

Mediators of innate immune recognition of bacteria concentrate in lipid rafts and facilitate lipopolysaccharide-induced cell activation

Martha Triantafilou¹, Kensuke Miyake², Douglas T. Golenbock^{3,*} and Kathy Triantafilou^{1,†}

¹University of Portsmouth, School of Biological Sciences, King Henry Building, King Henry I Street, Portsmouth, PO1 2DY, UK

²Department of Immunology, Saga Medical School, Nabeshima, Japan

³Boston University School of Medicine, Boston Medical Center, The Maxwell Finland Laboratory for Infectious Diseases, Boston, Massachusetts 02118, USA

*Present address: Department of Medicine, Division of Infectious Diseases, University of Massachusetts Medical School, Worcester, MA 01665, USA

†Author for correspondence (e-mail: ktrian@hotmail.com)

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Summary

The plasma membrane of cells is composed of lateral heterogeneities, patches and microdomains. These membrane microdomains or lipid rafts are enriched in glycosphingolipids and cholesterol and have been implicated in cellular processes such as membrane sorting and signal transduction. In this study we investigated the importance of lipid raft formation in the innate immune recognition of bacteria using biochemical and fluorescence imaging techniques. We found that receptor molecules that are implicated in lipopolysaccharide (LPS)-cellular activation, such as CD14, heat shock protein (hsp) 70, 90, Chemokine receptor 4 (CXCR4), growth differentiation factor 5 (GDF5) and Toll-like receptor 4 (TLR4), are

present in microdomains following LPS stimulation. Lipid raft integrity is essential for LPS-cellular activation, since raft-disrupting drugs, such as nystatin or MCD, inhibit LPS-induced TNF- α secretion. Our results suggest that the entire bacterial recognition system is based around the ligation of CD14 by bacterial components and the recruitment of multiple signalling molecules, such as hsp70, hsp90, CXCR4, GDF5 and TLR4, at the site of CD14-LPS ligation, within the lipid rafts.

Key words: Lipid rafts, LPS, Heat shock proteins, CXCR4, GDF5, TLR4

Introduction

The plasma membrane of mammalian cells was once believed to be homogeneous, but it is now clear that it is discontinuous, containing numerous microdomains that are essential for cellular function. Several studies of the plasma membrane have provided evidence for the existence of these microdomains (Damjanovitch et al., 1995; Jenei et al., 1997; Kenworthy and Edidin, 1998a; Jacobson et al., 1995; Jacobson and Dietrich, 1999). Imagining these microdomains as floating islands in the membrane, Simons and Ikonen (Simons and Ikonen, 1997) described them as 'lipid rafts'. From a biochemical point of view, lipid rafts appear as detergent-insoluble/resistant glycolipid-enriched membrane domains and are often termed as detergent-resistant membranes (DRMs), detergent-insoluble glycolipid-enriched complexes (DIGs) or glycosphingolipid-enriched membranes (GEMs) (Horejsi et al., 1998).

Even though lipid rafts are known to exist, their physiological significance is not yet clear. One of the most widely appreciated roles of lipid rafts (or microdomains) is in the recruitment and concentration of molecules involved in cellular signalling (Pralle et al., 2000). A large-scale accumulation of receptors and their signal transduction machinery in microdomains seems to enhance the signalling efficiency by providing a focusing effect (Vereb et al., 2000).

Recent studies have shown the importance of lipid raft

formation in the acquired immune response. MHC-restricted T-cell activation seems to be facilitated by lipid raft formation (Romagnoli and Bron, 1997; Xavier et al., 1998; Viola et al., 1999; Yashiro-Ohtani et al., 2000; Huby et al., 1999; Anderson et al., 2000). Although the importance of lipid raft formation for the acquired immune recognition is clear, the involvement of membrane microdomains in the innate immune response has not yet been investigated.

Our current understanding of the innate immune recognition of bacterial lipopolysaccharide (LPS) is based on the seminal discovery that LPS binds to the serum protein LPS-binding protein (LBP) (Tobias et al., 1986) and then the LPS-LBP complex binds to CD14 (Wright et al., 1990). Since CD14 is a glycosylphosphatidylinositol (GPI)-linked protein, it transduces the signal by associating with other signalling molecules. Toll-like receptor (TLR)-4 has recently been implicated as an LPS-signal transducing molecule (Poltorac et al., 1998; Chow et al., 1999; Lien et al., 2000) as well as heat shock proteins (hsps) (Byrd et al., 1999; Triantafilou et al., 2001a), CD55 (Heine et al., 2001), chemokine receptor 4 (CXCR4) and growth differentiation factor 5 (GDF5) (Triantafilou et al., 2001a).

CD14, the key molecule in innate bacterial recognition, is a GPI-linked protein. A characteristic feature of GPI-anchored proteins is that they are found in microdomains (Schroeder et

al., 1994). Pugin et al. have previously demonstrated that GPI-anchored CD14 is mostly localised in the Triton X-100-insoluble fraction of the plasma membrane, which is characteristic of microdomains (Pugin et al., 1998). Since CD14 is found in such microdomains on the cell surface, it is probable that the entire bacterial recognition system is based around the ligation of CD14 by bacterial components and the recruitment of multiple signalling molecules at the site of CD14-LPS ligation, within the lipid rafts. In order to test this hypothesis we investigated the existence of receptors identified as mediators of the innate immune recognition of LPS in lipid rafts. Using biochemical and fluorescence imaging techniques, we found that a complex of receptors is being formed upon LPS stimulation within the lipid rafts. This complex of receptors involves hsp70, hsp90, CXCR4, GDF5 and TLR-4. Furthermore we show that by disrupting lipid raft integrity using raft-disrupting drugs such as nystatin or methyl- β -cyclodextrin (MCD) we can inhibit LPS-induced cell activation. Taken together these results lead us to conclude that accumulation of these receptor molecules within lipid rafts following LPS stimulation serves to facilitate LPS signalling by concentrating LPS 'transducers' and their signalling machinery in specific regions of the plasma membrane for a focused signalling effect.

