previous reports on the importance of LBP and CD14 in the initiation of cellular response to LPS (18–20), no motion of LPS on the plasma membrane was observed in the absence of LBP or CD14−/− as shown in the video clips (Fig. 6 and supplemental Movies S4 and S5), indicating the essential role of LBP and CD14 receptors for the movement of LPS on the living cells.

Differences in the Molecular Action of LPS in TLR4−/− and MD2−/− Macrophages—Toll-like receptors are a family of pattern recognition receptors of various natural ligands. Among the TLR family, TLR4 is indispensable for LPS-stimulated signal transduction and various proinflammatory cytokine production (1, 21, 22). We also observed the differences of movements of LPS molecule in TLR4−/− macrophages (Fig. 7B and supplemental Movie S6). Although after 55–65 min of LPS administration, the fluorescence spots ranged in between 40 and 87 AU for wild type macrophage, spots were not found beyond 60 AU for TLR4−/− macrophage (Fig. 7B). In fact the mean FI of LPS at 60 min for TLR4−/− macrophages was the lowest among all the studied deficient macrophages (18% lower than wild type), suggesting the indispensability of TLR4 for the formation of oligomers of LPS on the plasma membrane (Fig. 8A). On the other hand, as seen in Fig. 8B, the mean logarithm of DC of LPS expressed in μm²/s after 60 min in TLR4−/− macrophages was higher (−0.283 ± 0.031, n = 498) than that of the wild type macro-
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Compared with the wild type macrophage, the specific pattern of the diagram of number of spots versus fluorescence intensity of LPS in MyD88−/− macrophages indicated formation of smaller oligomers. The mean FI of LPS after 60 min was 52.46 ± 0.33 AU (n = 535), the second highest value observed for all the deficient macrophages (Fig. 8A). On the other hand, the mean logarithm of DC of LPS expressed in μm²/s in the case of MyD88−/− macrophages showed the highest value (0.113 ± 0.033, n = 535), which was 76% higher than the mean value in wild type (Fig. 8B). It is known that LPS signaling consists of both MyD88-dependent and MyD88-independent pathways. MyD88-dependent signaling is essential for the production of inflammatory cytokines (25, 26). In our study, the importance of MyD88 was manifested by a higher diffusion rate of LPS on the cell surface in the case of MyD88−/− macrophages. Supplemental Movie S8 shows the motion of LPS molecules on the plasma membrane of MyD88−/− macrophage.

We further examined the dynamics of LPS molecules on the plasma membranes of peritoneal macrophages of TRIF−/− mouse (supplemental Movie S9). TRIF is responsible for MyD88-independent signaling, which is important for the maturation of dendritic cells and induction of IFN-inducible genes (25, 26). The diagram of the number of spots versus fluorescence intensity at 55–65 min showed a moderate range (wider than the diagram of TLR4−/− and MD2−/− but narrower than that of wild type) of fluorescence intensities (ranging up to 81 AU). Fewer peaks of higher FI in the same diagram indicated the lower extent of oligomerization than in the case of wild type macrophage (Fig. 7E). Fig. 8A further shows the difference in the values of mean fluorescence intensity in the case of wild type (59.53 ± 0.35 AU, n = 524) and TRIF−/− macrophages (53.40 ± 0.31 AU, n = 540). On the other hand, in contrast to a sharp decrease of DC in the case of wild type macrophage, the mean DC of LPS in the case of TRIF−/− macrophages remained virtually unchanged with time. When compared with the macrophages deficient in LPS receptors (TLR4 and MD2), the mean logarithm of DC of LPS expressed in μm²/s at 60 min for TRIF−/− macrophage (−0.175 ± 0.032, n = 540) showed significant difference (63% higher than wild type) with the corresponding value in the case of wild type macrophage (Fig. 8B), indicating the role of TRIF in lowering the DC of LPS on the cell surface.
DISCUSSION

In this study, TIRFM visualization of the molecular action of LPS on the peritoneal macrophages of wild type C57BL/6 as well as the mice deficient in other LPS receptors/adaptor molecules revealed the diversity of the molecular behavior of LPS.

Analysis of molecular kinetics of fluorescence-labeled LPS in wild type murine macrophages revealed an increase in the number of spots with higher FI and a decrease in DC in a time-dependent manner. This observation is in line with Iino et al. (27, 28), who showed that with the increase of fluorescence intensity, green fluorescent protein-conjugated integrin molecules tend to exhibit lower diffusion rates. They contended that this increase in fluorescence was due to oligomerization. Sako et al. (12) also showed that when epidermal growth factor (EGF) molecules bind with its receptor, the FI rises because of dimerization of EGF-EGF receptor complexes, which is the early step in the signal transduction of EGF. Accordingly, the time-dependent increase in the spots with higher FI accompanied by the increase in the spots with lower DC in our study can be comprehended as the result of the oligomerization of LPS in the plasma membrane of macrophages.

