Supplemental Table S1: Abundance of lipid species in uninfected *vs* HCMV infected HFF cells, related to the Figure 7.

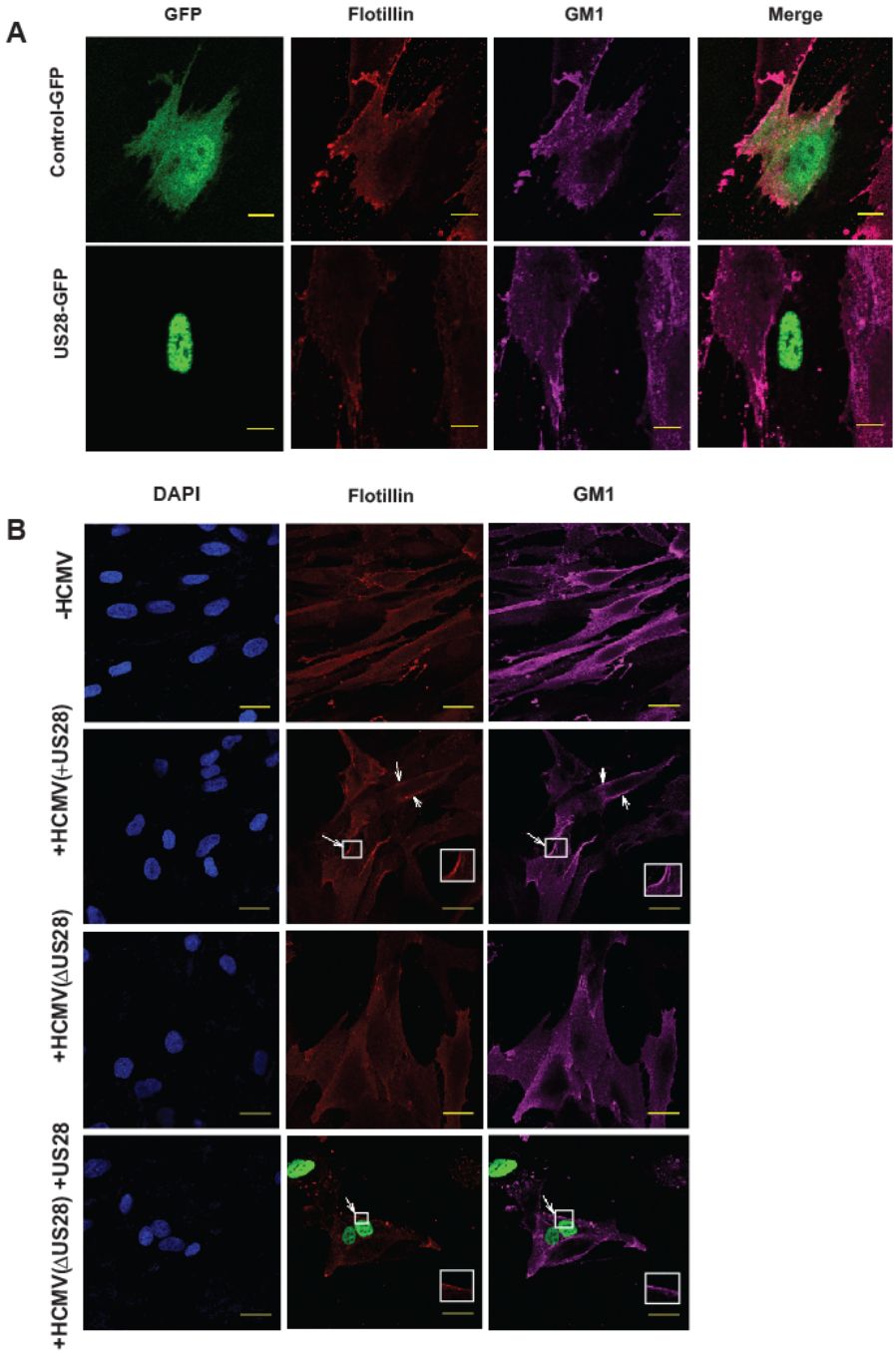
Abbreviations: CE, cholesteryl ester; Cer, ceramide; COH, cholesterol; DHC, dihexosylceramide; GM3, G_{M3} ganglioside; LPC, lysophosphatidylcholine; LPC(O), alkyllysophosphatidylcholine; LPE, lysophosphatidylethanolamine; LPI, lysophosphatidylinositol; MHC, monohexosylceramide; PC, phosphatidylcholine; PC(O), alkylphosphatidylcholine; PC(P), alkenylphosphatidylcholine; PE, phosphatidylethanolamine; PE(O), alkylphosphatidylethanolamine; PE(P), alkenylphosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine; SM, sphingomyelin; THC, trihexosylceramide

