Mammalian responses to LPS require the expression of Toll-like receptor 4 (TLR4), CD14, and MD-2. We expressed fluorescent TLR4 in cell lines and found that TLR4 densely localized to the surface and the Golgi. Similar distributions were observed in human monocytes. Confocal imaging revealed rapid recycling of TLR4-CD14-MD-2 complexes between the Golgi and the plasma membrane. Fluorescent LPS followed these trafficking pathways in CD14-positive cells. The TLR4-adapter protein, MyD88, translocated to the cell surface upon LPS exposure, and cross-linking of surface TLR4 with antibody induced signaling. Golgi-associated TLR4 expression was disrupted by brefeldin A, yet LPS signaling was preserved. We conclude that LPS signaling may be initiated by surface aggregation of TLR4 and is not dependent upon LPS trafficking to the Golgi.

The first line defense of multicellular organisms against bacterial pathogens relies upon germ line-encoded receptors that recognize a variety of conserved microbial structures on microorganisms (1–3). One family of such pattern recognition receptors is the type I transmembrane signaling receptors known as Toll-like receptors (TLRs).1 These receptors are all characterized by an intracellular signaling domain that is homologous to that of the IL-1 receptor and an extracellular domain with leucine-rich repeats (4, 5). The most extensively studied microbial product known to engage TLRs is lipopolysaccharide (LPS; endotoxin), a complex glycolipid that comprises the major portion of the outer leaflet of the outer membrane of Gram-negative bacteria (6). A potent immune response is orchestrated upon the recognition of LPS by mammalian cells, including the production and release of cytokines, activation of complement, and various other effects that result in the killing and clearance of the pathogen. Uncontrolled hyperinflammatory host responses to LPS may lead to life-threatening complications such as septic shock, multiorgan failure, and death (7). Toll-like receptor 4 is the signaling receptor for LPS and requires the small glycosylated protein MD-2 for optimal signaling (8–13). We have recently observed that a mutant form of MD-2 (C95Y) completely abrogated LPS responses and that wild-type MD-2 was able to confer LPS responsiveness in TLR4-positive cells lacking MD-2 expression (11, 12).

Whereas there is widespread agreement that CD14, TLR4, and MD-2 expression are necessary for optimal responses to LPS, the mechanism of cellular activation remains in doubt. Wright and co-workers (14, 25) observed that in the absence of internalization and movement of LPS to the Golgi, LPS did not activate mammalian cells (14). Wright proposed these events to be the critical initiators of signal transduction. TLR4 has now been reported to be localized to the Golgi in certain epithelial cells (15). Together, these data suggested that LPS stimulates innate immune responses by activating an internal receptor, TLR4, that normally resides in the Golgi apparatus.

Since the discovery and development of green fluorescent protein (GFP), the subcellular localization, trafficking, and fate of proteins can be studied in living cells using the fluorescent protein as a fusion tag (16–18). Moreover, spectral variants of GFP, such as yellow fluorescent protein (YFP) and cyan fluorescent protein (CFP), have been developed. These GFP variants have found wide use in cellular and molecular biology and will be important tools in further characterization of the TLR4-MD-2-CD14 complex in a process that is distinct from the initiation of signal transduction.

The abbreviations used are: TLR, Toll-like receptor; IL, interleukin; LPS, lipopolysaccharide; GFP, green fluorescent protein; CFP, cyan fluorescent protein; YFP, yellow fluorescent protein; HEK, human embryonic kidney; MALP-2, M. fermentans-derived membrane lipopeptide macrophage-activating lipopeptide of 2 kDa; mAb, monoclonal antibody; FRAP, fluorescent recovery after photobleaching; TNF, tumor necrosis factor; HRP, horseradish peroxidase; FACS, fluorescence-activated cell sorting; PBS, phosphate-buffered saline; TNFR, tumor necrosis factor receptor; ER, endoplasmic reticulum; PBMC, peripheral blood mononuclear cell; GM1, monosialo ganglioside GM1; APC, allophycocyanin; BODIPY, 4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-5-indanone; rLBP, recombinant lipopolysaccharide binding protein.
ants can be exploited for simultaneous visualization of two or more different proteins.

We have engineered a TLR-fluorescent protein chimera with CFP or YFP fused in frame to the C terminus of TLR2 and TLR4 and, as a control, fused to the TNF receptor p60. These cDNA constructs were stably expressed in human embryonic kidney 293 (HEK293) cells. HEK293 cells lack TLR2 or TLR4 expression, and expression of the fluorescent chimeric Toll receptor enables these cells to respond to the appropriate TLR ligand. We observed the subcellular localization, trafficking, and reorganization of TLR4, CD14, MD-2, and LPS in living cells by employing time lapse confocal microscopy of monocolor- and dual and triple color-labeled cells as well as fluorescent photo-bleaching techniques. We confirmed that LPS traffics to the Golgi and found that TLR4 is expressed in the Golgi of transfected HEK cells as well as native monocytes. However, TLR4 is also surface-expressed. Furthermore, LPS continued to traffic between the cell surface and the Golgi and the Golgi under experimental conditions where signal transduction does not occur. Conversely, TLR4 expression in the Golgi was not necessary for cells to respond to LPS. In addition, antibody cross-linking of surface TLR4 was able to trigger strong signaling both in TLR4-transfected cells and human monocytes. These findings provide strong evidence that LPS signaling is initiated on the plasma membrane. Localization of TLR4 in an intact Golgi network and the movement of LPS to this internal pool of TLR4 is neither necessary nor sufficient for signaling to occur.