The statistical mean FI was almost the same at 10 and 30 min, followed by a significant increase at 60 min after LPS administration (Fig. 5A). As mentioned under “Results,” however, the appearance of spots with FI levels of 70–75 AU and apparent reduction of the spots with FI levels of ~60 AU at 30 min compared with the result at 10 min (Fig. 4B) indicate that oligomerization of LPS had already started ~30 min after the administration in wild type macrophages. No significant difference between mean FI at 10 and 30 min in this study can be attributed to the considerable number of spots with FI below 55 AU still existing at 30 min. One probable explanation regarding this distribution of FI of fluorescence-labeled LPS at 30 min is that a number of LPS were still binding to the plasma membrane of macrophages, whereas some membrane-bound LPS had already started the formation of oligomers.
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On the other hand, a statistically significant increase in the mean FI at 60 min after LPS administration in wild type macrophages was due to a striking reduction in the number of spots with FI levels below 55 AU accompanied by a remarkable increase in the number of spots above 70 AU. This fact may indicate a decline of binding of LPS to plasma membrane and accelerated progression of oligomerization of formerly membrane-bound LPS.

Regarding the relationship between FI and DC of individual fluorescence-labeled LPS, we did not find any positive relationship between these two parameters; rather, a tendency of the increase in mean FI with decrease of DC was observed (Figs. 4 and 5B). It is worth mentioning here that DC is very sensitive and can be affected by certain factors, e.g. time scale of observation, type and mobility of the cells. Oligomers are also trapped in membrane skeletal fence (28). In our study, when we analyzed the MSD of 100 fluorescence-labeled LPS spots, a restricted movement pattern was observed. However, MSD of only two arbitrarily selected fluorescence-labeled LPS spots against time showed linear pattern (Fig. 3C). This indicates that, at the point of observation, although some of the molecules were moving freely on the surface, some had already bound with their receptors and signaling molecules, which caused the overall rate of diffusion to appear restricted.

Direct visualization of the role of LBP and CD14 in molecular dynamics of LPS on living cells is yet to be reported. Although the obligatory role of LBP and CD14 in LPS signaling has been reported in different studies, available reports only demonstrate the importance of interaction of LPS with LBP and CD14 in mediating cellular response (18–20). Using LPS with and without LBP in CD14−/− and wild type macrophages, respectively, our TIRFM observation revealed the absolute necessity of LBP and CD14 in the initiation of kinetics of LPS on the surface of the living cell.

Next, the differences in the values of mean FI and mean DC of LPS at different time intervals between wild type macrophages and TLR4−/−, MD2−/−, MyD88−/−, and TRIF−/− macrophages were shown (Fig. 8). The difference was most significant at 60 min of LPS stimulation. In the case of FI of fluorescence-labeled LPS spots, the mean values at 60 min of LPS administration varied in the following order: wild type (59.53 ± 0.35 AU) > TRIF−/− (53.40 ± 0.31 AU) > MyD88−/− (52.46 ± 0.33 AU) > MD2−/− (50.63 ± 0.27 AU) > TLR4−/− (48.78 ± 0.20 AU). The graph of spots/µm² of cells versus time followed the same order as that of mean FI of LPS spots at 60 min (Fig. 9). Our results suggest that among all of these signaling molecules, TLR4 plays the major role for oligomerization of LPS. The above comparison also indicates the more important role of surface molecules (TLR4 and MD2) for the dynamics of LPS over the intracellular adaptor molecules (MyD88 and TRIF) of LPS (Fig. 8A). In line with the available reports that LPS-initiated signaling begins with the cross-linking and clustering of surface TLR4-MD2 (29), a nondispersed pattern of FI and lower mean FI was demonstrated in the case of TLR4−/− and MD2−/− macrophages compared with wild type macrophages at 60 min after LPS administration, suggesting the failure of LPS molecules to form oligomers with its surface receptors (Figs. 7, B and C, and 8A).

Contrary to the time-dependent decrease of the mean value of DC in wild type macrophages, the mean values of DC at 60 min were more or less similar to their respective initial values in TLR4−/−, MD2−/−, MyD88−/−, and TRIF−/− macrophages. However, the values were significantly higher than the values in the case of wild type. DC of LPS in the case of TLR4−/−, MD2−/−, MyD88−/−, and TRIF−/− macrophages showed a trend reverse of the corresponding FI (Fig. 8). At 60 min of LPS administration, the mean logarithm of DC of LPS expressed in µm²/s varied in the following order: MyD88−/− (−0.113 ± 0.033) > TRIF−/− (−0.175 ± 0.032) > MD2−/− (−0.231 ± 0.032) > TLR4−/− (−0.283 ± 0.031) > wild type (−0.467 ± 0.036). Although the FI
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profiles in MyD88−/− and TRIF−/− macrophages indicated the formation of smaller oligomers, this oligomerization did not affect their DC; rather higher diffusion rates were observed. The mean values of DC in the absence of MyD88 and TRIF molecules were significantly higher than those of the cell surface receptors of LPS (TLR4 and MD2). Taking this observation into account, it may be stated that MyD88 and TRIF play a critical role in lowering the diffusion rate of LPS after 60 min in the case of wild type.

