Membrane Sorting of Toll-like Receptor (TLR)-2/6 and TLR2/1 Heterodimers at the Cell Surface Determines Heterotypic Associations with CD36 and Intracellular Targeting

Martha Triantafilou, Frederick G. J. Gamper, Rowenna M. Haston, Marios Angelos Mouratis, Siegfried Morath, Thomas Hartung, and Kathy Triantafilou

From the 1Infection and Immunity Group, School of Life Sciences, University of Sussex, Falmer, Brighton, BN1 9QG, United Kingdom and 2Department of Biochemical Pharmacology, University of Konstanz, D-78457 Konstanz, Germany

Toll-like receptors (TLRs) are receptors of the innate immune system responsible for recognizing pathogen-associated molecular patterns. TLR2 seems to be the most promiscuous TLR receptor able to recognize the most diverse set of pathogen-associated patterns. Its promiscuity has been attributed to its unique ability to heterodimerize with TLRs 1 and 6 and, most recently, to its association with CD36 in response to diacylated lipoproteins. Thus, it seems that TLR2 forms receptor clusters in response to different microbial ligands. In this study we investigated TLR2 cell surface heterotypic interactions in response to different ligands as well as internalization and intracellular trafficking. Our data show that TLR2 forms heterodimers with TLR1 and TLR6 and that these heterodimer pre-exist and are not induced by the ligand. Upon stimulation by the specific ligand, these heterodimers are recruited within lipid rafts. In contrast, heterotypic associations of TLR2/6 with CD36 are not preformed and are ligand-induced. All TLR2 receptor clusters accumulate in lipid rafts and are targeted to the Golgi apparatus. This localization and targeting is ligand-specific. Activation occurs at the cell surface, and the observed trafficking is independent of signaling.

The recent study by Hoebe et al. (23) has given us a clearer picture of receptor associations in response to different TLR2 microbial stimuli, questions still remain. Does TLR2 form different heterotypic associations in response to different stimuli? Is the mechanism of innate recognition via TLR2 similar to TLR4, which seems to form activation clusters in response to different stimuli composed of different receptors depending on the microbial ligand (24, 25)? Most importantly, do these heterotypic associations pre-exist, or are they induced by the ligand? Finally do the different TLR2 microbial ligands trigger signaling in a similar manner, from the surface or intracellularly?
In this study we have chosen to investigate the cell surface heterotypic interactions as well as the intracellular trafficking of TLR2 and associated molecules in response to different microbial ligands. Using fluorescent imaging techniques, we investigated the localization of TLR2, TLR1, TLR6, and CD36 at the cell surface as well as defined the subcellular localization and trafficking of these receptors. Here we show that different TLR2 microbial ligands induce the formation of different receptor clusters on the cell surface within lipid rafts. We demonstrate for the first time the direct association of TLR2/TLR6 and CD36 in response to bacterial LTA as well as diacylated lipoproteins, whereas TLR2/TLR1 heterodimers were formed in response to triacylated lipoproteins in the absence of CD36. The heterodimers formed between TLR2/TLR6 or TLR2/TLR1 seem to pre-exist and to internalize along with the corresponding ligand and rapidly targeted the Golgi apparatus independently of the clathrin-interacting endocytic machinery. In contrast, receptor clusters involving CD36 are not preformed but are formed within lipid rafts upon ligand binding and also targeted to the Golgi apparatus. Signaling is triggered at the cell surface, and thus, CD36 must act as a facilitator associating and presenting the ligand to the TLR heterodimer on the cell surface, and subsequently, the whole complex must be internalized and targeted to the Golgi apparatus.

Thus, it seems that TLR2 utilizes the same molecular mechanisms as TLR4. Similar to TLR4, TLR2 forms activation clusters composed of several receptors depending on the ligand; these clusters trigger signaling from the cell surface and subsequently are targeted to the Golgi in a lipid-raft dependent pathway.