Materials and Methods

Materials

ReLPS from *Salmonella Minnessota* Re595 was purchased from List Labs (CA, USA). All fine chemicals and human pooled serum were purchased from Sigma Chemical Co. (St Louis, MO). Hybridoma cells secreting 26ic (anti-CD14) were obtained from the American Type Culture Collection (ATCC, MD). The monoclonal antibody against human hsp90 α was obtained from Bioquote (York, UK). The hsp70-specific monoclonal antibody, MyD88 goat polyclonal serum and Rac-1 rabbit polyclonal serum were obtained from Autogen Bioclear (Wiltshire, UK), whereas the CXCR4-specific mouse monoclonal was purchased from Serotec (Oxford, UK). Phospho-specific SAPK/JNK rabbit polyclonal serum, which detects SAPK/JNK only when activated by phosphorylation, was obtained from New England Biolabs (USA). Swine anti-rabbit Ig conjugated to HRP and donkey anti-goat Ig conjugated to HRP were obtained from Dako (UK). Goat polyclonal serum directed against GDF5 was obtained from Research Diagnostics Inc. (USA), along with the TNF- α ELISA kit. FITC-CD25 monoclonal antibody was obtained from Serotec (UK).

Repurification of LPS preparation

Commercial LPS was resuspended in endotoxin-free water containing 0.2% triethylamine followed by vortexing. LPS was repurified using a modified phenol-water extraction procedure followed by ethanol precipitation as described previously (Tapping et al., 2000; Hirschfeld et al., 2000; Manthey and Vogel, 1994). Recovery of LPS was determined by 3-deoxy-D-manno-octulosonic acid assay.

Cells

Chinese hamster ovary (CHO) cells transfected with hCD14 and hTLR4 cDNA in a reporter background were constructed as previously described (Delude et al., 1998). CHO cells were maintained in Ham's F12 from Gibco BRL supplemented with 2 mM L-glutamine, 7.5% FCS and 500 μ g/ml gentamycin sulfate (G418, Sigma). Cells were grown in 80 cm³ tissue culture flasks (Nunc).

Trypsin/EDTA (0.05% Trypsin/0.53 mM EDTA) was used for passaging the cells.

The MonoMac-6 cell line was obtained from the Institute of Immunology, University of Munich, Munich, Germany. MonoMac-6 cells were cultured in 5% CO₂ at 37°C in Iscove-modified Dulbecco medium (Gibco, BRL) containing 10% foetal calf serum.

Isolation of human monocytes

Monocytes were isolated from human A+ buffy coats. Adherent cell monolayers (1 \times 10⁵ to 2 \times 10⁵ monocytes/well) were cultured in 24-well plates in serum free medium (Gibco) supplemented with 0.01% L-glutamine and 40 μ g of gentamicin/ml.

Isolation of lipid rafts

MonoMac-6 or CHO/CD14/TLR4 cells (1 \times 10⁶) were lysed in 500 μ l of MEB buffer (150 mM NaCl, 20 mM MES, pH 6.5) containing 1% Triton X-100 and protease inhibitors (500 μ M PMSF and 5 mM iodoacetamide) for 1 hour on ice. The cells were mixed with an equal volume of 90% sucrose in MEB and placed at the bottom of a centrifuge tube. The sample was overlaid with 5.5 ml of 30% sucrose and 4.5 ml of 5% sucrose in MEB and centrifuged at 100,000 g for 16 hours. Fractions (1 ml) were gently removed from the top of the gradient and *n*-octylglucoside was added to each fraction (60 μ M final) to solubilise rafts. For isolation of cellular membranes following LPS stimulation, MonoMac-6 or CHO/CD14/TLR4 cells were stimulated with 100 ng/ml of LPS in 5% HPS for 30 minutes at 37°C prior to solubilisation in MEB buffer.

Western blotting

Equal portions of each fraction were analysed by SDS-PAGE and transferred onto a nitrocellulose filter (Schleicher-Schuell, Germany) or Immobilon P membranes (Millipore) for 1 hour at 220 mA in the presence of transfer buffer (20 mM Tris-acetate, 0.1% SDS, 20% isopropanol, pH 8.3). After transfer, the membrane was blocked for 1 hour in blocking solution (5% low fat dried milk dissolved in PBS-T) and washed with PBS-T (two rinses, a 15 minute wash and two 10 minute washes). Membranes were probed with the appropriate dilution of primary antibody for 1 hour followed by washing with PBS-T. Membranes were incubated with HRP conjugated to either swine anti-rabbit Ig (1:4000), donkey anti-goat Ig or rabbit anti-mouse Ig for 1 hour. After extensive washing with PBS-T, the antigen was visualised using the ECL procedure (Amersham) according to the manufacturer's instructions.

Fluorescent probes

Cholera-toxin conjugated to rhodamine was purchased from List Labs (CA, USA). Antibodies against the molecules of interest were conjugated with FITC using the FITC labelling kits from Molecular Probes Europe.

Cell labeling for FRET

CHO/CD14/TLR4 or MonoMac-6 cells on microchamber culture slides (Lab-tek, Gibco) were labelled with 100 μ l of a mixture of donor-conjugated antibody (FITC) and acceptor-conjugated cholera toxin (rhodamine). The cells were rinsed twice in PBS/0.02% BSA prior to fixation with 4% formaldehyde for 15 minutes. The cells were fixed in order to prevent potential reorganisation of the proteins during the course of the experiment.

Confocal imaging

Cells were imaged on a Carl Zeiss confocal microscope (with an

Axiovert 100 fluorescent microscope) using a 1.4 NA 63× Zeiss objective. The images were analysed using LSM 2.5 image analysis software (Carl Zeiss). FITC and Rhodamine were detected using the appropriate filter sets. Using typical exposure times for image acquisition (less than 5 seconds), no fluorescence was observed from a FITC-labelled specimen using the rhodamine filters nor was rhodamine fluorescence detected using the FITC filter sets.

