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Limited cholesterol depletion causes aggregation of plasma membrane lipid rafts inducing T cell activation

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ABSTRACT

Acute cholesterol depletion is generally associated with decreased or abolished T cell signalling but it can also cause T cell activation. This anomaly has been addressed in Jurkat T cells using progressive cholesterol depletion with methyl-beta-cyclodextrin (MBCD). At depletion levels higher than 50% there is substantial cell death, which explains reports of signalling inhibition. At 10-20% depletion levels, tyrosine phosphorylation is increased, ERK is activated and there is a small increase in cytoplasmic Ca^{2+} . Peripheral actin polymerisation is also triggered by limited cholesterol depletion. Strikingly, the lipid raft marker GM1 aggregates upon cholesterol depletion and these aggregated domains concentrate the signalling proteins Lck and LAT, whereas the opposite is true for the non lipid raft marker the transferrin receptor. Using PP2, an inhibitor of Src family kinase activation, it is demonstrated that the lipid raft aggregation occurs independently of and thus upstream of the signalling response. Upon cholesterol depletion there is an increase in overall plasma membrane order, indicative of more liquid ordered domains forming at the expense of liquid disordered domains. That cholesterol depletion and not unspecific effects of MBCD was behind the reported results was confirmed by performing all experiments with MBCD-cholesterol, when no net cholesterol extraction took place. We conclude that non-lethal cholesterol depletion causes the aggregation of lipid rafts which then induces T cell signalling.

Keywords: actin; cholesterol; colocalisation; lipid rafts; membrane order; methyl-beta-cyclodextrin; T cell signalling

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Abbreviations used: Amplex Red, 10-acetyl-3,7-dihydroxyphenoxazine; CT-B, cholera toxin B subunit; ERK, extracellular-regulated kinase; laurdan, 6-dodecanoyl-2-dimethyl-aminonaphthalene; MBCD, methyl-beta-cyclodextrin; lo, liquid ordered; PMA, phorbol 12-myristate 13-acetate; PTKs, protein tyrosine kinases, TCR, T cell antigen receptor; TX-DRMs, Triton X-100 DRMs

1. Introduction

Lipid rafts is the inclusive term for a range of membrane nanodomains implicated in cellular processes as diverse as cell signalling, endocytosis and protein sorting in the Golgi. Lipid rafts are thought to form by the self-aggregation of cholesterol and sphingolipids [1] and are believed to exist in a liquid ordered (lo) like state that resembles both the liquid disordered state, in that the lipids are fluid, and the solid phase gel state, in that the lipids are highly organised. Lipid rafts can be enriched due to their insolubility in non-ionic detergents at 4°C - a procedure that generates detergent-resistant membranes (DRMs). Triton X-100 DRMs (TX-DRMs) are enriched in cholesterol, glycosphingolipids, sphingomyelin and saturated glycerophospholipids [2] and these lipids can by themselves form a lo-like state at 37°C, where acyl chains are tightly packed, highly ordered and extended, which supports the raft hypothesis [3]. Although TX is more selective than other detergents, it is however clear that not even TX-DRMs represent a true physiological entity, but nonetheless TX-DRMs are a useful tool for lipid raft partitioning [4-6].

Cholesterol depletion is commonly employed to establish the involvement of lipid rafts in a cellular process. Most frequently, this is achieved using methyl-beta-cyclodextrin (MBCD), which acutely extracts cholesterol from the exoplasmic leaflet of the plasma membrane by harbouring cholesterol in a hydrophobic cavity in a 2:1 ratio [7, 8]. We have developed a protocol for progressive cholesterol depletion that does not affect cell viability and have recently demonstrated that MBCD is not, in contrast to the current dogma, specific for lipid raft cholesterol [9]. Furthermore, we have shown that only a minor fraction of a T cell's cholesterol resides in its plasma membrane and that plasma membrane cholesterol is replenished from intracellular stores when depleted by MBCD.

The role of cholesterol in T cell signalling is much debated. Studies early in the lipid raft era showed that cholesterol depletion could abolish T cell signalling [10, 11] but also that cholesterol depletion could lead to T cell activation [11]. Cholesterol depletion has also been reported to

activate ERK in fibroblasts, the epidermal growth factor receptor in a panel of cell types and protein kinase D in neuronal cells [12-14] although cholesterol depletion is generally associated with decreased signalling. While abolition of signalling is easy to explain, considering the importance of cholesterol for cell function, the signal induction is intriguing. When the fraction of cholesterol is decreased in a three component lipid phase diagram, the system can go from consisting of lo phase only to consisting of both lo and ld phases [15]. If this holds for cell plasma membranes, cholesterol depletion could lead to the aggregation of lipid rafts. That cholesterol and the capability to form raft-like liquid ordered domains are important for membrane condensation (the existence of ordered domains), at the contact site between T cells and beads coated with T cell antigen receptor (TCR) stimulating antibodies, has been demonstrated in fixed cells [16, 17].

Ligation of the TCR results in the rapid tyrosine phosphorylation of multiple intracellular proteins, mediated by the membrane-associated Src-family protein tyrosine kinases (PTKs) Lck and Fyn and the soluble PTKs ZAP-70 and Syk. The tyrosine phosphorylation triggers downstream signalling pathways including Ca^{2+} mobilisation, activation of the Ras/extracellular-regulated kinases (ERK) and hydrolysis of phosphoinositide polyphosphates [18]. Stabilisation or formation of large lipid rafts has been demonstrated to trigger all these pathways, since they can be activated by the aggregation of the ganglioside GM1 using cholera toxin B subunit (CT-B) and anti-cholera toxin [19, 20]. This links reorganisation of lipids to T cell signaling, as does a recent study where it was shown that raft lipids, like cholesterol and sphingomyelin, accumulate where beads coated with TCR activating antibodies interact with a T cell [21]. However, the role of lipid rafts in TCR signalling is continuously being questioned [22-24].

In this study we have investigated the role of progressive cholesterol depletion on T cell signalling at levels where cell viability was maintained. Our suspicion was that in many previous studies a lot of dead cells were

inadvertently included, making their conclusions questionable. We thus wished to revisit the effect of cholesterol depletion on T cell signalling and present results resolving the apparent paradox mentioned above.

