# Lateral diffusion of Toll-like receptors reveals that they are transiently confined within lipid rafts on the plasma membrane

**Martha Triantafilou[1], Siegfried Morath[2], Alan Mackie[3], Thomas Hartung[2] and Kathy Triantafilou[1,*]**

[1]Infection and Immunity Group, School of Life Sciences, University of Sussex, Falmer, Brighton, BN1 9QG, UK
[2]Department of Biochemical Pharmacology, University of Konstanz, Universitätsstr. 10, 78457 Konstanz, Germany
[3]Department of Food Biophysics, Institute of Food Research, Norwich Research Park, Colney Lane, Norwich, NR4 7UA, UK
*Author for correspondence (e-mail: k.triantafilou@sussex.ac.uk)

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## Summary

**The innate immune system utilises pattern recognition receptors in order to recognise microbial conserved molecular patterns. The family of Toll-like receptors (TLRs) has been shown to act as the main pattern recognition receptors for the innate immune system. Using biochemical as well as fluorescence imaging techniques, TLR2 and TLR4 were found to be recruited within microdomains upon stimulation by bacterial products. Furthermore their lateral diffusion in the cell membrane as determined by fluorescence recovery after photobleaching revealed that upon stimulation by bacterial products TLRs encounter barriers to their lateral movement, thus supporting the notion that specialised domains on the plasma membrane facilitate the innate recognition.**

Key words: LTA, Toll-like receptors, LPS-activation cluster, Innate recognition, FRAP

## Introduction

The innate immune system has evolved the capacity to recognise a broad spectrum of pathogens and to discriminate between 'infectious non-self' and 'non-infectious self' (Medzhitov and Janeway, 2002). The basis of microbial recognition lies in the ability of the innate immune system to recognise conserved microbial components that are specific to the micro-organisms but not to the host. These conserved microbial components are referred to as pathogen-associated molecular patterns (PAMPs). Most widely known PAMPs include lipopolysaccharide (LPS) from Gram-negative bacteria and lipoteichoic acids (LTA) as well as peptidoglycan from Gram-positive bacteria. Many different types of receptors participate in the microbial detection by the innate immune system and they are called pattern-recognition receptors (PRRs). Such receptors include CD14 (Wright et al., 1990), integrins such as CD11c/CD18 (Ingalls and Golenbock, 1995) and CD11b/CD18 (Perera et al., 2001), CD55 (Heine et al., 2001; Heine et al., 2003), heat shock proteins (Byrd et al., 1999; Triantafilou et al., 2001a), but most importantly the Toll-like receptor (TLR) family that has been shown to participate in the recognition of microbial pathogens in several organisms including humans, mice and flies (Underhill and Ozinsky, 2002). The best characterised members of the TLR family include TLR4 and TLR2. TLR4 has been shown to recognise LPS from Gram-negative bacteria (Poltorak et al., 1998), and TLR2 has been shown to mediate responses against various fungal, Gram-positive and mycobacterial components (Qureshi et al., 1999; Ozinsky et al., 2000; Means et al., 1999).

Although TLRs have been shown to be the key PRRs for the innate immune system, it is not yet clear what is the exact mechanism that these conserved molecules utilise in order to recognise such a broad spectrum of microbial components. The challenge has now changed from simply identifying the PRRs responsible for recognising microbial products to determining the exact mechanism of receptor engagement.

In order to provide the first dynamic picture of TLR engagement we investigated the diffusion of TLRs on the cell surface before and after stimulation by their ligands. The measurement of the lateral diffusion of TLRs was performed by fluorescence recovery after photobleaching (FRAP), which provided us with a diffusion coefficient, D, which shows us how fast TLR molecules are diffusing in the plasma membrane before and after stimulation. Both TLR2 and TLR4 were found to have similar diffusion coefficients. In addition, FRAP also provided us with the extent of fluorescence recovery, which indicates the percentage of molecules that are moving into the area, and also the existence of membrane microdomains. Both TLRs exhibited reduced percentage recovery upon stimulation by their respective ligand, thus indicating that upon stimulation by bacterial products TLRs encounter barriers to their lateral movement. Our data supports the notion that association with rafts is not only restricted to TLR4, but is a common feature of the TLR family and that specialised domains on the plasma membrane facilitate the innate recognition.

## Materials and Methods

### Materials

ReLPS from *Salmonella minessota* Re595 was purchased from List Labs (CA, USA) and repurified as previously described (Manthey and Vogel, 1994). LTA from *Staphylococcus aureus* was prepared as

previously described (Morath et al., 2002). All fine chemicals and human pooled serum was purchased from Sigma Chemical Co. (St Louis, Mo., USA). Hybridoma cells secreting 26ic (anti-CD14) and W6/32 secreting MHC class I-specific monoclonal antibody (mAb), were obtained from the American Type Culture Collection (ATCC, MD). TLR2-specific mAb was obtained from Cambridge Biosciences (Cambridge, UK). HTA125, TLR4-specific mAb was a gift from K. Miyake. Oregon Green 488 as well as X-Rhod-1 and Pluronic F-127 were obtained from Molecular Probes (Netherlands).