The rise in diffusion rates after 30 min of LPS administration compared with 10 min of LPS administration were observed in TLR4−/−, MD2−/−, MyD88−/−, and TRIF−/− macrophages (Fig. 8B). The respective mean DC values at 30 min for TLR4−/−, MD2−/−, MyD88−/−, and TRIF−/− were −0.079 ± 0.030, −0.172 ± 0.032, −0.086 ± 0.032, and 0.071 ± 0.033, and these values are statistically different (p < 0.01) from the value for wild type cell (−0.295 ± 0.033) at the same time point. The reason for the rise of DC in these knock-out macrophages after 30 min of LPS administration is not clear. One probable explanation may be that the failure of LPS to associate with these molecules critically contributed to the rise in DC of LPS on the plasma membrane in these knock-out macrophages at this time point. It is worth noting here that our TIRFM observation also revealed significantly higher DC of Alexa 594 LPS in HEK293 cells solely transfected with human CD14 gene than in human CD14/TLR4/MD2 cotransfected HEK 293 (data not shown). On the other hand, diverse reasons may have contributed to the decrease in DC at 60 min compared with 30 min in these knockout macrophages. As indicated by previous studies, the DC of molecules on plasma membrane is affected by a variety of factors, and it may not follow any certain rule. It can be affected by a variety of different factors like membrane skeletal fence, time scale of observation, and type and mobility of the cells (28).

To clarify the effects of oligomerization in subcellular internalization of LPS, we performed confocal microscopy using wild type and TLR4−/−, MD2−/−, MyD88−/−, and TRIF−/− peritoneal macrophages. It has been reported that TLR4 and MD2 are essential for signaling but not for internalization of LPS (29–31). In line with the available reports, our data also showed that in the case of wild type as well as LPS receptors and signaling molecule-deficient macrophages, LPS molecules start to get entry into the Golgi apparatus and endosomal vesicles within a few minutes of LPS administration (data not shown). It should, however, be noted that confocal microscopy does not provide quantitative information about the extent of internalization. TIRFM data confirmed the differences in the diffusion rate of LPS in TLR4−/−, MD2−/−, MyD88−/−, and TRIF−/− macrophages, and under our experimental conditions the diffusion rates were such that even after 60 min, internalization was not complete, and accordingly oligomers were observed on the surface of the macrophages. Recently, endosome was reported to be the pivotal site for TRIF-dependent signal transduction of LPS (32). It was also shown that the TRIF-related adaptor molecule is essentially involved in the translocation of LPS/TLR4 complex into the endosomal vesicles. That study, however, did not exclude the possibility of the interaction of TRIF with the LPS-TRL4 complex at the plasma membrane level. The change in molecular action of LPS on the plasma membrane of TRIF−/− macrophages in our direct observation by TIRFM potently indicates the association of TRIF with the LPS-TLR4 complex in the plasma membrane of cells.

This study was carried out to directly visualize the molecular action of LPS on the plasma membrane of mouse peritoneal macrophages. LPS is known to play a dominant role in life-threatening disease sepsis. According to our knowledge, no study unveiling the differences of molecular dynamics of LPS in a wide variety of LPS signaling molecule-deficient cells is available. This is the first comprehensive investigation carried out to observe the differences of kinetics of LPS in a wide variety of receptor and adopter molecule knock-out mice employing the TIRFM technique. In our study, through direct visualization, we observed marked differences in molecular action of LPS in the case of wild type, TLR4−/−, MD2−/−, MyD88−/−, and TRIF−/− macrophages. From our observation we propose a hypothesis that LPS receptor complexes (TLR4 and MD2) play a major role in oligomerization, whereas intracellular signaling molecules such as MyD88 and TRIF are important for lowering the diffusion rates of LPS on the surface of the cell. Based on the findings in this study, it is likely that not only LPS but the receptors with the intracellular domain of other various ligands are responsible for the oligomerization of the ligands, and the association and activation of intracellular signaling molecules may be involved in lowering the diffusion rate of the ligands on the plasma membrane of living cells. Further studies employing TIRFM technique regarding the molecular dynamics of other ligands on the surface of living cells would reveal the validity of this speculation.

Acknowledgments—We thank Yuya Terashima and Mikiya Otsuji (Department of Molecular Preventive Medicine, Graduate School of Medicine, The University of Tokyo) for kind assistance in operating TIRFM.

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