EXPERIMENTAL PROCEDURES

Reagents—Reagents were obtained from Sigma unless otherwise indicated. PBS, Dulbecco’s modified Eagle’s medium, G418, and trypsin/ versene mixture were from BioWhittaker (Walkersville, MD). Low endotoxin fetal bovine serum was from Hyclone (Logan, UT). Ciprofloxacin was a gift from Miles Pharmaceuticals (West Haven, CT). LPS derived from Escherichia coli strain 0111:B4 was purchased from Sigma and re-extracted by phenol chloroform as described (19). Human IL-1β and TNF-α were purchased from Genzyme Pharmaceuticals (Cambridge, MA). The Mycoplasma fermentans-derived membrane macrophage-activating lipopeptide of 2 kDa (MALP-2) was obtained from C. Peladi (Institut Pasteur, Paris, France).

Fluorescent TLR Chimeras and Expression Plasmids—The cDNA for human TLR4 was provided in the vector pcDNA3 by Drs. C. Janeway and R. Medzhitov (Yale University, New Haven, CT). The expression plasmid pRK7-TLR2 was obtained from Dr. C. Kirschning (Technical University of Munich). The vector pCDNA3.1 was modified to include either CFP or YFP as C-terminal epitope tags in frame with a cloning site; these vectors were provided by Drs. F. Chan and M. Lenardo (20). The same investigators also supplied epitope-tagged p60 TNFR (20). Polyomavirus early region 2 response of TLR2 and TLR4 was performed on pRK7-TLR2 and on pCDNA3-TLR4 in order to construct chimeric fluorescent cDNAs. The upper and lower primers for TLR2 were 5’-GAAGACGAGTTCATCAGCTACAATGTTTG-3’ and 5’-GGCTGCAAGGAGGTATACCGTCCAGAAGA-3’. The upper and lower primers for TLR4 were 5’-GATGATGGATCCATGATGTCTGCCGAGATG-3’ and 5’-ATTTTTGGCTCGAGGATAGATGTTGCTTCC-3’. The PCR fragment were digested with BamHI and XhoI and cloned in frame into pcDNA3-CFP and pcDNA3-YFP. The hMD-2 mammalian expression plasmid pEFBOS containing C-terminal FLAG and His epitope was a gift of Dr. K. Miyake (University of Tokyo). The Golgi subcellular localization vector consisting of the targeting sequence of human β-galactosidase transferase fused to CFP was purchased from ClONTECH (Palo Alto, CA) using the following upper and lower primers for MyD88: 5’-CAGCGGAGGATCAGGTCCGAGGATC-3’ and 5’-GAAACGGTGCAGGCGGACAAAGGC-3’. The PCR fragments were trimmed with BamHI and SalI and cloned in frame into pcDNA3-CFP or pcDNA3-YFP. Respectively.

Stable Cell Lines—Stable cell lines of HEK293 cells expressing the fluorescent protein TLR constructs were engineered by calcium phosphate transfection (21), selection of bulk populations of cells in the neomycin analog G418 (1 mg of total drug/ml), and positive selection by fluorescence-activated cell sorting (BD Vantage, Becton Dickinson Immunocytemetry). Clonal cell lines were established by limiting dilution. The fluorescent HEK293 cells lines were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum and 0.5 mg/ml G418 in a 5% saturated CO2 atmosphere at 37 °C. A cell line stably expressing both TLR4 and MD-2 was generated by retroviral transduction of HEK-TLR4YFP cells as described with a retrovirus encoding human MD-2 (12).

