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Membrane cholesterol is essential for triterpenoid saponin augmentation of a saporin-based immunotoxin directed against CD19 on human lymphoma cells

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ABSTRACT

Triterpenoid saponins from Saponinum Album (SA) exert potent lytic effects on eukaryotic cell plasma membranes and, when used at sub-lytic concentrations, significantly augment the cytotoxicity of saporin-based immunotoxins (IT). To help elucidate the mechanism(s) behind these two phenomena we investigated the role of cholesterol to both.

Human Daudi lymphoma cells were lipid deprived using a combination of three different approaches. Following treatment, the total cellular lipid content was analyzed by electrospray ionization mass spectrometry (ESI-MS) and plasma membrane (PM) cholesterol content measured using the lipophilic fluorescent probe NR12S. Maximal lipid deprivation of cells resulted in a complete loss of sensitivity to lysis by SA. Similarly augmentation of the anti-CD19 immunotoxin (IT) BU12-SAPORIN by SA was lost but without a concomitant loss of intrinsic IT cytotoxicity. The lytic activity of SA was restored following incubation of lipid deprived Daudi cells with Synthecol or LDL. The augmentative effect of SA on IT cytotoxicity for Daudi cells was restored following repletion of PM cholesterol levels with LDL. NR12S fluorescence and ESI-MS analysis of cellular lipids demonstrated that restoration of SA lytic activity by Synthecol was entirely due to increased PM cholesterol levels. Restoration of cellular and PM cholesterol levels by LDL also restored the augmentative effect of SA for IT, an effect associated with repletion of PM cholesterol with minor changes in some phospholipid species. These results indicate that the lytic and IT augmentative properties of SA are cholesterol-dependent in contrast to intrinsic IT cytotoxicity that is at least partially cholesterol independent.

1. Introduction

Devising means of improving the therapeutic index of targeted therapeutics such as immunotoxins (IT) or antibody drug conjugates (ADC) would be an important advance for these novel classes of drug. Various approaches have been used previously in an attempt to achieve this and range from the use of viral vectors [1,2] to peptides [3,4] and small molecules [5] that modify intracellular vesicular trafficking or disrupt the endolysosomal limiting membrane allowing for increased efficiency of drug cargo release into the cytosol. Reducing off-target and improving on-target cytotoxin payload delivery through the judicious selection of the target antigen whilst simultaneously improving the efficiency with which the cytotoxic payload is delivered to the cell interior would almost certainly result in a marked improvement in the therapeutic index of such targeted therapeutics.

Augmentation of an EGF-saporin targeted toxin for human carcinoma cells by saponins from gypsophila plant species was first described by Heisler et al. [6] and later independently confirmed by others for saporin-based immunotoxins directed against a variety of target molecules on carcinoma [7] and haematological cell lines [8]. Not all purified triterpenoid saponin species present in Saponinum Album exhibit augmentative properties for targeted saporin cytotoxicity [9]. Similarly not all ribosome inactivating toxins are augmented by saponins [10]. These and other observations led to the hypothesis that a targeted toxin such

Abbreviations: EGF, epidermal growth factor; ESI, electrospray ionisation; MS, mass spectrometry; IT, immunotoxin; ADC, antibody drug conjugate; RIP, ribosome inactivating protein; RME, receptor mediated endocytosis; MβCD, methyl-β-cyclodextrin; SA, Saponinum Album; SAP, saporin; PC, phosphatidylcholine; PE, phosphatidyethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol; LBPA, lyso bis phosphatidic acid; NPC, Niemann-Pick type C; Ab, antibody.

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as EGF-SAP internalized together with the SA saponin species 1641 into a common intracellular vesicle following receptor mediated endocytosis leads to the formation of complexes between SA 1641 and the saporin component of the targeted toxin as the pH within the lumen of the maturing endosome becomes progressively more acidic. It is speculated that the complex then undergoes a conformational change rendering it lytic for the endolysosomal limiting membrane consequently releasing the targeted toxin cargo into the cytosol [8,11]. The precise mechanism driving augmentation is poorly understood but we speculate that it involves interaction of saporin-saponin complexes with cholesterol resident in the luminal inner leaflet of the endosomal membrane [8].

Various models have been proposed for the mechanism by which saponins perturb the plasma membrane of eukaryotic cells which include pore formation [12], membrane vesiculation [13] and membrane lipid domain disruption [14]. Molecular dynamic modeling with the steroidal saponin dioscin has revealed that it is the possible non-covalent associations of the steroidal aglycone moiety of this saponin species with PM cholesterol that ultimately leads to membrane disruption [14]. The contribution of the saponin sugar moieties to membrane disruption is less clear though one early view considered the saccharide side chains to be directly involved in the formation of an aqueous pore following the formation of two dimensional micellar-type structures comprised of saponin and cholesterol in the PM [15,16]. This led to suggestions that saponin assembles into complexes with cholesterol in a dose-dependent manner, and these then accumulate into matrices or plaques causing membrane curvature and the subsequent formation of pores or hemitubular protrusions that can lead to sterol extraction via vesiculation [17]. Structure-function studies of saponins have not previously been accurately predictive of their lytic properties [18]. Molecular modeling *in silico* suggests that the three dimensional structure formed by the saponin saccharide side chains is likely to be an important factor consequent to the distortion of membrane structure [14]. It is currently not known whether the augmentative properties of SA saponins for saporin-based IT cytotoxicity are linked to their lytic activity. It is possible that SA saponins that enter the endolysosomal compartment following cellular uptake by an endocytic process then interact with cholesterol in the luminal leaflet of the endolysosomal limiting membrane thus destabilizing it. In the present study we have investigated the contribution of PM and cellular cholesterol to both the lytic and augmentative properties of SA saponins exerted against the human lymphoma cell line Daudi. Understanding the mechanistic basis for saponin augmentation of IT cytotoxicity is particularly important if this or similar approaches are to be exploited for clinical application.

2. Materials and methods

2.1. Cell lines

Fully validated Daudi human Burkitt lymphoma cells [19] were obtained from the European Collection of Cell Cultures (ECACC, Porton Down, Salisbury, UK). Cells were routinely passaged and grown in RPMI 1640 medium containing 10% foetal calf serum (FCS) supplemented with 2 mM glutamine and 2 mM sodium pyruvate (referred to hereafter as R10) at 37 °C in 7% CO_2 in a humidified environment.

2.2. Saponinum Album (SA)

SA is a commercial preparation from Merck (Darmstadt, Germany) and has been characterized as described previously [20]. SA contains a mixture of several saponins all possessing the same aglycone core but with variations in the carbohydrate side chain structure. Fig. 1C shows the chemical structures for two of the most abundant saponin species present in SA: SA1641 and SA1657.

2.3. Delipidation of foetal calf serum

Five hundred ml batches of foetal calf serum (FCS) (Sigma Chemical Co, Poole) were delipidated according to the method of Sprong et al. [21] and filter sterilized by passage through a sterile 0.2 μm filter.

2.4. Culture media

All experiments were conducted in standard RPMI 1640 (Sigma Chemical Co, Poole, UK) or in phenolphthalein-free RPMI 1640 for XTT assays. Both media were supplemented with 2 mM glutamine and 2 mM sodium pyruvate containing either 10% standard FCS (R10) or 10% delipidated FCS (referred to as dR10).

2.5. Flow cytometry

Cell surface expression levels of CD19 were determined by single colour flow cytometry on an Epics XL instrument (Beckman Coulter, FL) running EXPO32 ADC analytical software or on a Cytoflex Flow Cytometer (Beckman Coulter) using CytExpert software. Cells were stained with a saturating concentration of the relevant antibody followed by a 1:50 dilution of fluorescein isothiocyanate labeled rabbit anti-mouse IgG (STAR 9B, Bio-Rad Laboratories, Kidlington, UK).

2.6. Plasma membrane permeabilisation assay

Cell membrane permeabilisation was quantified in treated Daudi cells by propidium iodide (PI) uptake using single colour flow cytometry. SA was diluted in RPMI, from a 1 mg/ml stock solution stored at −80 °C, and added to flow cytometry tubes. Cells (1×10^6/ml) were added in an equal volume of RPMI and the samples were left for up to 5 min at room temperature. Samples were centrifuged at 1500 rpm for 5 min, the supernatant was aspirated off and 200 μl of 1 μg/ml propidium iodide was added to the tubes. Cells were identified using forward scatter and side scatter and settings were adjusted to allow easy identification of cells showing an upward shift in fluorescence. Gates were set appropriately to measure the percentage of total cells with high fluorescence in each sample.

2.7. Lipid and cholesterol depletion of Daudi cells

Daudi cells were starved of lipids and cholesterol by treatment with various combinations of methyl-β-cyclodextrin (MβCD) [22,23] (for extraction of plasma membrane cholesterol), continuous culture in dR10 medium (for depletion of cellular lipids and cholesterol) and continuous lovastatin treatment [24] (for inhibition of biosynthetic cellular cholesterol production). The treatment schedules we employed are shown in Fig. 1. Daudi cells were treated with 1 mM MβCD in non-supplemented medium (RPMI) or in RPMI alone for 1 h at 37 °C in a humidified environment of 5% CO_2. After centrifugation and washing twice with RPMI the pulse treated cells were incubated in R10, R10 containing 1 μM lovastatin, dR10 or dR10 containing 1 μM lovastatin for between 1 and 30 h at 37 °C, 7% CO_2.

2.8. Cholesterol repletion

Lipid deprived or mock treated control cells were washed twice by centrifugation and re-suspension in RPMI before being re-suspended in R10, dR10, dR10 containing 0.016 mg/ml LDL (Bio-Rad Laboratories, Kidlington, UK), dR10 containing 0.032 mg/ml Synthechol (a synthetic soluble form of cholesterol from Sigma-Aldrich) or dR10 containing MβCD complexed cholesterol (Sigma-Aldrich) and incubated for 24 h at 37 °C in 7% CO_2.