FRET measurements

FRET is a non-invasive imaging technique used to determine molecular proximity. FRET can occur over 1-10 nm distances and effectively increases the resolution of light microscopy to the molecular level. It involves nonradiative transfer of energy from the excited state of a donor molecule to an appropriate acceptor (Wu and Brand, 1994). The rate of energy transfer is inversely proportional to the sixth power of the distance between the donor and the acceptor (Kenworthy and Edidin, 1998a; Kenworthy and Edidin, 1998b). The efficiency of energy transfer (E) is defined with respect to r and R_0 , the characteristic Forster distance by:

$$E = 1/[1 + (r/R_0)^6].$$

In the present study, FRET was measured using a previously described method (Kenworthy and Edidin, 1998a; Kenworthy and Edidin, 1998b). Briefly, samples were labelled with donor- and acceptor-conjugated antibodies, and energy transfer was detected as an increase in donor fluorescence (dequenching) after complete photobleaching of the acceptor molecule.

Cells labelled only with the 26ic-Cy5 probe were used in order to determine the minimum time required to bleach Cy5. Cy5 was bleached by continuous excitation with an arc lamp using a Cy5 filter set for 10 minutes. Under these conditions, Cy3 was not bleached.

FRET images were calculated from the increase in donor fluorescence after acceptor photobleaching by:

$$E(\%) \times 100 = 10,000 \times \frac{[(\text{Cy3 postbleach} - \text{Cy3 pre-bleach})/\text{Cy3 postbleach}]}{10,000}.$$

The scaling factor of 10,000 was used in order to expand E to the scale of the 12-bit images.

TNF induction

Human monocytes (5×10^5) were mixed with 50 μ l of serial dilutions of LPS in the presence of 1% HPS in serum free medium. After 2.5 hours, the supernatant was collected and analysed for TNF- α using an enzyme-linked immunosorbent assay (Research Diagnostics Inc). For inhibition experiments, cells were treated with either 60 μ g/ml nystatin or 10 mM MCD for 10 minutes prior to LPS stimulation.

Results

Existence of receptor molecules involved in LPS-cellular activation in lipid rafts prior to LPS stimulation

In order to determine whether receptor molecules involved in the innate immune recognition of bacterial LPS were constitutively present in membrane rafts, plasma microdomains were isolated from MonoMac-6, a human monocytic cell line, and from CHO/CD14/TLR4 cells, on the basis of their insolubility in Triton X-100 and low buoyant density in sucrose gradients (Brown and Rose, 1992). MonoMac-6 or CHO/CD14/TLR4 cells were treated with 1% triton X-100 in buffer for 1 hour on ice and then subjected to sucrose density centrifugation as described in the Materials and Methods. Fractions were collected from the

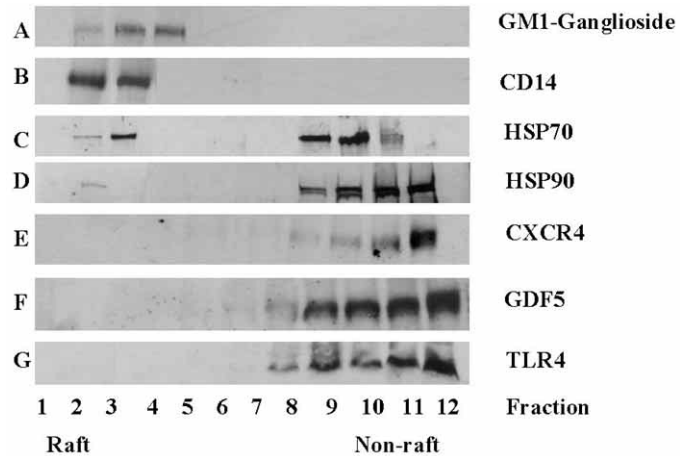


Fig. 1. Receptor molecules implicated in LPS-cellular activation are present in lipid rafts. MonoMac-6 cells were treated with 1% Triton X-100 buffer for 1 hour on ice and then subjected to sucrose density gradient centrifugation. Fractions were collected from the top of the gradient; 1% *n*-octylglucoside was added to each fraction; and equivalent portions of each fraction were analysed by SDS-PAGE and immunoblotting. The lipid raft marker was detected using HRP-conjugated cholera toxin (A), the nitrocellulose membranes were also probed with 26ic (CD14-specific mAb) (B), hsp70 (C), hsp90 (D), CXCR4 mAbs (E) and GDF5 polyclonal serum (F), as well as with the HTA125 TLR4-specific mAb (G). The relative positions of the raft and non-raft (soluble) fractions are indicated.

top, and 1% *n*-octylglucoside was added to each fraction (to solubilise lipid rafts). Equivalent portions of each fraction were analysed by SDS-PAGE electrophoresis and immunoblotting.

GM-1 ganglioside, a raft-associated lipid, was detected using HRP-conjugated cholera toxin. We found that GM-1 ganglioside migrated near the top of the sucrose gradient (fractions 2-5), indicating that this procedure was effective in separating membrane rafts from the rest of the cellular membrane (Fig. 1A).

In order to test whether CD14, a key molecule in the innate recognition of LPS, was present in microdomains prior to LPS stimulation, we immunoblotted the nitrocellulose membranes with 26ic, a CD14-specific monoclonal antibody (mAb), then added HRP-conjugated rabbit anti-mouse Ig. We found that CD14 was present in fractions two to three of the gradient, thus CD14 was constitutively present in lipid rafts (Fig. 1B). Control experiments utilising HRP-conjugated rabbit anti-mouse Ig in the absence of a primary antibody revealed no protein bands, thus demonstrating the specificity of the antibodies.

The presence of other receptors involved in LPS recognition in lipid rafts was also investigated. Nitrocellulose membranes were immunoblotted with hsp70, hsp90, CXCR4 and GDF5 mAbs, as well as TLR-4-specific mAb HTA125 in order to investigate their existence in lipid rafts prior to LPS stimulation. We found that hsp70 and hsp90 were also associated with lipid rafts (Fig. 1C,D), whereas CXCR4, GDF5 and TLR4 were not found in microdomains (Fig. 1E,F,G). Control experiments with just HRP-conjugated antibody revealed no protein bands. Furthermore, western blots of CHO cells transfected with the empty vector, and thus not expressing

TLR4, also revealed no protein bands, verifying the specificity of the antibodies used.