Western Blot and Immunoprecipitation—Cells were analyzed by Western blot and immunoprecipitation as previously described (12). Briefly, MD-2-transduced HEK-TLR4YFP or HEK-TLR2YFP cells were grown on 10-cm dishes and washed in ice-cold Hank’s balanced salt solution, and surface proteins were labeled at 4 °C for 30 min in 5 ml of a solution of sulfosucinimidobiotin (1 mg/ml; Pierce) in Hank’s balanced salt solution. Cells were washed and then incubated in 1 ml of glycine, pH 9, for 5 min, washed again, and lysed in 1 ml of buffer (173 mM NaCl, 20 mM Tris-HCl, pH 7.4, 1 mM EDTA, 0.5% Triton X-100) containing 60 mM n-octylglucoside, 25 mM iodoacetamide, and a mixture of protease inhibitors (10 μg/ml leupeptin and aprotonin and 1 μg/ml phenylmethylsulfonyl fluoride). Lysates were centrifuged, pre cleared, and washed for 1 h in 40 μl of packed protein A-Sepharose (Amersham Biosciences), and immunoprecipitated with 2 μg of a GFP polyclonal antibody (Molecular Probes, Inc., Eugene, OR) per ml and 20 μl of protein A-Sepharose for 16 h at 4 °C. Pellets were washed four times in lysis buffer, resolved by 4–15% SDS-PAGE under reducing conditions, and transferred to nitrocellulose membranes. These membranes were blocked in 5% powdered milk (Difco) and blotted with either the MD-2-HRP mAb (Sigma), anti-GFP mAb (CLONTECH), or Avidin-HRP (Bio-Rad). The blots were then incubated with HRP substrate (enhanced chemiluminescence substrate; Amersham Biosciences) and developed by exposure to film (Hyperfilm; Amersham Biosciences).

Dual Luciferase Reporter Assays for NF-κB Activation—HEK293 cells that stably express TLR4YFP, TLR2YFP, TNFRYFP, or empty vector (pcDNA) were seeded into 96-well tissue culture plates at a density of 2 × 10⁴ cells/well. The following day, cells were transiently transfected with luciferase reporter genes using Genejuice (Novagen) per the manufacturer’s recommendations. In order to assess NF-κB activation, an NF-κB-luciferase reporter gene consisting of an artificial promoter composed of a multimer of five NF-κB sites driving the firefly luciferase gene, was co-transfected with a constitutively active Renilla-luciferase reporter gene (Promega, Madison, WI). The following day, the cells were stimulated as indicated. When necessary, HEK-TLR4YFP cells were either co-transfected with MD-2, stimulated in the presence of soluble MD-2 in conditioned medium, or retrovirally transduced with the cDNA for MD-2 (12). Note that we have established that all three of these methodologies for expressing MD-2 comparably enhance TLR4-mediated responses to LPS. After 4–6 h of stimulation, the cells were lysed in passive lysis buffer (Promega), and reporter gene activity was measured using a plate reader luminesimeter (Victor2, PerkinElmer Life Sciences) using the Dual-Luciferase Assay Reporter System (Promega) and luciferase assay kit (Promega). All values are given as relative luciferase units and are the mean ± S.D. of triplicate samples.

Confocal Microscopy—Confocal microscopy was performed with a Zeiss Axiovert 100-H inversion microscope equipped with an LSM 510 laser-scanning unit. A Zeiss 40× and a 63×1.4 numerical aperture plan Apochromat oil immersion objective (Zeiss) was used. Cells were seeded on 35-mm glass bottom g-irradiated tissue culture dishes (MatTek Corp., Ashland, MA). CFP-tagged proteins were visualized using the 455-nm argon laser line; for YFP and GFP, the 514- or 488-nm line of a 25-milliwatt argon laser was used. Red fluorophores were excited with a 1.6-milliwatt helium/neon laser emitting at 633 nm. Alexa 484 or Cy5-LPS were excited with a helium/neon laser emitting at 633 nm. Band pass or long pass filters were chosen to optimally separate the fluorescence emissions between the different photomultipliers using single-labeled samples of the probes as controls. When crossover of the fluorescence signal was measured to be more than 5%, two or more photomultipliers were used. Measurements were made by PCR of MyD88 and the respective detector channel active at each time. Fluorescent recovery after photobleaching (FRAP) experiments were performed by selecting an area of interest and rapidly applying 99 consecutive scans using the 514-nm line of a 25-milliwatt argon laser at full laser power. Fluorescence recovery was observed under low illumination over time and imaged in the figure. Additional FRAP experiments were done after pretreatment with 200 μg/ml cycloheximide (Sigma) in complete tissue culture medium for 2 h. Live cell images and confocal time lapse

E. Latz and A. Visintin, unpublished data.
fluorescence imaging were performed at 37 °C using a warm stage apparatus (Zeiss).

Labeling with Antibody and Fluorescent Dyes—Indirect immunofluorescence staining for FACS was done using purified mouse monoclonal antibodies (TLR4, HTA125; TLR2, TL2.1; CD14, 3C10) and isotype-matched control antibodies (Sigma) as primary antibodies. Cells were counterstained with APC-conjugated goat anti-mouse secondary antibody (Caltag). Cells were analyzed by flow cytometry (Becton Dickinson LSRII) using the argon laser at 488 nm for excitation of YFP fluorescence and the helium/neon laser emitting at 633 nm to excite APC. Under these conditions, no spectral overlap of the fluorophores was observed.