2. Materials and methods

2.1 Materials

CT-B-Alexa Fluor 594, anti-rabbit-Alexa Fluor 488, anti-mouse-Alexa Fluor 488, fluo-4 AM and 6-dodecanoyl-2-dimethyl-aminonaphthalene (laurdan) were from Molecular Probes (Invitrogen, Carlsbad, CA). Anti-CD3 monoclonal antibodies (OKT3 and UCHT1), and anti-pTyr monoclonal antibody (4G10) were generous gifts from S. Ley (The NIMR, London). Anti-Lck rabbit antiserum 2166 [25] was a kind gift from T. Magee (Imperial College, London) and anti-LAT rabbit antiserum (M41) was a kind gift from M. Turner (The Babraham Institute, Cambridge). Anti-p44/42 MAP kinase and anti-phospho-p44/42 MAP kinase were from Cell Signaling Technology (Danvers, MA). Anti-Lck, clone 3A5, was from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-goat, anti-mouse and anti-rabbit horse raddish peroxidase conjugated antibodies were from Amersham Biosciences (GE Healthcare, Bucks, UK). Anti-CT-B, PP2 and PP3 were from Calbiochem (San Diego, CA). 10-acetyl-3,7-dihydroxyphenoxazine (Amplex Red) was from Synchem OHG (Felsberg, Germany). Unless otherwise stated, chemicals were from Sigma (St Louis, MO).

2.2 Cell culture

Jurkat T cells (clone E6.1) and Jurkat T cell clones J.RT3-T3.5 and p116 were obtained from ATCC and the JCam2 clone was a kind gift of Arthur Weiss (University of California, CA). All cells were cultured in RPMI supplemented with 5% v/v heat inactivated fetal bovine serum, 100 U/ml penicillin, 100 µg/ml of streptomycin, 2 mM glutamine and 25 mM HEPES maintained at 37°C in humidified incubator under 5% CO₂.

2.3 Cholesterol depletion

To achieve roughly 10%, 20%, 30%, 40% and

50% total cholesterol depletion, cells at 10×10⁶/ml were treated with 2.5mM MBCD for 2.5, 5.5, 9 and 14.5 min or with 5 mM MBCD for 5 min. MBCD was dissolved freshly in RPMI 1640 supplemented with 25 mM HEPES before each experiment and the cell density was kept constant at 10×10⁶ cells/ml. Lysates were prepared and fixation was performed immediately after MBCD-treatment to minimise cholesterol repletion from intracellular stores [9].

2.4 Cholesterol equilibrium

For equilibrium experiments, MBCD-cholesterol complexes were principally prepared as described [26]. Briefly, 193 ml cholesterol in chloroform:methanol (1:1) at 25 mg/ml in a glass tube was heated to 80°C to evaporate the solvents. 2.5 or 5 mM MBCD was added, the mix was sonicated, vortexed heftily for 3 min and left at 37°C overnight with constant stirring resulting in 100% saturated MBCD-cholesterol complexes. The complexes were diluted with freshly made 2.5 or 5 mM MBCD 0.3-3X and used to establish at what conditions there was no net extraction of cholesterol from Jurkat T cells at 10×10⁶ cells/ml. Cholesterol concentration was measured using Amplex Red as described previously [9].

2.5 Electrophoresis and Western blotting

MBCD-treated and control Jurkat T cells were subjected to brief centrifugation and lysed in 1% TX-containing buffer (50 mM Tris, 150 mM NaCl, 1 mM EDTA, 10 mM NaF, 10 mM Na₄P₂O₇, 1 mM PMSF and 5µg/ml each of chymostatin, leupeptin, antipain and pepstatin). Where indicated, cells were activated by OKT3 antibodies for 5 min at 37°C prior to lysis. Cells were treated with phorbol 12-myristate 13-acetate (PMA) for 5 min as a positive control for ERK activation. Lysates were analysed by SDS-PAGE on 12% or 4-12% NuPAGE gradient gels (Invitrogen, Carlsbad, CA) and wet-blotted to nitrocellulose membranes from Amersham Biosciences (GE Healthcare, Bucks, UK). Protein concentration of total lysates was estimated visually by Coomassie Brilliant Blue staining of filter papers containing aliquots of the lysates alongside BSA standards. Gels were loaded on an equal protein basis. For analysis of pTyr,

membranes were blocked in 3% BSA in TBST and for all other analyses membranes were blocked in 5% milkpowder in PBS. Blots were developed using enhanced chemiluminescence (Pierce Biotechnology, (Thermo Fisher Scientific, Rockford, IL).

2.6 Cellular fractionation

50x10⁶ cells treated with MBCD at 37°C as described above were lysed for 15 min on ice in 1 ml MNE (25 mM MES pH 6.5, 150 mM NaCl, 2 mM EDTA) containing 1% TX, protease inhibitors (5 µg/ml each of antipain, leupeptin, chymostatin and pepstatin and 1 mM PMSF), 5 mM NaF and 1 mM Na₃VO₄. The sucrose density gradient was made up of 2 ml 40% sucrose, 2 ml 30% sucrose and 1 ml 5% sucrose, all in MNE. The gradients were centrifuged at 46000 rpm in a Sorvall AH-650 rotor for 16-18 hrs. TX-DRMs were collected from the 5-30% sucrose interface and pelleted by centrifugation at 100 000 x g for 1 h. The resulting TX-DRM pellet was rinsed and suspended in MNE as was the pellet from the sucrose density gradient tube. The bottom 1.5 ml of the gradient was named the TX-soluble fraction and the 1.5 ml above the intermediate fraction.

2.7 FACS analysis

Control cells and cells treated with MBCD were stained with 2.5 mg/ml CT-B-Alexa Fluor 594 in PBS containing 2% BSA for 30 min at rt at a cell density of 5x10⁶/ml. The cells were analysed with a FACSCalibur (BD Biosciences, San José, CA) with excitation at 595 nm and a 660/8 emission filter. For Ca²⁺-flux measurements 10x10⁶ cells were labeled with Flou-4 AM and cholesterol was depleted at room temperature using 2.5 mM MBCD. Cells were analysed by flow cytometry for intracellular Ca²⁺ release.