**EXPERIMENTAL PROCEDURES**

**Materials**—LTA from *Staphylococcus aureus* was prepared as previously described (26). The N-terminal part of the lipoprotein LP44 of *Mycoplasma salivarium* (FSL-1) and N-palmitoyl-S-dipalmitoylglycerol (Pam₃) Cys-Scr-(Lys)₄ (CSK₄) were obtained from EM Microcollections GmbH (Tubingen, Germany). EEA1-specific polyclonal serum was obtained from HyCult (Denmark). TLR6-specific mAb were obtained from the American Type Culture Collection (ATCC, Manassas, VA). TLR2-specific antibody, TLR2.1, as well as TLR1-specific antibody were purchased from HyCult (Denmark). TLR6-specific antibodies were purchased from Santa Cruz. GM130, Golgi-specific antibody, was purchased from BD Biosciences. The antibodies were conjugated to either Cy3 or Cy5 using Cy3 and Cy5 labeling kits from Amersham Biosciences. Cholera toxin was purchased from List Labs.

**Stable Cell Lines**—Stable transfections of HEK293 cells with pcDNA3, GFP-TLR2, or CD14 were performed using Superfect transfection reagent (Qiagen) according to the manufacturer’s recommendations as previously described (27). Positive selection by fluorescence-activated cell sorting was performed. Clonal cell lines were obtained by limiting dilution. Transfected cell lines were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 0.5 units/ml penicillin, 0.5 µg/ml streptomycin, 400 µg/ml G418 for HEK/pcDNA3, HEK/GFP-TLR2, HEK/CD14, HEK/GFP-TLR2/CD14, and 400 µg/ml hygromycin for HEK/GFP-TLR2/CD14.

Stably transfected cells were transiently transfected with puno-TLR1, TLR6, and/or CD36 plasmids (Invitrogen) using Lipofectamine 2000 according to the manufacturer’s recommendations.

**Isolation of Human Monocytes**—Monocytes were isolated from human A⁺ buffy coats. Adherent cell monolayers (1 × 10⁵ to 2 × 10⁶ monocytes/well) were cultured in 24-well plates in serum free medium (Invitrogen) supplemented with 0.01% L-glutamine and 40 µg/ml gentamicin/ml.

**Cytokine Assays**—Stably transfected HEK cells or human monocytes were plated on 24-well dishes (Nunc, Germany) at a concentration of 1 × 10⁵/ml in 300 µl of Dulbecco’s modified Eagle’s medium and 10% fetal calf serum. The next day cells were stimulated for the indicated time period. The supernatants were collected and frozen until the cytokine assays were performed. The BD Biosciences bead array system was used to determine the level of TNF-α.

**Cell Labeling for Fluorescence Resonance Energy Transfer (FRET)**—Human monocytes were labeled with 100 µl of a 1:1 mixture of donor-conjugated antibody (Cy3) and acceptor-conjugated antibody (Cy5). For control experiments, to determine whether FRET is dependent on donor and acceptor surface density, the donor:acceptor ratio was varied to 1:2 and 1:4. The cells were rinsed twice in phosphate-buffered saline, 0.02% bovine serum albumin before fixation with 4% formaldehyde for 15 min. The cells were fixed to prevent potential re-organization of the proteins during the course of the experiment.

**FRET Measurements**—FRET is a non-invasive imaging technique used to determine molecular proximity. FRET can occur over more than 1–10-nm distances and effectively increases the resolution of light microscopy to the molecular level. It involves non-radiative transfer of energy from the excited state of a donor molecule to an appropriate acceptor. The rate of energy transfer is inversely proportional to the sixth power of the distance, between donor and acceptor. The efficiency of energy transfer (E) is defined with respect to r and Rₒ, the characteristic Forster distance by \( E = 1/(1 + (r/Rₒ)^6) \). In the present study, FRET was measured using a method as previously described (28, 29).

**Fluorescent Imaging**—HEK/GFP-TLR2/CD14 transiently transfected cells with TLR1, TLR6, and/or CD36 were grown on microchamber culture slides (Labtek slides, Nunc). Cells were stimulated with either 10 µg/ml LTA, Pam₃CSK₄, or FSL-1 for 30 min. After stimulation they were fixed with 4% formaldehyde for 15 min and labeled with fluorescent antibodies in phosphate-buffered saline, 0.02% bovine serum albumin, 0.02% saponin. Then they were washed three times with phosphate-buffered saline. The slides were mounted on Prolong antifadant (Molecular probes).