Transient transfection of cells observed with confocal microscopy was done on cells plated in 35-mm glass bottom tissue culture dishes using Eukaryotic Transfection Reagent (Quiagen) according to this manufacturer’s recommendations. Golgi stain by BODIPY-TR-conjugated ceramide (Molecular Probes, Inc., Eugene, OR) was done per the manufacturer’s recommendations. MD-2 was stained for confocal analysis by use of M2 (Sigma) as a primary antibody and Alexa 647-conjugated goat anti-mouse as the secondary antibody (Molecular Probes); mCD14 was stained by Tricolor-conjugated anti-CD14 antibody (Caltag). Monocytes were isolated by plastic adherence of PBMCs in confocal Petri dishes (MatTek) and cultivated in RPMI1640 with 5% human A serum (University Hospital, Trondheim, Norway) for 48 h before they were washed three times in Hanks’ solution and fixed in 2% formaldehyde (Merck) in PBS for 15 min on ice. The cells were then washed twice in PBS with 1% A serum (PBS/A) before they were treated with acetone (−20 °C) for 10 min followed by a careful wash with PBS/A.

Nonspecific binding was blocked by adding PBS with 20% A+ serum for 20 min. The cells were then stained for 30 min at room temperature with 10 μg/ml HTA125 conjugated with Alexa 546 (Molecular Probes) or a control murine IgG (Caltag) also conjugated with Alexa 546. After three washes with PBS/A+, the cells were examined in the confocal microscope with a 543-nm excitation.

Membrane expression of TLR4 and TLR2 in monocytes was measured by flow cytometry (BD LSRII). Freshly isolated PBMCs were surface-stained with an APC-labeled anti-CD14 antibody (Caltag) together with biotinylated anti-TLR4, anti-TLR2, or isotype control antibodies (all from Amersham Biosciences) after preincubation in 20% human serum for 20 min. Biotinylated antibodies were developed with fluorescein isothiocyanate-conjugated streptavidin (Sigma), and CD14-positive cells (gated according to their forward and sideward scatter characteristics) were analyzed for TLR expression.

LPS Uptake Experiments—E. coli 0111:B4 LPS was subjected to the use of GFP or the spectral variants CFP and YFP as a protein tag allows protein function and dynamics to be investigated within the environment of the living cell. The tagged constructs were designed so that the subcellular localization of the TLR receptors remained directed by their native signal sequences rather than by a signal sequence contained in the vector, as is commonly done when employing FLAG-tagged constructs, expression of these chimeric proteins at the C terminus did not result in appreciable constitutive NF-κB activation (data not shown).

Thus, fusion proteins of TLR4 and TLR2 with fluorescent tags at the C terminus are functional signaling receptors that confer both specific and sensitive recognition of their cognate ligands in HEK293 cells.

Subcellular Distribution of TLR4—The use of GFP or the spectral variants CFP and YFP as a protein tag allows protein function and dynamics to be investigated within the environment of the living cell. The tagged constructs were designed so that the subcellular localization of the TLR receptors remained directed by their native signal sequences rather than by a signal sequence contained in the vector, as is commonly done when employing FLAG-tagged constructs. Cells were grown on glass bottom tissue culture dishes and observed at 37 °C. TLR4YPF was primarily expressed in two different subcellular localizations. We observed plasma membrane expression and localization in a defined juxtanuclear area. Surface staining for TLR4 using a monoclonal antibody (HTA125) revealed a clear surface expression of the protein independently of the fluorochrome tag used (Fig. 1C). Similarly, human monocytes expressed significant levels of both TLR4 and TLR2. Surface biotinylation and immunoprecipitation with anti-GFP antibody, followed by Western blot with avidin-HRP, revealed that only the heavily glycosylated mature forms of TLR4 and MD-2 (Fig. 2B) are expressed on the cell surface. We tested whether TLR2 would bind to MD-2 or whether TLR4 would bind MD-1 under identical experimental conditions but failed to observe co-localization of these protein pairs (data not shown) (12).

These results suggest that both TLR4 and MD-2 follow the trans-Golgi secretory pathway and reside on the cell surface as a mature protein complex.

We next sought to identify the intracellular compartment that is enriched in TLR4. A fluorescent subcellular localization marker for the Golgi complex consisting of the Golgi-targeting sequence of human α-galactosyltransferase fused to CFP co-localized with the juxtanuclear compartment positive for TLR4 (Fig. 2A). Likewise, fluorescent ceramide, which is known to enrich in Golgi membranes (23), also co-localized with juxtanuclear TLR4YPF (Fig. 2B). These studies identify the juxtanuclear region enriched in TLR4 as the Golgi apparatus.

To determine the localization pattern of TLR4 was an artifact of transfection and overexpression or reasonably reflected the distribution of TLR4 in native cells, we stained...
human monocytes with a monoclonal anti-TLR4 antibody. FACS analysis of monocytes revealed detectable TLR4 on the surface of these cells (Fig. 1C). Intracellular staining of purified monocytes clearly showed that TLR4 is also expressed in a defined juxtanuclear region (Fig. 2C), consistent with the conclusion that TLR4 resides both on the cell surface and in the Golgi.