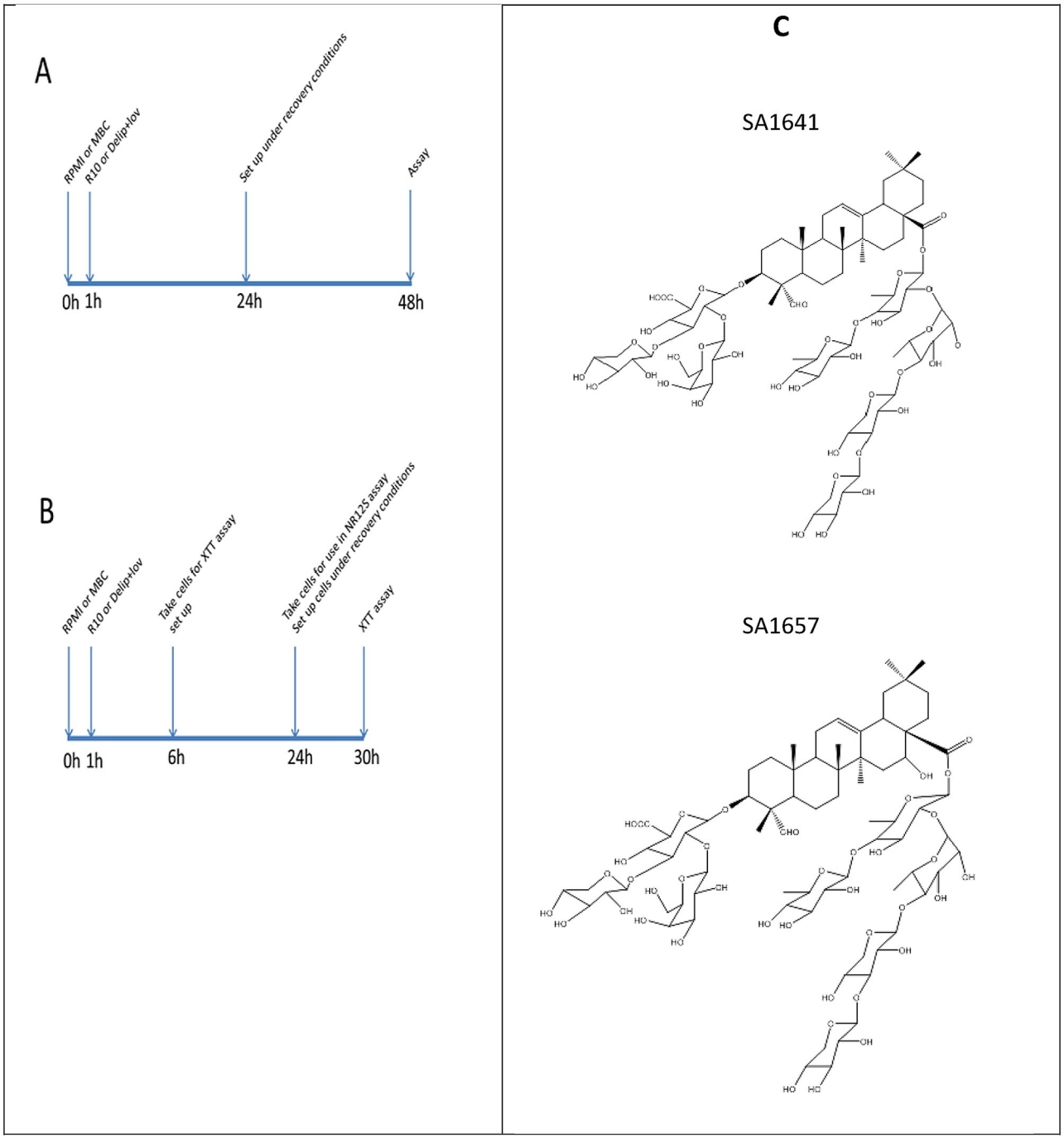


Fig. 1. A and B: Schematic diagrams showing the schedules for lipid depletion and repletion of Daudi cells. (A) Diagram demonstrating the standard depletion and lipid repletion procedure used. Daudi cells were incubated in the presence of 1 mM MβCD in RPMI or RPMI alone for 1 h at 37 °C, 7% CO_2. Cells were then washed twice in RPMI and re-suspended in dR10 containing 1 μM lovastatin or R10 respectively. The treated cells were incubated for 24 h at 37 °C, 7% CO_2 before being used in the relevant assay. In repletion studies treated cells were washed and re-suspended in dR10, dR10 containing LDL, dR10 containing Synthechol or R10 and incubated for 24 h at 37 °C, 7% CO_2. (B) Specific delipidation and repletion schedule used when performing the XTT and NR12S assay in tandem. Daudi cells were incubated in the presence or absence of 1 mM MβCD for 1 h at 37 °C, 7% CO_2. Cells were washed twice in RPMI, re-suspended in dR10 containing lovastatin or R10, and incubated at 37 °C, 7% CO_2. After 6 h samples of cells were taken and used to set up the XTT assay. XTT plates were incubated at 37 °C, 7% CO_2 for 24 h. The remaining cell populations were incubated for a further 18 h at which time samples from each treatment were taken for use in the NR12S assay. The remaining lipid deprived cells were washed and re-suspended in either dR10, dR10 containing LDL or R10 and incubated at 37 °C, 7% CO_2. After 6 h samples of treated cells were taken for use in the XTT assay. XTT plates were incubated at 37 °C, 7% CO_2 for 24 h. The remaining cells were incubated for a further 18 h at which point they were used in the NR12S assay. Fig. 1C: Two of the major bidesmosidal triterpenoid saponins, SA1641 and SA1657 present in Saponinum Album.

2.9. NR12S assay for plasma membrane cholesterol

NR12S was used as a fluorescent membrane probe to indirectly monitor changes in the levels of plasma membrane cholesterol in Daudi cells, after exposure to lipid depleting treatments, according to the method of Darwich et al. [25]. NR12S is a Nile Red based compound that can be used as a fluorescent membrane probe to measure lipid order changes in the cell plasma membrane. The fluorescence emission spectra of the compound change in response to lipid order shifting towards shorter wavelengths when incorporated into a liquid ordered

phase compared to a liquid disordered phase [25]. The zwitterionic head group and long alkyl chain mean that the compound selectively stains the outer leaflet of model and cell plasma membranes [26]. Cells were washed twice in RPMI by centrifugation and re-suspended at 2×10^6/ml in RPMI. An equal volume of 0.04 μM NR12S, diluted just before use in RPMI, was added to the cells which were incubated in the dark at room temperature for 7 min. The cells were then washed twice in RPMI by centrifugation, re-suspended at 1×10^7 cells/ml in RPMI and100 μl aliquots were added to the wells of a black 96 well plate. The intensity ratio of the NR12S emission spectra at 560 and 630 nm using an excitation wavelength of 520 nm was determined on a BMG Fluostar Omega plate reader (BMG Labtech, Aylesbury, UK) equipped with the appropriate filter sets.

2.10. Lipid extraction and mass spectrometric analysis

Total lipids were extracted from 4×10^6 cells using a procedure based on the method of Bligh and Dyer [27]. Solvents were all HPLC grade from Fisher. 1,2-Dimyristoyl-sn-glycero-3-phosphocholine (DMPC),1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine (DMPE), 1,2-dimyristoyl-sn-glycero-3-phosphate (DMPA),1,2-dimyristoyl-sn-glycero-3-phosphoserine (DMPS) and cholesterol 2.2.3.4.4.6.D^6 were obtained from Avanti Polar Lipids. Neutral lipids were separated from the total phospholipid fraction by passing through aminopropyl bond elute columns. Cholesterol derivatization was carried out on the neutral lipid fractions based on the method of Liebisch et al. [28].

Phosphatidylcholine (PC), phophatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylinositol (PI) and derivatized cholesterol were analysed by ESI-MS using a Xevo TQ mass spectrometer, (Waters UK, Wilmslow, UK). Lipid extracts were dissolved in solvent (methanol:DCM:water:ammonia 66:30:3:1 v:v) and analysed by direct infusion tandem MS/MS at a flow rate of 8 μl/min using a syringe pump. PC was selectively determined by precursor scans of the phosphocholine fragment (m/z 184) in positive ionization mode. PE species were quantified from neutral loss fragments (m/z 141) in positive ionization mode and PS species from neutral loss fragments (m/z 87) in negative ionization mode. For PI composition analyses precursor scans of m/z 241 were conducted in negative ionization mode. Derivatized cholesterol was analysed by precursor scans of m/z 77 and 369 in positive ionization mode.

Data were acquired and processed using Mass Lynx NT software employing a macrodeveloped in house by one of the authors (GK). MS were smoothed, base line subtracted, converted to centroid format and exported to individual Excel sample files which were imported into an analyser programme and corrected for the $[M + 1]^+$ and $[M + 2]^+$ ions generated by the natural abundance of ^{13}C (~1%).

2.11. XTT cytotoxicity assay

The cytotoxicity of BU12-SAP or non-targeted saporin used individually or in the presence of SA was determined for quadruplicate cultures of target cells using a modified XTT assay first described by Scudiero et al. [29]. The plates were read on a BMG Fluostar plate reader using a spectral scan from 300 to 650 nm.

2.12. Confocal microscopy

Control or MSL treated Daudi cells were incubated with BU12 antibody (10 μg/ml) on ice for 30 min. Samples were washed and incubated in culture medium at 4 °C or 37 °C for up to 90 min. Cells were fixed in 4% PFA, set in 2% low melting point agarose, permeabilised, incubated with Chicken anti EEA1, washed, incubated with Goat anti-mouse Alexa Fluor 488 and Goat anti-chicken Alexa fluor 555, washed and mounted. Images were acquired using a Leica TCs-SP8 laser scanning confocal microscope on DMi8 inverted microscope stand with a HC PL APO CS2 63×/1.30 glycerol immersion objective zoom 2.25 and Leica LAS-X acquisition software. Excitation wavelengths of 488 and 561 nm were used for Alexa Fluor 488 and Alexa Fluor 555 respectively.

2.13. Statistical analyses

Results are presented as mean and standard deviations. The number of data points used to determine the mean and standard deviation used in each figure is given in the individual figure legends. The number of independent experiments carried out is also given in the figure legend. The Student's t test was used to determine p values for the BU12 binding data in Fig. 6A.

3. Results

3.1. Changes in PM and total cellular cholesterol levels of lipid starved Daudi cells

We investigated the effects of lipid deprivation of Daudi cells on their PM and total cellular cholesterol content following three different treatments. Treatment with methyl-β-cyclodextrin (MβCD) alone consisted of incubation with MβCD for 1h followed by incubation in R10 for 24 h. Lipid starvation plus lovastatin involved incubation of Daudi cells in RPMI for 1 h followed by 24 h incubation in RPMI supplemented with delipidated FCS, 2 mM glutamine and 2 mM sodium pyruvate (dR10) containing lovastatin for 24 h. The third treatment comprised of combined MβCD treatment for 1 h with incubation in dR10 containing lovastatin for 24 h. Details of the treatment schedules employed for the various experiments described here are schematically shown in Fig. 1.

