Emerging approaches to study cell-cell interactions

Poulcharidis, D.

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Identifying membrane lipid exchange between immune cells

4.1 Introduction

The transfer of cell membrane components such as surface lipids and proteins between immune cells is an area of intense investigation.[1] Cells communicate with a wide variety of mechanisms including both exosome secretion and direct cell-cell contact.[2-4] Cellular membrane components can also be exchanged with the formation of gap junctions, nanotubes, and processes such as direct membrane component transfer and trogocytosis.[5-8] During antigen presentation, lymphocytes and antigen-presenting cells (APCs) form a rigidly structured contact; i.e. immune synapse (IS) as a mechanism of communication.[9,10] The exact mechanism for cellular membrane exchange or which components can be exchanged is still unknown. However, it has been

suggested that direct contact or close distance between the two cells plays an essential role.[11,12] Between immune cells, the membrane exchange of proteins via direct contact in CD8+ T cells was first described by Hudrisier *et al.* and later by others.[13–18] Recently, Daubeuf *et al.* have described the ability of T cells to "steal" components from the membranes of target cells.[1,19] However, the role of specific lipids in this exchange – as well as the rate at which non-proteins are exchanged – remains unknown, as does the directionality of the approach.

Chapter 3 described how forced cell-to-cell contacts between non-exchanging cells can result in the exchange of lipids and glycans.[20] It was hypothesized that the synaptic contact between a T cell is a natural variant of such a forced contact. The cells often remain in very close proximity for more than 3 hours with the contact surface between the two cells not being atypical.[21–23] As such, it was postulated that synapse formation could also enhance membrane exchange. This hypothesis was further fuelled by the observations that T cells are in serious need of nutrients and lipids to ensure their proper activation.

To date this exchange of membrane lipids along the immunological synapse has not been reported on. In the past lipophilic fluorescent chemical tracers have been used between cells, suggesting membrane compound exchanges. However, a possible complication with these dyes is the fact that they show a severe fluorescent loss due to modifications. Additionally, many different experimental steps, such as fixation, dye concertation or cell concentration, might affect the final fluorescent intensity of lipophilic dyes.

Bioorthogonal chemistry can be used instead to visualise non-genetically templated biomolecules in cells incorporating a small

biologically inert chemical group into a biomolecule class of choice and visualising these at the end of an experiment using tag-selective ligation chemistry.[24–26] The benefit of this approach is that the small and stable bioorthogonal groups can be incorporated into non-templated molecules, and they can hijack the biosynthetic pathways of these molecules. This approach has been used extensively to label many different cell biomolecules, such as glycans, lipids and nucleotides.

The exchange of plasma membrane components has been used as the fundamentals in assays for trogocytosis analysis protocols (TRAP) for detecting fluorescent components such as proteins, lipids or glycoconjugates.[27–30] In this chapter an adaptable strategy based on TRAP assays is presented, in which fluorescent and clickable lipids were used to determine the membrane component exchange between APC and T cells in co-culture. A range of sterols and aliphatic acids were screened in an attempt to learn more about the effect of their biochemical characteristics and structure on their behaviour in trogocytosis tests, and possibly to shed light on the still unanswered question of the cellular and molecular mechanisms of membrane component exchange between immune cells.

4.2 Results and discussion

This work aimed to develop a methodology that would allow the facile quantification of the exchange of membrane components between immune cells. Chapter 3 described the optimisation of the kinetic study of cholesterol exchange between mammalian cells using flow cytometry.[20] The occurrence of membrane compound exchange can be demonstrated by labelling the membrane of APCs using modified lipids or fluorescent sterols and observing the existence of these probes onto the target cells; i.e. lymphocytes after their co-culture. Membrane

component exchange was calculated based on the fluorescent intensity of the acceptor cell upon co-culture with donor cells.