Rapid Exchange between Plasma Membrane and Golgi Pools of TLR4—The Golgi complex is an intracellular compartment that is specialized for secretory traffic. Newly synthesized proteins and lipids are received from the ER by the Golgi complex and are covalently modified in preparation for delivery to their final destination (such as plasma membrane, lysosomes, secretory granules) or to be recycled back to the ER. In addition to these sorting and filtering capabilities, the Golgi complex recycles plasma membrane components that are retrieved by endocytosis (17, 24).

The physical properties of GFP allow the GFP chimera to be used in a technique known as FRAP. After GFP or its spectral variants are excited with very high illumination, these fluorophores are readily photobleached, a process that irreversibly extinguishes the fluorescence. When performing FRAP, a small area of interest in the cell is rapidly photobleached by applying scans with a high powered laser beam. Thereafter, the movement of nonbleached fluorophores into the photobleached area can be recorded at low illumination. Fluorescence recovery gives insights in the diffusional properties of the reporter chimera.

We employed FRAP to investigate whether membrane-expressed TLR4YPF would cycle back to the Golgi complex after photobleaching of Golgi-associated TLR4YPF. Application of repetitive scans with full laser power to the Golgi almost completely extinguished the Golgi-localized fluorescence, whereas neither the surrounding areas nor the neighboring cells were affected (Fig. 3A). Observation of fluorescence recovery revealed a fast and complete recovery of Golgi fluorescence. Over 90% of the Golgi-associated fluorescence was recovered within 4 min after photobleaching, indicating rapid lateral movement of TLR4YPF between cell compartments.

We next questioned whether the recovery of Golgi fluorescence after FRAP is primarily due to new protein synthesis or represents the recycling of TLR4YPF from the membrane pool to the Golgi apparatus. To address this question, cells were preincubated with the protein synthesis inhibitor cycloheximide for 2 h prior to FRAP. The recovery of Golgi fluorescence observed in the cycloheximide-treated cells was nearly identical to that seen in untreated cells, indicating that rapid transport of TLR4 from the plasma membrane replenished TLR4 in the Golgi area (Fig. 3B).

These data provide evidence that TLR4 is a highly mobile protein that rapidly and continuously recycles between its two major cellular pools, the Golgi complex and the plasma membrane.

Subcellular Localization and Trafficking of MD-2 and CD14—Activation of TLR4 by LPS requires the presence of MD-2, a glycosylated protein with a cleavable signal sequence. MD-2 has been suggested to assemble with TLR4 in the intracellular compartment. We have recently reported that MD-2 can be released from cells into culture supernatants, where it retains its ability to enable TLR4 responses to LPS (11, 12). Soluble MD-2 conferred LPS responsiveness to TLR4-positive cells, whereas a single point mutant, MD-2c955V, failed to enable LPS responses (11).

We transiently expressed a FLAG-tagged version of MD-2 in...
HEK293 cells that stably expressed either TLR4<sup>YFP</sup>, TLR2<sup>YFP</sup>, or TNFR<sup>YFP</sup> and visualized MD-2 by confocal microscopy in order to investigate the localization and trafficking of MD-2. Aggregation of MD-2 by antibody surface staining co-aggregated TLR4<sup>YFP</sup> but not TLR2<sup>YFP</sup> or TNFR<sup>YFP</sup>, suggesting that MD-2 forms a stable and tight complex with TLR4 on the cell membrane (Fig. 4<sup>A</sup> and data not shown). We next asked whether MD-2 also recycled between the membrane and the Golgi pools of TLR4. HEK-TLR4<sup>YFP</sup> cells that were transfected with MD-2 were surface-stained for MD-2 and observed by time lapse confocal microscopy at 37 °C. We observed that, like TLR4, MD-2 rapidly recycled between the cell membrane and the Golgi and that MD-2 co-localized with TLR4 at all stages during this process (Fig. 4<sup>B</sup>; data not shown). In order to determine whether CD14 followed the same pattern of movement from the cell membrane to the Golgi, we used mAb to CD14 to track its movements. Like MD-2, CD14 initially stained on the cell surface and was found to be rapidly inter-

**FIG. 2. Localization of TLR4 to the Golgi complex.** HEK-TLR4<sup>YFP</sup> cells were transiently transfected with a Golgi subcellular localization vector (A) or stained with BODIPY-TR ceramide (B). Confocal imaging shows extensive overlay of areas positive for Golgi stain and the intracellular paranuclear TLR4<sup>YFP</sup> pool. C, human monocytes were intracellularly stained for TLR4 using HTA125 antibody directly conjugated with Alexa 546. A control antibody did not label the cells. Shown are representative images of confocal sections of experiments performed at least three times. Scale bar, 10 μm.