Daudi cells exposed to a combined treatment of MβCD and lipid starvation plus lovastatin (referred to hereafter as MSL) for 24 h produced a decrease in the NR12S 560 nm/630 nm intensity ratio of approximately 30% (Fig. 2A), indicating a significant reduction in plasma membrane lipid order which we take to be an indirect measure of cholesterol content, relative to mock treated control cells. The NR12S probe specifically binds the outer plasma membrane leaflet of living cells and changes its emission ratio (FIR560/630nm) as a function of cholesterol content [26]. Daudi cells incubated in RPMI for 1 h followed by 24 h in dR10 containing 1 μM lovastatin showed a 10% reduction in lipid order in the PM, as measured by NR12S, representative of an equivalent reduction in cholesterol whilst cells treated with MβCD only and then grown for a further 24 h in R10 medium showed only a 5% reduction (Fig. 2A).

Total cellular cholesterol levels were determined in lipid deprived Daudi cells using ESI MS. The amount of cholesterol measured in MSL treated cells was 60% lower than that found in mock treated control cells (Fig. 2B). The level of cholesterol in cells incubated in dR10 containing lovastatin for 24 h following 1 h incubation in RPMI was reduced by 35% (Fig. 2B). These results are in concordance with those obtained using the NR12S assay to indirectly monitor PM cholesterol and show that Daudi cells treated with MβCD and grown in dR10 with lovastatin contain less cholesterol than those exposed only to dR10 containing lovastatin. Daudi cells incubated in the presence of MβCD for 1 h and then returned to R10 for 24 h contained an increased amount of total cellular cholesterol compared to that seen in control cells. The loss of cholesterol from the plasma membrane is likely to lead to the up regulation of *de novo* cholesterol synthesis and/or release from cholesteryl ester stores. Returning the cells to serum containing medium then allows cells to take cholesterol from the medium producing an increase in total cellular cholesterol. The NR12S assay is only an indicator of cholesterol present in the PM whereas MS measures total cellular cholesterol. Therefore the MS data is likely to show an increase in total cellular cholesterol due to up regulation of cholesterol biosynthesis whereas the NR12S assay does not detect any increase in internal cellular cholesterol levels. This explanation accounts for the differences between Fig. 2A and B.

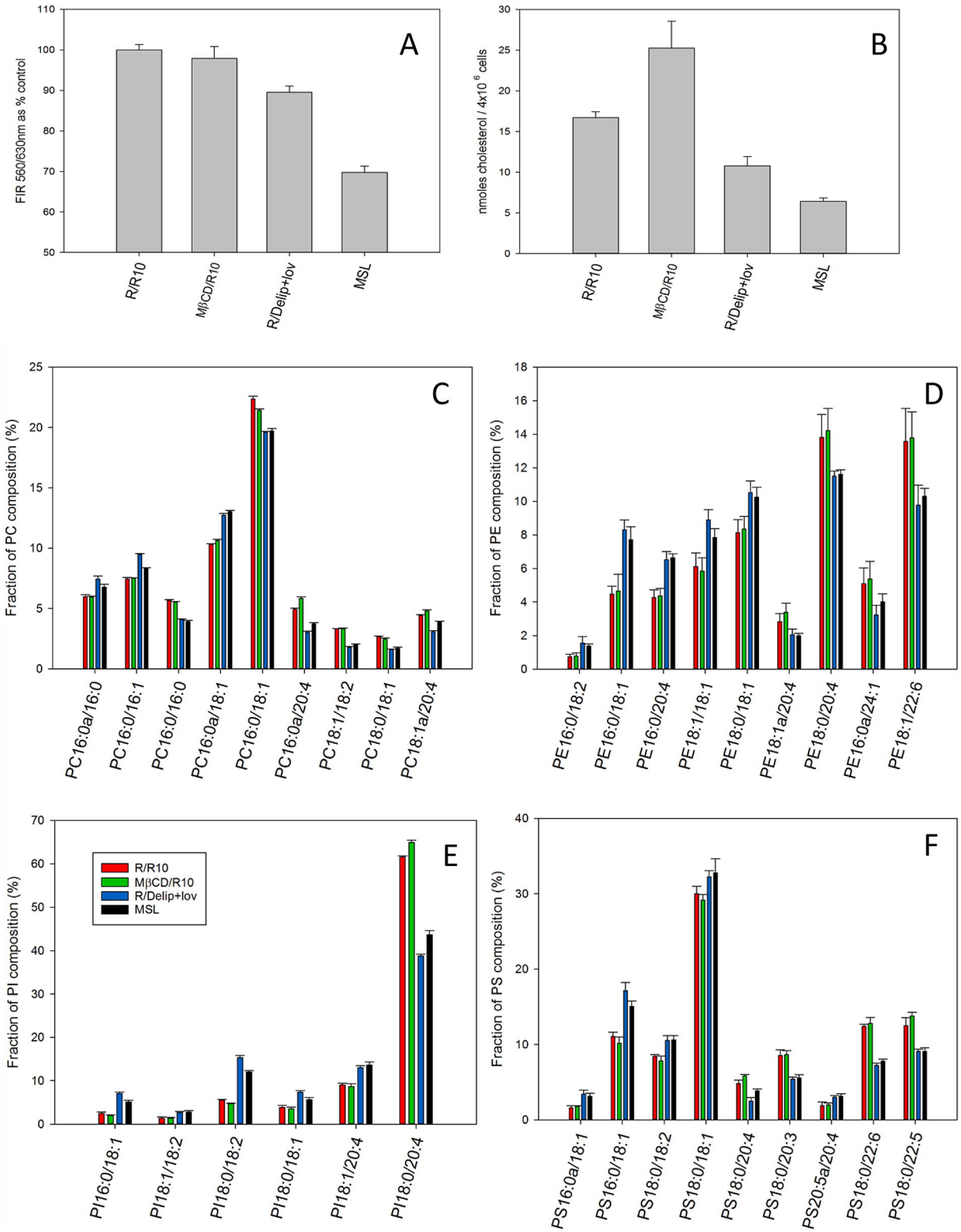


Fig. 2. Lipid starvation of Daudi cells lowers their cholesterol content and alters their phospholipid molecular species composition. (A) Monitoring the plasma membrane cholesterol level in mock treated and lipid deprived Daudi cells after 24 h using the NR12S probe. The ratios of the emission intensities of NR12S at 560 and 630 nm, representative of lipid order indicative of cholesterol content, are presented for lipid depleted cells as a percentage of the intensity ratio obtained for mock treated control cells exposed to RPMI and incubated in R10 at the corresponding time point. Values are the average of 5 replicates. NR12S was used at a concentration of 0.02 μM. (B) Cholesterol levels in Daudi cells exposed to control or lipid depleting conditions for 24 h, measured by ESI MS. Values are the average of 5 replicates. Profiles of selected phosphatidylcholine (PC) (C), phosphatidylethanolamine (PE) (D), phosphatidylserine (PS) (E) and phosphatidylinositol (PI) (F) molecular species for mock treated and lipid starved Daudi cells exposed to RPMI/R10, RPMI/dR10 plus lovastatin, MβCD /R10 and MSL treated for 24 h. Values are the average of 5 replicates and the data is representative of three independent experiments. Error bars represent standard deviation either side of the mean for quintuplicate samples. Standard lipid notation has been used for the phospholipid molecular species, as per the published lipid classification system [30].

3.2. Changes in the lipid content of Daudi lymphoma cells following lipid starvation

We determined the effects of lipid deprivation on the lipid composition of Daudi cells using ESI MS. Results for selected lipid species are shown in Fig. 2C–F. The full composition datasets for all lipid classes and species are given as supplemental data in Fig. S1. Incubation of Daudi cells with 1mM MβCD for 1 h followed by incubation in dR10 containing 1 M lovastatin for 24 h affected the levels of nine phosphatidylcholine (PC) species, eight phosphatidylethanoloamine species (PE), five phosphatidylinositol species (PI) and seven phosphatidylserine (PS) species compared to those measured in untreated control cells. The levels of several arachidonate species were altered across the lipid classes. PC16:0a/20:4, PC18:1a/20:4 (Fig. 2C), PE 18:0/20:4, PE18:1a/20:4 (Fig. 2D) and PI18:0/20:4 (Fig. 2 E) all decreased whereas PE16:0/20:4 increased (Fig. 2D). There were also decreases in the amounts of PS18:0/22:3, PS18:0/22:6 and PS18:0/22:5 and a concomitant increase in the amount of PS16:0/18:1 (Fig. 2E). The major changes in PI species occurred in 16:0/18:1, 18:0/18:2 and 18:0/20:4 (Fig. 2F), the greatest change being a 40% decrease in the arachidonate species PI18:0/20:4 in MSL treated cells.

Daudi cells incubated in dR10 containing lovastatin but without an initial MβCD treatment showed near identical phospholipid composition levels to cells that had an initial MβCD pulse treatment (Fig. 2C, D, E, F). The proportions of phospholipid molecular species measured in cells treated with MβCD and then incubated in R10 for 24 h were very similar to those found in control cells. Our results demonstrate that treatment with MβCD alone had no effect on the phospholipid composition of Daudi cells. However, cells incubated in dR10 containing lovastatin for 24 h showed clear changes in phospholipid molecular species composition regardless of whether or not cells had been pre-treated with MβCD. The molecular species phospholipid composition of Daudi cells in cells incubated in MβCD for 1 h, then washed and lipid extracted, was almost identical to that of cells incubated in RPMI for 1 h (see Supplementary Fig. S2) indicating that the possible extraction of PM phospholipids by MβCD in vesicular form was limited.

3.3. The effects of lipid starvation on SA-mediated PM permeabilisation of Daudi cells

We conducted experiments to determine the effects of the three different lipid deprivation treatments (MβCD alone, lipid starvation plus lovastatin or MSL) for varying lengths of time (1 h, 3 h, 9 h, 12 h, 18 h and 24 h) on the PM permeabilisation effects of SA in Daudi cells. Control cells were mock treated at the same time points with serum-free/serum containing medium as appropriate. PM permeabilisation due to the different concentrations of SA was quantified in treated Daudi cells using flow cytometry to measure propidium iodide ingress.