Means ± SD are shown. Concentrations are given in pmol/mg

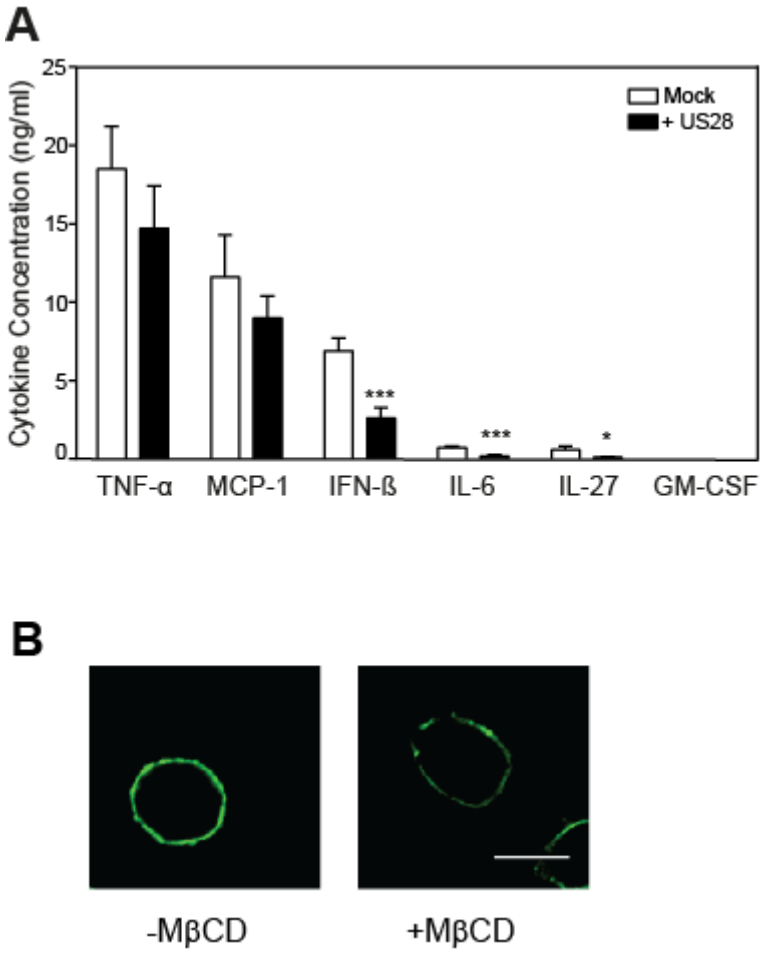
* $p < 0.05$ *vs* Control; ** $p < 0.01$ *vs* Control; *** $p < 0.001$ *vs* Control

† Statistical significance analysed using Student's *t*-test. When results were corrected for multiple comparisons using the Benjamini-Hochberg method, there was no significant difference between Control *versus* +HCMV.

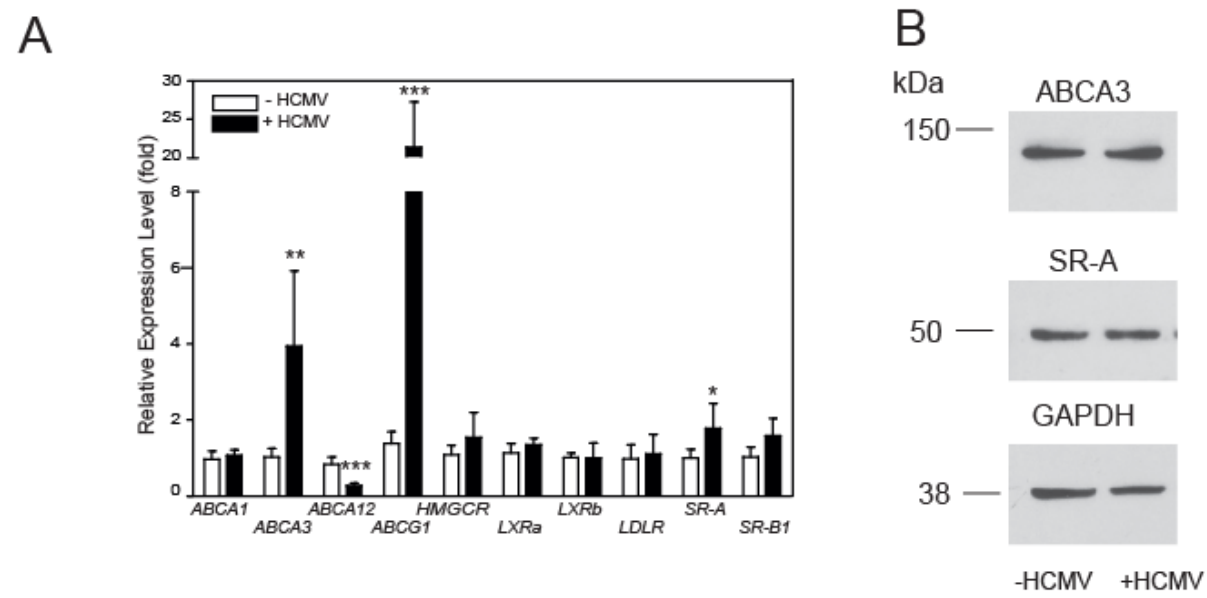
Table is presented separately as an Excel file.