**FIG. 3. Constitutive cycling of TLR4 between the Golgi apparatus and the plasma membrane.** A, the Golgi pool of TLR4 recovers rapidly after photobleach. Living HEK-TLR4<sup>YFP</sup> cells were observed by confocal microscopy at 37 °C, and Golgi-associated TLR4<sup>YFP</sup> was selectively photo-bleached by repeated illumination with high laser intensity. Shown are representative images before (prebleach), immediately after photobleach, and minutes after photobleach as indicated. Golgi areas unaffected by photobleaching are indicated by the arrows. Shown is a representative experiment of several independent experiments. B, quantification of fluorescence recovery after photobleaching and influence of cycloheximide treatment. Mean fluorescence intensities of Golgi and non-Golgi pools were measured at the time points indicated and expressed as percentage of the ratio obtained before bleaching. To block protein synthesis, cells were pretreated with cycloheximide, and fluorescence recovery after photobleaching was analyzed as above. Scale bar, 10 μm.
nalized to the Golgi area (Fig. 5D; Supplemental Materials, video 1). These observations lead to the conclusion that the LPS signaling receptor complex, consisting of TLR4, MD-2, and CD14, is formed on the plasma membrane and that this protein complex is rapidly recycled between the plasma membrane and the Golgi. A slightly modified interpretation of this hypothesis is that surface CD14, TLR4, and MD-2 primarily reside in lipid rafts and that this residency is enhanced by the presence of LPS. These rafts recycle between the cell membrane and the Golgi, carrying all of their resident proteins, including raft-localized receptors and, when present, their cognate ligands.

LPS Internalization Follows the Path of CD14 and Is Independent of Cellular Activation—To address the role of MD-2 and CD14 in LPS binding and internalization, we transiently transfected HEK cells expressing TLR4YFP with CD14 and/or MD-2 and assessed binding and internalization of Cy5-labeled LPS. Rapid binding and uptake of Cy5-LPS, but not Cy5-labeled bovine serum albumin (data not shown), was observed in cells transfected with CD14 (Fig. 5, A and C). Co-transfection of CD14 with MD-2 did not significantly enhance Cy5-LPS binding or uptake in comparison with cells transfected solely with CD14. Transfection of MD-2 alone had little effect on the uptake of Cy5-LPS (Fig. 5A).

Under the same conditions, CD14-mediated uptake of LPS occurred in the apparent absence of signaling. Treatment of CD14-positive (and MD-2-null) HEK-TLR4YFP cells with LPS did not result in the activation of NF-κB (Fig. 5B) or the release of IL-8 (data not shown), despite the ability of these cells to internalize large amounts of LPS. In contrast, MD-2 expression conferred LPS responsiveness despite the absence of membrane CD14. Under these conditions (MD-2 transfection only), LPS binding and uptake were only minimally detectable after 30 min of incubation (Fig. 5A), a time point that is after the completion of many LPS-inducible events. Note that whereas the FACS assay employed in Fig. 5A does not necessarily distinguish between total cellular LPS binding and LPS uptake, confocal microscopy was also performed. The results confirmed our assessment that minimal LPS binding and internalization occurred in MD-2-transfected cells (data not shown).

We transiently transfected HEK-TLR4YFP with CD14 and/or MD-2 and analyzed these cells using confocal microscopy. Cy5-LPS bound rapidly (within 10 seconds) to the cell surface of CD14-transfected cells, whereas LPS binding was scarcely detectable in CD14-null cells, thus again confirming the results obtained by flow cytometry (Fig. 5A). CD14-positive cells internalized large quantities of Cy5-LPS, and large vesicles of LPS were visualized shortly after cells were exposed to LPS (Fig. 5C). This type of bulk endocytosis of LPS leads to profound redistribution of TLR4YFP around these LPS-containing vesicles, an observation that was not made in HEK cells expressing TLR2YFP or TNFRYFP (Fig. 5C).

LPS and CD14 Trafficking in Living Cells—We employed time lapse confocal imaging in living HEK cells expressing TLR4-CD14, TLR2-CD14, or CD14 alone to define the kinetics of the LPS internalization process and to decipher the possible role of TLR4 in intracellular LPS trafficking. We employed supernatants rich in recombinant MD-2 to enable TLR4-mediated signaling in order to minimize the effects of transient transfection upon our results. We confirmed our previous observation that soluble recombinant MD-2 could enable TLR4 signaling in response to endotoxin. Furthermore, we found that soluble MD-2 failed to enable LPS responses in HEK cells stably transfected with either CD14 or CD14 plus TLR2 (data not shown).

HEK-CD14 cells were incubated with Cy5-LPS while sitting on the confocal stage at 37 °C. Within 10 seconds after the addition of LPS, strong membrane co-localization of LPS and CD14 was observed. Thereafter, CD14- and LPS-containing
vesicles were observed to rapidly recycle between the plasma membrane and an intracellular compartment. The vesicle movement from the cell membrane to the juxtanuclear area of the cell interior was repeatedly measured to require between 4 and 8 s, a time that corresponds to a movement of $1-2 \mu m/s$.