The particular lipids were selected based on their ability to be incorporated in cell membranes, and for their structure or biochemical properties to not be affected by the presence or absence of a fluorophore. The studies outlined previously revealed a bodipy-cholesterol (TopFluor®[31]) and cholesterol-alkyne[32] analogue which lead to very efficient incorporation of the sterols into live cells. It was found that between mammalian cells, membrane sterol of the plasma membrane tend to exchange in different rates between cells upon direct contact.[20] Therefore, the tendency of sterols or palmitate lipid to efficiently incorporate into immune cells such as bone marrow derived dendritic cells (BMDCs) and T cells was examined. Indeed it was found that certain lipids have a much stronger tendency to exchange between cells than others.

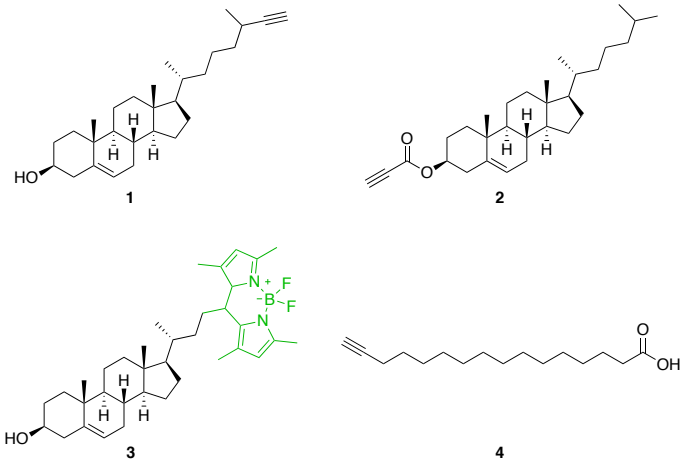

Figure 1 *Library of different lipids used: (1) Alkyne-cholesterol, cholesterol-click, Avanti Polar Lipids; (2) cholesterol propiolic acid, O-Click; (3) TopFluor® Cholesterol, bodipy-cholesterol, Avanti Polar Lipids; (4) palmitic acid (15-yne), palmitic-click, Avanti Polar Lipids. Cells were incubated for 24 h with 10 uM of each lipid.*

Initially a possible exchange of membrane lipids (Figure 1) between these cells as a result of their close direct contact was investigated. To study the lipid exchange between live cells, either naïve T cells (splenocytes) were labelled with clickable or fluorescent lipids and co-cultured with BMDC cultures or vice versa for 48 hours. Azides and alkynes are the archetypal bioorthogonal group due to their absence and small size respectively in biological systems.[33] The former can be modified using different bioorthogonal chemical reactions such as the copper-(I)-catalysed cycloaddition with terminal alkynes (CuAAC) with the smallest available modification highly suitable for lipid modification.[34–36] Flow cytometry of the population of T cells co-cultured for 48 hours with labelled-BMDC showed that T cells acquired membrane sterol from BMDC. Strikingly, this experiment showed a potent lipid-type dependence of exchange between them.

