[課程―２]

審査の結果の要旨

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Sepsis describes a complex clinical syndrome that results from dysregulation in host response to infection. The resulting immunosuppression has been suggested to be a major contributing factor in sepsis induced mortality. Lipopolysaccharide (LPS) or endotoxin plays a pivotal role in initiation of sepsis; it maintains the structural and functional integrity of gram negative bacterial outer membrane. LPS is carried into the body with a specific carrier protein—LPS binding protein (LBP). Then it interacts with CD14, a receptor on macrophages/monocytes and neutrophils. TLR4, with the help of another co-receptor MD2 mediates intracellular invasion of LPS. MyD88 and TRIF are known as intracellular signaling molecules which are essential for transcription of numerous genes of pro-inflammatory mediators in response to LPS.

Evanescent wave microscopy, also termed total internal reflection fluorescence microscopy (TIRFM), has shed new light on important cellular processes taking place near the plasma membrane. For example, this technique can enable the direct observation of membrane fusion of synaptic vesicles and the movement of single molecules during signal transduction. TIRFM typically involves very thin (less than 100 nm) optical sectioning which means that the signal to noise (S/N) ratio is much better than with confocal images, and cellular photodamage and photobleaching are also minimal. It has proven useful for quantitative analysis of the dynamics and kinetics of cell signaling reactions.

The aim of this study is to reveal the molecular kinetics of LPS on the plasma membrane of wild type and $CD14^{-/-}$, $TLR4^{-/-}$, $MD2^{-/-}$, $Myd88^{-/-}$ or $TRIF^{-/-}$ murine peritoneal macrophages by total internal reflection fluorescence microscope (TIRFM) which is an ideal means to study the molecular mechanism of biological interaction of cellular molecules in vitro.

The results of this study have been described below:

1. We stimulated mouse peritoneal macrophages with commercially available Alexa 594-labeled LPS (100% fluorescence labeled LPS) and unlabeled LPS of same strain. Our data showed that both fluorescence labeled and unlabeled LPS triggered almost the same amount of TNF-α. It proves that fluorescence labeled LPS that we used in our experiment is immunologically functional.

2. The molecular action of Alexa 594-labeled LPS from E.coli was examined on the living peritoneal macrophages of C57BL/6 mice by TIRFM, and the molecular kinetics of LPS was analyzed. 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. When we analyzed MSD of only 2 Alexa 594-labeled LPS spots against time, our graph showed 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 molecules diffusion becomes restricted.

3. Compared with 5-15 mins after LPS administration, the number of spots in the higher fluorescence range progressively increased for the case of 25-35 mins and 55-65 mins after LPS administration. At 5-15 mins the range of FI was 40-70 A.U.. The range extended up to 75 at 25-35 mins along with apparent decrease in the number of spots with FI around 60 A.U. in comparison to the result at 5-15 mins after LPS administration, while many spots with FI below 55 A.U. still could be found at 25-35 mins. Compared with the case of 5-15 mins after LPS administration, the diagram of number of spots vs. FI after 55-65 mins of LPS administration showed a more diffused pattern with several distributed peaks, which indicates formation of larger oligomers at 55-65 mins. 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 diffusion coefficient (DC). The statistical analysis of the data at different time intervals confirmed the apparent time dependent change of both FI and DC. The mean FI of 55.73±0.31 A.U. (N=520) after 10 mins increased to 59.53±0.35 A.U. (N=524) after 60 mins of LPS administration, while the corresponding mean logarithms of DC values expressed in μm^2/sec were -0.156±0.026 (N=520) and -0.467±0.036 (N=524), respectively. The number of fluorescence labeled LPS spots/μm^2 of wild type macrophages showed gradual increase with time indicating higher binding of LPS spots with its concerning receptors and adaptor molecules.

4. In our experiments, consistent with the previous reports on the importance of LBP and CD14 in the initiation of cellular response to LPS, no motion of LPS on the plasma membrane was observed in absence of LBP or $CD14^{-/-}$ cases indicating the essential role of LBP and CD14 receptors for the movement and oligomerization of LPS on the living cells.

5. While after 55-65 mins of LPS administration the fluorescence spots ranged in between 40-87 A.U. in case of wild type macrophage, spots were not found beyond 60 A.U. in case of $TLR4^{-/-}$ macrophage. In fact the mean FI of LPS at 60 mins in case of $TLR4^{-/-}$ 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. On the other hand, the mean logarithm of DC of LPS expressed in μm^2/sec after 60 mins in $TLR4^{-/-}$ macrophages was higher (-0.283±0.031, N=498) than that of the wild type macrophages (-0.467±0.03, N=524). The number of fluorescence labeled LPS spots/μm^2 incase of $TLR4^{-/-}$ macrophage was only 3 at 60 mins of LPS administration whereas in wild type macrophages the number was 10 spots/μm^2 at the same time point. It is likely that binding of fluorescence labeled LPS spots with its receptors is the lowest incase of $TLR4^{-/-}$ among all the studied macrophages.

In our study, as seen in $TLR4^{-/-}$ macrophages, a narrower range of FI of LPS spots (40-69 A.U.) in comparison to the case of wild type (40-87 A.U.) was also observed in case of $MD2^{-/-}$ macrophages. Among all the studied deficient macrophages, the mean FI

of LPS after 60 mins in case of $MD2^{-/-}$ macrophages was the second lowest (50.63±0.27 A.U, N=523).

6. A broader range of FI (up to 76 A.U.) as well as DC of LPS spots in comparison to the cases of LPS receptors (TLR4, MD2) was observed in absence of MyD88. As compared with the wild type macrophage, the specific pattern of the diagram of number of spots vs. fluorescence intensity of LPS in $MyD88^{-/-}$ macrophages indicated formation of smaller oligomers. On the other hand, the mean logarithm of DC of LPS expressed in μm^2/sec in 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. In our study, the importance of MyD88 was manifested by higher diffusion rate of LPS on the cell surface in case of $MyD88^{-/-}$ macrophages.

In case of $TRIF^{-/-}$ macrophages the diagram of number of spots vs. fluorescence intensity at 55-65 mins 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 A.U.). Fewer peaks of higher FI in the same diagram indicated lower extent of oligomerization than in the case of wild type macrophage. When compared to the macrophages deficient in LPS receptors (TLR4, MD2), the mean logarithm of DC of LPS expressed in μm^2/sec at 60 mins in case of $TRIF^{-/-}$ 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, indicating the role of TRIF in lowering DC of LPS on the cell surface.

7. Data of our experiment clearly showed that LPS molecules get entry into the golgi apparatus and endosomal vesicles within a few minutes of LPS administration. After 30 mins of stimulation with E.coli LPS, fluorescence labeled LPS molecules co-localized with lysosome marker in wild type and other knock out macrophages.On the other hand, after 20 mins LPS molecules were clearly detectable in Golgi apparatus of wild type as well as other knock out macrophages where Alexa-594 labeled LPS merged with BIODIPY C_5 Ceramide stained Golgi complex.

The presence of all the receptors is not obligatory for the internalization of the LPS molecules into the organelles of a living cell.

This is the first comprehensive investigation carried out to observe the differences of kinetics of LPS in a wide variety of receptor and adaptor molecule knock out mice employing TIRFM technique. Based on the findings in this study, it is likely that not only LPS but the receptors with 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.

This study contributes significantly to the knowledge base of molecular interaction of LPS with its concerning receptors as well as the role of adaptor molecules in signal transduction. The current study bears an important significance for future research by revealing the respective roles of the LPS receptors and signaling molecules in the molecular behavior of LPS on the living cell surface by direct visualization.