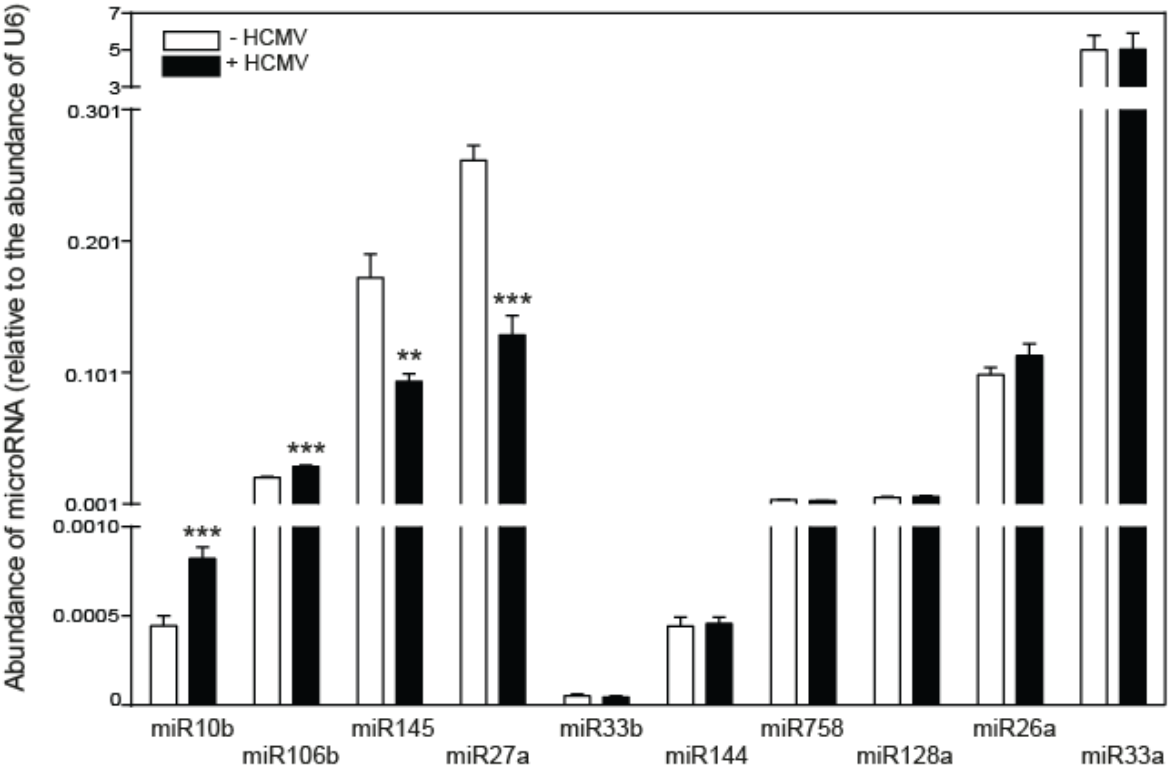


Supplemental Figure S1, related to the Figure 5: A – The effect of transfection of fibroblasts with mock-GFP or US28-tGFP on abundance and co-localization of flotillin and GM1; B - Abundance and distribution of flotillin and GM1 in uninfected cells, cells infected with +US28HCMV, ΔUS28HCMV or infected with ΔUS28HCMV and transfected with US28-tGFP.