### Preparation of fluorescent probes

In order to create the smallest possible fluorescent probes purified IgG was digested to gain Fab and Fc fragments. Since protein A has a high affinity for the Fc region of IgG, Fab fragments were purified from the Fc by protein-A affinity chromatography. Fab fragments of TLR2- and TLR4-specific mAbs were conjugated to Oregon Green (OG) 488 (Molecular Probes, Europe), with a succinimidyl ester linkage according to the manufacturer's instructions, in order to be used as probes for FRAP measurements. Briefly 0.5 mg/ml of Fab were mixed with 20% by volume of sodium bicarbonate buffer (1 M, pH 8.0) and freshly prepared Oregon Green (OG) succinimide in DMSO (5 mg/ml). The mixture was placed in the dark for 1 hour at room temperature. The reaction was terminated by adding 10% by volume of hydroxylamine (1.5 M, pH 8.5) and incubating for a further hour in the dark. The TLR2-OG or TLR4-OG conjugate was separated from the unconjugated Fab and unreacted OG by gel exclusion chromatography using a PD10 column (Amersham Pharmacia). The conjugated Fab appeared in the second or third 1 ml fraction and was dialysed against PBS.

In addition, for FRET measurements TLR2-specific mAb as well as cholera toxin were conjugated to either Cy3 or Cy5 (Amersham Pharmacia) according to the manufacturer's instructions.

### Cells

Monocytes were isolated from human A+ buffy coats. Adherent cell monolayers ($1\times10^5$ to $2\times10^5$ monocytes/well) were cultured in 24-well plates in serum-free medium (Gibco) supplemented with 0.01% L-glutamine and 40 μg of gentamicin/ml.

Chinese hamster ovary (CHO) cells transfected with hCD14 and hTLR4 cDNA or hTR2 cDNA in a reporter background (Delude et al., 1998) were also utilised. These cells provide us with the perfect reporter systems for studying LPS- and LTA-induced cellular activation, since there is surface expression of the human Tac Ag (a-chain of the IL-1R; CD25) following NF-κB activation in response to either LPS or LTA. CHO cells were maintained in Ham's F12 from Gibco-BRL supplemented with 2 mM L-glutamine, 7.5% FCS, 500 μg/ml gentamycin sulfate (G418; Sigma).

### Biochemical isolation of lipid rafts

Biochemical isolation of lipid rafts was performed as previously described (Triantafilou et al., 2001a). Monocytes ($1\times10^8$) were lysed in 500 μl of MEB buffer (150 mM NaCl, 20 mM MES, pH 6.5) containing 1% Triton X-100 and protease inhibitors (500 μM 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 μM final concentration).

### 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 plus 0.1% Tween-20; PBST) and washed with PBST. Membranes were probed with the appropriate dilution of primary antibody for 1 hour followed by washing with PBST. Membranes were incubated with HRP conjugated to rabbit anti-mouse Ig for 1 hour. For detection of GM-1 ganglioside, membranes were incubated with HRP-conjugated cholera toxin (Quadratech, UK). After extensive washing with PBST, the antigen was visualised using the ECL procedure (Amersham Pharmacia) according to the manufacturer's instructions.

### FRET measurements

FRET involves non-radiative transfer of energy from the excited state of a donor molecule to an appropriate acceptor (Wu and Brand, 1994). The rate of energy transfer (E) is inversely proportional to the sixth power of the distance r between the donor and the acceptor:

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

In the present study FRET was measured using a previously described method (Kenworthy and Edidin, 1998a). Briefly, human monocytes were cultured on microchamber culture slides (Lab-tek, Gibco), and labelled with 100 μl of a mixture of donor-conjugated antibody (Cy3) and acceptor-conjugated cholera toxin (Cy5). Energy transfer was detected as an increase in donor fluorescence (dequenching) after complete photobleaching of the acceptor molecule. Cells were imaged on a Carl Zeiss, Inc. LSM510 META confocal microscope (with an Axiovert 200 fluorescence microscope) using a 1.4 NA 63× Zeiss objective. Cy3 was excited with a helium/neon laser line emitting at 543 nm, whereas Cy5 was excited with the 633 nm laser line. Tracks were scanned sequentially with only one laser and respective detector channel active per scan. The images were analysed using LSM 2.5 image analysis software (Carl Zeiss, Inc.). FRET was calculated from the increase in donor fluorescence after acceptor photobleaching by:

$$E\% \times 100 = 10{,}000 \times [(\text{donor postbleach-donor prebleach})/\text{donor postbleach}] .$$