2.8 Live cell imaging

Cells were labeled with 5µM laurdan for 30 min at 37°C, washed twice and suspended in serum-free RPMI medium containing 7000U/ml of catalase and 16U/ml of glucose oxidase. Cells were attached to TESPA-coated coverslip and imaged live at 37°C with a Zeiss Axiovert 200M microscope (Carl Zeiss MicroImaging GmbH,

Göttingen, Germany) equipped with a Cascade 1 camera and a dual viewer (Photometrics, Tuscon, AZ), a 63X water objective lens (NA 1.3) and a DG4 (Sutter Instrument, Novato, CA) with 350/50 and 577/20 excitation filters. The emission was split by a ms-470 LDX dichroic and emission filters 425/40 and 51018m dual were used (Chroma, Rockingham, VT). Focus was adjusted under transmitted light and laurdan images were acquired without prior exposure to uv-light to minimise photobleaching. Series of eleven 200 nm z-stack images were taken at the equatorial plane of the cells. Single cells were followed throughout the experiment and imaged prior to as well as 2 and 15 minutes after the addition of 0.02 mM MBCD.

2.9 Immunofluorescence staining

For colocalisation analysis, cells were washed in PBS and attached to TESPA-coated coverslips by incubation at 37°C for 5 min (2.5 x 10⁵ cells/coverslip). Fixation was performed in 4% PFA/PBS at 37°C for 15 min. The cells were then blocked with 2% BSA/PBS on ice for at least 15 min followed by incubation with primary antibodies (1-10 mg/ml in 2% BSA/PBS) at room temperature for 30 min. After washing in PBS, the cells were incubated with Alexa Fluor-conjugated secondary antibodies in 2% BSA/PBS at room temperature for 15 min followed by washing in PBS. Cells were mounted in AF1 (Citifluor Ltd, London UK).

2.10 Quantification of cellular protrusions

Cells were attached to coverslips, incubated and fixed for 5 minutes at 37°C using a solution that preserves actin filaments (1% Triton X-100, 0.75% glutaraldehyde, 0.137M NaCl, 5mM KCl, 1.1mM Na₂HPO₄, 0.4mM, KH₂PO₄, 4mM NaHCO₃, 5.5mM glucose, 2mM MgCl₂, 2mM EGTA, 5mM PIPES pH 6.0) [27]. Cells were then stained with 200 nM FITC-phalloidin for 30 min at room temperature. Z-sections at 0.25 µm intervals were acquired throughout the cells using a Zeiss Axiovert 200M microscope. The number of protrusions in a cell was obtained by sequentially examining Z series deconvolved, using the nearest neighbour algorithm in Slidebook (Intelligent Imaging Innovations, Göttingen,

Germany), and noting the first appearance of each protrusion.

2.11 Image processing and analysis

Cells selected for image analysis met the criteria of having no near neighbours and nuclei clearly discernable under phase contrast. Imaging was performed at the equatorial plane of the cells with gain and offset set to stay within the dynamic range. Each experiment was repeated at least three times and about ten cells meeting the criteria were quantitatively analysed from each repeat. Regions of the plasma membrane in intimate contact with the nuclear membrane were excluded from the analysis. The images were both acquired and analysed blindly to minimise operator bias. The unitary scale bar was used to display distances [28]. The colocalisation analysis was performed using RBNCC (replicate based noise corrected correlation) [29] with the Pearson correlation coefficient. All in house designed software was built around a Semper6w kernel (Synoptics Ltd, Cambridge, UK). Maximum intensity projection images were generated using Slidebook (Intelligent Imaging Innovations, Göttingen, Germany). Displayed images were prepared using Adobe Photoshop 7.0 software.

2.12 Estimation of filamentous actin at the plasma membrane

Cholesterol depleted and control cells were fixed and blocked as described above. Cells were then stained with 200 nM FITC-phalloidin for 30 min at room temperature. Cells were washed three times in PBS and mounted in AF1. Images were acquired using an UltraView ERS spinning disc confocal system (Perkin Elmer, Waltham, MA) connected to an Axiovert 200M microscope (Carl Zeiss MicroImaging GmbH, Göttingen, Germany). To avoid bleaching affecting the image quality, focus was adjusted under transmitted light and FITC-phalloidin images immediately acquired when the excitation source was turned on. The image of CT-B-Alexa Fluor 594 was used to define the plasma membrane which was delineated manually with sequentially marked points that were joined automatically [20]. Once delineated,

the mean fluorescence intensity per pixel of the corresponding image of FITC-phalloidin was calculated. The software was built around a Semper6w kernel (Synoptics Ltd, Cambridge, UK).

2.13 GM1 distribution in the plasma membrane

Cholesterol depleted and control cells were fixed at 37°C in 4% paraformaldehyde for 15 min. Cells were blocked with 2% BSA/PBS on ice for at least 15 min followed by incubation with CT-B-Alexa Fluor 594 for 30 min at rt. Images were acquired using a spinning disc confocal microscope (Perkin Elmer, Waltham, MA) and analysed as described earlier [30]. Briefly, the standard deviations of the intensity of the pixels in the perimeter trace were expressed as a percentage of the mean perimeter fluorescence. For assessment of Src family kinase involvement, cells were pretreated with 10 mM PP2 or PP3 for 5 min, with a final DMSO concentration at 0.06 %. The compounds then remained present at the same concentration when MBCD was applied.

2.14 Ratiometric analysis

Image stacks from both laurdan channels were deconvolved together with an image stack showing a plasma membrane molecule using AutoQuantATM (Media Cybernetics, Bethesda, MD). The images were then checked for alignment using a cross correlation function and the plasma membrane was demarcated as described above. The locations of the points were optimized by searching over a short distance around the manually entered pixel along a line drawn between the initial position and the centre of the cell, for the most intense pixel. A single line of 4-connected pixels, between sequential points, was used to select pixels corresponding to the plasma membrane. The background intensity, based on an area outside the cell, was subtracted. The standard deviation of the background intensity was around 1. The calculation of the ratio between the two channels was based on the generalised polarisation formula:

$$GP = \frac{I_{(385-470)} - I_{(470-508)}}{I_{(385-470)} + I_{(470-508)}}$$

The average ration over the whole membrane was obtained from the arithmetic mean of the ratios for individual pixels.