Cells were imaged on a Carl Zeiss, Inc. LSM510 META confocal microscope (with an Axiovert 200 fluorescent microscope) using a 1.4 NA 63× Zeiss objective. The images were analyzed using LSM 2.5 image analysis software (Carl Zeiss, Inc.). The different fluorophores were detected using the appropriate filter sets.
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![Graph showing TNF-α secretion in response to different bacterial ligands.](image)

**FIGURE 1.** TNF-α secretion in response to different bacterial ligands. Human monocytes as well as HEK cells transfected with CD14/CD36/TLR2/1/6 were either not stimulated (white histograms) or stimulated with 10 μg/ml LTA (black histograms), 100 ng/ml LPS (checked histograms), 10 nM Pam3CSK₄ (striped histograms), or 10 nM FSL-1 (gray histograms) for 4 h at 37 °C under 5% CO₂. TNF-α content was determined using the Th1/Th2 Cytometric Bead array system (BD Biosciences). The mean and S.D. from a number of independent experiments is presented.

**Luciferase Reporter Assays for NF-κB Activation**—HEK293 cells transfected with CD14, GFP-TLR2, TLR6/1, and CD36 were seeded into 96-well plates. The following day the cells were transiently transfected with an NF-κB luciferase reporter gene using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. The next day the cells were stimulated as indicated, and after 6 h of stimulation, the cells were lysed in passive lysis buffer (Promega). Luciferase activity was measured using a plate reader luminometer.

**RESULTS**

**TNF-α Secretion in Response to Different TLR2 Ligands**—Before investigating whether different TLR2 ligands induce the formation of different receptor clusters, we investigated cytokine production in response to the different ligands that we were going to utilize in this study. Human monocytes were stimulated with LTA, PAM₃CSK₄ (triacyl-lipopeptide), and FSL-1 (bisacyl-lipopeptide) as well as LPS as a control.

We measured TNF-α production in response to the different TLR2 ligands (Fig. 1). We found that all TLR2 ligands (LTA, PAM₃CSK₄, FSL-1) triggered similar levels of TNF-α secretion.

In addition to human monocytes, in this study we transfected HEK293 cells with CD14/GFP-TLR2, TLR6/1, and CD36 and either TLR1 or TLR6 to be able to define the subcellular localization and trafficking of TLR2 receptor complexes before and after stimulation by different TLR2 ligands. HEK293 cells are normally unresponsive to microbial ligands since they lack TLR14 and TLR expression.

To establish that transfection of HEK293 cells with CD14 and TLR2/1 or TLR2/6 rendered them able to respond to the different ligands, we measured TNF-α secretion in HEK293, HEK/CD14, HEK/CD36, HEK/TLR2/1, HEK/TLR2/6, HEK/CD14/CD36/GFP-TLR2/1, HEK/CD14/CD36/GFP-TLR2/6 cells after stimulation by LTA, PAM₃CSK₄, or FSL-1. In parallel, as a control HEK293, HEK/CD14, HEK/CD36, HEK/TLR2/1, HEK/TLR2/6, HEK/CD14/CD36/GFP-TLR2/1, HEK/CD14/CD36/GFP-TLR2/6, and human monocytes were stimulated with 10 μg/ml LTA and 100 ng/ml LPS. After stimulation, supernatants were collected and analyzed for TNF-α using a flow cytometric bead array system.

As expected it was shown that HEK293 cells were incapable of responding to any of the microbial ligands. Transfection with CD14, TLR2/1, or TLR2/6 alone did not make them responsive to bacterial products, whereas transfection with CD14 and TLR2/1 rendered them responsive to Pam3CSK4 but not to LPS (Fig. 1, striped bars). In contrast, transfection with TLR2/6 rendered them responsive to LTA and FSL-1 but not to LPS (Fig. 1, black and checked bars). From these data we conclude that transfection of HEK293 cells with both CD14 and TLR2/1 renders them responsive to triacylated lipopeptides, whereas transfection with CD14, CD36, and TLR2/6 enables them to respond to diacylated lipopeptides.

**Heterotypic Interactions of TLR2**—It has previously been demonstrated using biochemical techniques as well as knockout mice that TLR2 forms heterodimers with either TLR1 or TLR6 in response to different ligands (13, 21) as well as CD36 (23). One of the questions that remains is whether these associations are preformed or whether they are ligand-induced. In this study we set out to investigate TLR2 heterotypic associations before and after stimulation by LTA, PAM₃CSK₄, or FSL-1 by utilizing FRET.