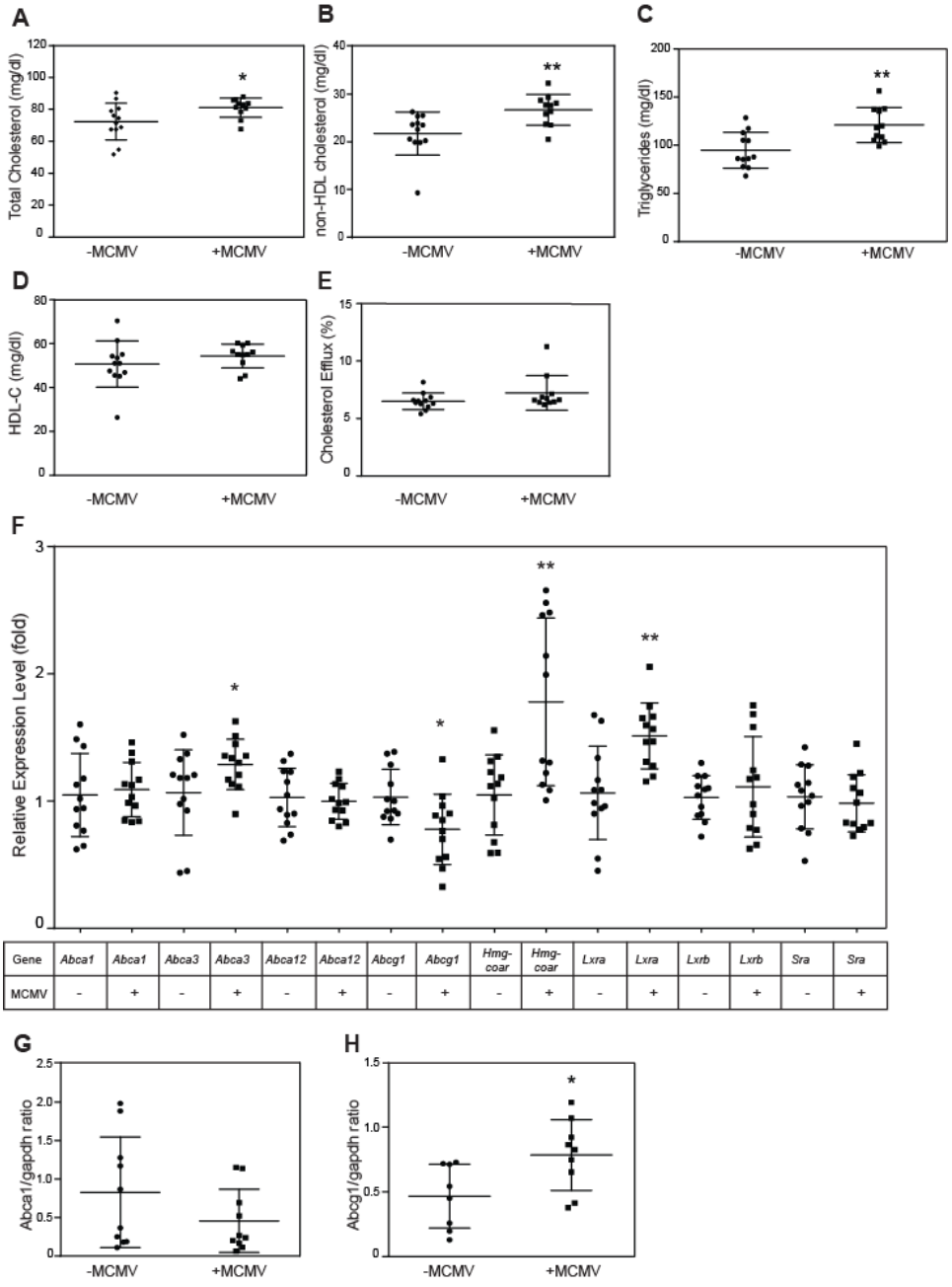


Supplemental Figure S2, related to the Figure 6: A – The effects of transfection with US28 on cytokine secretion from unstimulated RAW 264.7 macrophages. Concentration of cytokines in the conditioned medium after incubation with unstimulated cells for 24h are shown (Means ± SD, n=4). Concentrations of IL-23, IL-1α, INF-γ, IL-12p70, IL-1β, IL-10 and IL-17α were below the detection level. *$p < 0.05$; *** $p < 0.001$ (*versus* mock-transfected cells). B –Analysis of the effect of MβCD on raft abundance in RAW 264.7 cells with confocal microscopy (staining with CT-B for GM1).