3. Results

MBCD acutely depletes cells of cholesterol by binding cholesterol at a ratio of 2:1 in its hydrophobic cavity [7, 8]. Acute cholesterol depletion has been reported to abolish as well as to promote T cell signalling [10, 11, 31]. To resolve this paradox, conditions for controlled, progressive cholesterol depletion using MBCD were employed to test the hypothesis that the extent of cholesterol depletion determines the signalling response, membrane order and arrangement. In Jurkat T cells, cholesterol depletion beyond 50% results in substantial cell death [9]. To restrict studies to conditions where cell viability is maintained is self evidently important.

Fig. 1. Cholesterol equilibrium during MBCD-treatment. Jurkat T cells at 10×10^6 cells/ml were treated for 15 min with 2.5 mM MBCD mixed with 2.5 mM MBCD-cholesterol complexes at 37°C . The cells were then washed and extracted with chloroform:methanol:water. After evaporation of the organic solvents, the residue was dissolved in assay buffer containing HRP, cholesterol oxidase and Amplex red. Fluorescence was read after 150 min with excitation at 544 nm and emission at 590 nm. Control cells were defined as containing 100% cholesterol. Data shown are means $\pm$ s. e. m., $n=4$. # $p=0.767$ for a two tailed t-test.

3.1 Cholesterol equilibrium

MBCD does not exclusively bind cholesterol and its hydrophobic cavity can accommodate a range of lipids. It is therefore important to establish that any effects of MBCD-treatment are actually due to the depletion of cholesterol and not of other membrane components. To this end, cholesterol saturated MBCD was mixed with MBCD at different ratios to establish the ratio

that caused no net cholesterol extraction. In our assay, this condition was met at a ratio of MBCD-cho: MBCD at 1:0.8 (Fig. 1) which is in the same range as that reported for several different cell types [32]. Treating the Jurkat T cells with MBCD-cho only increased cell cholesterol content by 300%. Only a minor fraction of this excess cholesterol is however likely to end up in the plasma membrane. Furthermore, it was confirmed that 2.5 mM MBCD-treatment for 15 min results in the extraction of about 40% of total cell cholesterol.

Fig. 2. Cholesterol dependence of tyrosine phosphorylation and ERK activation in Jurkat T cells. Cells at 10×10^6 cells/ml were treated with MBCD to achieve 0, 10, 20, 30, 40 and 50% cholesterol extraction. Cell lysates were analysed by Western Blotting for (A) tyrosine phosphorylation (4G10) or (B) ERK activation (anti-phospho-p44/42 MAP kinase). PMA was used as a positive control. Gels were loaded on an equal protein basis and membranes were probed with anti-p44/42 MAP kinase as an additional loading control. Molecular mass markers are indicated (kDa). Fluorographs shown are representative of four experiments.

Cholesterol equilibrium controls were performed for all experiments, ensuring that the results obtained were due to changes in cholesterol content.

3.2 T cells are activated by moderate cholesterol depletion

Two of the most widely used readouts for T cell activation are increased tyrosine phosphorylation of signalling proteins alongside the activation of the MAP kinase ERK by serine and tyrosine phosphorylation. We have previously developed protocols resulting in roughly 10%, 20%, 30%, 40% and 50% cholesterol extraction [9], which were used in the present study. At 10% cholesterol depletion, there was a small increase in pTyr, particularly in polypeptides with molecular masses 21-23, 35-38, 56-58, 71 and about 105 kDa (Fig. 2A) confirming previous work [11]. These bands most likely correspond to CD3z, LAT, Lck and Fyn Src-family PTKs, ZAP-70 and Vav, all known to participate in T cell signalling. A comparable pattern of increased tyrosine phosphorylation was observed upon stimulation with OKT3, a CD3-binding and T cell activating antibody (Supplemental Fig. 1). The increase was maintained up to 50% cholesterol depletion.

Table 1
Cholesterol depletion induces actin polymerisation at the cell periphery

Population	Fluorescence intensity in plasma membrane pixels (relative values)	n	p
Control	23.1 ± 4.9	26	
10% depletion	32.5 ± 5.4	24	0.040
30% depletion	31.4 ± 5.9	27	0.064
MBCD-chol	24.5 ± 2.9	23	0.812

Cholesterol depleted and control Jurkat T cells were fixed, blocked and stained with FITC-phalloidin and CT-B-Alexa Fluor 594. Confocal images were acquired at the equatorial plane of the cells. The image of CT-B-Alexa Fluor 594 was used to define the plasma membrane which was delineated manually with sequentially marked points that were joined automatically. Once delineated, the mean fluorescence intensity per pixel of the corresponding image of FITC-phalloidin was calculated. Data shown are means ± s.e.m. p-values are for a two-tailed t-test for comparisons with the control.

Cholesterol loading did not have any effect on pTyr (not shown). In TCR deficient JRT.3-T3.5 cells, a similar increase was observed differing only in the lack of the CD3ζ corresponding band (Supplemental Fig. 2A). LAT deficient JCam2 cells

Fig. 3. Cholesterol depletion induces cell spreading. Control and cholesterol depleted Jurkat T cells were fixed and stained with CT-B-Alexa Fluor 594 to visualise GM1 (A) in the membrane touching the coverslip. (B) Maximum intensity projections were generated from confocal z-stacks of cells fixed to preserved actin filaments and stained with FITC-phalloidin. (C) Cells were pretreated with 10 μM PP2 or PP3, which then remained present throughout the experiment. Cells were fixed and stained with CT-B-Alexa Fluor 594 to visualise GM1.

and ZAP-70 deficient p116 cells displayed no increase in pTyr upon cholesterol extraction

(Supplemental Fig. 2A). In Jurkat T cells, ERK was substantially activated at 10% cholesterol depletion, returned to baseline at 20%, became completely inactivated at 30-40% depletion to return to baseline at 50% depletion (Fig. 2B). ERK activation upon cholesterol depletion was not detected in any of the mutant cell lines (Supplemental Fig. 2B) or upon cholesterol loading (not shown).