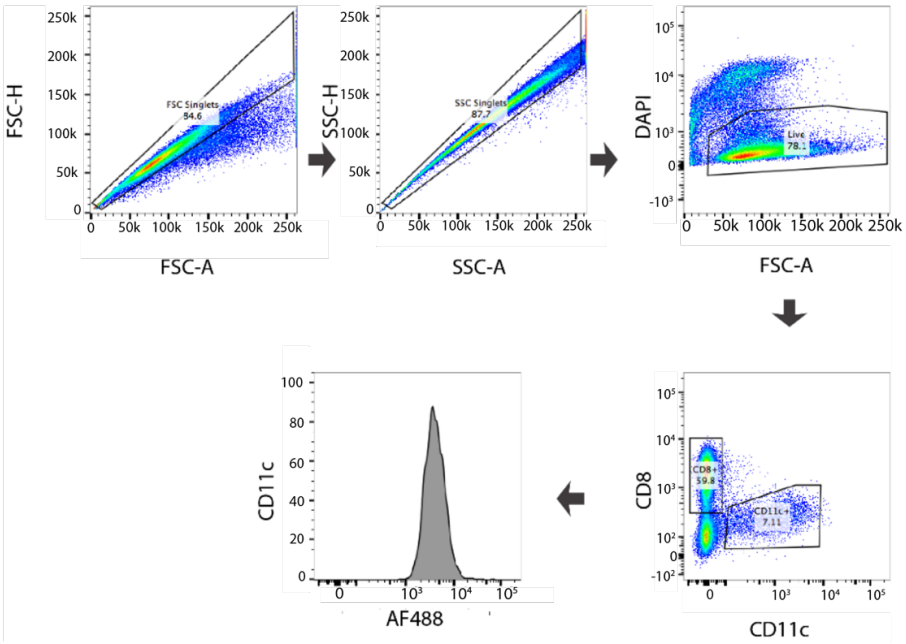

Figure 2 *The gating strategy used for the analysis of bone marrow derived dendritic cells (BMDCs) co-incubated with T-cells (splenocytes). Initially, cells were analysed for FSC-A versus FSC-H and SSC-A versus SSC-H to exclude doublet cells. The cells were then analysed with a live/dead (DAPI) staining versus FSC-A, and a gate was drawn to include all dye-negative cells. The combination of these gates served to analyse CD8 versus CD11c staining in the various samples. The mean fluorescent intensity (MFI) of the acceptor cell was measured, calculating the amount of the exchanged compounds.*

The aim of co-culturing BMDCs (acceptor) with naïve T cells (donor) was to gather evidence regarding the significance of their membrane component interactions *in vitro*. BMDCs co-cultured with T cells stimulate the generation of mature cells and influence certain stages of immune cell development (Figure 2). To determine whether cell-cell contact, soluble lipids or exosome exchange[37–39] was the predominant route of exchange, the unlabelled and labelled populations of cells were separated from one another in a trans-well assay.[20] In this system, the rates of exchange were found to be abolished when the cells were separated by a 0.4 μm membrane. The results showed that, in order for

the BMDCs to apply those interactions with T cells, direct cell-cell contact must exist between them. Fixation of the donor cell's membrane using paraformaldehyde (PFA) inhibits the transfer of membrane components between cells (Figure 3). Cells treated with unmodified bodipy-488 and co-cultured at 37 °C for 24 hours with unlabelled cells showed no exchange of fluorescence (Figure 3).