The rate of vesicle movement and the fate of LPS with respect to its final destination were similar between cells expressing CD14 alone and those that expressed CD14 in combination with TLR4 or TLR2 (Fig. 5D; Supplemental Materials, video 2). Note that in the absence of either MD-2 or TLR4 expression (or both), there is no evidence that HEK cells can be activated by LPS. Thus, the intracellular transport of LPS appears not to be sufficient to result in cellular activation.

**Initiation of LPS Signaling on the Plasma Membrane**—Whereas it is clear that the movement of LPS to the Golgi is not sufficient to activate signal transduction, several authors have postulated that LPS internalization and trafficking to the Golgi are necessary for LPS-induced signaling to occur (15, 25). We evaluated the subcellular site of LPS signaling by the expression of a CFP-tagged version of MyD88 in TLR4-YFP and MD-2-positive cells in order to address this question. MyD88 binds to the intracellular signaling domain of TLRs to induce the downstream signaling processes (26) and is required for full responses to LPS, such as the production and release of cytokines. In cells unexposed to LPS, the fluorescently tagged MyD88 was expressed in cytoplasmic aggregates. Upon LPS stimulation, a portion of the tagged MyD88 intracellular pool was observed to translocate to the cytoplasmic side of the plasma membrane (Fig. 6). This finding is consistent with the hypothesis that MyD88 is recruited to the TIR domain of surface TLR4, where signal transduction is initiated.

Brefeldin A reversibly inhibits the small GTPase Arf, which leads to retraction of the Golgi membranes back into the ER (17, 27). We pharmacologically disrupted the Golgi by adding brefeldin A to HEK-TLR4-YFP cells in culture to examine further the functional significance of Golgi-localized TLR4. Under these conditions, the release of cytokines such as IL-8 was entirely blocked (data not shown). Confocal imaging of brefeldin A-pretreated cells confirmed that TLR4-YFP disappeared from the perinuclear area; consequently, TLR4-YFP fluorescence was greatly enhanced in the ER (Fig. 7A). However, despite the clear morphological changes in the subcellular localization of TLR4, NF-κB signaling in brefeldin A-treated cells was not diminished (Fig. 7B). This result suggests that TLR4-mediated signaling does not require Golgi localization.

Finally, we sought to determine whether surface-localized TLR4 was capable of transducing a signal under conditions where it could be stated with a reasonable degree of certainty that Golgi-localized TLR4 could not be engaged. In order to ask this question, we coated tissue culture grade high protein binding plastic dishes with monoclonal antibodies to TLR4 or irrelevant antigens. The dishes were washed extensively with PBS, and transfected HEK293 cells were allowed to settle onto the plastic, where their surface proteins could become cross-linked.
accumulations cannot account for the stimulatory capacity of the Golgi apparatus. This report demonstrates the surprising rapidity with which this movement takes place; the interval from membrane to Golgi localization can be measured in seconds, not minutes as was previously assumed. However, it has never been clear whether the mechanism of LPS-induced cellular activation was in any way related to the movement of LPS, although a variety of strongly held opinions existed on this topic.

The discovery of Toll receptor 4 as the primary LPS signal transducer (at least for endotoxins derived from enterobacteria), initially seemed to answer the question of how LPS activates immune cells. Like all of the TLRs, TLR4 has striking homology to the IL-1 receptor. This receptor is known to initiate signal transduction by forming heteromeric complexes with the IL-1-associated receptor; this complex has a marked affinity for the cytoplasmic linker molecule MyD88, and once this molecule is recruited to the TIR domain of the IL-1r, signaling proceeds rapidly. However, despite the homology of TLRs to the IL-1r, it has yet to be established whether TLRs function in the same way. The issue appeared even more complex when Hornef et al. (15) reported that TLR4 is localized in the Golgi in intestinal epithelial cells and demonstrated colocalization of LPS with TLR4 in this location. This report was highly suggestive that signaling might begin in the Golgi and that the endotoxin receptor was truly an intracellular receptor.

We engineered fusion proteins of yellow and cyan fluorescent protein with several TLRs in order to be able to determine the subcellular localization of TLRs without the ongoing need to permeabilize cells for antibody staining. This approach has allowed us to monitor not only the location of TLR4 at any given time but also how that location was related in time to the presence of LPS. These chimera are fully functional receptors whose expression in HEK293 cells results in the types of immune responses to bacterial products that one would anticipate from native cells that express TLR2 and/or TLR4. TLR4_YFP can clearly be seen to be located within the Golgi of transfected HEK293 cells, but, unlike intestinal epithelial cells, it is also clearly present on the cell surface. It is worth noting that whereas the expression of TLR4 in fixed and permeabilized human monocytes only can be documented in the perinuclear area that strongly resembles the Golgi, FACS analysis of monocytes has consistently demonstrated surface expression of TLR4 (Fig. 1C). This may, in fact, be due to the disruption of the mammalian outer membrane that occurs whenever cells are treated with a detergent. We propose that the Golgi pool of TLR4 normally serves as a steady-state pool of TLR4 protein and that cells may regulate the proper surface expression of TLR4 by either enhancing or diminishing the retention of TLR4 in the Golgi (32, 33). We suspect that careful inspection of the intestinal epithelial cell outer membrane will also result in the discovery of TLR4 in these cells, especially because there is no reason to believe that the recycling of membrane components from the surface to the Golgi and back again is unique to HEK293 cells.