Existence of receptor molecules involved in LPS-cellular activation in lipid rafts after LPS stimulation

In order to investigate whether the distribution of molecules involved in LPS-cellular activation changes after LPS stimulation, we stimulated MonoMac-6 or CHO/CD14/TLR4 cells with LPS and lysed them in 1% triton X-100 buffer for 1 hour on ice and then subjected them to sucrose density centrifugation as described in the Materials and Methods. Fractions were collected from the top, and 1% *n*-octylglucoside was added to each fraction (to solubilise lipid rafts). Equivalent portions of each fraction were analysed by SDS-PAGE electrophoresis and immunoblotting.

We tested again the distribution of GM-1 ganglioside, a lipid raft marker that is detected using HRP-conjugated cholera toxin. We found that GM-1 ganglioside migrated near the top of the sucrose gradient (fractions 2-5), indicating that LPS treatment did not affect raft integrity (Fig. 2A).

The presence of CD14 in lipid rafts after LPS stimulation was also investigated. CD14 was found to be present in lipid rafts after LPS stimulation (Fig. 2B). Other LPS receptors such as hsp70 and hsp90 that were associated with lipid rafts prior to LPS stimulation were still present in microdomains after LPS treatment (Fig. 2C,D). In contrast CXCR4, GDF5 and TLR4, which were not associated with lipid rafts prior to LPS stimulation, were recruited in microdomains following LPS treatment (Fig. 2E,F,G). No protein bands were detected in

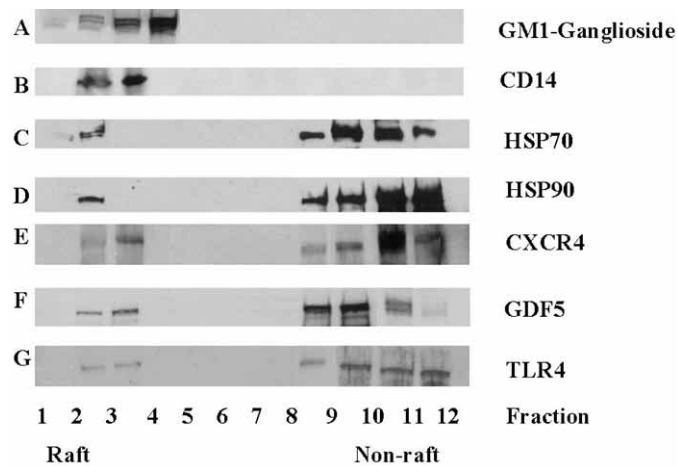


Fig. 2. Receptor molecules implicated in LPS-cellular activation present in lipid rafts after LPS stimulation. MonoMac-6 cells were stimulated with 10 ng/ml LPS in 5% HPS for 30 minutes prior to solubilisation with 1% Triton X-100 buffer for 1 hour on ice and then subjected to sucrose density gradient centrifugation. Fractions were collected from the top of the gradient, 1% *n*-octylglucoside was added to each fraction, and equivalent portions of each fraction were analysed by SDS-PAGE and immunoblotting. The lipid raft marker was detected using HRP-conjugated cholera toxin (A), the nitrocellulose membranes were also probed with 26ic (CD14-specific mAbs) (B), hsp70 (C), hsp90 (D), CXCR4 mAbs (E) and GDF5 polyclonal serum (F), as well as with the HTA125 TLR4-specific mAbs (G). The relative positions of the raft and non-raft (soluble) fractions are indicated.

control experiments utilising just the HRP-conjugated secondary antibody.

In order to rule out the possibility that impurities in the LPS preparation were responsible for the events observed, we re-purified our commercial LPS using a protocol shown previously to remove contaminants from LPS preparations (Hirschfeld et al., 2000; Tapping et al., 2000) and used it to stimulate cells prior to raft isolation. Results similar to the commercial preparations were obtained with the re-purified LPS.

FRET imaging of lipid rafts

The concentration of receptor molecules involved in LPS-induced cell activation in lipid rafts was also examined using fluorescence resonance energy transfer (FRET). FRET is a biophysical method used to determine proximity of molecules on the cell surface of living cells under conditions very close to the physiological state of the cells (Stryer, 1978; Szollosi et al., 1989; Wu and Brand, 1994). Lipid rafts tend to aggregate into distinct patches on the cell membrane that can be visualised by confocal microscopy using rhodamine-conjugated cholera toxin. By performing FRET experiments we could investigate whether the receptors of interest were colocalising with lipid rafts.

We measured FRET in terms of dequenching of donor fluorescence after complete photobleaching of the acceptor fluorophore. Increased donor fluorescence after destruction of the acceptor indicated that donor fluorescence was quenched in the presence of the acceptor because of energy transfer. We tested the energy transfer efficiency in our system using a positive control, that is, energy transfer between mAbs to different epitopes on CD14 molecules, showing that the maximum energy transfer efficiency (E%) was 39 ± 1.0 (Table 1). A negative control between FITC-26ic (the mAb specific for CD14) and rhodamine-W6/32 (the mAb specific for the major histocompatibility complex (MHC) class I) was also used, which revealed no significant energy transfer (4 ± 1.0). This background FRET value is probably caused by random FRET as both species are present at high concentrations.

Table 1. Energy-transfer efficiency values (E) between donor-acceptor pairs before and after LPS stimulation

Donor (FITC)	Acceptor (Rhodamine)	E \pm Δ E (%)
Before LPS stimulation		
CD14	CD14	39 \pm 1.0
CD14	GM-1 ganglioside	32 \pm 1.2
Hsp70	GM-1 ganglioside	17 \pm 1.2
Hsp90	GM-1 ganglioside	13 \pm 1.0
TLR4	GM-1 ganglioside	6 \pm 0.8
CXCR4	GM-1 ganglioside	7 \pm 2.0
GDF5	GM-1 ganglioside	3 \pm 1.0
After LPS stimulation		
CD14	GM-1 ganglioside	32 \pm 1.8
Hsp70	GM-1 ganglioside	31 \pm 2.0
Hsp90	GM-1 ganglioside	28 \pm 0.5
TLR4	GM-1 ganglioside	34 \pm 0.8
CXCR4	GM-1 ganglioside	26 \pm 1.0
GDF5	GM-1 ganglioside	19 \pm 1.2

Energy transfer between different pairs was detected before and after LPS stimulation from the increase in donor fluorescence after acceptor photobleaching. Data are means \pm s.d. values from several independent experiments.