Representative results obtained from at least three independent experiments are shown in Fig. 3A–D. The lytic activity of SA on mock

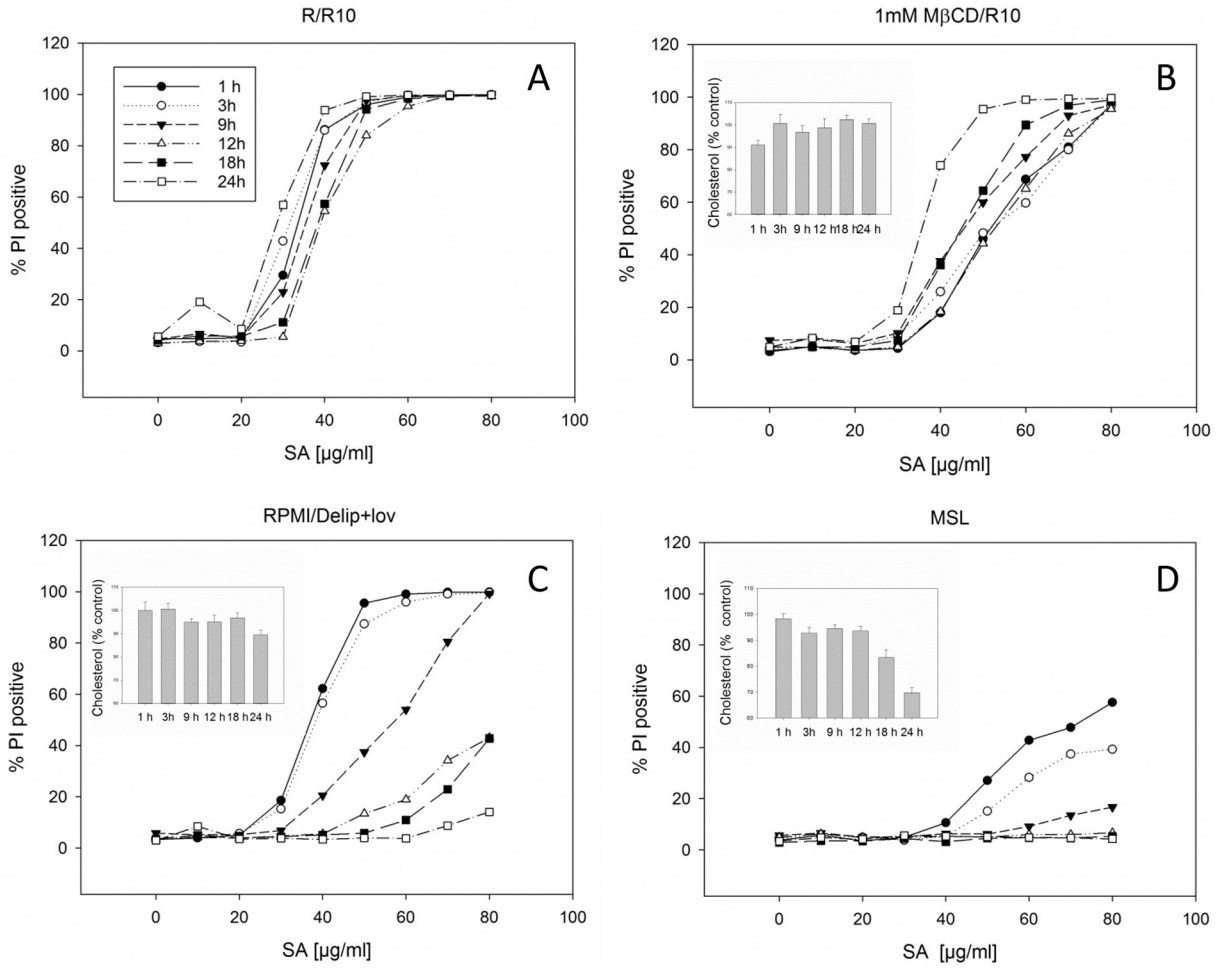


Fig. 3. Lipid starvation of Daudi cells abrogates SA-mediated lysis of Daudi cells in a concentration and time dependent manner also determined by the extent of lipid deprivation. Daudi cell membrane permeabilisation with varying concentrations of SA was quantified by flow cytometry using propidium iodide at varying time points ranging from 1 h to 24 h after commencement of lipid starvation and results reported as a percentage of propidium iodide positive cells within the appropriately gated population to yield the dose-response curves shown. Dose-response curves to SA for each time point studied for each of the four treatments (A) mock treated (B) MβCD treated (C) 24 h dR10 plus lovastatin treated and (D) MSL treated. The data is representative of three independent experiments. The chartlets inset show PM cholesterol levels at the various time points as determined using the NR12S assay.

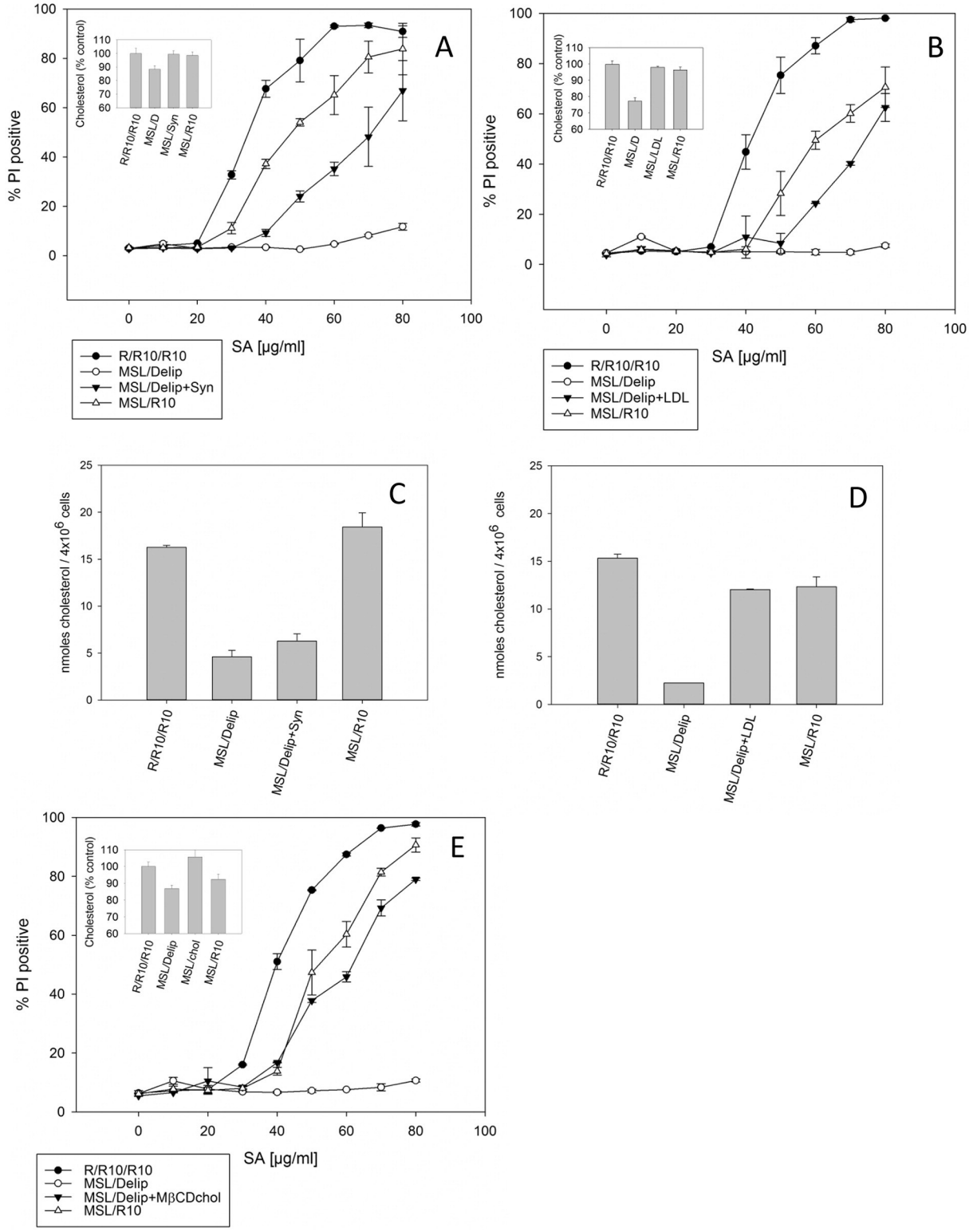
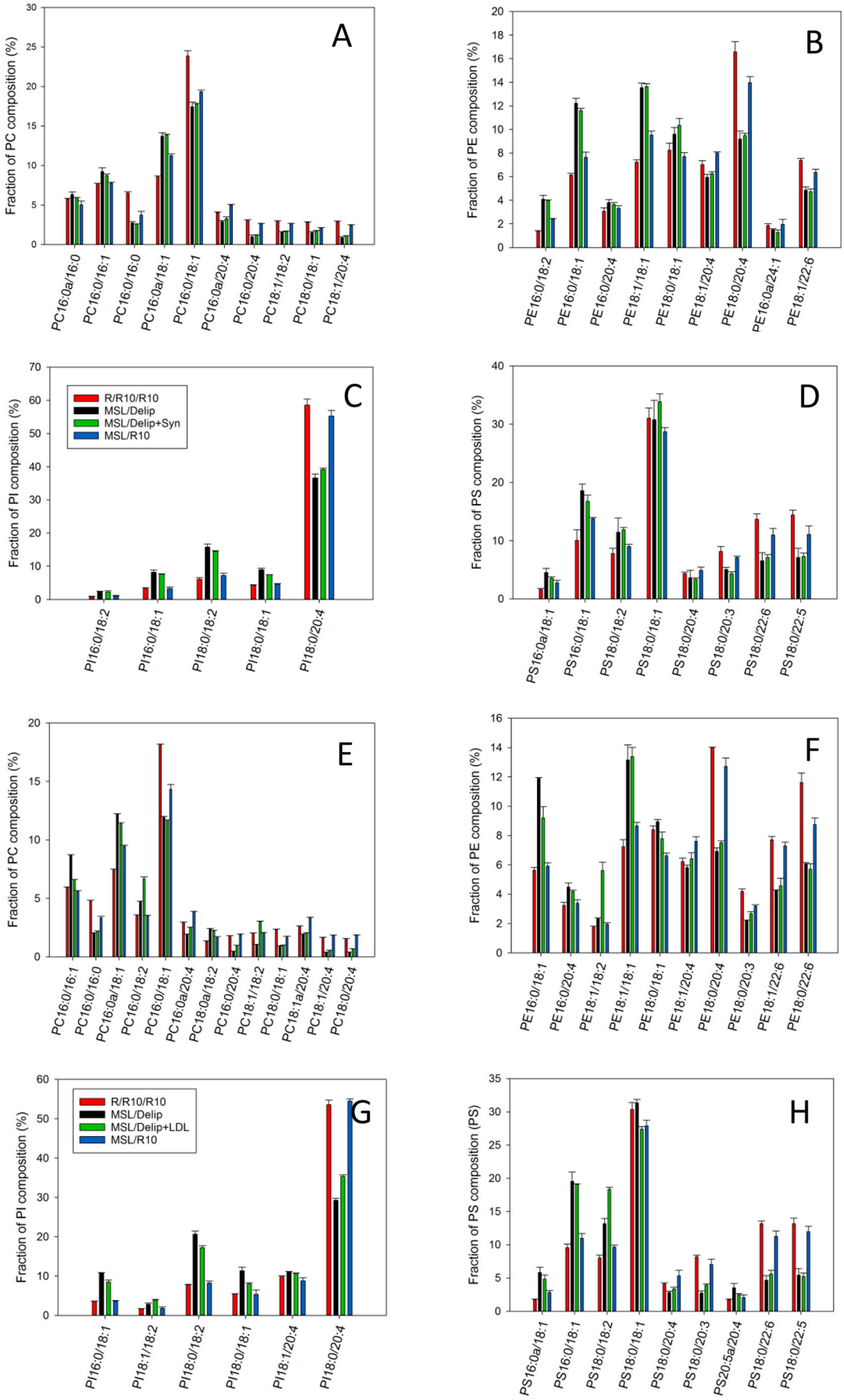