We measured FRET in terms of dequeenching of donor fluorescence after complete photobleaching of the acceptor fluorophore. Increased donor fluorescence after complete destruction of the acceptor indicated that the donor fluorescence was quenched in the presence of the acceptor because of energy transfer. We tested the energy transfer efficiency in our system using as a positive control the energy transfer from mAbs Cy3-W6/32 and Cy5-MCA1115 to two different epitopes on major histocompatibility complex class I molecules, which showed that the maximum energy transfer efficiency ($E$) was 35 ± 1.0.

To determine TLR2 heterotypic interactions before and after stimulation by bacterial products, we proceeded to measure FRET on human monocytes before stimulation between TLR2 (using Cy3-specific TLR2 mAb) and TLR1, TLR6, or CD36 (Cy5-specific mAbs). TLR2 was found to associate to some extent with TLR1 and TLR6 before stimulation, thus suggesting that TLR2/1 and TLR2/6 heterodimers pre-exist (Fig. 2, white bars). In contrast, TLR2 was found not to associate with CD36 before stimulation by bacterial products (Fig. 2); thus, TLR2 associations with CD36 are ligand-induced.

To determine whether TLR2 associated with these molecules after stimulation, human monocytes were isolated from periph-
eral blood of healthy donors and stimulated for 10 min at 37 °C with either 10 µg/ml LTA, 10 nM FSL-1, or 10 nM Pam₃CSK₄ before fixation and labeling with the fluorescent probes. Energy transfer between TLR2-Cy3 and Cy5-labeled antibodies against CD14, TLR1, TLR6, or CD36 was measured. As expected upon LTA stimulation, TLR2 associated with CD14, TLR6, and CD36 since large dequenching was observed after Cy5-CD14, Cy5-TLR6, and Cy5-CD36 photobleaching (Fig. 2), suggesting that upon stimulation there is further clustering of these molecules. Similar findings were found upon stimulation with the diacylated lipopeptide FSL-1. We observed large dequenching (E = 32 ± 1.0%) between TLR2 and TLR6 and between TLR2 and CD14 (E = 34 ± 2.0%) as well as TLR2 and CD36 (E = 30 ± 1.0%). In contrast, when cells were stimulated with Pam₃CSK₄, it was shown that TLR2 clustered with TLR1 but not with CD36 (Fig. 2).

To rule out the possibility that the FRET observed was due to random distribution, we varied the ratio of donors and acceptors used to label the proteins of interest (data not shown). E was found to be independent on acceptor density, to be sensitive to the donor:acceptor ratio, and not to go to zero at low surface density, thus suggesting that the FRET values observed were due to clustered molecules and not random associations.

TLR1, TLR6, and CD36 Associate with Lipid Rafts—Because TLR2 has been previously shown to reside within lipid rafts upon stimulation by LTA (27, 30), we proceeded to investi-

**FIGURE 3.** Associations of receptors with GM1 ganglioside before and after stimulation with bacterial products. Energy transfer between different receptors (Cy3-mAb) and GM1 ganglioside (Cy5-cholera-toxin) before (white histograms) and after stimulation by LTA (black histograms), Pam₃CSK₄ (striped histograms), FSL-1 (gray histograms), or LPS (checked histograms). Energy transfer (E%) can be detected by the increase in donor fluorescence after acceptor photobleaching. The percentage of energy transfer and S.D. was calculated from three independent experiments.