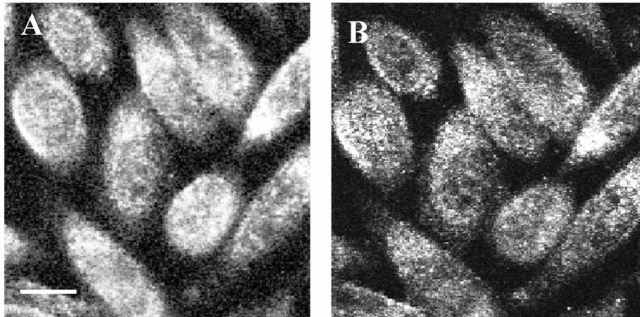


Fig. 3. CD14 and GM-1 ganglioside FRET measurements. Energy transfer between CD14 (FITC-26ic) and GM-1 ganglioside (rhodamine-cholera-toxin) can be detected by the increase in donor fluorescence after acceptor photobleaching. Donor (FITC) after (A) acceptor photobleaching and (B) E image. Bar, 5 μ m.

In order to visualise whether CD14 molecules were localised in lipid rafts prior to LPS stimulation, CD14 molecules were labelled with FITC-26ic and GM-1 ganglioside, a raft-associated lipid, was labelled with rhodamine-cholera toxin. We proceeded to measure FRET between CD14 (FITC-26ic) and GM-1 ganglioside (rhodamine-cholera-toxin) on CHO/CD14/TLR4 cells (Fig. 3). Large dequenching was observed once the rhodamine was photobleached ($E=32\pm 1.2\%$), suggesting that CD14 resides in lipid rafts. Similar results were obtained when FITC-hsp70 was used ($E=17\pm 1.2\%$). Hsp90 also gave similar results although there was less FRET than the one observed between CD14 and GM-1 ganglioside, $E=13\pm 1.0\%$ (Table 1). In contrast TLR4, CXCR4 and GDF5 were not found to be associated with lipid rafts prior to LPS stimulation, giving FRET values of $6\pm 0.8\%$, $7\pm 2.0\%$ and $3\pm 1.0\%$ respectively (Table 1).

We also examined whether CD14, hsp70, hsp90, CXCR4, GDF5 and TLR4 were located in lipid rafts after LPS stimulation. In agreement with our biochemical data, we found GM-1 ganglioside to associate with CD14 ($E=32\pm 1.8\%$), hsp70 ($E=31\pm 2.0\%$) and 90 ($E=28\pm 0.5\%$) after LPS stimulation (Table 1). Similar FRET levels were also observed between TLR4 (FITC-TLR4) and GM-1 ganglioside (rhodamine-cholera toxin) ($E=34\pm 0.8\%$) (Fig. 4), and CXCR4 (FITC-CXCR4) and the lipid raft marker (rhodamine-cholera toxin) ($E=26\pm 1.0\%$), as well as GDF5 ($E=19\pm 1.2\%$). Our data shows that TLR4, CXCR4 and GDF5 do not reside in lipid rafts but are recruited there after LPS stimulation. As a control, cells were stimulated with Phorbol myristate acetate (PMA). Similar FRET values to the ones observed before LPS stimulation were obtained. In conclusion our confocal data was in agreement with the biochemical results obtained. Similar results were obtained from both cell lines.

In order to rule out the possibility that the FRET observed was caused by randomly distributed molecules and not clustered molecules in microdomains, we decided to test the theory of Kenworthy et al. (Kenworthy and Edidin, 1998a) and measure the dependence of FRET on donor and acceptor surface density. Thus we varied the ratio of donors and acceptors used to label the proteins of interest and plotted FRET efficiency (E) against acceptor concentration. E was found to be independent on acceptor surface density, to be sensitive to donor:acceptor ratio and not to go to zero at low surface density,

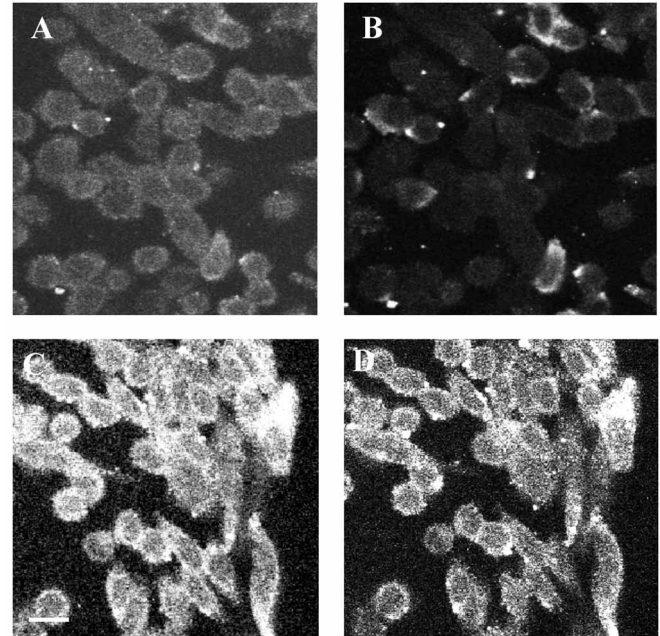


Fig. 4. TLR4 and GM-1 ganglioside FRET measurements. Energy transfer between TLR4 (FITC-HTA125) and GM-1 ganglioside (rhodamine-cholera-toxin) before (A,B) and after (C,D) LPS stimulation. Energy transfer can be detected by the increase in donor fluorescence after acceptor photobleaching. Donor (FITC) after (A,C) acceptor photobleaching and (B, D) E image. Bar, 10 μ m.

thus suggesting that the FRET values observed were caused by clustered molecules and not random associations.