Fig. 4. Cholesterol repletion of lipid starved cells restores SA mediated lysis of Daudi cells. (A) Dose response curves for lipid deprived cells exposed to dR10 (○), R10 (Δ) or dR10 (▼) containing Synthechol for 24 h in comparison to mock treated control cells (●) with plasma membrane cholesterol levels, as determined using the NR12S assay, inset. (B) Dose response curves for lipid deprived cells exposed to dR10 (○), R10 (Δ) or dR10 (▼) containing LDL for 24 h in comparison to mock treated control cells (●) with plasma membrane cholesterol levels as determined using the NR12S assay inset. Values are the average of duplicate samples and representative of two experiments. PM cholesterol levels were determined using the NR12S assay on samples of the same cells as the lytic experiment. (C) Cholesterol levels, as determined by ESI-MS for mock treated control cells after 48 h and MSL treated cells after 24 h in dR10, dR10 containing Synthechol or R10. Errors bars represent the standard deviation either side of the mean for triplicate samples. (D) Cholesterol levels, as determined by ESI-MS for mock treated control cells after 48 h and MSL treated cells after 24 h in dR10, dR10 containing LDL or R10. Errors bars represent the standard deviation either side of the mean for triplicate samples. (E) Dose response curves for lipid deprived cells exposed to dR10 (○), R10 (Δ) or dR10 (▼) containing MβCD complexed cholesterol for 24 h in comparison to mock treated control cells (●) with plasma membrane cholesterol levels as determined using the NR12S assay inset. Error bars represent the standard deviation either side of the mean for duplicate samples.


A
Fraction of PC composition (%)
PC16:0a/16:0
PC16:0/16:1
PC16:0/16:0
PC16:0a/18:1
PC16:0/18:1
PC16:0a/20:4
PC16:0/20:4
PC18:1/18:2
PC18:0/18:1
PC18:1/20:4
B
Fraction of PE composition (%)
PE16:0/18:2
PE16:0/18:1
PE16:0/20:4
PE18:1/18:1
PE18:0/18:1
PE18:1/20:4
PE18:0/20:4
PE16:0a/24:1
PE18:1/22:6
C
R/R10/R10
MSL/Delip
MSL/Delip+Syn
MSL/R10
Fraction of PI composition (%)
PI16:0/18:2
PI16:0/18:1
PI18:0/18:2
PI18:0/18:1
PI18:0/20:4
D
Fraction of PS composition (%)
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PS16:0/18:1
PS18:0/18:2
PS18:0/18:1
PS18:0/20:4
PS18:0/20:3
PS18:0/22:6
PS18:0/22:5
E
Fraction of PC composition (%)
PC16:0/16:1
PC16:0/16:0
PC16:0a/18:1
PC16:0/18:2
PC16:0/18:1
PC16:0a/20:4
PC18:0a/18:2
PC16:0/20:4
PC18:1/18:2
PC18:0/18:1
PC18:1a/20:4
PC18:1/20:4
PC18:0/20:4
F
Fraction of PE composition (%)
PE16:0/18:1
PE16:0/20:4
PE18:1/18:2
PE18:1/18:1
PE18:0/18:1
PE18:1/20:4
PE18:0/20:4
PE18:0/20:3
PE18:1/22:6
PE18:0/22:6
G
R/R10/R10
MSL/Delip
MSL/Delip+LDL
MSL/R10
Fraction of PI composition (%)
PI16:0/18:1
PI18:1/18:2
PI18:0/18:2
PI18:0/18:1
PI18:1/20:4
PI18:0/20:4
H
Fraction of PS composition (PS)
PS16:0a/18:1
PS16:0/18:1
PS18:0/18:2
PS18:0/18:1
PS18:0/20:4
PS18:0/20:3
PS20:5a/20:4
PS18:0/22:6
PS18:0/22:5

treated Daudi cells is shown in Fig. 3A and only small variations in the lysis curves were observed for the various time points studied. Treatment with MβCD alone for 1h had only a minor effect on cell lysis across the range of SA concentrations used and by 24 h the lytic sensitivity of cells cultured in R10 had returned to near control levels (Fig. 3B). Use of the NR12S assay showed indirectly that plasma membrane cholesterol levels were reduced by 10% 1 h following MβCD treatment but this was transient with levels returning to near control levels after 3 h (Fig. 3B inset). In contrast lipid starvation plus lovastatin has a significantly greater effect on reducing the lytic sensitivity of cells to SA that occurred in a ranked time-dependent manner with cells treated for 24 h showing almost complete loss of lytic sensitivity to SA (Fig. 3C). These cells showed a reduction in cell membrane cholesterol occurring after only 9 h reaching a minimum at 24 h (Fig. 3C inset). The most pronounced loss of SA lytic sensitivity was seen in MSL treated Daudi cells. In this instance cells treated for 12 h, 18 h and 24 h demonstrated a complete loss of lytic sensitivity to SA and a clearly ranked sensitivity to SA lysis for the other time points studied (Fig. 3D). In this instance the NR12S assay indicated that PM cholesterol levels remained just below control levels until 18 h when they declined to 85% of the control level, reaching the lowest 70% of control level by 24 h. There is thus an imperfect correlation between PM cholesterol levels as indicated by NR12S fluorescence and sensitivity to SA-induced lysis in this particular model system.

3.4. Cholesterol repletion of Daudi cells with Synthecol or LDL restores their lytic sensitivity to SA

Having shown that MSL treatment of Daudi cells completely abolishes the lytic activity of SA (see Fig. 3D) we next investigated whether restoring cholesterol to the PM of these cells also restored the lytic activity of SA. We used Synthecol or low density lipoprotein (LDL) as two separate reagents with which to restore cholesterol to the PM. The results obtained for Synthecol and LDL are shown in Fig. 4A and B, respectively. Mock treated Daudi cells retained full lytic sensitivity to SA and as anticipated combined MSL treated cells almost completely lost their lytic sensitivity to SA. Both Synthecol (Fig. 4A) and LDL (Fig. 4B) partially restored the lytic sensitivity of MSL treated Daudi cells to SA after culturing with each of these for a 24 h period. Synthecol performed marginally better than LDL at restoring lytic sensitivity to SA. Incubation of MSL treated cells in R10 medium (containing 10% standard FCS) for the same duration also partially restored the lytic sensitivity of cells to SA but to a greater extent than either Synthecol (Fig. 4A) or LDL (Fig. 4B). The NR12S assay was used to indicate that Synthecol and LDL both restored plasma membrane cholesterol to very similar levels and to the same extent as R10 medium (Fig. 4A & B insets, respectively). Total cellular cholesterol levels were not fully restored in MSL treated cells after incubation with Synthechol but were after 24 h incubation in R10 (Fig. 4C) or dR10 containing LDL (Fig. 4D). We also replenished cellular cholesterol using the commonly used method of MβCD loaded cholesterol [31]. The results presented in Fig. 4E show that MβCD complexed cholesterol partially restored the lytic sensitivity of MSL treated Daudi cells to SA supporting our Synthechol and LDL repletion data. MβCD cholesterol restored the plasma membrane cholesterol level in lipid depleted Daudi cells to a higher level than that seen in lipid depleted Daudi cells incubated in R10.

3.5. The effects of cholesterol repletion on Daudi cell lipid content

We investigated whether restoring cholesterol with Synthecol or LDL to the plasma membrane of lipid deprived cells also affected their phospholipid content. The phospholipid composition of MSL treated Daudi cells incubated in dR10, R10, delipidated R10 containing Synthechol or delipidated R10 containing LDL for a further 24 h was determined using ESI MS. The results for selected lipid species are presented in Fig. 5A–H and the full composition data are shown as supplemental data in Fig. S3. MSL treated Daudi cells incubated in R10 for 24 h show full or partial recovery of all PC and PE species measured (Fig. 5A and B respectively). PC16:0/16:0, 16:0/18:1 and 18:0/18:1 showed the least response in returning to the levels found in control cells. Incubation of MSL treated cells for 24 h in R10 led to the levels of PS and PI species returning to those measured in Daudi cells grown under control conditions (Fig. 5C and D respectively). Incubation with Synthechol did not restore the lipid molecular species content of PC, PE, PS or PI in lipid deprived cells to that found in untreated control cells. Fig. 5A–D shows that the lipid molecular species composition of lipid depleted cells incubated with Synthechol is consistent with that of cells further incubated solely in delipidated R10. In summary our results show that lipid deprived cells exposed to full medium for 24 h were able to partially restore their lipid content towards the levels found in control cells but those treated only with Synthechol were not. This strongly suggests that PM cholesterol is of central importance to the lytic effects of SA saponin.