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

### FRAP measurements

Cells were cultured on microchamber slides and labelled with either TLR2-OG or TLR4-OG, subsequently FRAP measurements were performed as previously described (Ladha et al., 1994; Triantafilou et al., 2001b; Edidin, 2001). Briefly, slides containing labelled cells were placed onto a temperature controlled microscope stage (Physitemp, Model TS-4) and allowed to equilibrate to the desired temperature for FRAP measurements. The beam of an argon ion laser (Innova, Palo Alto, CA; 100-10) was focused onto the desired area on the cell. The laser beam was of Gaussian cross-sectional intensity, with a half-width at $1/e^2$ height of the laser beam at its point of focus equal to 1.24 or 2.15 μm spot radius. The wavelength of 488 nm was used for bleaching the TLR2-OG or TLR4-OG probes. For each set of conditions ten curves were collected and averaged before analysis. FRAP data were analyzed by non-linear least squares fitting to the equation:

$$F(t) = \frac{F(0) + F(\infty)(t/\beta\tau_D)}{1 + (t/\beta\tau_D)} ,$$

where $F(t)$ is the measured fluorescence as a function of time $t$, $F(0)$ is the measured pre-bleach fluorescence and $F(\infty)$ is the value to which the fluorescence finally recovers. The parameter β is dependent on the

depth of bleach and $\tau_D$ is the diffusion time. The diffusion coefficient, $D$ is given by $D= \omega^2/4\tau_D$, where $\omega$ is the radius at $1/e^2$ height of the illuminating laser spot. The analysis assumes the laser illumination to be circular with a Gaussian intensity profile.

### Cytokine assay

Human monocytes ($5\times10^5$) were mixed with 50 μl of serial dilutions of LTA 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 μg/ml nystatin or 10 mM MCD for 10 minutes prior to LTA stimulation.

### Calcium signalling

CHO cells transfected with CD14 and TLR4 or TLR2 were grown in microchamber slides for 48 hours prior to the experiments. Cells were incubated in growth medium supplemented with 2 μM of the acetoxymethyl (AM) ester of the $Ca^{2+}$ indicator X-Rhod 1 and 25 mg/l Pluronic F-127 (Molecular Probes) for 30 minutes. The cells were subsequently labelled with TLR4-OG or TLR2-OG and Cy5-cholera toxin followed by washing with PBS in order to remove the unbound probes. Once the cells were labelled they were stimulated with 100 ng/ml of either LPS or pLA and viewed using a Zeiss Axiovert 200M microscope with an LSM 510 laser scanning head and a META detector. X-Rhod 1 is a rhodamine-based dye that is non-ratiometric and simply increases in fluorescence on binding $Ca^{2+}$. The increase in fluorescence after stimulation was measured using arbitrary units for fluorescence. Triple labelling of TLR4, the lipid raft marker and X-Rhod-1 was visualised using the appropriate filter sets as well as the META detector.

In addition, FRAP experiments were performed by bleaching a region of interest using the 488 laser line at 100% and subsequent monitoring using no more than 4% laser power.

## Results

### Biochemical isolation of lipid rafts

Since we have previously shown that TLR4 is recruited in lipid rafts after LPS stimulation, we wanted to directly test the hypothesis that this might also be true for other TLRs. Initially biochemical isolation of lipid rafts based on their insolubility in Triton X-100 and low buoyant density in sucrose gradients (Brown and Rose, 1992) was utilised in order to determine whether TLR2 was present in 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. GM-1 ganglioside was found to fractionate near the top of the sucrose gradient (fractions 2-5) (Fig. 1A), whereas the rest of the cellular membrane was found in the lower fractions. Immunoblotting with a TLR2-specific mAb revealed that TLR2 was concentrated in the lower fractions and thus was not constitutively present in lipid rafts (Fig. 1B). Upon stimulation with lipoteichoic acid (LTA) from *Staphylococcus aureus*, TLR2 was found to be recruited into lipid rafts (Fig. 1C), thus suggesting that similarly to TLR4, TLR2 must enter confined microdomains upon stimulation by Gram-positive bacterial products.

### FRET imaging of lipid rafts

Since isolation of the membrane microdomains, which are rich in phospholipids, sphingolipids and cholesterol, based on their insolubility in Triton X-100 and low buoyant density in sucrose gradients might not be a true reflection of membrane organisation, we also utilised fluorescence resonance energy transfer (FRET; a non-invasive imaging method) in order to investigate whether TLR2 associates with lipid raft markers such as GM-1 ganglioside. FRET measurements revealed that there was little energy transfer (E=5±1.0%) between TLR2 and GM-1 ganglioside prior to stimulation with LTA from *S. aureus* (Fig. 2B). Large increase in fluorescence (dequenching) was observed between TLR2 and GM-1 ganglioside after LTA stimulation (E=34±3.0%) (Fig. 2D).

In order to rule out the possibility that the FRET observed was due to randomly distributed molecules and not clustered molecules in microdomains, we decided to measure the dependence of FRET on donor and acceptor surface density (Kenworthy and Edidin, 1998b). 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. 2E), thus suggesting that the FRET values observed were due to clustered molecules and not random associations.

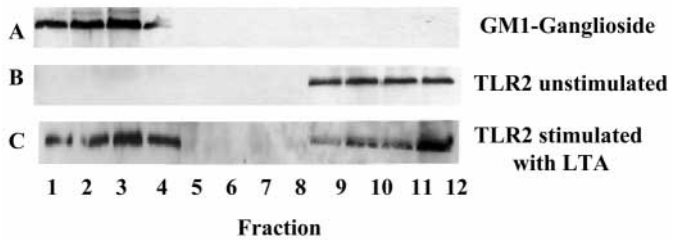


**Fig. 1.** TLR2 is present in lipid rafts following LTA stimulation. Monocytes were either not stimulated (A,B) or stimulated with 100 mg/ml LTA in 5% HPS for 30 minutes (C) prior to solubilisation with 1% Triton X-100 buffer for 1 hour on ice and then subjected to sucrose density gradient centrifugation. Fractions were collected from the top of the gradient 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 TLR2 specific mAb (B,C). The relative positions of the raft and non-raft (soluble) fractions are indicated.