When T cells are activated, actin polymerisation is initiated to enable major plasma membrane rearrangement. That monomeric actin inhibits the activity of DNase 1, whereas filamentous actin does not, was used to differentiate between the two pools of actin [33]. The changes observed were small and not significant (data not shown), which is not surprising since actin is a very abundant protein and any changes in actin dynamics incurred by T cell activation will predominately take place at the cell periphery. We therefore quantified filamentous actin at the cell edge using FITC-phalloidin staining and used CT-B as a membrane marker. The intensity of the filamentous actin staining increased by 41% and 36% upon 10% and 30% cholesterol depletion, respectively, confirming that substantial actin polymerisation took place at the plasma membrane (Table 1).

Fig. 4. Commonly used cholesterol depletion protocols result in substantial cell death. Cells at $2\text{-}20 \times 10^6$ cells/ml were treated with 10-24 mM MBCD for 20-30 min as indicated. Cells were examined for viability with an automatic cell counter using the trypan blue exclusion method. Several hundred cells were counted from each population. The results shown are the means of three experiments $\pm$ s. d..

MBCD-chol complexes at equilibrium conditions did not induce actin polymerisation. Moreover, cell shrinkage was not the reason for the increase in peripheral actin, rather the cells with 10% and 30% of their cholesterol removed had a

somewhat larger diameter at their equatorial planes than the control cells.

Cell spreading, a sign of cell activation, accompanied the increase in actin filaments at the plasma membrane after cholesterol depletion. Whereas control cells were round and had a small contact area with the coverslip, the cholesterol depleted cells were flattened and had a large contact area with the coverslip (Fig. 3A). Control cells had a rounded morphology, whereas cholesterol depletion induced the formation of membrane protrusions visualized in 3D projections of image stacks (Fig. 3B). The fixation solution was designed to preserve details of filamentous structures [27]. The number of protrusions per cell was increased from two in control cells to 14 in cells depleted of 30% of their cholesterol (Table 2).

Table 2
Membrane Protrusions on Jurkat T Cells

Treatment	Protrusions per cell	n	p
None	2.08 ± 0.22	53	
30% depletion	14.1 ± 0.83	53	<0.0001
MBCD-chol	2.18 ± 0.20	53	0.717

Control and MBCD-treated Jurkat T cells were fixed at 37°C to preserve actin filaments. Cells were then stained with FITC-phalloidin. Z-stack images were deconvolved and the number of protrusions counted manually by going through the stacks. Data shown are means $\pm$ s.e.m. p-values are for a two-tailed t-test compared with the untreated cells.

To investigate if the cell spreading occurred as a result of the initiation of signalling or was the initiator of the signalling response seen after non-lethal cholesterol depletion, cells were treated with the Src family kinase inhibitor PP2 or its inactive analogue PP3. This treatment prevents the phosphorylation and activation of Lck and Fyn, the kinases responsible for the initial phosphorylation events in T cell signalling, and blocks all downstream signalling. Treatment with PP3 did not, as expected, have any effect on cell spreading whereas PP2 treatment resulted in a clear reduction in cell spreading (Fig. 3C), implying that the activity of Lck and Fyn are necessary to initiate the actin polymerisation.

Fig. 5. Cholesterol depletion induces the aggregation of GM1 in the plasma membrane. Control and cholesterol depleted Jurkat T cells were fixed and stained with CT-B-Alexa Fluor 594 to visualise GM1 at (A) the equatorial plane of the cells. (B) The plasma membrane of confocal images was delineated by manually marking sequential points, which were then automatically joined by line segments. The intensity variation along individual plasma membranes was quantified using one standard deviation, expressed as a percentage of the mean intensity. The arithmetic mean of the s.d. for the control cells was normalised to 100. 38-42 cells from each population were analysed and means are presented $\pm$ s.e.m. ** $p < 0.002$ and # $p = 0.974$ for a two tailed t-test. All populations were compared with the control group.

An increase in intracellular Ca^{2+} is an early signalling response upon receptor engagement. When Jurkat T cells were subjected to MBCD-treatment at room temperature a small, but reproducible, Ca^{2+} -response was observed (Supplemental Fig. 3). Compared with the Ca^{2+} -response upon GM1-CT-B patching and anti-CD3 stimulation, the MBCD-induced response had a slow onset and was minor. It has repeatedly been reported that cholesterol depletion inhibits the Ca^{2+} -response. Reproducing the cholesterol depletion conditions used in some of these studies [10, 11, 22] and checking the cells for viability, it was clear that the treatment was far too brutal for cell survival (Fig. 4). At these cholesterol extraction conditions, there was no Ca^{2+} -response when the cells were stimulated with anti-CD3 (data not shown).

Limited cholesterol depletion causes an early T cell signalling response analogous to that caused by stimulation of the TCR.

3.3 Cholesterol depletion results in lipid raft aggregation

Lipid rafts are membrane nanodomains with a higher order than the surrounding membrane that concentrate signalling molecules, especially those carrying lipid modifications and their aggregation triggers T cell signalling [19, 30]. To determine if the T cell activation triggered by moderate cholesterol depletion altered the distribution of signalling molecules two lines of investigation were used; TX-DRM partition and fluorescence microscopy.