Inhibition of raft formation

In order to test whether we can disrupt lipid raft formation we utilised MCD or nystatin. MCD or nystatin treatment of cells leads to the redistribution of molecules out of lipid rafts (Keller and Simon, 1998; Kilsdonk, 1995; Scheiffele et al., 1997; Cheng et al., 1999; Xavier et al., 1998). CHO/CD14/TLR4 cells were treated either with MCD or nystatin before or after LPS stimulation followed by raft isolation using sucrose density centrifugation as described in the Materials and Methods. Equivalent portions of each fraction (collected from the top of the gradient) were analysed by SDS-PAGE electrophoresis and immunoblotting. It was found that MCD (and to a lesser extent nystatin) inhibited raft formation when nitrocellulose

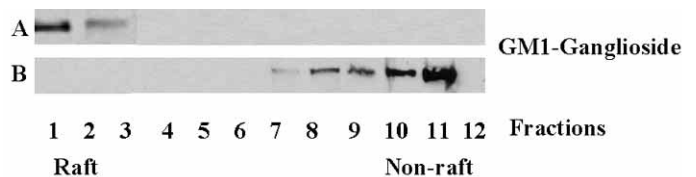


Fig. 5. MCD disrupts lipid raft formation. MonoMac-6 cells were either not treated (A) or treated (B) with 10 mM MCD for 10 minutes, before solubilisation in 1% Triton X-100 buffer, followed by raft and non-raft separation by sucrose density gradient centrifugation. The GM-1 ganglioside distribution was visualised using HRP-conjugated cholera toxin.

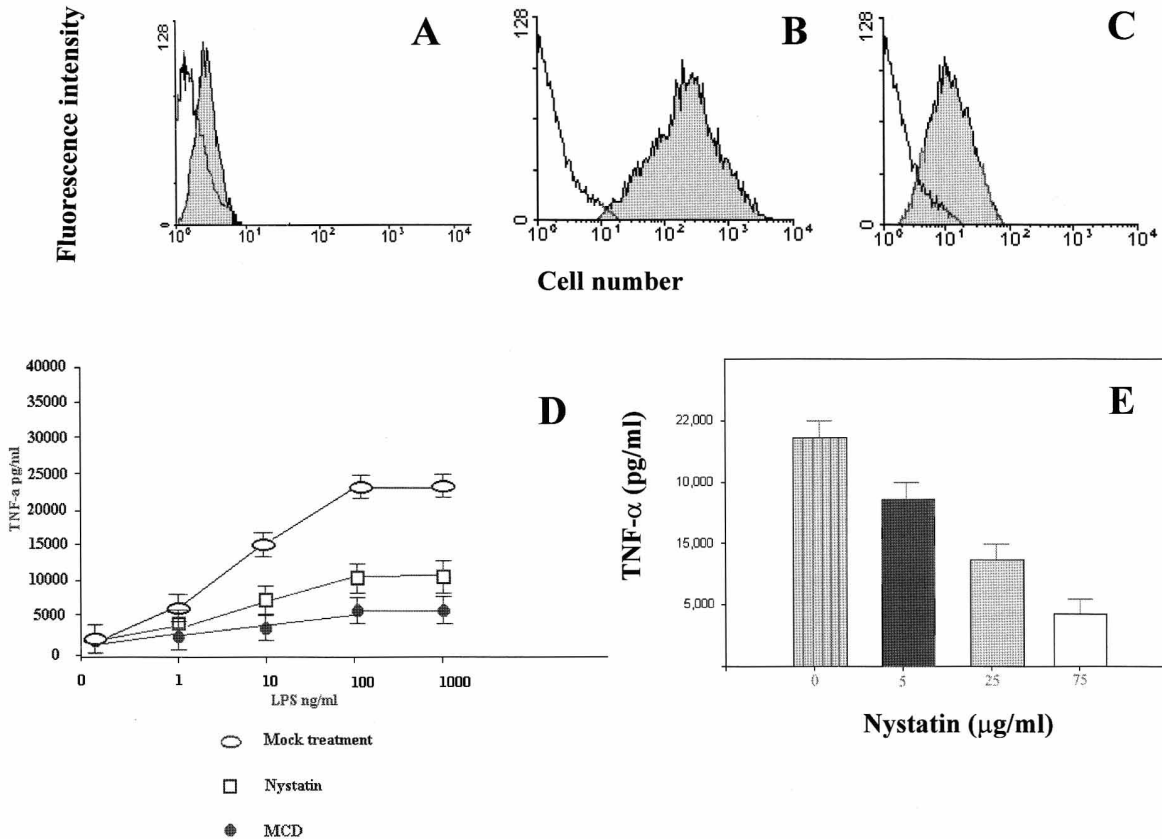


Fig. 6. Disrupting lipid raft integrity inhibits LPS-mediated cellular activation. CHO/CD14/TLR4 reporter cell line was either not stimulated (A) or stimulated with 10 ng/ml of LPS in 5% HPS for 30 minutes either in the absence (B) or presence (C) of 60 μ g/ml nystatin. The induction of CD25 surface expression was detected with FITC-CD25. Fluorescence was detected using a FACSCalibur (Becton Dickinson), counting 10,000 cells per sample. The effect of raft-disrupting drugs on TNF- α secretion was measured (D) by treated monocytes isolated from the blood of healthy donors with 10 mM MCD (black circles) or 60 μ g/ml nystatin (white squares) prior to stimulation with 10 ng/ml LPS in 5% HPS. Control experiments with cells stimulated with LPS in the absence of raft-disrupting drugs were also performed (eclipse). The effect of different concentrations of nystatin on LPS-induced TNF- α secretion was also measured (E). TNF- α secretion was measured using an ELISA. Each data point represents a number of independent experiments.

membranes were treated with HRP-cholera toxin in order to visualise GM1-ganglioside, the lipid raft marker (Fig. 5B). MCD was found to inhibit CD14 (Fig. 5B), hsp70, hsp90, CXCR4, GDF5 and TLR4 association with lipid rafts. Similar results were obtained when MonoMac-6 cells were used.

Functional significance of lipid raft integrity

In order to investigate the functional significance of lipid raft integrity we evaluated the ability of LPS to stimulate cells that had been treated with raft-disrupting agents. The CHO/CD14/TLR4 LPS-reporter cell line was used, which upregulates surface expression of the human Tac Ag (α -chain of the IL-2R; CD25) following exposure to LPS. CD25 expression was measured before (A) and after (B) LPS stimulation. CD25 was found to be specifically upregulated in response to LPS (Fig. 6B). In contrast if the cells were pre-treated with raft-disrupting drugs, prior to LPS stimulation, CD25 surface expression was significantly reduced (Fig. 6C).