### Lateral diffusion of TLRs

FRAP is a biophysical method that measures the lateral diffusion of a population of molecules on a micrometer scale. The diffusion coefficient gives us information as to how fast the molecules of interest are moving in the plasma membrane. In addition FRAP provides us with a percentage of molecules that are recovering within the photobleached area. This gives us information on whether the molecules are diffusing freely and also on the existence of segregated plasma membrane regions or microdomains. Thus FRAP provides us with the perfect tool to unravel the mechanism by which TLRs are engaged by their ligands. If TLR molecules associate with immobile or slowly diffusing proteins upon ligand stimulation, then their diffusion coefficient is expected to be lower than the one observed before stimulation. In addition, if the molecules

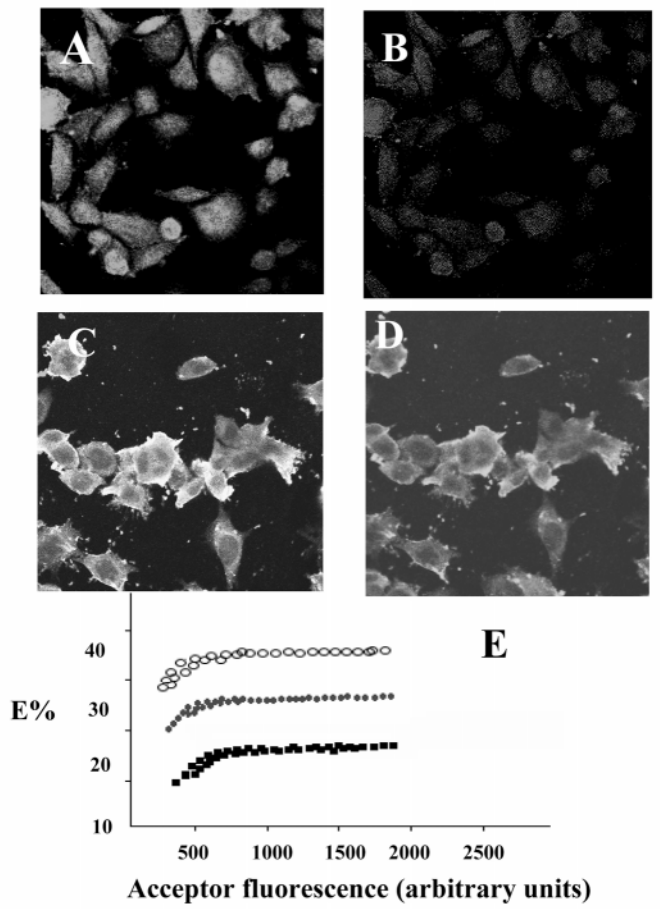


**Fig. 2.** TLR2 and GM-1 ganglioside FRET measurements before and after LTA stimulation. Energy transfer between TLR2 (Cy3-TLR2) and GM-1 ganglioside (Cy5-cholera toxin) before (A,B) and after (C,D) stimulation by LTA can be detected by the increase in donor fluorescence after acceptor photobleaching. (A,C) Donor (Cy3) image after acceptor photobleaching, (B,D) E image (image resulting from the subtraction of the Cy3 image before photobleaching from the Cy3 image after photobleaching). E as a function of fluorescence, for D:A of 1:1 (squares), 1:2 (closed circles) and 1:4 (open circles) (E). Scale bar: 5 μm.

accumulate in membrane microdomains then this should result in low percentage recovery or mobile fraction.

For our experiments a spot diameter of 1.24 μm was used initially in order to determine the mobility of TLRs. At 37°C the diffusion coefficient of TLR4 prior to LPS stimulation was found to be $(2.01\pm0.2)\times10^{-9}$ cm$^2$/second with 62±3% fluorescence recovery. Upon stimulation with 100 ng/ml of LPS, approximately 30% of the TLR4 pool became immobile, whereas the remaining 70% had a diffusion coefficient of $(9.73\pm0.2)\times10^{-10}$ cm$^2$/second with 57±8% fluorescence recovery. The TLR immobility suggests not only confinement, but also association of TLR4 with a receptor complex or an immobile receptor. This is in good agreement with our previous findings where we had observed that FITC-LPS becomes progressively immobile on the plasma membrane (Triantafilou et al., 2001b).

Similarly TLR2 was found to have a diffusion coefficient of $(1.71\pm0.3)\times10^{-9}$ cm$^2$/second with 52±14% fluorescence recovery prior to LTA stimulation. Upon LTA stimulation, in 86% of the cells examined TLR2 was found not to recover, whereas the remaining 14% had a diffusion of $(4.16\pm0.6)\times10^{-10}$ cm$^2$/second with 35±6% fluorescence recovery. This suggests that TLR2 must associate with other molecules, either slow-moving or immobile, upon ligand stimulation. This is in good agreement with the work of Ozinsky et al. who have demonstrated that TLR2 associates with TLR1 and TLR6 in order to discriminate between different ligands (Ozinsky et al., 2000).