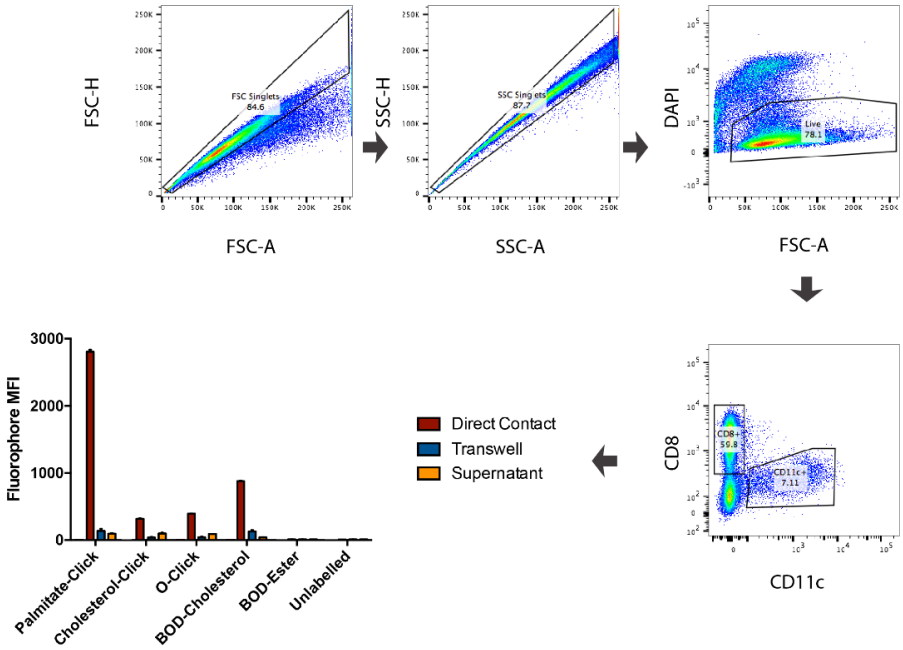

Figure 3 *Cells were analysed based on the protocol described in Figure 2. The MFI of the acceptor cell was measured, calculating the amount of the exchanged compounds. Red: direct contact between BMDC (acceptor) and CD8 (donor); Blue: transwell assay; labelled T cells were separated by a 0.4 µm membrane from unlabelled BMDC and incubated for 48 h. Flow cytometry analysis showed no lipid exchange; Yellow: supernatant exchange. T cells were incubated with different lipids: (1) Alkyne-Cholesterol, Cholesterol-Click; (2) Cholesterol propiolic acid, O-Click ;* (3) *TopFluor® Cholesterol, Avanti Polar Lipids, BOD-Cholesterol; (4) palmitic acid (15-yne), Avanti Polar Lipids, Palmitic-Click) all at final concertation 10 µM. After 1 h, cells were washed 3 times and the supernatant collected and added to an unlabelled population of splenocytes for 48 h. The absence of labelling was observed. Cells labelled with a live/dead dye, antibody surface staining (CD11c for BMDC and CD8 for T cells), and after PFA 1% fixation, with CuAAC. A control fluorophore bodipy ester at final concertation of 10 µM used. Data expressed as mean ± SEM (n=3) and is representative of 3 independent experiments. **p <0.01, unpaired t-test.*

Thereafter the previous protocol was applied in order to study whether the presence of an epitope and the subsequent activation of T cells was playing a role in the lipid exchange. Thus, OT-I transgenic T cells, which express a T-cell receptor (TCR) that recognises SIINFEKL, were co-cultured with BMDC. OT-I cells were treated with **1** (Alkyne-cholesterol, Ch-Alk) or **4** (palmitic acid (15-yne)) at final concertation of 10 μM with or without the addition of 10 nM SIINFEKL and co-cultured with BMDC for 24 hours. Fluorescence microscopy and flow cytometric analysis was used in order to confirm the importance of the epitope in the membrane exchange as well as to localise the uptaken lipids. At the end of the incubation, cells were gently removed, labelled with CuAAC and tested by flow cytometry for fluorescence increase of BMDC as a result of lipid exchange with OT-I. Lipid transfer occurred only upon the presence of the epitope SIINFEKL, indicating that the T-cell activation is vital for the membrane compound exchange. Cells were also co-cultured in a microscopy chamber plate in order to visualise the exchanged lipids. After the T cells were removed, the residual adherent BMDCs were directly examined by fluorescence microscopy to investigate the localisation of the transferred lipids from the OT-I to the BMDC (Figure 4).