Partition into TX-DRMs is a strong indication that a molecule is found in lipid rafts, although the two should not be equated [4, 5]. TX-DRM partition of the lipid raft markers the Src family kinase Lck, the adaptor protein LAT and the ganglioside GM1 was studied. Lysed cells were split into three fractions; TX-DRMs containing unsolubilised cellular components with a low protein to lipid ratio, the TX-soluble fraction containing fully solubilised cellular components and the intermediate fraction containing unsolubilised material with a higher protein to lipid ratio than TX-DRMs [9]. Cholesterol depletion of 10% and 20% produced an increase in the level of the more slowly migrating form of

Lck in TX-DRMs (Supplemental Fig. 4). At 30% cholesterol depletion and above, Lck was gradually lost from TX-DRMs and increased in the TX-soluble fraction. The amount of Lck in the intermediate fraction remained unchanged. The level of LAT in TX-DRMs was not altered after 10% cholesterol depletion but, at 20% depletion and higher, LAT was lost from TX-DRMs as its level in the TX-soluble fraction was increased (Supplemental Fig. 4). LAT was not found in the intermediate fraction. At cholesterol equilibrium conditions, the distribution of LAT and Lck were not affected (not shown). Regardless of the treatment, the non lipid raft marker the transferrin receptor (TfR) was never found in either TX-DRMs or the intermediate fraction (data not shown). GM1 was lost from TX-DRMs at 40% and 50% cholesterol depletion (Supplemental Fig. 4). Surprisingly, this was not accompanied by a GM1 increase in any of the other fractions, suggesting that GM1 was lost from the cells. FACS analysis confirmed that GM1 was progressively extracted upon MBCD-treatment (Supplemental Fig. 5).

Table 3
Localisation of Lck, LAT and the TfR in relation to GM1

Protein	Treatment	Correlation	n	p
Lck	None	0.076±0.029	35	
Lck	10% depletion	0.167±0.025	35	0.029
Lck	30% depletion	0.139±0.035	36	0.205
Lck	MBCD-chol	0.097±0.025	35	0.596
LAT	None	0.102±0.047	35	
LAT	10% depletion	0.238±0.037	35	0.035
LAT	30% depletion	0.206±0.038	36	0.103
LAT	MBCD-chol	0.069±0.034	35	0.438
TfR	None	0.086±0.027	35	
TfR	10% depletion	0.035±0.029	36	0.184
TfR	30% depletion	0.057±0.022	35	0.391
TfR	MBCD-chol	0.116±0.019	35	0.289

Control and MBCD-treated Jurkat T cells were fixed at 37°C and stained with CT-B-Alexa Fluor 594 and antibodies against Lck, LAT or TfR that were visualized by staining with appropriate secondary Alexa Fluor 488 conjugated antibodies. Confocal images were acquired at the equatorial plane of the cells and the level of colocalisation determined using RBNC using the Pearson correlation coefficient. Data shown are means ± s.e.m. p-values are for a two-tailed t-test compared with the untreated cells.

The distribution of the lipid raft marker GM1 in intact cells was studied to investigate the possibility that acute cholesterol depletion affects the plasma membrane organization. In control

cells GM1, stained with cholera toxin B Alexa Fluor 594, was homogenously distributed in the plasma membrane (Fig. 5A). This was not altered by the presence of cholesterol-loaded MBCD-complexes at equilibrium conditions. When 10 % cholesterol was extracted by MBCD the GM1 distribution became patchy, with regions of the plasma membrane depleted of staining. A patchy GM1 distribution was also observed after 30% and 50% cholesterol depletion. GM1 clustering was quantified using the spread of intensities around the mean intensity of a cell perimeter trace. The relative patchiness increased by about 50% after 10% cholesterol depletion and remained similarly elevated after 30% and 50% cholesterol depletion (Fig. 5B).

Fig. 6. Aggregation of GM1 upon cholesterol depletion is not dependent on T cell signalling. Cells were treated with 10 μ M PP2 or PP3 and then depleted of 30% of their cholesterol with MBCD. PP2/PP3 were present throughout the experiment. Cells were fixed and stained with CT-B-Alexa Fluor 594 to visualise GM1. The plasma membrane of confocal images was delineated by manually marking sequential points, which were then automatically joined by line segments. The intensity variation along individual plasma membranes was quantified using one standard deviation, expressed as a percentage of the mean intensity. The arithmetic mean of the s.d. for the control cells was normalised to 100. 18-28 cells from each population were analysed and means are presented ± s.e.m. **p<0.002 for a two tailed t-test. (B) Representative images of the equatorial plane of the cells.

To investigate whether the aggregation of GM1 occurred as a result of the initiation of signalling or was the initiator of the signalling response seen after non-lethal cholesterol depletion, cells were treated with PP2 or PP3. Neither treatment prevented GM1 aggregation (Fig. 6), implying

that lipid raft aggregation is the cause rather than the consequence of cholesterol depletion induced signalling.

The distributions of Lck and LAT in the TX-DRM preparations indicate that they are progressively lost from lipid rafts upon cholesterol depletion. We therefore set out to determine their colocalisation with GM1. As expected, the colocalisation of Lck and LAT with

Fig. 7. Cholesterol depletion increases the fraction of ordered membrane domains. Jurkat T cells were stained with 5 μ M laurdan for 30 min at 37°C after which they were imaged at 37°C. The cells were then treated with MBCD or MBCD-chol and reimaged two and 15 minutes after the start of the treatment. After deconvolution of the image series, the generalised polarisation was calculated. The time zero GP values were normalised to zero and the absolute changes from the time zero value at two and 15 minutes are displayed $\pm$ s.e.m.. The difference between the changes for the MBCD or MBCD-chol populations were assessed using a two tailed t-test, ** $p < 0.007$.

GM1 increased upon limited cholesterol depletion (Table 3 and Supplemental Fig. 6), but at 30% extraction the increase was no longer statistically significant which is consistent with the ERK activation occurring at 10% cholesterol depletion only (Fig. 2). The colocalisation between the non-lipid raft marker, the TfR, and GM1 appeared to decrease upon cholesterol extraction, but the decrease was not statistically significant.

In conclusion, cholesterol depletion induced lipid raft aggregation precedes the T cell signalling response.

3.4 Cholesterol depletion increases the proportion of ordered domains in the plasma membrane

Lipid rafts should exist in a lo-like state and to investigate if cholesterol depletion had an effect on membrane order the fluorophore laurdan was used. Laurdan experiences an emission red shift of about 50 nm upon transfer from lo to ld phase. The experiments were performed on live cells since fixation affects membrane order and dead cells tend towards equilibrium [34]. For live cell experiments, conditions (0.02 mM MBCD, 8.33×10^5 cells/ml) resulting in 8-15% of total cholesterol extraction after 15 minutes were used

Fig. 8. Generalised polarisation of live cells depleted of cholesterol. Representative GP images of (A) time series of MBCD-treated cells and (B) time-series of MBCD-chol treated cells.