**Table 1. Lateral diffusion of TLRs**

| Receptor | Temperature (°C) | Stimulation | Diffusion coefficient | Percentage recovery |
|---|---|---|---|---|
| TLR4 | 37 | – | $(2.01\pm0.2)\times10^{-9}$ | 62±3 |
| TLR4 | 37 | MCD | $(2.14\pm0.2)\times10^{-9}$ | 62±7 |
| TLR4 | 37 | LPS | 28% immobile, 72% $(9.73\pm0.2)\times10^{-10}$ | 57±8 |
| TLR4 | 37 | MCD + LPS | $(2.83\pm0.2)\times10^{-9}$ | 64±5 |
| TLR2 | 37 | – | $(1.71\pm0.3)\times10^{-9}$ | 52±14 |
| TLR2 | 37 | MCD | $(1.39\pm0.1)\times10^{-9}$ | 62±11 |
| TLR2 | 37 | LTA | 86% immobile, 14% $(4.16\pm0.6)\times10^{-10}$ | 35±6 |
| TLR2 | 37 | MCD + LTA | $(1.58\pm0.6)\times10^{-9}$ | 61±8 |

The values are the mean±standard deviation from several determinations (*f*10).

In order to determine whether the TLR immobility observed upon stimulation was the result of their confinement within lipid rafts, which in turn promoted large cluster formation, we examined the lateral diffusion of TLR2 and TLR4 before and after stimulation by their respective ligand, after treatment with methyl-β-cyclodextrin (MCD), a lipid raft inhibitor (Keller and Simons, 1998). MCD treatment did not affect TLR2 or TLR4 mobility on the plasma membrane of unstimulated cells (Table 1). In contrast, MCD treatment affected TLR lateral diffusion in cells stimulated by bacterial products (Table 1). TLR4 was found to have a lateral diffusion of $(2.83\pm0.2)\times10^{-9}$ cm$^2$/second with 64±5% fluorescence recovery, almost identical to that observed in unstimulated cells. Similarly, upon LTA stimulation, TLR2 was found to have a diffusion coefficient of $(1.58\pm0.6)\times10^{-9}$ cm$^2$/second with 61±8% fluorescence recovery. Stimulation by bacterial products, following MCD treatment, did not lead to an immobile TLR2 or TLR4 pool, or to lower diffusion coefficients as observed in the absence of MCD, suggesting that TLR molecules must be confined within lipid rafts upon stimulation. This confinement must promote the formation of receptor clusters, or association of TLR2 or TLR4 with an immobile receptor, which results in the immobile TLR pool that we observe (Table 1). It seems that disruption of lipid rafts must release TLR molecules to diffuse on the plasma membrane without any confinement or formation of immobile clusters.

## Mobility of TLRs reveals confinement within microdomains

Patches, corals or lateral domains in the plasma membrane were first identified as structures that hinder protein diffusion in the plasma membrane as detected by FRAP (Yechiel and Edidin, 1987; Edidin and Stoynowski, 1991; Pfeiffer et al., 2001). It is believed that the domains on the plasma membrane arise through the confinement of diffusible membrane proteins

**Table 2. Lateral diffusion of TLRs**

| Receptor | Spot radius (μm) | Stimulation | Diffusion coefficient | Percentage recovery |
|---|---|---|---|---|
| TLR4 | 1.24 | – | $(2.95\pm0.3)\times10^{-9}$ | 65±10 |
| TLR4 | 1.24 | LPS | $(2.10\pm0.2)\times10^{-9}$ | 49±12 |
| TLR4 | 2.15 | – | $(8.25\pm0.4)\times10^{-9}$ | 57±6 |
| TLR4 | 2.15 | LPS | $(3.61\pm0.4)\times10^{-9}$ | 39±8 |
| TLR2 | 1.24 | – | $(2.06\pm0.2)\times10^{-9}$ | 64±5 |
| TLR2 | 1.24 | LTA | $(7.51\pm0.8)\times10^{-10}$ | 60±14 |
| TLR2 | 2.15 | – | $(2.07\pm0.2)\times10^{-9}$ | 54±5 |
| TLR2 | 2.15 | LTA | $(1.39\pm0.2)\times10^{-9}$ | 40±6 |

The values are the mean±standard deviation from several determinations (*f*10).

(Edidin, 2001). Using FRAP we can determine the percentage of molecules that are recovering within the photobleached area, and so obtain information on whether the molecules are diffusing freely. With this in mind, if we compare the diffusion coefficient of the two TLR molecules before and after stimulation by bacterial products, we can see that they are very similar. In contrast if we compare the percentage recovery before and after stimulation we can detect a difference, and thus evidence of confinement after stimulation (Table 1).