Accumulating evidence exists that TLR4 requires MD-2 in order to initiate LPS signal transduction. Furthermore, it seems apparent that MD-2 tightly binds to the extracellular domain of TLR4 (10, 35) and that this tight binding has functional significance. MD-2 is a secreted protein that can enable LPS responsiveness in cells that express TLR4 but lack MD-2 (11, 12). By employing time lapse confocal dual color imaging, we observed that the TLR4-MD-2 receptor complex was preassembled at the plasma membrane and that MD-2 together with TLR4 rapidly recycles between the plasma membrane and the Golgi apparatus in the absence or presence of ligand. CD14 also appears to move from the cell surface to the Golgi, despite
previous reports to the contrary (36). In fact, our data confirm previous observations that the entire cholesterol-rich, ganglioside-rich "lipid raft," in which TLR4, CD14, and MD-2 all are thought to reside, is constantly shuffling back and forth from the surface to the Golgi (24, 37, 38). Our recent studies confirm this report, since we have found that when HEK293 cells were incubated with the rhodamine-labeled cholera toxin subunit B, which binds to GM1 gangliosides present in lipid rafts, the cholera toxin rapidly moved from the plasma membrane to the Golgi indistinguishably from CD14, MD-2, TLR4, and LPS (data not shown).

Whereas the observation that LPS is internalized into cells and traffics to the Golgi under conditions where cell signaling does not occur is consistent with the hypothesis that cellular activation begins on the cell surface, these data alone could never be considered to be conclusive. The ability of cells to become activated by LPS after brefeldin A treatment clearly demonstrates that Golgi localization of TLR4 is not necessary to observe activation, although it might be argued that these unusual conditions simply change the important location of interaction from the Golgi to the ER. We found that after LPS stimulation, Myd88 will translocate from an intracellular organelle to the plasma membrane in living cells, again giving additional credence to the concept that signaling does occur on the plasma membrane. Perhaps most importantly, surface TLR4 is capable of initiating a signal to cells, since the antibody cross-linking experiment shown in Fig. 7, C and D, employs surface-immobilized monoclonal antibodies that are physically incapable of interacting with Golgi-localized TLR4. Surface TLR4 would be expected to be the pool of TLR4 that first interacts with LPS as bacteria are encountered by phagocytes. Thus, it seems that the response to LPS begins at the cell surface but may have the potential to continue at other locations within the cell, including the Golgi and perhaps other organelles.

Ideally, it would be interesting to inhibit LPS uptake and to test whether LPS could nevertheless activate cells. Unfortunately, such experiments are difficult to design and interpret. Whereas cytochalasins effectively block LPS internalization, we and others, have found that they interfere with many other aspects of cellular function (39, 40). As an alternative to the use of pharmacological agents, we previously used genetic means to prevent bacterial internalization via CD11b/CD18 in Chinese hamster ovary cells by transfecting these cells with truncated ("tailless") LPS-binding receptors that were missing those portions of these receptors that interact with the cytoskeleton. Under these conditions, the TLR4-mediated response was entirely unaffected by the lack of ligand internalization (41).

Recently, members of the Nod/CARD protein family have been suggested to mediate signals to LPS that finds its way into the cytosol, especially by pathogens that have the ability to invade mammalian cells. This class of receptors may, in fact, be true intracellular signaling molecules that represent a specialized form of host defense against certain intracellular pathogens (34, 42). Regardless of the final outcome of the study of Nod proteins, it is clear that the majority of medically relevant responses to LPS do not occur via this intracellular pathway but rather via the Toll pathway.

The data presented herein suggest that the movement of LPS into the cell and toward the Golgi reservoir of TLR4 is neither necessary nor sufficient for signal transduction to occur. We propose that although LPS is internalized by the action of CD14 and is dragged to the Golgi along with the CD14-rich lipid raft, this movement of LPS has little immunological importance. Conversely, our data support the hypothesis that LPS-initiated signaling begins with the cross-linking and clustering of surface TLR4-MD-2, which we have observed to be sufficient to initiate signal transduction.
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Lipopolysaccharide Rapidly Traffics to and from the Golgi Apparatus with the Toll-like Receptor 4-MD-2-CD14 Complex in a Process That Is Distinct from the Initiation of Signal Transduction

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