**FIGURE 4.** Intracellular distribution of TLR2, TLR1, TLR6, and CD36 in response to LTA. HEK/CD14/CD36/GFP-TLR2/1/6 cells were incubated with LTA and subsequently labeled with a Cy3-labeled anti-Golgi antibody (red) and antibodies against either TLR1, TLR6, or CD36 (Cy5). The cells were fixed and imaged using the Zeiss 510 META confocal microscope. The merged image shows extensive overlay of areas positive for Golgi, TLR2, and either TLR6 or CD36 (shown as white). Scale bar, 20 µm.
gate whether these TLR2-receptor clusters were formed in lipid rafts. We measured FRET on monocytes between the different receptor molecules and GM1 ganglioside, a raft-associated lipid (using Cy5-cholera toxin). Initially we investigated whether TLR6 and CD36 were present within lipid rafts before stimulation. It was found that CD36 was present in lipid rafts before stimulation, but TLR6 was not, since there was no energy transfer (Fig. 3). In contrast, upon stimulation with either LTA or FSL-1, large dequenching was observed once the Cy5 was photobleached, with $E = 26 \pm 0.5\%$ and $30 \pm 1.0\%$, respectively, suggesting that TLR6 concentrates in lipid rafts upon stimulation with bacterial products (Fig. 3). Control experiments were performed after Pam3CSK4 or LPS stimulation. The results showed that there was minimal energy transfer, thus suggesting that only stimulation with diacylated lipoproteins induces the association of TLR6 with lipid rafts and the subsequent clustering with CD14 and CD36, which are resident lipid-raft proteins.

We similarly examined whether TLR1 receptor molecules localize in lipid rafts before Pam3CSK4 stimulation by using Cy3-TLR1-specific fluorescent probe and Cy5-cholera toxin for the GM1 ganglioside. Our results showed that there was no energy transfer observed between TLR1 and GM1 ganglioside ($6 \pm 1.0\%$); thus, TLR1 was not present in lipid rafts before stimulation by bacterial products (Fig. 3). In contrast, there was large dequenching between TLR1 and GM1 ganglioside after Pam3CSK4 stimulation ($27 \pm 1.5\%$), suggesting that similarly to TLR6, TLR1 does not reside in lipid rafts before stimulation but is recruited there after ligand binding (Fig. 3). Control experiments investigating the association of TLR1 with GM1 ganglioside after LTA, FSL-1, or LPS stimulation were also performed. It was shown that TLR1 was not recruited in lipid rafts after LTA, FSL-1, or LPS stimulation, since there was no energy transfer observed between TLR1 and GM1 ganglioside after stimulation by these ligands (Fig. 3). In addition, control experiments were performed investigating the recruitment of TLR4 in lipid rafts in response to different bacterial stimuli (LTA, FSL-1, Pam3CSK4, and LPS). It was shown that TLR4 associated with GM1 ganglioside only in response to LPS, thus demonstrating that recruitment to lipid rafts is specific to the microbial ligand.

**Internalization of TLR2 Receptor Clusters after LTA Stimulation**—Because TLR2 seemed to form different receptor clusters in response to different stimuli, we proceeded to investigate whether the complexes formed followed similar targeting routes. We had previously investigated the internalization of TLR2 and CD14 in response to LTA and had found that they are both targeted to the Golgi apparatus (27). In this study we...
investigated whether the whole complex comprising CD14, TLR2, TLR6, and CD36 internalizes and is targeted to the same intracellular compartment.

HEK/GFP-TLR2/CD14 cells transiently transfected with CD36, TLR1, and TLR6 were incubated for 30 min with 10 nM Pam$_3$CSK$_4$ (triacyl-lipopeptide) and subsequently labeled with a Cy3-labeled anti-Golgi antibody (red) and antibodies against either TLR1, TLR6, or CD36 (Cy5). The cells were fixed and imaged using the Zeiss 510 META confocal microscope. The merged image shows extensive overlay of areas positive for Golgi, TLR2, and TLR1 (seen as white). Scale bar, 20 μm.

**FIGURE 6. Intracellular distribution of TLR2, TLR1, TLR6 and CD36 in response to Pam$_3$CSK$_4$.** HEK/CD14/CD36/GFP-TLR2/1/6 cells were incubated with 10 nM Pam$_3$CSK$_4$ (triacyl-lipopeptide) and subsequently labeled with a Cy3-labeled anti-Golgi antibody (red) and antibodies against either TLR1, TLR6, or CD36 (Cy5). The cells were fixed and imaged using the Zeiss 510 META confocal microscope. The merged image shows extensive overlay of areas positive for Golgi, TLR2, and TLR1 (seen as white). Scale bar, 20 μm.