In order to determine whether they are indeed confined in small areas (or microdomains) and whether their diffusion is hindered by barriers we also investigated the long-range diffusion of TLR2 and TLR4 by increasing the spot radius in our FRAP experiments to 2.15 μm. By varying the spot size we can determine whether TLR molecules are confined in lipid rafts, since this would result in a low percentage recovery or mobile fraction as the radius of the measuring and bleaching spots exceeds the radius of the microdomain. Upon increase of the spot size, the diffusion coefficient of TLR4 was $(8.25\pm0.4)\times 10^{-9}$ cm$^2$/second with 57±6% fluorescence recovery. After LPS stimulation, in 30% of the cells examined TLR4 was immobile, whereas the remaining 70% had a diffusion coefficient of $(3.61\pm0.4)\times 10^{-9}$ cm$^2$/second with 39±8% fluorescence recovery, suggesting that its long-range diffusion was restricted after LPS stimulation (Table 2). Similarly, when we utilised the larger spot radius the diffusion coefficient of TLR2 was $(2.07\pm0.2)\times10^{-9}$ cm$^2$/second with 54±5% fluorescence recovery. Stimulation with 10 μg/ml of LTA resulted in TLR2 immobility in 30% of the cells examined, whereas the remaining 70% had a diffusion coefficient of $(1.39\pm0.2)\times10^{-9}$ cm$^2$/s with 40±6% fluorescence recovery (Table 2).

If we look at the mobile fraction or percentage recovery of the molecules after stimulation by bacterial products in relation to the spot size used, it is clearly higher when the smaller radius was used (Table 2). This suggests that TLRs must be confined within microdomains following stimulation. Control experiments measuring the mobility of TLR4 after addition of LTA demonstrated that the mobile fraction of TLR4 remained unaffected before and after stimulation. Similar results were obtained when we performed control experiments measuring the mobility of TLR2 after addition of LPS.

### Significance of raft integrity

In order to determine the functional significance of these membrane microdomains for the innate recognition of Gram-positive bacteria, the ability of LTA to stimulate cells that had been treated with raft-disrupting drugs was evaluated. Initially the CHO/CD14/TLR2 reporter cell line was used, which upregulates surface expression of the human Tac Ag (a-chain of the IL-1R; CD25) following exposure to LTA. CD25 expression was measured before (Fig. 3A), and after (Fig. 3B) LTA stimulation. CD25 was found to be specifically upregulated in response to LTA (Fig. 3B). Control experiments using a TLR2-independent stimulus (i.e. LPS) did not upregulate CD25 expression on the cell surface (data not shown), thus demonstrating the specificity of the reporter cell line. In contrast, in cells that were pre-treated with raft-disrupting drugs, prior to LTA stimulation, CD25 surface expression was significantly reduced (Fig. 3C). Furthermore the possibility that raft-integrity affected LTA-induced TNF-α secretion was also investigated. Pre-incubation of human monocytes with raft-disrupting drugs, such as nystatin, dramatically inhibited LTA-induced TNF-α secretion (Fig. 3D). Control experiments were performed in order to investigate whether the cell surface expression of TLRs was affected by raft-disrupting drugs. These showed that the cell surface expression of TLRs remained unaffected. Furthermore we tested whether MCD or nystatin was toxic to the cells. Our results showed that the viability of the cells was not affected after nystatin treatment, since drug-treated cells excluded Trypan Blue.

### Signalling in lipid rafts

To further investigate the link between raft association and signalling function we measured calcium signalling in response to TLR activation during our FRAP experiments and correlated it with the observed diffusion properties on a cell to cell basis. CHO cells transfected with hCD14 and TLR4 or TLR2 were incubated with Cy5-labelled cholera toxin, as well as 2 μM X-Rhod 1/AM (a rhodamine-based dye which increases in fluorescence on binding $Ca^{2+}$) and Pluronic F-127, then stimulated with either LPS or LTA while performing FRAP experiments. We found TLR4 to co-localise with both the lipid raft marker and X-Rhod 1 after LPS stimulation (Fig. 4). FRAP experiments on a cell to cell basis in the same field, revealed that cells that exhibited an immobile TLR4 fraction also had a strong $Ca^{2+}$ signal (Fig. 4E,F, solid line), whereas cells that exhibited a confined TLR4 lateral mobility had a less intense $Ca^{2+}$ signal (Fig. 4E,F, dashed line). Similar results were obtained for TLR2.

Our data suggests that the confinement of TLR molecules within lipid rafts and subsequent clustering in response to bacterial products is crucial for signalling. In addition, the cell to cell variability in TLR immobilization after stimulation must be attributed to a variability in responsiveness to the stimulation. Cells that exhibit receptor immobilization must be the most responsive to stimulation, promoting TLR-lipid raft association and formation of clusters at a faster pace. The cells exhibiting confined TLR diffusion must be slower in responding to the stimulation. In those cells TLR molecules must be in the process of associating with lipid rafts but have not yet formed activation clusters.