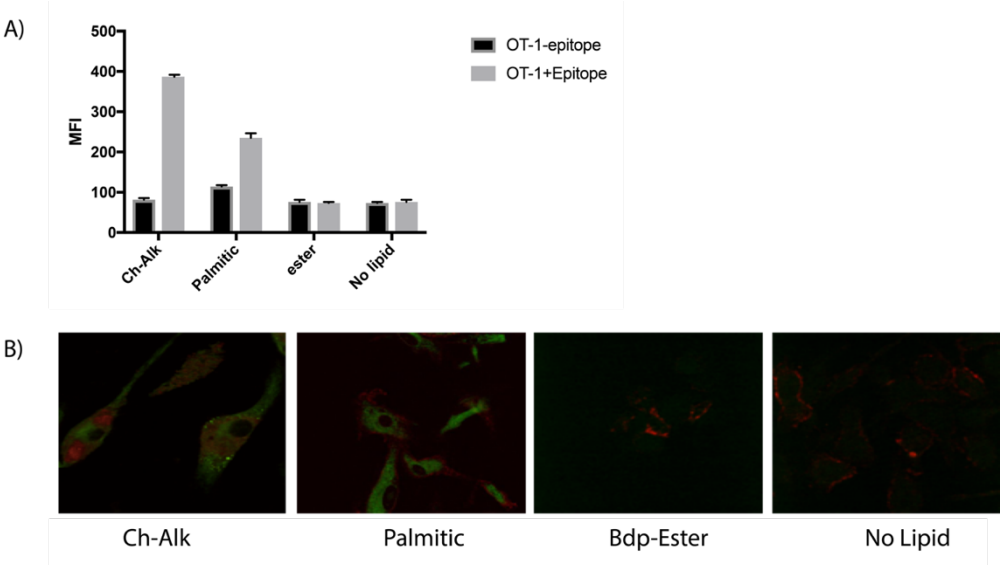

Figure 4 *Membrane transfer between OT-I and BMDC cells increased in the presence of SINFEEKL epitope. OT-I cells were labelled with different lipids (**1** Alkyne-cholesterol, Ch-Alk, Avanti Polar Lipids; **4** palmitic acid (15-yne), Avanti Polar Lipids) at final concentration of 10 μM, with or without the addition of 10nM SIINFEKL, and co-cultured with BMDC for 24 h. Cells labelled with CuAAC (click solution comprised of 1 mM CuSO4, 100 μM TTMA ligand, 2 mM sodium ascorbate and 2 μM Alexa Fluor® 488 azide). (A) MFI of unlabelled BMDC after co-culture. Increase of the fluorescence intensity indicated the lipid transfer from the OT-I cells. Data shows the MFI of BMDC cells expressing CD11C. (B) Confocal microscopy images indicate the localisation of the transferred lipids from the OT-I to the BMDC after SIINFEKL treatment. A control fluorophore bodipy ester at final concentration of 10 μM was used. Data expressed as mean ± SEM (n=3) and is representative of 3 independent experiments. **p <0.01, unpaired t-test.*

4.3 Conclusions

Previous results suggested that cell membrane lipids might not be equally transferred during cell-cell contact. However, there does appear to be a specific rate of selectivity in the classes of cell membrane components transferred from antigen-presenting cells to T cells. The precise mechanism of exchange between these cells requires further study, but likely requires the formation of a synapse after direct contact between donor and acceptor cells. It has been shown that abnormalities in T-cell development could be affected by the capture

of membrane fragments such as lipids. This study demonstrates that BMDCs can perform capture of membrane lipids. These findings could lead to alternative approaches to the exchange of membrane lipids in immune cells, focusing on manipulating the targeting lipids for restricting undesirable processes, in order to understand immunotherapies.

4.4 Experimental methods

Reagents

Cholesterol and all other chemical reagents were purchased at the highest grade available from Sigma-Aldrich and used without further purification. All solvents were purchased from Biosolve. Phosphate buffered saline (PBS): 5 mM KH_2PO_4, 15 mM K_2HPO_4, 150 mM NaCl, pH 7.4. Silica gel column chromatography was performed using silica gel grade 40-63 μm (Merck). TLC analysis was performed using aluminium-backed silica gel TLC plates (60_F 254, Merck), visualisation by UV absorption at 254 nm and/or staining with $KMnO_4$ solution. NMR spectra (^1H and ^{13}C) were measured on a Bruker AV-400MHz spectrometer at ambient temperature at the Leiden Institute of Chemistry NMR facility. Electrospray LC-MS analysis was performed on a PE SCIEX: API 3000 LC/MS/MS system using a Gemini 3u C18 110A analytical column (5μ particle size, flow: 1.0 ml/mins), on which the absorbance was measured at 214 and 254 nm. Solvent system for LC-MS: A: 100% water, B: 100% acetonitrile, C: 1% TFA (aq). TFA (aq). Fluorophore Alexa Fluor® 488 Azide was purchased from Thermo Fisher Scientific. Compounds 1 (Alkyne-Cholesterol, Cat No: 700146, CAS 1631985-09-5, Avanti Polar Lipids), 3 (TopFluor® Cholesterol, Cat No: 810255, CAS: 878557-19, Avanti Polar Lipids) and 4 (Palmitic acid (15-yne), Cat No: 900400, CAS: 99208-90-9, Avanti Polar Lipids) were

commercially available. Compound 2 was prepared in full accordance with the reported procedure.[40] Samples were imaged with a Leica TCS SP8 confocal microscope (63x oil lens, N.A.=1.4).