(data not shown). Two minutes after the addition of MBCD, the overall order of the plasma membrane was increased in 23 out of 27 cells (Supplemental Fig. 7A), meaning that a larger fraction was in a lo-like state. There was a further increase in overall membrane order 15 minutes after the addition of MBCD (Fig. 7). The spread in the population was higher at the later time point, possibly due to the bleaching of laurdan throughout the experiment. MBCD-chol at equilibrium conditions did not cause any change in the plasma membrane order (Fig. 7 and Supplemental Fig. 7B). Representative images of the generalised polarisation show that the membrane order is heterogenous and domains of higher order exist (Fig. 8). From these results, we conclude that lipid rafts form upon limited cholesterol depletion.

4. Discussion

Although MBCD is widely used for cholesterol extraction, there is considerable variability in the details of its use [32]. Many of the protocols are likely to cause high depletion levels which severely affect cell viability. However, cell viability after MBCD-treatment is rarely assessed and controls to exclude effects of MBCD other than cholesterol depletion are even rarer. In the present study, it was ascertained both that the cells were still viable after MBCD-treatment and that the results were caused by cholesterol depletion rather than an unspecific effect of MBCD, paving the way for clarifying some misinterpretations when it comes to the role of cholesterol in T cell signalling. The gentle cholesterol depletion method should set the standard for the proper way of using MBCD in a cellular context.

The role of lipid rafts in T cell signalling is repeatedly being challenged [23, 35-37] although there is substantial evidence for their involvement in this process [16, 19, 38, 39]. In several studies it was concluded that lipid rafts are involved in T cell signalling because acute cholesterol depletion abolished T cell signalling [10, 11, 40]. We found that these studies employed cholesterol depletion protocols where the bulk of the cells die, which means that the involvement of lipid rafts in the process cannot

be assessed. At 50% acute cholesterol depletion the cell viability of Jurkat T cells falls and this could explain the herein observed ERK activation and actin depolymerisation at this level of depletion, both of which are signs of apoptosis [41-43].

That cholesterol dependence may be different to lipid raft involvement in T cell signalling was suggested by the demonstration that MBCD caused membrane depolarisation and inhibited Ca^{2+} -signalling, while TCR ζ and PLC γ 1 could still be tyrosine phosphorylated through stimulation by CD3 [22]. The cholesterol depletion conditions used in this study undoubtedly caused substantial cell death. Clearly, leaky and dead cells cannot maintain ion gradients, precluding Ca^{2+} -signalling. Despite the cells being leaky, the aggregation of GM1-domains may have been sufficient to colocalise the molecules necessary for pTyr when stimulated by an antibody inducing a favourable conformational change in the TCR [44]. This is further supported by the finding that pTyr is maintained long after cell death [11]. In the present study, we observed a small increase in intracellular Ca^{2+} , which can be attributed to the aggregation of lipid rafts, upon non-lethal cholesterol depletion.

Lipid rafts are predicted to be more ordered than the surrounding membrane. That the T cell plasma membrane of fixed cells that are in contact with beads coated with anti-CD3 antibody is more ordered than the rest of the T cells plasma membrane, has been demonstrated by studying laurdan generalised polarisation [16, 17]. In a very recent study by the same group, it was demonstrated that lipids expected to form lipid rafts like cholesterol, sphingomyelin and PC with saturated acyl chains accumulate where T cells interact with beads covered in antibodies against the T cell receptor [21]. Intriguingly, accumulation of the same lipids was seen also when T cells interacted with beads coated with antibodies against the non lipid raft marker the TfR. This suggests that the cell-bead interaction attracts certain lipids rather than that the TCR in resting cells was present in lipid rafts. That cells are capable of large scale phase separation has been demonstrated in membrane blebs protruding from cells both at subphysiological

and at physiological temperature [34, 45] as well as in giant unilamellar vesicles isolated from plasma membranes [45, 46]. A common feature of these systems is that they can reach equilibrium, since they are no longer parts of viable cells, which suggests that phase separation in the plasma membrane is prevented because viable cells are not at equilibrium. However, large scale domain coalescence can be induced in live cells by cross-linking of lipid raft components, decreases in temperature [19, 30] and, as we demonstrate, by limited cholesterol depletion; all events that affect lipid phase behaviour.

It is intriguing that the more slowly migrating isoform of Lck appeared upon limited cholesterol depletion and that this isoform was solely responsible for the cholesterol depletion induced increase of Lck in TX-DRMs. This isoform represents a serine phosphorylated version of Lck [47] that may participate in communication with actin filaments since it is also found in the pellet fraction upon anti-CD3 stimulation of T cells (I. Parmryd, unpublished result). Polymerisation of actin was one of the first processes described upon lipid raft aggregation [48] and, in the present study, we observed an increase in cell peripheral actin polymerisation upon moderate cholesterol depletion - treatment that also caused lipid raft aggregation. The finding that PP2 did not prevent lipid raft aggregation is in line with microcluster formation proceeding T cell signalling [49], but the aggregates formed upon cholesterol depletion appear to be larger than those reported previously. That PP2 inhibited cell spreading demonstrate that Lck and Fyn work upstream of the actin polymerisation machinery which support previous data [50].

Clear phase separation is a likely outcome, if cholesterol depletion results in the transfer from a one liquid phase region to a two liquid phase coexistence region in a three component phase diagram [51], and is a plausible explanation for the aggregation of GM1 domains. This would also explain why cholesterol loading did not result in T cell signalling and why cholesterol loading does not trigger phase separation [52]. That cholesterol depletion can lead to large scale membrane domain segregation has been shown

in several cell types containing caveolae [53]. However, unlike the present study, where the lipid raft marker GM1 stained with CT-B resided in large aggregates, after limited cholesterol depletion, the folate receptor, a GPI-anchored protein and thus also a lipid raft marker, only formed small aggregates [53]. It could be that the levels of cholesterol depletion differed or that there is a limit to the maximum domain size a receptor destined for internalisation could reside in or that only a fraction of GPI-anchored proteins form clusters [54].