## Discussion

A large leap forward in our understanding of the innate immune system came when work on *Drosophila* revealed that

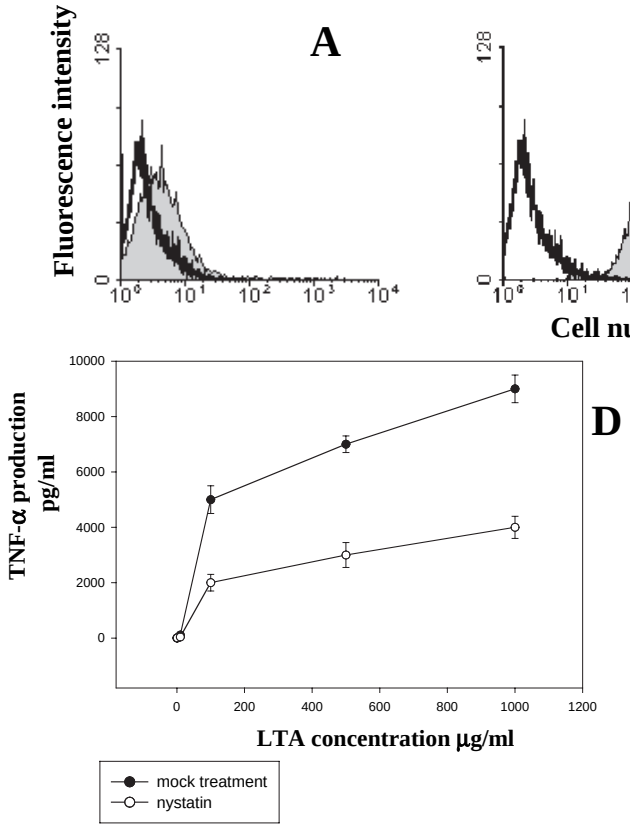


**Fig. 3.** Disrupting lipid raft integrity inhibits LPS-mediated cellular activation. CHO/CD14/TLR2 reporter cell line was either not stimulated (A) or stimulated with 100 mg/ml of LTA in 5% HPS for 30 min either in the absence (B) or presence (C) or 60 μg/ml nystatin. The induction of CD25 surface expression was detected with FITC-CD25. Fluorescence was detected using a FACSCalibur (BectonDickinson), counting 10,000 cells per sample. (D) The effect of raft-disrupting drugs on TNF-α secretion was measured in treated monocytes isolated from the blood of healthy donors with 60 μg/ml nystatin (open circles) prior to stimulation with LTA. Control experiments with cells stimulated with LTA in the absence of raft-disrupting drugs were also performed (closed circles). TNF-α secretion was measured using ELISA. Each data point represents the mean±s.d. of three independent experiments.

two members of the TLR family are responsible for the anti-fungal and anti-bacterial response in flies (Williams et al., 1997). The discovery of a human homologue of Toll (Medzhitov et al., 1997) changed completely our perception on how microbes are recognised by the innate immune system. Since then an onslaught of research has demonstrated that the family of Toll-like receptors represents the most ancient host defence system and plays a crucial role in the host defence of mammals, insects and plants. In mammals TLRs have been found to recognise diverse microbial products such as LPS (Poltorak et al., 1998), LTA (Yoshimura et al., 1999), peptidoglycan (Schwandner et al., 1999), flagellin (Hayashi et al., 2001), unmethylated CpG dinucleotides in bacterial DNA (Hemni et al., 2000), as well as double-stranded viral RNA (Alexopoulou et al., 2001).

The question that remains is how is the immune system able to discriminate among such a diverse pathogenic environment in order to direct a response that is tailored to the specific threat. It has been recently suggested that combinational assocations of TLRs (Ozinsky et al., 2000) or even combinational associations of TLRs with other receptors (Triantafilou and Triantafilou, 2002) might determine the repertoire of recognition of the innate immune system. The possibility that these associations are achieved by confinement of pattern recognition receptors in membrane microdomains has been suggested (Wang et al., 1996). Recently, we have found that TLR4 is recruited in membrane microdomains following stimulation by LPS (Triantafilou et al., 2002) and that subsequent lipid raft integrity is crucial for LPS-induced cellular activation. Here, for the first time we looked at the dynamic engagement of TLRs by their ligands using FRAP and FRET.

Membrane microdomains or ‘lipid rafts’ have been proposed as lateral structural components of the plasma membrane (Simons and Ikonen, 1997). This lateral plasma membrane asymmetry is maintained by tight junctions, which act as diffusion barriers for membrane bound proteins. To understand better the role of lipid rafts in innate immune recognition, a characterisation of their dynamics is needed. Thus although we isolated lipid rafts biochemically we also employed FRAP in order to investigate for the first time the lateral mobility of TLR4 and TLR2, as well as their confinement in membrane microdomains. FRAP provided us with the perfect tool to investigate the dynamic association of TLRs with lipid rafts, since it provided the diffusion coefficient of TLRs as well as the percentage of molecules that are recovering within the photobleached area. FRAP measurements before and after stimulation by bacterial products revealed that upon stimulation a pool of TLR molecules becomes immobile, whereas the remaining molecules exhibit a confined diffusion.