Flow cytometry[41]

Flow cytometry assays were performed using the Merck Guava® easyCyte 12HT Benchtop Flow Cytometer and all flow cytometry data was analysed using FlowJo™ v10.1 (FlowJo, LLC). Counting and characterization was performed by measuring 10,000 events in triplicate and concatenation of this data. For manual gating, the outermost ring of the dot plot was selected. Quadrants were manually selected to illustrate fluorescence plots.

Co-culturing BMDC with naïve T cells

All mice were bred and maintained under specific pathogen-free conditions under protocols approved by the Animal Welfare Committee of the Max Planck Institute of Immunobiology and Epigenetics, Freiburg, Germany. Mice used in all *in vitro* and *in vivo* experiments were 6–10 weeks of age and were age/sex matched.

Bone marrow derived dendritic cells (BMDC) were generated from 6 mice bone marrow. Bone marrow was flushed from femurs and tibia and cells were cultured in a complete RPMI supplemented with 8% heat-inactivated foetal calf serum, 20 μM 2 Mercaptoethanol, 2 mM glutamax, 50μg/mL streptomycin and 100 l.U./mL penicillin and in the presence of 20 ng/mL GM-CSF (Peprotech, cat #315-03-250). Medium was replaced on day 3 of culture and the cells were used between day 6 and 7.

Spleens were isolated from the same mice and single-cell suspensions prepared by filtering through a 70 μm filter, followed by red blood cell lysis (RBC Lysis Solution, Qiagen). For the T-cell stimulation, 5 ug/mL aCD3 (BioXCell, clone 17A2, BE0002) in PBS were used. Thereafter aCD28 was added, at 0.5 ug/mL(BioXCell, clone 37.51, product number BE0291). Human IL-2 was used at a final concentration of 100 U/mL (Peprotech, cat #200-02-1000).

Splenocytes were then incubated with lipids **1**, **2**, **3** and **4** (final concentration 10 μM) for 24 hours. The cells were washed and co-cultured with BMDC for 48 hours, before being collected and labelled with a live/dead dye, antibody surface staining (CD11c for BMDC and CD8 for T cells), and after PFA 1% fixation, with CuAAC (for the cells treated with lipids **1**, **2** and **4**). The click solution comprised of 1 mM CuSO4, 100 μM TTMA [(Tris((1-((O-ethyl)carboxymethyl)-(1,2,3-triazol-4-yl))methyl)amine] ligand, 2 mM sodium ascorbate and 2 μM Alexa Fluor® 488 azide (Invitrogen). After 20 minutes, the cells were washed three times with PBS, prior to incubation with 3% BSA (bovine serum albumin) for 30 minutes to remove unreacted fluorophore. The cells were then washed and flow-cytometry was performed.

Co-culturing BMDC with OT-I T cells (SINFEEKL)

Immature dendritic cells were obtained from the bone marrow of 12-week-old C57BL/6 mice under specific pathogen-free conditions. The mice were euthanized by cervical dislocation; bone marrow of tibiae and femurs was flushed out and washed with PBS. Cells were grown in dendritic cell selection medium (IMDM containing granulocyte-macrophage colony stimulating factor (GM-CSF) (2:1 v/v) containing 8% FCS, penicillin/streptomycin (100 units/mL), glutamax (2 mM) and β-mercaptoethanol (20 μM). The cells were incubated in non-

adhesive petri dishes at 37 °C, 5% CO_2, under humidified air. Cells were selected for 10 days (37 °C; 5% CO2) and subcultured every two to three days before use in the assays.