It is an apparent paradox that moderate cholesterol depletion results in an increase in the fraction of ordered membrane domains, since cholesterol generally has an ordering effect on phospholipids in the fluid state. Lipids are by number the predominant component of a cell membrane but the presence of proteins will have a great impact on phase boundaries when considering the system as a three-component phase diagram. The increase in the fraction of ordered membrane domains is likely a result of the increase in actin polymerisation at the cell periphery, which is part of T cell activation. Jasplakinolide treatment, which stabilises actin filaments, thus leading to their accumulation, also results in a higher fraction of ordered membrane domains (J. Dinic and I. Parmryd, unpublished data). In addition, actin filaments have been shown to have a stabilising effect on phase separation in PIP₂-containing model membranes [55]. A link between cholesterol content and actin filaments, via the PH-domain binding membrane lipid PIP₂, has been reported previously [56]. Interestingly, the cholesterol depletion-induced increase in the number of membrane protrusions after cholesterol depletion, is almost identical to that observed in Jurkat T cells upon increasing the lipid raft associated PIP₂ pool [57]. These findings offer further support for an intimate relationship between cholesterol, PIP₂ and actin filaments.

Cholesterol depletion has repeatedly failed to demonstrate any consistent difference in diffusion behaviour between molecules that partition to lipid rafts and those that do not [58-60]. Using high resolution STED-FCS it was recently shown that the diffusion behaviour of a

lipid raft marker was sensitive to cholesterol depletion, whereas an artificial lipid expected to have no preference for lipid rafts, was not. This was taken as evidence for the formation of cholesterol-mediated molecular complexes [61], but we think that membrane topography can help explain this finding. With limited cholesterol depletion we observed an increase in actin polymerisation at the plasma membrane and, after substantial cholesterol depletion, cells have been shown to flatten [62]. The propensity of different membrane molecules to enter highly curved regions of the membrane differs and cholesterol depletion thus will affect their partitioning as it changes the cell topography. Furthermore, all results where a three dimensional surface is interpreted only in two dimensions should be treated with care [63].

In summary, we have demonstrated that limited cholesterol depletion causes both aggregation of plasma membrane lipid rafts and an increase in their abundance in the plasma membrane. The aggregation precedes Src-family kinase activation, leads to increased colocalisation between signalling molecules and results in an early T cell signalling response accompanied by striking actin polymerisation at the plasma membrane. Our findings also emphasise that viability tests are crucial for avoiding misleading interpretations.

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Supplemental Fig. 1. Cells at 10×10^6 cells/ml were stimulated with anti-CD3 (OKT3) at 1 μ g/ml for 5 min at 37°C. Cell lysates were analysed by Western Blotting for tyrosine phosphorylation (4G10). Untreated cells are shown for comparison. Gels were loaded on an equal protein basis. Molecular mass markers are indicated (kDa). The fluorograph shown is representative of three experiments.

Supplemental Fig. 2. The genetic requirement for T cell activation caused by cholesterol depletion. Jurkat-derived cell lines J.RT3-T3.5 (TCR/CD3 deficient), JCam2 (LAT deficient) and p116 (ZAP70 deficient) were examined for pTyr and activation of ERK. Cells at 10×10^6 cells/ml were treated with MBCD to achieve 0, 10, 20, 30, 40 and 50% cholesterol depletion. Cell lysates were analysed by Western Blotting for (A) tyrosine phosphorylation (4G10) or (B) ERK activation (anti-phospho-p44/42 MAP kinase). PMA was used as a positive control. Gels were loaded on an equal protein basis and membranes were probed with anti-p44/42 MAP kinase as an additional loading control. Molecular mass markers are indicated (kDa). Fluorographs shown are representative of three experiments.

Supplemental Fig. 3. Moderate cholesterol depletion induces a small calcium response. 10×10^6 Flou-4 AM labelled Jurkat T cells were used per sample. The fluorescence intensity was monitored at room temperature upon the addition of anti-CD3 antibody (OKT3), anti-CT-B to cells labelled with CT-B on ice or cholesterol depletion using 2.5 mM MBCD. Cells were analysed for intracellular Ca^{2+} release by flow cytometry. The traces shown are the means of three experiments $\pm$ SD.

Supplemental Fig. 4. Subcellular Distribution of Lck, LAT and GM1 upon Cholesterol Extraction. TX-DRMs, the TX-soluble and the intermediate fractions were prepared from control and MBCD-treated cells. 50×10^6 cells were used for each sample. The fractions were analysed by SDS-PAGE followed by Western blotting and gels were loaded on an equal cell number basis. Lck in TX-DRMs, the intermediate and the TX-soluble fractions. LAT in TX-DRMs and the TX-soluble fraction. GM1 in TX-DRMs, the intermediate and the TX-soluble fractions. GM1 was detected by subsequent incubation with CT-B, anti-CT-B and anti-goat horse radish peroxidase antibodies. Fluorographs shown are representative of at least three experiments.

Supplemental Fig. 5. GM1 is Lost from T Cells upon MBCD-treatment. Control cells and cells treated with MBCD were stained with CT-B-Alexa Fluor 594. The cells were analysed with a FACSCalibur and relative changes are displayed. Data shown are means $\pm$ s.e.m., n=3.

Supplemental Fig. 6. Control and MBCD-treated Jurkat T cells were fixed at 37°C and stained with CT-B-Alexa Fluor 594 and antibodies against (A) Lck, (B) LAT or (C) TfR that were visualized by staining with appropriate secondary Alexa Fluor 488 conjugated antibodies. Confocal images were acquired at the equatorial plane of the cells and the level of colocalisation determined using RBNCC and the Pearson correlation coefficient.

A

B

Supplemental Fig. 7. Cholesterol depletion increases the fraction of ordered membrane domains. Jurkat T cells were stained with 5 μ M laurdan for 30 min at 37°C after which they were imaged at 37°C. The cells were then treated with MBCD or MBCD-chol and reimaged 2 and 15 minutes after the start of the treatment. After deconvolution of the image series, the generalised polarisation was calculated. (A) Individual cells after MBCD-treatment. (B) Individual cells after MBCD-chol treatment at equilibrium conditions.