In order to investigate whether raft integrity affected TNF- α secretion, we attempted to disrupt lipid raft formation. Human monocytes were either cultured for 5 minutes with nystatin, a fungal metabolite that binds membrane cholesterol

and disrupts raft integrity (Rothberg, 1992), or for 10 minutes with MCD, a compound that disrupts protein association with lipid rafts (Keller and Simon, 1998). Pre-incubation of monocytes with either nystatin or MCD dramatically inhibited LPS-induced TNF- α secretion (Fig. 6D). In order to verify that raft integrity is necessary for LPS-cellular activation, titration experiments with nystatin were performed. It was shown that TNF- α secretion was inhibited in nystatin-concentration-dependent manner (Fig. 6E). Similar data were obtained with MCD.

Cell surface expression of receptor molecules involved in LPS cellular activation is not altered by raft disrupting drugs

Since nystatin and MCD treatment greatly inhibited LPS-induced TNF- α secretion, we examined the effect of these drugs on the cell surface expression of molecules involved in LPS-cellular activation. Treatment of MonoMac-6 cells with nystatin or MCD under conditions that greatly inhibited TNF- α secretion did not affect the cell surface expression of CD14, hsp70, hsp90, CXCR4, GDF5 or TLR4 (Fig. 7). Therefore raft-disrupting drugs do not cause loss or shedding of these molecules.

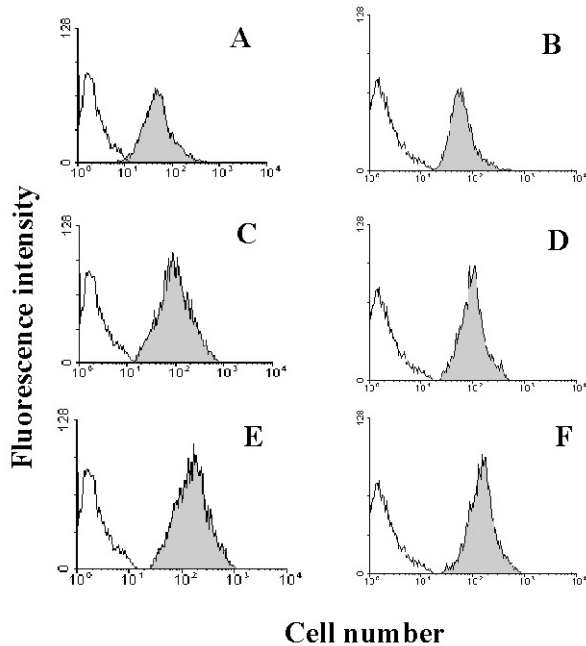


Fig. 7. Raft-disrupting drugs do not alter surface expression of molecules involved in LPS-induced cellular activation. MonoMac-6 cells were either mock-treated (A,C,E) or treated with 10 mM MCD (B,D,F) for 10 minutes before washing with buffer. Surface expression of CD14 (A,B), CXCR4 (C,D) or TLR4 (E,F) was determined by flow cytometry. All primary antibodies were visualised with FITC-conjugated secondary antibodies and analysed by flow cytometry utilising a FACScalibur (Becton Dickinson) counting 10,000 cells per sample.

Furthermore we tested whether MCD or nystatin was cytotoxic to the cells. Our results showed that the viability of the cells was not affected after MCD or nystatin treatment, as drug-treated cells excluded trypan blue.

Signalling in lipid rafts

One of the most widely appreciated roles of lipid rafts is in the recruitment and concentration of molecules involved in cellular signalling (Horejsi et al., 1999). Many signalling molecules involved in T-cell activation, such as the Src family kinase Lck, have been found to be associated with rafts, (Rodgers and Rose, 1996; Kabouridis et al., 1997). With this in mind we addressed the possibility that lipid rafts concentrate signalling molecules implicated in LPS-cellular activation. We immunoblotted nitrocellulose membranes with MyD88, a signalling molecule that is employed by TLR4 for signalling. We found that we could detect MyD88 in fractions two to three of LPS-stimulated MonoMac-6 cells, suggesting that it is indeed associated with lipid rafts (Fig. 8A), whereas MyD88 was not detected in fractions isolated from non-stimulated MonoMac-6 cells (Fig. 8B). We also probed nitrocellulose membranes with Rac1-specific and phospho-specific Jun N-terminal kinase/Stress-activated protein kinase (JNK/SAPK) polyclonal sera; these sera only recognise the activated form of the molecule. Rac1 has recently been implicated in TLR2-mediated activation (Arbibe et al., 2000), whereas JNK/SAPK is required for LPS stimulation (Weinstein et al., 1990; Hambleton et al., 1996; Swantek et al., 1997). We found that Rac1 was not concentrated in lipid rafts

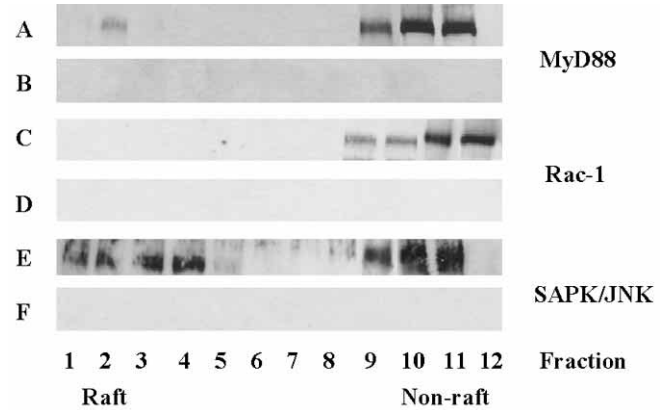


Fig. 8. LPS signalling in lipid rafts. MonoMac-6 cells were either stimulated (A,C,E) or not stimulated with LPS (B,D,F) prior to treatment with 1% Triton X-100 buffer for 1 hour on ice and then subjected to sucrose density gradient centrifugation. Fractions were collected from the top of the gradient, 1% *n*-octylglucoside was added to each fraction, and equivalent portions of each fraction were analysed by SDS-PAGE and immunoblotting. The nitrocellulose membranes were probed with MyD88 (A,B), Rac-1 (C,D) or SAPK/JNK (E,F) phospho-specific antibodies followed by incubation of HRP-conjugated secondary antibodies. The relative positions of the raft and non-raft (soluble) fractions are indicated.

either before (Fig. 8D) or after LPS stimulation (Fig. 8C). Whereas when we used a phospho-specific JNK/SAPK antibody we found that JNK/SAPK was concentrated in lipid rafts only following LPS stimulation (Fig. 8E), lending more support to the idea that LPS signalling occurs within lipid rafts.