OT-I T cells were provided by M. Camps (Leiden University Medical Centre) from OT-I/CD45.1 mice spleen. OT-I cells were labelled with different lipids (**1** Alkyne-Cholesterol, Ch-Alk, Avanti Polar Lipids; **4** palmitic acid (15-yne), Avanti Polar Lipids at final concentration 10 μM) for 24 hours with or without the addition of 10 nM SIINFEKL (kindly provided by Dr. Joanna B. Pawlak). Cells were washed and then co-cultured with BMDC for 48 hours. Thereafter cells were collected and labelled with an antibody surface staining (CD11c for BMDC and CD8 for T cells), and after PFA 1% fixation, with CuAAC (for the cells treated with lipids **1**, **2** and **4**). The click solution comprised of 1 mM CuSO4, 100 μM TTMA [(Tris((1-((O-ethyl)carboxymethyl)-(1,2,3-triazol-4-yl))methyl)amine] ligand, 2 mM sodium ascorbate and 2 μM Alexa Fluor® 488 azide (Invitrogen). After 20 minutes, the cells were washed three times with PBS, prior to incubation with 3% BSA for 30 minutes to remove unreacted fluorophore. The cells were then washed and flow cytometry and confocal microscopy performed.

Transwell assay

Donor T cells were prevented from being in direct contact with BMDC cells using a transwell 0.4 μm-pore membrane (Costar®). Splenocytes were seeded in a 6-well plate with full RPMI media with the addition of lipids **1**, **2**, **3** and **4** and cells incubated for 24 hours at 37 °C. T cells were washed and then re-suspended in DMEM media. Treated T cells (in 0.3 mL of medium) were added in the upper compartment (done in 6-well plates) and unlabelled BMDC cells (in 0.5 mL of medium)

placed in the lower chamber separated from targets. The inserts were then picked up using gloves and transferred onto the top of the unlabelled BMDC cell culture with the addition of 2 ml of fresh media into the inserts. The cells were incubated for 48 hours at 37 °C. Cells were then collected and labelled with a live/dead dye, antibody surface staining (CD11c for BMDC and CD8 for T cells), and after PFA 1% fixation, with CuAAC (for the cells treated with lipids **1**, **2** and **4**). The click solution comprised of 1 mM CuSO4, 100 μM TTMA [(Tris((1-((O-ethyl)carboxymethyl)-(1,2,3-triazol-4-yl))methyl)amine] ligand, 2 mM sodium ascorbate and 2 μM Alexa Fluor® 488 azide (Invitrogen). After 20 minutes, the cells were washed three times with PBS, prior to incubation with 3% BSA for 30 minutes to remove unreacted fluorophore. The cells were then washed and flow-cytometry was performed.

Synthesis of cholesterol propiolic acid (O-Chol) (2)

Cholesterol propiolic acid (**2**) was synthesised in full accordance with the reported procedure.[40] A solution of DMAP (0.01 eq) and DCC (1 eq) in dichloromethane was added slowly over 30 minutes by syringe to a solution of propiolic acid (1 eq) and a cholesterol (1.1 eq) in dichloromethane at 0 °C to give a dark reddish suspension. The mixture was allowed to stir at room temperature until the acid was consumed (determined by TLC). Upon completion, the mixture was filtered through a layer of celite, the filtrate concentrated in vacuo. The crude product was purified by column chromatography (Rf=0.5, hexane/EtOAc 9:1) to afford **2** as a yellowish solid (40%). 1H NMR (300 MHz, $CDCl_3$): d=5.4 (d, 1H), 4.70 (dd, 1H), 2.80 (s, 1H), 2.40 (dd, 2H), 2.00–0.80 (m, 38H), 0.7 ppm (s, 3H); LC-MS (ESI): m/z calcd for $C_{30}H_{46}O_2$: 439.35 [M+H]+; found: 439.36.

4.5 References

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