**TLR2/TLR6/CD36 Are Targeted to the Golgi**
with either filipin or nystatin prevented TLR6/CD36 colocalization to the Golgi (Fig. 7), leading us to believe that the internalization of the receptor complex is dependent on lipid raft integrity. Similarly, we investigated whether lipid-raft-disrupting drugs could inhibit TLR2/TLR1 targeting to the Golgi apparatus. It was shown that treatment with either filipin or nystatin prevented TLR2/TLR1 colocalization with the Golgi in response to triacylated lipoproteins (Pam3CSK4) (data not shown). Thus, it seems that membrane partitioning at the cell surface determines the subsequent internalization route that the complex is going to follow. The cells viability was not affected either by nystatin or filipin treatment because the drug-treated cells excluded trypan blue.

**TLR2/6 Trafficking in the Presence and Absence of CD36**—To determine whether the trafficking and targeting of TLR2/6 heterodimers depended on CD36, we proceeded to investigate the trafficking of TLR2/6 in the absence of CD36.

In the absence of CD36, in HEK-CD14/TLR2/6 cells there was rapid targeting to the Golgi apparatus. Using triple labeling, we were able to visualize co-localization of TLR2, TLR6, and the Golgi (Fig. 8), thus suggesting that the intracellular targeting to the Golgi is not CD36-dependent.

Receptor Clustering on the Cell Surface Initiates Signaling—To verify that the signaling is initiated from the plasma membrane, we investigated the cell surface TLR6 distribution in response to LTA. HEK/CD14/GFPTLR2/6 cells were stimulated with 10 μg of LTA and imaged by confocal microscopy. It has shown that before stimulation, the distribution of TLR6 was diffuse on the cell surface (Fig. 9A). Upon LTA stimulation, TLR6 seems to form clusters. To investigate whether signaling was initiated via these clusters we analyzed the location of MyD88 before and after LTA stimulation. Before LTA stimulation, MyD88 was found to be expressed in diffused in the cytoplasm (Fig. 9A). Upon LTA stimulation, it was found that MyD88 was recruited close to the cell surface and co-localized with the TLR6 (Fig. 9B), thus suggesting that signaling is initiated via clustering of receptor molecules on the cell surface. Similar results were obtained with TLR2/1 heterodimers in response to Pam3CSK4 (data not shown).

**DISCUSSION**

In this study we set out to characterize TLR2 heterotypic associations in response to different ligands, such as LTA and diacylated (FSL-1) as well as triacylated (Pam3CSK4) lipoproteins. Initially we investigated using transfected cells which receptors are required for innate immune responses against these ligands. It was shown that LTA-induced responses as well as responses against diacylated lipoproteins required the presence of CD14, CD36, TLR2, and TLR6. CD14 or CD36 alone could not trigger responses against microbial diacylglycerides, suggesting that cellular activation is a result of synergic interactions among these molecules. In contrast, innate immune responses against triacyl lipoproteins required the presence of CD14, TLR2, and TLR1 but not CD36.

Because we had established which molecules were required...
for responses against different ligands, we proceeded to investigate whether there are molecular associations between these receptors in response to the different ligands. FRET experiments revealed that similarly to TLR4, TLR2 forms combina-
tional associations with receptors depending on the microbial stimuli. The only difference was that TLR2 existed in heterodimers with either TLR1 or TLR6 before stimulation. These heterodimers formed bigger oligomers in response to their corresponding ligands as well as further heterotypic associations with other receptors (CD14 and/or CD36). When we investigated the distribution of these receptor clusters on the plasma membrane, another similarity with TLR4 was found, in that these clusters were formed within membrane microdomains or lipid rafts. CD14, which is a glycosylphosphatidylinositol-linked protein, as well as CD36 were found to be constitutively localized in lipid rafts (34, 35), whereas TLR2, TLR1, and TLR6 were found not to reside in lipid rafts before stimulation but to increasingly accumulate in the raft upon the presence of the corresponding microbial ligand. Thus, it seems that it is common for TLR molecules not to be present in lipid rafts but to be recruited there upon stimulation. Associations with lipid-raft-resident molecules, such as CD14 and CD36, seem to be another common feature of the TLR mechanism of activation. Lipid-raft resident molecules seem to associate with the ligand and to recruit TLR molecules to the specialized area of the plasma membrane.