Discussion

Our understanding of the structure of the plasma membrane of mammalian cells was based on the Singer-Nicolson fluid mosaic membrane model, where all the constituents of the membrane move freely (Singer and Nicolson, 1972). In recent years the Singer-Nicolson membrane model has been changed by demonstrations of lateral heterogeneities, patches and domains in the plasma membrane (Edidin, 1996; Jacobson et al., 1995; Kusumi and Sako, 1996). These lateral heterogeneities of the plasma membrane, often called 'lipid rafts', are emerging as sites of cellular signalling.

In this study we decided to investigate the significance of raft formation in LPS-mediated cellular activation. Using biochemical techniques in order to isolate lipid rafts on the basis of their insolubility in Triton X-100 and low buoyant density centrifugation, we isolated lipid rafts before and after LPS stimulation. Certain molecules involved in the innate recognition of bacteria, such as CD14 and hsp70 and 90, were constitutively found in membrane microdomains. In contrast molecules, such as TLR4, CXCR4 and GDF5, which have been recently implicated in LPS-cellular activation (Triantafilou et al., 2001a), were not found constitutively in lipid rafts, but were recruited there following LPS stimulation. Similar results were obtained with FRET imaging using dual labelling GM1-ganglioside, a lipid raft marker, and the receptor molecules of interest we investigated whether they were associated before and after LPS stimulation. We found that a cluster of receptors accumulated in microdomains after LPS stimulation.

These results are in agreement with our previous data obtained with fluorescence recovery after photobleaching (FRAP) that suggested that LPS quickly transfers from CD14 to a complex of receptors (Triantafilou et al., 2001b). Furthermore these results are in good agreement with our previous fluorescence resonance energy transfer (FRET) studies that suggested physical proximity between hsp70, 90, CXCR4 and GDF5 (Triantafilou et al., 2001a) as well as CD14 and TLR4 (Jiang et al., 2000) following LPS stimulation. This physical proximity that was previously observed is obviously induced by the concentration of these molecules in membrane microdomains after LPS ligation.

Since our data suggested that the receptors involved in the innate recognition of bacteria are recruited in lipid rafts following LPS stimulation, we decided to investigate the functional significance of raft integrity for LPS-mediated cellular activation. We found that by disrupting lipid raft formation we could inhibit LPS-induced TNF- α production. Our findings clearly demonstrate that the effects of the raft-disrupting drugs, such as nystatin or MCD, can be directly attributed to their ability to displace the complex of receptors involved in LPS activation (CD14, hsp70, hsp90, CXCR4, GDF5 and TLR4) rather than to other nonspecific effects, such as alteration of the cell surface expression of these molecules or reduction of cell viability.

Furthermore we found that certain signalling molecules that have been implicated in LPS-mediated cellular activation, such as MyD88 and JNK/SAPK, were recruited in lipid rafts following LPS stimulation. Raft-disrupting drugs were found not only to displace the receptor complex involved in LPS signalling but also their signalling machinery, lending more support to the notion that lipid rafts are areas of concentrated receptor signalling.

Ever since the discovery of the immunological synapse and the plethora of receptors and microdomains involved in the acquired immune recognition (Grakoui et al., 1999; Monks et al., 1998), it has become clear that the mechanism of action of a single receptor molecule is an oversimplified model in many cases. In the case of innate immune recognition and particularly in LPS recognition, accumulating evidence suggests that it involves the dynamic association of multiple receptors within microdomains. Given the diverse range of receptors that have been reported to be involved in LPS recognition, it is possible that the transient association of different receptors within the activation cluster could give rise to the recognition/discrimination of different ligands. This potential has been demonstrated in recent studies where the association of CD11b/CD18 with CD14 and TLR4 has been shown to be required for the expression of a full repertoire of LPS/taxol-inducible genes (Perera et al., 2001). In addition TLR2 interacts with either TLR1 or TLR6 in response to phenol-soluble modulin (Hajjar et al., 2001), whereas CD14 co-clusters with different receptor molecules depending on the ligand (Pfeiffer et al., 2001), thus suggesting that the innate recognition of bacteria can not be attributed to a single molecule but rather to a cluster of receptors.

On the basis of our findings we propose a model where LPS initially binds to CD14. Following ligation of CD14 by LPS, different signalling molecules are recruited at the site of the ligation within lipid rafts, where LPS is then briefly released into the lipid bilayer where it finally interacts with a complex

of receptors, which involves hsp70, hsp90, CXCR4, GDF5 and TLR4. This is in good agreement with Schromm et al., whose data suggest that LPS cellular activation occurs in the plasma membrane by lateral diffusion of the intercalated LPS molecules to transmembrane proteins that then initiate signalling by steric stress (Schromm et al., 2000). This is also in good agreement with Pfeiffer et al. (Pfeiffer et al., 2001), who have recently shown that LPS and ceramide can provoke ligand-specific receptor clustering in rafts.

In conclusion, the combined biochemical fractionations of cellular membranes and FRET studies suggest that CD14 and hsp normally reside in microdomains. Following LPS ligation, other signalling molecules, such as CXCR4, GDF5 and TLR4, are recruited to the site of LPS ligation within the lipid rafts. The LPS 'transducing' molecules and their signalling machinery concentrate in the small receptor islands, form a complex and enhance LPS-mediated signalling by providing a focused signalling event. Thus, this receptor cluster on both the molecular and the submicron level underlies the efficiency of signalling in LPS-stimulated cells and plays a significant role in the directed secretion of pro-inflammatory cytokines, such as TNF- α .

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