MSL treated Daudi cells that were incubated in dR10 containing LDL for 24 h showed very few differences in lipid composition compared to those incubated in dR10 only for 24 h (see Fig. 5E, F, G and H). It is noticeable that the small changes in molecular species observed across the lipid classes were in phospholipids containing the acyl chain 18:2, namely PC16:0/18:2, PC18:1/18:2, PE18:1/18:2 and PS18:0/18:2. Several PI species showed a slight recovery towards the levels found in control cells (Fig. 5H).

We determined the phospholipid composition of the LDL reagent used in the supplementation experiments and the data are presented as supplementary material in Fig. S4. The major phospholipid components of LDL were PC species present at over a twenty-fold greater amount than PE species (240 nmol compared to 11 nmol in the sample tested). PC16:0/18:2 comprised 26% of the PC species measured whilst PC 18:1/18:2 contributed to 5% of the PC species measured. The variety of PC and PE species measured in the LDL was limited compared to the number detected in Daudi cells. However, the full complement of PI species found in Daudi cells were detected in the LDL. No PS species were detectable.

3.6. The effects of lipid starvation and cholesterol repletion on the expression level and internalisation of CD19 in Daudi lymphoma cells

In order to exclude the possibility that cholesterol depletion reduced CD19 expression and subsequent internalisation following ligation by antibody and that this might have been wholly or partially responsible for the loss in the augmentative effect of SA, we investigated the effects of lipid starvation on the surface expression level of CD19 and internalisation kinetics in Daudi cells using flow cytometry. There was a significant 40% reduction ($p < 0.01$ Student's t test with a 2 tailed distribution) in the MFI values obtained for BU12 antibody binding in MSL treated Daudi cells compared to mock treated control cells (Fig. 6A). By 30h BU12 antibody binding had decreased further to 45% of that measured in control cells. There was no significant difference in BU12 binding between cells pulsed for 1 h in MβCD and mock treated control cells pulsed in RPMI prior to the incubation in delipidated R10 containing lovastatin. Our results show that the apparent level of CD19 expression is reduced in MSL treated Daudi cells but unaffected following partial removal of cholesterol from the PM with MβCD only. Flow cytometric

Fig. 5. Cholesterol repletion alone for 24 h does not restore the phospholipid molecular species content of lipid deprived Daudi cells to that of control cells. Profiles of selected PC (A), PE (B), PS (C) and PI (D) molecular species for lipid starved Daudi cells exposed to R10, dR10 or dR10 containing Synthechol for 24 h compared to R/R10 treated control cells. Error bars represent standard deviations either side of the mean for triplicate cultures. Profiles of selected PC (E), PE (F), PS (G) and PI (H) molecular species for lipid starved Daudi cells exposed to R10, dR10 or dR10 containing LDL for 24 h compared to mock treated control cells. Error bars represent the standard deviation either side of the mean for triplicate samples.

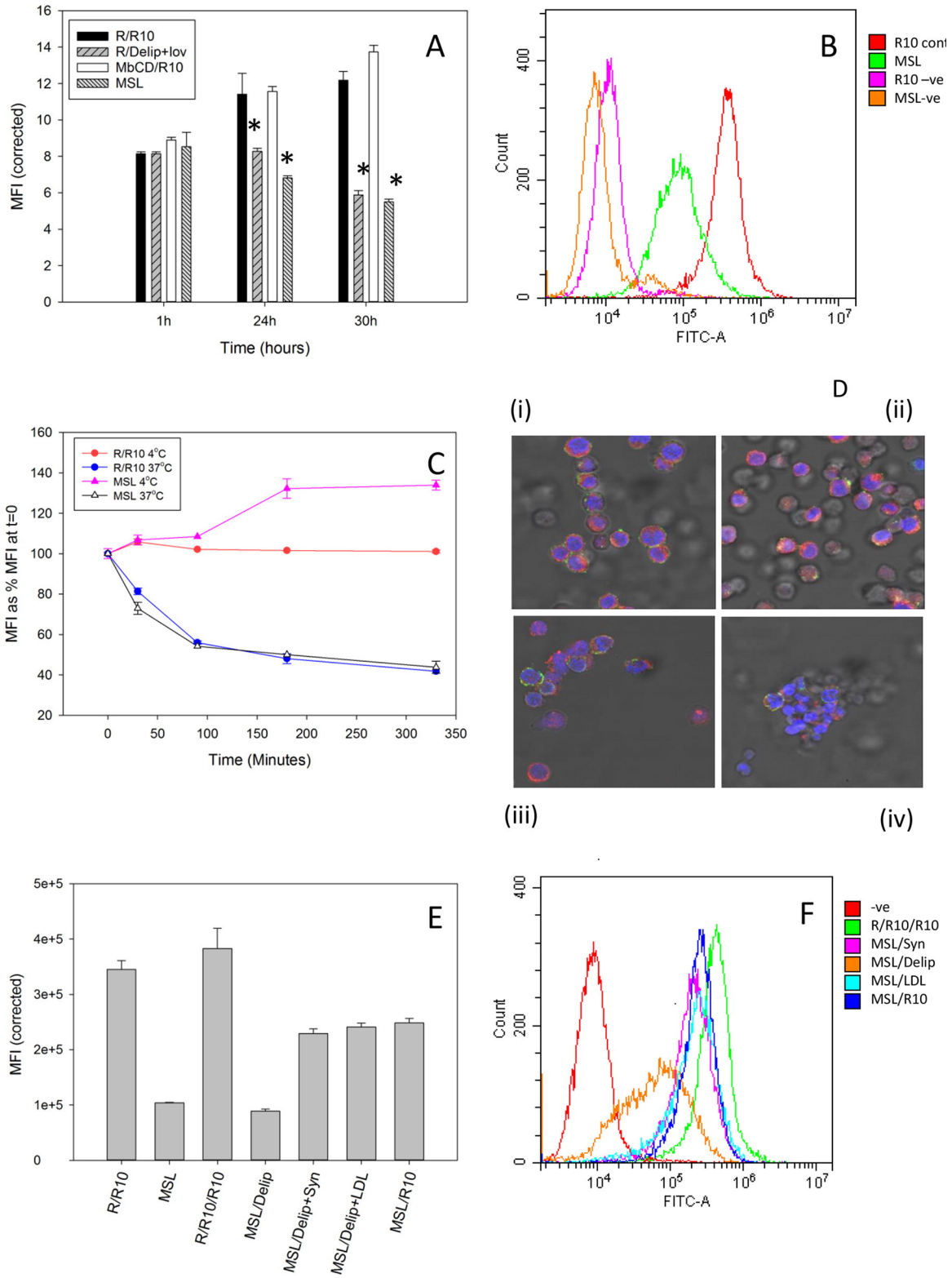

Fig. 6. Lipid starvation and cholesterol repletion affects the expression level of CD19 in Daudi cells. (A) BU12 binding in mock treated and lipid deprived Daudi cells exposed to RPMI / R10, RPMI / delipidated R10 plus lovastatin, MβCD / R10 or MSL for 1h, 24 h and 30 h. Results are plotted as mean fluorescence intensity (MFI). Values are the average of five replicates and representative of four separate experiments. * $p < 0.01$ Student's t test relative to control. (B) Flow cytometric traces showing BU12 Ab binding to the surface of control (red) and MSL treated (green) Daudi cells with 2° Ab only for mock treated control (magenta) and MSL treated cells (orange). (C) Time course of BU12 internalisation in mock treated control and lipid deprived cells. Results are expressed as a percentage relative to the MFI at time 0 h for each treatment. The data is representative of at least three independent experiments and the values presented are the average of duplicate cultures. (D) Internalisation of CD19 in lipid deprived and mock treated control cells after binding of BU12 viewed by confocal microscopy. (i) Control cells at 37 °C for 0 min, (ii) control cells at 37 °C for 90 min, (iii) MSL treated cells at 37 °C for 0 min, (iv) MSL treated cells at 37 °C for 90 min. (E) BU12 binding in lipid deprived cells incubated in R10, dR10, dR10 containing Synthechol or dR10 containing LDL for 24 h compared to mock treated control cells incubated in R10 for 24 h. Results are plotted as MFI. Error bars represent standard deviations either side of the mean for quadruplicate cultures. (F) Flow cytometric traces showing BU12 Ab binding to MSL treated cells incubated for 24 h in; dR10 containing Synthechol (magenta), dR10 (orange), dR10 containing LDL (cyan), R10 (navy) compared to mock treated control cells (green). Cells exposed only to 2° Ab are shown in red. Results are representative of two independent experiments.

histograms showed that expression levels of CD19 were markedly decreased in MSL treated cells with a wider distribution of expression levels amongst the total population compared to mock treated control cells (Fig. 6B). Fig. 6C shows that CD19 was lost from the cell surface at a similar rate in MSL treated and mock treated control cells even though the initial level of BU12 binding measured appeared to be reduced in lipid depleted cells. Internalisation occurred after 30 min and reached a plateau after 90 min. The internalisation of antibody into lipid starved and mock treated control cells was confirmed by fluorescence confocal microscopy. Fig. 6D shows a loss in cell surface material and internalisation of antibody in control cells after 90 min (ii) with a small amount of antibody co-localising with the endosome marker EEA1. There was a general reduction in the surface expression of antibody in MSL treated cells with some co-localisation with EEA1 (iii). However, there are sporadic cells that show higher surface expression supporting the flow cytometry data which had a broad distribution with a small number of cells exhibiting fluorescence levels equivalent to those measured in control cells (iv).

We next investigated whether restoring cholesterol to the plasma membrane of lipid deprived cells restored the apparent expression level of CD19. MSL treated Daudi cells were incubated for 24 h in R10, dR10, dR10 containing Synthechol or dR10 containing LDL. Fig. 6E shows that both LDL and Synthechol partially restored BU12 antibody binding in lipid deprived cells to 70% of that measured in mock treated control cells. Incubation of lipid deprived cells in R10 also restored the apparent expression level of CD19 to the same degree as LDL or Synthechol alone. However, lipid deprived cells cultured in delipidated R10 for a further 24 h maintained a BU12 antibody binding level equivalent to that measured in Daudi cells grown under delipidating conditions for 24 h.

Flow cytometric histograms of MSL treated cells incubated in dR10 for a further 24 h showed a considerably reduced but broad distribution of expression levels compared to control cells (Fig. 6F). MSL treated cells incubated for a further 24 h in R10, dR10 containing Synthechol or dR10 containing LDL show an increase in fluorescence intensity with a tighter distribution of expression levels. These results demonstrate that CD19 apparent expression levels in lipid deprived Daudi cells are partially restored by repletion of cellular and PM cholesterol.