We measured the mobile fractions and diffusion coefficients of TLRs using different laser spot sizes of the $1/e^2$ radius ranging from 1.24 to 2.15 μm. We found that the diffusion coefficient and the mobile fraction of both TLRs under investigation varied with the size of the laser spot used. In particular, we found that the mobile fraction of these molecules decreased as the spot radius increased, suggesting that they were encountering barriers on their long-distance lateral movement. The confinement within these domains must promote the formation of clusters that eventually trigger signalling. This hypothesis is further strengthened by the fact that when we use lipid raft disrupting drugs prior to stimulation

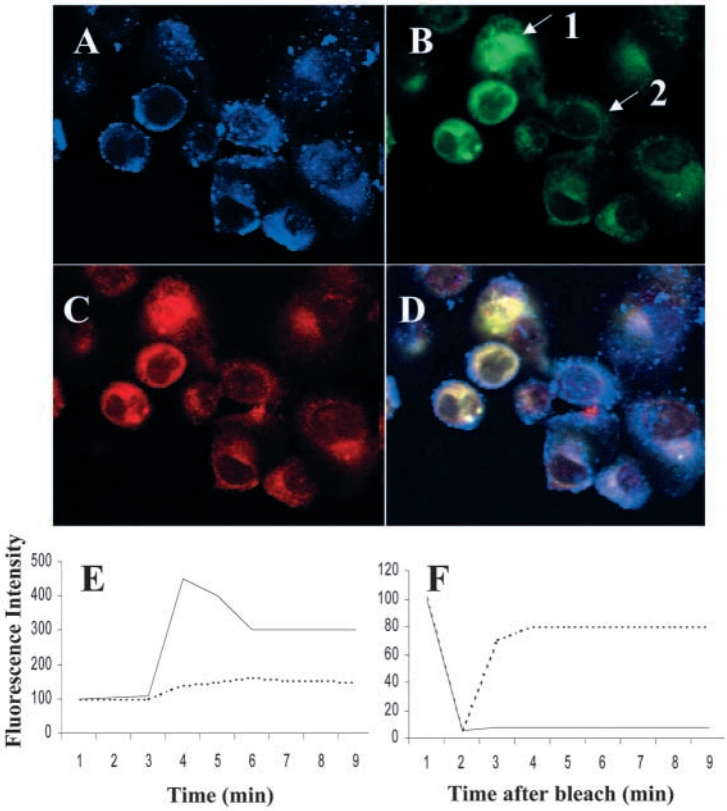


**Fig. 4.** Calcium signalling in lipid rafts. CHO cells transfected with CD14 and TLR4 were incubated for 30 minutes in medium supplemented with 2 μM X-Rhod-1 1/AM and 25 mg/l Pluronic F-127. LPS was added at zero time, resulting in the production of $Ca^{2+}$, which was detected as an increase in X-Rhod 1 fluorescence. Lipid rafts were visualised using Cy5-cholera-toxin (A), TLR4 was visualised using OG-TLR4 (B); $Ca^{2+}$ signal was visualised by the fluorescence of X-Rhod-1 (C); merged image (D). (E) $Ca^{2+}$ responses monitored by X-Rhod-1 fluorescence in cells expressing clustered (cell 1, solid line) or not clustered (cell 2, dashed line) TLR4 molecules. (F) Mobility of TLR4 measured by FRAP in cells exhibiting clusters (cell 1, solid line), as well as diffuse (cell 2, dashed line) TLR molecules.

by bacterial products, the lateral diffusion of TLRs remains unchanged. TLR lateral diffusion is no longer restricted, there is no formation of immobile clusters, and cytokine secretion is dramatically inhibited. In addition when we proceeded to investigate the link between raft association and signalling function by measuring calcium signalling in response to TLR activation during our FRAP experiments, it was revealed that cells with an immobile TLR4 fraction also had a strong $Ca^{2+}$ signal, whereas cells in which lateral mobility of TLR4 was confined had a less intense $Ca^{2+}$ signal. Thus our data suggests that the confinement of TLR molecules within lipid rafts and subsequent clustering in response to bacterial products is crucial for signalling. The cell to cell variability in TLR immobilization after stimulation must be attributed to a variability in responsiveness to the stimulation. Cells, which are highly responsive to the stimulation recruit TLRs within lipid rafts and form signalling clusters much faster than less-responsive cells. In the latter the TLR molecules display a restricted lateral diffusion as they are in the process of forming the signalling clusters.

Another important question is how big are the microdomains where TLR molecules are confined. The size of reported lipid rafts seems to range from 'vanishing small', which are so small they have no biological function (Jacobson and Dietrich, 1999), to intermediate rafts ~100 nm scale (Harder et al., 1998), to larger-scale macrodomains (~500 nm or larger) (Friedrichson and Kurzchalia, 1998; Mayor and Maxfield, 1995). Our data suggests that TLRs seem to be diffusing in the larger-scale microdomains. It is possible that the membrane constitutively contains many 'vanishing small' domains and that it can facilitate different functions by forming even larger macrodomains via coalescence of numerous smaller rafts. The driving force behind raft formation are lipid interactions that are weak and thus the formation of such domains would be transient (Harder and Simons, 1997). Generation of the different types of rafts will depend on the cell type and possibly the stimulus. It is feasible that upon stimulation by bacterial products we have transient joining of multiple small patches of the membrane in order to create the bigger macrodomains. These changes of the plasma membrane structure would be conceived by the cell as a signal, most likely an important element in TLR signalling. Following recognition, these specialised domains of the plasma membrane might further facilitate the internalisation and transport of the bacterial products along with the TLR molecules to the Golgi apparatus, as it has recently been shown (Latz et al., 2002). The formation of such domains seem to be transient on a biological timescale, but survive for long enough to create a local environment that enhances molecular associations leading to the recognition of both Gram-negative and Gram-positive bacteria and the associated signalling that leads to septic shock.

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