Lipid rafts do not only facilitate receptor interactions by compartmentalization of the plasma membrane but also seem to play a role in the internalization and intracellular targeting of the TLR activation clusters. In this study we utilized fluorescent imaging to track the entry route of the TLR2 receptor clusters within the cell. We utilized triple labeling fluorescent imaging to visualize TLR2, TLR1, and TLR6 CD36 as well as markers for intracellular compartments. We found that TLR2, TLR6, and CD36 were rapidly targeted to the Golgi apparatus within 30 min after LTA or dicycled lipoprotein stimulation. CD14 followed the same route, whereas TLR1 did not. In response to triacylated lipoproteins, TLR2/1 heterodimers were recruited in lipid rafts instead of TLR2/6 heterodimers,
and this recruitment determined their intracellular targeting. TLR2/1 heterodimers were internalized and targeted to the Golgi apparatus in response to triacylated lipoproteins, suggesting that the location of the activation cluster at the plasma membrane determined its intracellular fate. These data are in good agreement with previous studies performed with bacterial LPS (36, 37) as well as LTA (27).

In addition, TLR2-receptor cluster internalization seemed to be lipid raft-dependent. In the presence of lipid raft disrupting drugs, such as nystatin or filipin, targeting of CD14/TLR2/6/CD36 or CD14/TLR2/1 receptor clusters to the Golgi network was inhibited upon disruption of the lipid raft integrity. Our studies suggest that lipid raft formation is crucial for TLR2 receptor internalization and targeting. Thus, it is emerging that this membrane partitioning might play a major role in protein uptake and intracellular routing. It is becoming more apparent that this differential sorting on the cell surface might predispose the intracellular fate of a given molecule. The selective recruitment of receptors within the raft determines the responses that are going to be triggered in response to a particular ligand, and subsequently this differential sorting at the cell surface determines the fate of the entire complex (Fig. 10). The entire receptor complex seems to internalize and to be targeted to the Golgi apparatus.

The question that remains is whether this internalization and trafficking that we observe is crucial for TLR2-induced signaling. We had previously shown that LTA-induced TLR2 activation is independent of the internalization process (27), but a recent study by Stuart et al. (38) has suggested that responses against S. aureus require CD36-dependent phagocytosis. In this study we proceeded to investigate whether this was the case for other TLR2-ligands that employed CD36 as a co-receptor. To answer this question, we performed confocal experiments with HEK cells lacking CD36. These cells were shown to be able to internalize bacterial LTA and diacylated lipoproteins. In response to microbial diacylglycerides, TLR2 and TLR6 were internalized and rapidly targeted to the Golgi in the absence of CD36, thus suggesting that CD36 did not play a crucial role in the internalization and intracellular targeting of these receptor complexes. Brefeldin A disruption of the Golgi showed that TLR2/TLR6 localization in the Golgi was not essential for signaling and cytokine secretion. Similar to TLR4 (37), TLR2 was found to form clusters and to engage its ligand on the cell membrane where signaling was initiated. Thus our data are in agreement with Latz et al. (37), who have demonstrated that LPS internalization is independent of signal transduction but are in contrast to the recent report by Stuart et al. (38), who have showed that the internalization of S. aureus to the phagosome is dependent on CD36 and plays a crucial role for TLR2 signal transduction. The reason for these conflicting reports might be the fact that Stuart et al. (38) have used whole bacteria and not bacterial products, such as LTA or lipoproteins, in their study. It is possible that phagocytosis of whole bacteria, due to the difference in particle size, involves a completely different internalization mechanism when compared with bacterial products. Engagement of receptors and formation of clusters in response to whole bacteria could be completely different to those concerning bacterial products.

Our study, which utilizes bacterial products, demonstrates a common mechanism of activation that seems to be involved in TLR activation. Our study suggests that TLR2 forms combinational clusters in response to different microbial stimuli. Similarly to TLR4, these clusters are formed within lipid rafts. Lipid rafts seem to provide a confined space that accommodates
these receptor associations and their intracellular signaling machinery as well as a sorting mechanism that determines the internalization route that the receptor complex is going to follow. Selective recruitment of molecules within lipid rafts, depending on the ligand, determines innate immune responses as well as the intracellular fate of the receptor complex (Fig. 10). These findings highlight the fact that TLRs expressed on the cell surface utilize a common activation mechanism that involves the orchestration of multiple receptor molecules and plasma microdomains that lead to an extended repertoire of innate immune recognition.

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