3.7. Lipid starvation of Daudi cells abolishes SA augmentation of saporin immunotoxins

We asked the question whether lipid starvation of Daudi cells affected their sensitivity to SA-mediated augmentation of BU12-SAP IT cytotoxicity or to the intrinsic cytotoxicity of this IT *per se*. We achieved this using the XTT assay to quantify cytotoxicity following titration of increasing amounts of BU12-SAP in the absence and presence of an augmentative but sub-lytic concentration of SA against either sham treated cells or against cells that had been lipid starved. The dose-response curves obtained with the BU12-SAPORIN IT and unconjugated saporin for control and MSL lipid starved Daudi cells are shown in Fig. 7A and B, respectively. The results show that the augmentation of both IT and saporin cytotoxicity by SA is completely abolished in lipid starved cells. Lipid starvation resulted in a relatively small reduction in cytotoxicity of unconjugated saporin. Lipid starvation also reduced the cytotoxicity of the IT compared to control cells but was not completely abrogated. Analysis of total cellular phospholipid and cholesterol contents of control and lipid starved Daudi cells described previously demonstrated the occurrence of upwards and downwards shifts in some phospholipid molecular species, but the major effect was a 60% decrease in total cholesterol in lipid starved cells.

3.8. SA augmentation of IT cytotoxicity is restored by cholesterol repletion of lipid starved Daudi cells

Having demonstrated that cholesterol repletion of the PM restored the lytic sensitivity of lipid starved Daudi cells to SA we next investigated whether cholesterol repletion would also restore the SA-mediated augmentation of IT cytotoxicity. We used the XTT cytotoxicity assay to determine whether lipid starved Daudi cells repleted of their cholesterol with LDL regained SA-mediated augmentation of BU12-SAP cytotoxicity. Unlike the restoration of lytic activity of SA in lipid starved Daudi cells described previously (see Fig. 4) we were unable to use Synthecol or MβCD complexed cholesterol to replete PM cholesterol because both inhibit SA activity in the XTT assay, probably by competing for SA. However, LDL does not inhibit this activity in the XTT assay. Incubation of MSL treated cells with LDL for 24 h restored the augmentation of BU12 SAPORIN cytotoxicity by SA as shown in Fig. 8. The level of SA mediated augmentation of BU12 SAPORIN measured in MSL lipid starved cells incubated in R10 for 24 h or dR10 containing LDL for 24 h was consistent with that found in mock treated control cells incubated in R10. MSL treated cells incubated for a further 24 h in delipidated R10 showed no SA augmentation of BU12 SAPORIN cytotoxicity.

ESI MS analysis showed that the total amount of cholesterol in MSL treated cells incubated for 24 h in dR10 containing LDL was equivalent to the level measured in MSL treated cells returned to R10 for 24 h control cells and to that found in control cells incubated in R10 for 24 h. There were very minor changes in several phospholipid molecular

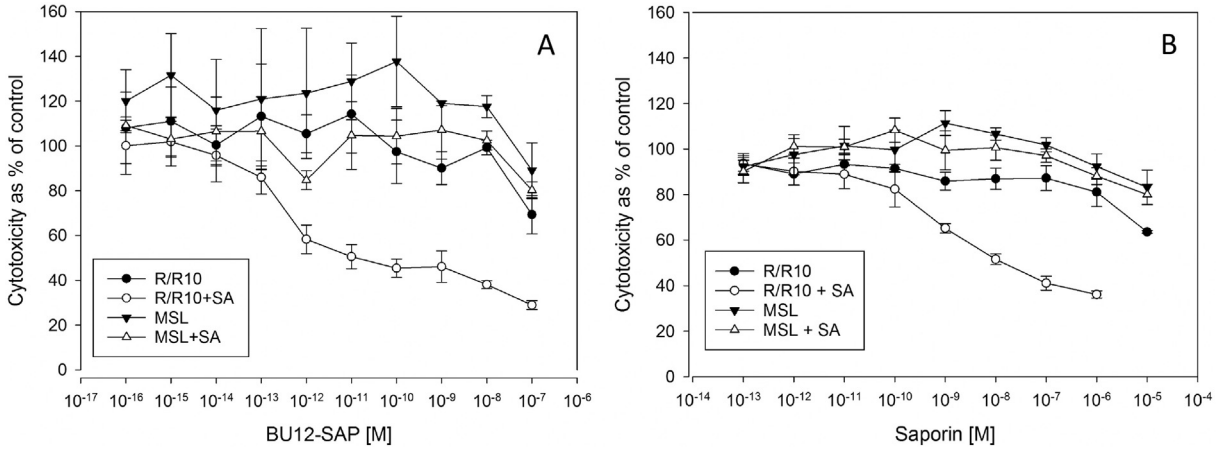

Fig. 7. Lipid starvation of Daudi cells abrogates the SA-mediated augmentation of BU12-SAP IT cytotoxicity. Dose-response curves obtained by XTT cytotoxicity assay for mock treated cells in the absence (●) or presence (○) of 1 μg/ml SA and lipid starved Daudi cells in the absence (▼) or presence (Δ) of 1 μg/ml SA exposed to increasing concentrations of (A) BU12-SAP or (B) unconjugated saporin. Samples were blank corrected and the absorbance at 470 nm–650 nm was calculated for each well. Results are expressed as a percentage of control cells cultured in the relevant medium alone. Error bars represent the standard deviation either side of the mean for quadruplicate cultures and the data is representative of four independent experiments.

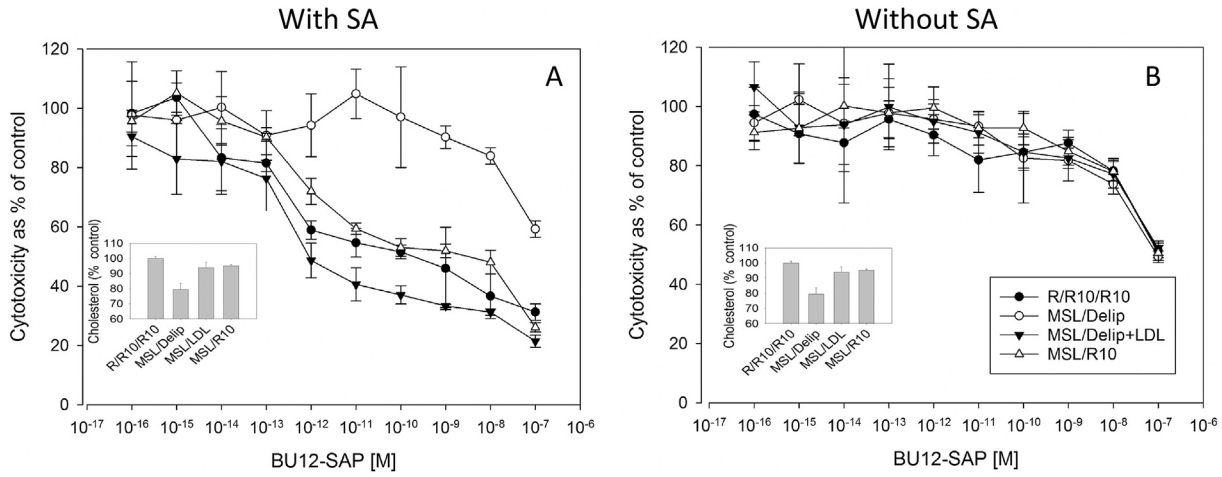

Fig. 8. Repletion of plasma membrane and cellular stores of cholesterol to lipid starved Daudi cells restores SA-mediated augmentation of BU12-SAP IT cytotoxicity. Dose-response curves determined by XTT assay for lipid deprived cells (○), lipid deprived cells repleted with LDL (▼) or R10 (Δ) compared to mock treated control cells (●) in the presence (A) or absence (B) of SA. The chartlets inset show plasma membrane cholesterol levels for the various treatments, as determined using the NR12S assay. Error bars represent the standard deviation either side of the mean for quadruplicate cultures and the data is representative of two independent experiments.

species that closely paralleled the phospholipid species present in the LDL particle as analysed by ESI MS. The minor changes observed were not consistent with the type of changes measured in MSL treated cells returned to full R10 for 24 h.

4. Discussion

The primary aim of this study was to establish the role that cellular lipids and particularly PM cholesterol contribute to the lytic and augmentative properties of SA saponins. Schulman & Rideal [32] were the first to show that saponin and cholesterol interacted non-covalently to form insoluble complexes. The membrane lytic properties of saponins towards eukaryotic cell membranes have been known for many years and early electron microscopic studies seemed to demonstrate that saponins created ring-like structures presumed to be pores [15] or discontinuities [33] in biological membranes. The precise mechanism behind the membrane lytic properties of saponins is still incompletely understood but is thought to at least partly result from a non-covalent interaction between the polycyclic ring structures contained within the saponin and cholesterol molecules. Structure-function studies indicate that in some instances but not always, the carbohydrate moieties attached to the aglycone core structure of some saponins are essential to the lytic characteristics of any given saponin species [18,34].

In order to investigate the role of cholesterol to the lytic and augmentative characteristics of SA saponins we chose to use controlled lipid starvation of Daudi lymphoma cells followed by repletion of their cholesterol content with either Synthecol or LDL. A requirement of this study was to deplete cells of cholesterol and maintain them throughout the experimental period with depleted levels of cholesterol whilst maintaining their viability and proliferative capacity. MβCD has been widely used to reduce membrane cholesterol levels [31,35,36]. However care must be taken to optimise conditions as MβCD can adversely affect cells causing loss of membrane fluidity and leakage of vital cellular components [23]. The use of MβCD alone removes only PM cholesterol leaving intracellular stores of cholesterol untouched. In order to prevent cellular cholesterol biosynthesis occurring during the experiments described here, lovastatin, an inhibitor of HMG Co A reductase and thus cholesterol biosynthesis [24], was used. At higher concentrations (10–50 μM) lovastatin can cause inhibition of cell proliferation and result in cell death [37]. Additional experimental evidence suggests that this is related to the lowering of cellular cholesterol levels resulting in cell cycle arrest in G1 [38]. We therefore optimized our cholesterol depletion conditions and finally elected to use a combined treatment comprised of 1 h incubation in 1 mM MβCD followed by culturing for 24 h in delipidated R10 containing 1 μM lovastatin.

Through the use of ESI MS analysis and the fluorescent membrane probe NR12S we showed that we could successfully reduce PM and cellular cholesterol levels whilst maintaining cell viability for the duration of the experimental period. However, we have also seen that that culturing Daudi cells in lipid depleted medium changes their phospholipid molecular species content, particularly the arachidonate species. Arachidonic acid is a major potent cell signalling molecule regulated through storage within phospholipid species such as PE, PS, PI and PC and released from storage by cPLA2 [39]. Therefore a lack of external lipid supply for cells grown in lipid depleted medium is likely to lead to loss of the arachidonate phospholipid species as cells up regulate release from storage. We also observed a general decrease in the amount of longer chain phospholipids across the lipid classes and an increase in the amount of some shorter chain phospholipids under delipidating conditions. When mammalian cells are cultured in the presence of serum, cholesterol and fatty acids are taken up from the medium and there is little endogenous cholesterol and fatty acid lipid synthesis [40,41]. If cells are transferred to lipid poor medium there is a reorganisation of lipid metabolism and cells switch to a process in which membrane lipid precursors are generated through hydrolysis of intracellularly stored cholesteryl esters and triglycerides by active synthesis from small metabolic precursors. Synthesis of phospholipids is likely to now be via aceto acetyl co A and malonyl co A forming palmitic acid [42] and thus shorter chain phospholipids will initially be produced. Therefore the observed changes in phospholipid molecular species composition that we observed in our lipid deprived Daudi cells were to be expected.

The role of cholesterol to the lytic process with saponins has been discussed in the literature but much of the research to date has been carried out in liposomes [43], model membrane systems [44] or red blood cells [45]. See [46] for a comprehensive review of this. There is a paucity of research in this area for nucleated cells. Wassler et al. [47] demonstrated that five times the amount of saponin was required to permeabilise the endoplasmic reticulum (ER) in comparison to the PM of rat hepatocytes and speculated that this could be due to the lower cholesterol content of ER membranes. In the present study we manipulated the cholesterol content of Daudi cells and showed that depletion of cholesterol leads to a complete loss of SA lytic activity for these cells. We clearly demonstrated that the lytic activity of SA is entirely cholesterol dependent since restoration of PM cholesterol levels with Synthecol, LDL or cholesterol complexed with MβCD restored the lytic activity of SA for lipid deprived Daudi cells. ESI MS analysis of lipid deprived cells

repleted with Synthechol showed that the phospholipid molecular species content of these cells is consistent with lipid deprived cells. We can conclude therefore that the compositional changes in phospholipid molecular species were not responsible for the lytic behavior of SA. The lytic activity of SA was restored to a greater extent using MβCD complexed with cholesterol compared to LDL repletion of cholesterol levels in Daudi cells. This is probably because MβCD cholesterol complexes will result in the direct insertion of cholesterol into the plasma membrane whereas lipoprotein-derived cholesterol will be taken up by the cell by receptor mediated uptake via LDL receptors that then requires intracellular trafficking to other locations of which the PM is only one [48,49]. Thus repletion using LDL will result in a more heterogeneous replenishment of cholesterol within the cell.

The critical finding of this study is that the augmentative properties of SA saponins for BU12-SAP IT cytotoxicity are also cholesterol dependent demonstrable through the restoration of the augmentative effect of SA when lipid deprived cells are treated with LDL. We consider that the very small changes in phospholipid composition, introduced in lipid deprived cells incubated in the presence of LDL, are unlikely to account for the restoration of the augmentative activity of SA. The phospholipid molecular species composition of cells is dependent on the medium in which cells are cultured [50]. Hague et al. [51] showed how phospholipid molecular species composition changes as the cell progresses through the cell cycle. Thus phospholipid composition is in a constant state of flux. In the experiments described here even lipid deprived cells incubated in full medium for 24 h did not fully restore their lipid content, in terms of molecular species, to that found in control cells. However, we have shown that the total cholesterol content of lipid deprived cells incubated in the presence of LDL had returned to approximately the same level as seen in control cells. We therefore propose that it is the repletion of cholesterol in lipid deprived Daudi cells exposed to LDL that restores the augmentative effect of SA to the cytotoxic effect of BU12-SAP.

Reducing the cholesterol content of the PM disrupts lipid raft structures [52] bringing about perturbations in expression levels and/or the functionality of a variety of membrane proteins [53]. In the case of CD20, the use of statins to reduce PM cholesterol levels results in an apparent down regulation in this therapeutically important molecule on target lymphoma cells not because of a reduction in copy number but because reduced cholesterol levels in the PM elicit a conformational change in the CD20 molecule that leads to epitope loss [54]. Changes in plasma membrane cholesterol levels will influence membrane curvature and rigidity [55]. The four ring structure of cholesterol confers biophysical properties that increase the ordering of neighboring lipids decreasing fluidity and reducing the permeability of polar molecules [48]. The observed down-regulation in CD19 expression in our lipid deprived Daudi cells was restorable following repletion of PM cholesterol using Synthecol or LDL leading us to speculate that similar conformational changes in CD19 might be responsible for the apparent down regulation we observed, though our limited data does not allow us to fully confirm this. It is noteworthy that the CD19 molecule does not possess CRAC, CARC or tilted domains that would allow it to interact with cholesterol directly [56]. However, our study has shown that lipid starvation does not affect the rate of CD19 internalisation from the Daudi cell surface which was near identical to that seen in non-lipid deprived control Daudi cells following ligation by BU12 antibody. Moreover confocal microscopy qualitatively revealed that the route of entry of BU12 antibody in lipid deprived Daudi cells was also nearly identical to that of control cells. Furthermore, lipid deprivation of Daudi cells resulted in a significant increased expression level of CD22 accompanied by a complete loss of SA augmentation for the anti-CD22 IT 4KB128-SAP (WSS unpublished observations). These combined observations lead us to conclude firstly that it is the decreased cholesterol content of the PM that is responsible for the apparent down regulation in CD19. Secondly our IT internalization studies indicate that the subsequent uptake rate of CD19 is the same in lipid depleted and control cells therefore making this an unlikely cause of the loss of the augmentative effect of SA on IT cytotoxicity.

The fact that a sublytic concentration of SA possesses augmentative activity for IT cytotoxicity indicates that the mechanism of augmentation does not require PM permeabilisation. However, this does not exclude the possibility that SA saponins may interact with PM cholesterol which may in turn initiate PM repair mechanisms [57] that may themselves then play a role in the augmentation process. We are currently exploring this particular avenue in this laboratory. We have previously suggested that it is an absolute requirement for saponin to be taken up into the same endocytic vesicle as the IT in order to achieve any augmentative effect [8]. Saponins have been shown to bind to saporin and other highly basic toxins at pH values below 6.0 that recapitulate the increases in luminal acidity as cargo progresses through the endolysosomal system [10]. This in turn led us to speculate that the interaction of saponin and saporin to form complexes at lower pH results in a conformational change to the saponin molecule that renders it lytic for the endolysosomal membrane [8]. Using confocal microscopy Weng et al. [11] showed that the gypsophila-derived saponin species SA1641 caused the leakage of saporin from the endosome lumen into the cytosol of the human bladder cancer cell line ECV-304 thus supporting the hypothesis that it is the endosomal membrane disruptive properties of saponins that confer their augmentative property. This however may be only a partial explanation with other mechanisms such as membrane repair processes leading to massive endocytosis [58] together with other cholesterol-dependent endocytic processes possibly also contributing [59]. The importance of membrane bound cholesterol to cell permeabilisation or penetration has also been described in several different systems [60–63]. Only certain toxins or targeted toxins constructed with these are augmented by SA saponins, indicating that it likely that an association between a given saponin species and the toxin is necessary to achieve augmentation of IT cytotoxicity.

The cholesterol content of the endosomal limiting membrane progressively decreases as these vesicles mature along the endolysosomal pathway with late endosomes containing approximately half the content of the PM [64] and lysosomal membranes even less [48]. The mechanism of cholesterol redistribution between different intracellular membranes has not been clearly defined. Late endosomal membranes rich in lyso bis phosphatidic acid (LBPA) are important regulators of cholesterol transport [65]. Two proteins Niemann-Pick C1 (NPC1) and NPC2, found in multi vesicular late endosomes, also appear to be crucial for moving cholesterol out of the endosomal system. Cheruku et al. [66] investigated the rate of cholesterol transfer from NPC2 to model phospholipid membranes and found that cholesterol transfer was most rapid at acidic pH and that the presence of LBPA in the membrane dramatically increased the rate of transfer. It is feasible that the lower cholesterol content of endosomal membranes combined with their lower luminal pH environment plays an important role in the way with which SA saponins interact with the bilayer that in some way confers the augmentative property. In this respect studies investigating the action of saponins towards large unilamellar vesicles led to the proposal of a curvature driven permeabilisation mechanism dependent on the interaction of saponins α and δ hederin with sterols [67]. Cholesterol plays a vital role in membrane structure and interaction of SA saponins with cholesterol may induce curvature which consequently brings saponin into closer physical proximity to saporin. Less cholesterol in the membrane could result in less membrane curvature and saponin may no longer be correctly orientated for interaction with saporin thus reducing the augmentative effect of SA.

The levels of fatty acids, phospholipids and cholesterol are increased and actively biosynthesized in cancer cells and tumours [68]. This may make cancer cells more sensitive to treatment that uses SA to augment immunotoxin cytotoxicity. However, lipid metabolism in cancer cells is not well characterised. We do not know how chronic lymphocytic leukaemia and acute lymphoblastic leukaemia cells will respond in terms of PM cholesterol levels after aggressive chemotherapy treatments

and there may be additional changes to cellular cholesterol levels that alter their susceptibility to SA augmentation of immunotoxin based treatments. Our present study has not established the cholesterol content of the various endolysosomal vesicles and further investigation of this is likely to shed further light on the mechanism by which saponins augment IT cytotoxicity and provide insights into the way that this might be exploited to deliver clinical benefits.

Conflict of interest

The authors have no conflicts of interest to declare.

Author contributions

DJF conceived and coordinated the study. WSS and DJF wrote the paper. WSS designed, performed and analysed the experiments shown in Figs. 2, 4, 5, 6 and 8. EB designed, performed and analysed the experiment in Fig. 3. EB and WSS designed, performed and analysed the experiment in Fig. 7. SEH helped with the conception of the study. GK contributed to the design and results analysis of the MS experiments. ANH provided assistance with MS and contributed to the discussion. DAJ contributed to the confocal microscopy experiment design, microscope operation and analysis. SUF assisted with the coordination of the study and helped prepare the figures. All authors approved the final version of the manuscript.

Transparency document

The Transparency document associated with this article can be found, in online version.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbamem.2017.02.013.

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