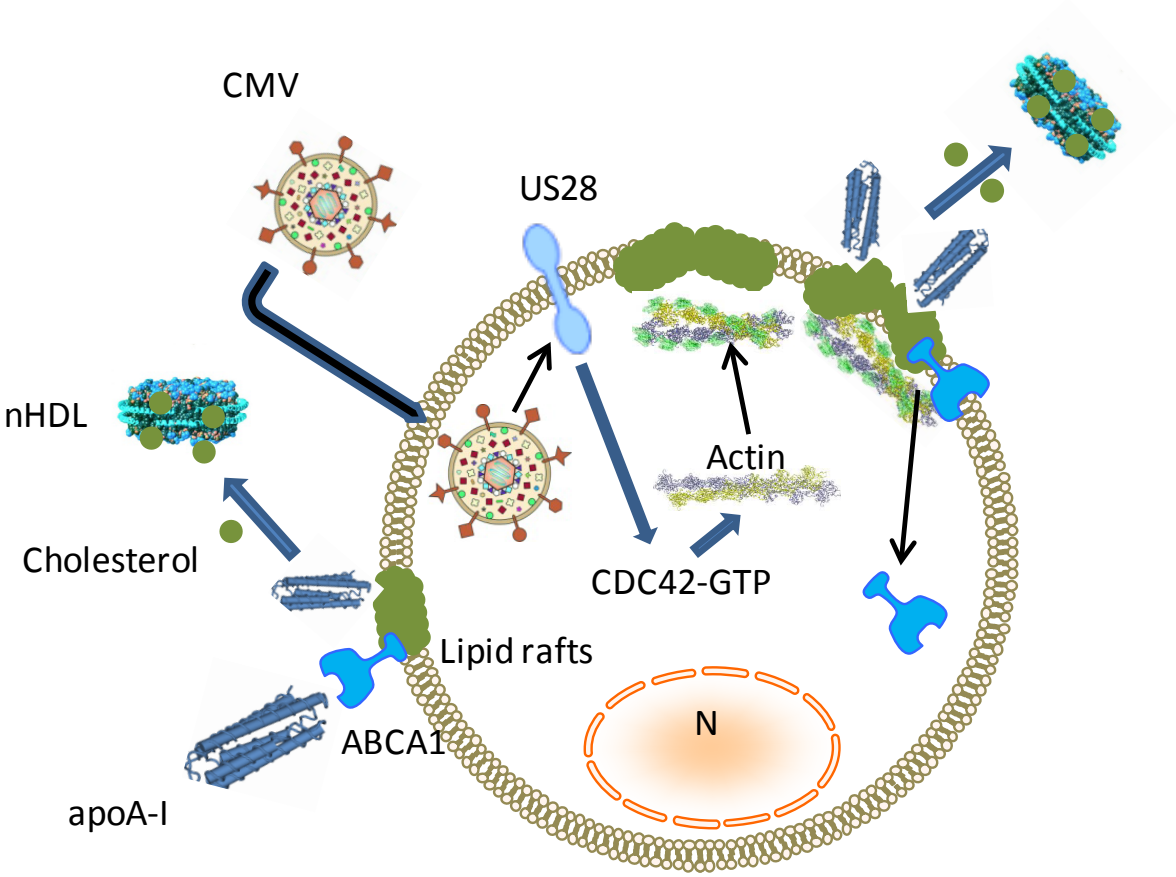


Supplemental Figure S3, related to the Figure 7: A –the effect of HCMV infection (48h) on the expression of genes related to cholesterol metabolism; **B** - The effect of CMV infection on the abundance of ABCA3 and SR-A; **C** – The effect of CMV infection on abundance of miRNAs implicated into regulation of ABCA1.



Supplemental Figure S4 – Animal studies, related to the text describing animal studies.

A-D - Plasma lipoprotein levels in mice infected with MCMV; **A** – total cholesterol; **B** – non-HDL-cholesterol; **C** – Triglycerides; **D** – HDL cholesterol; **E** – [^{3}H]cholesterol efflux from mouse macrophages RAW 264.7 to 1% mouse plasma; **F** – expression of genes related to cholesterol metabolism in the liver of mice infected with MCMV or controls; **G** – Abundance of Abca1 in the liver tissue of mice infected or not with MCMV; **H** – Abundance of Abcg1 in the liver tissue of mice infected with MCMV or controls. *p<0.05; **p<0.01.



Supplemental Figure S5. The proposed mechanistic model of the effect of HCMV on cellular cholesterol efflux, related to Discussion.

Under normal circumstances apoA-I binds to ABCA1 changing properties of adjacent regions of plasma membrane creating sites capable of apoA-I binding and cholesterol efflux. HCMV through US28 activates CDC42 causing remodelling of actin, its association with lipid rafts and reorganization of lipid rafts. Apparently new structures are able to bind apoA-I and support cholesterol efflux independently of ABCA1, the latter may be displaced from cell surface. See text for details.

Supplemental Experimental Procedures

Virus

The low passage Toledo strain of HCMV was used in the experiment described in this study unless indicated otherwise. This strain was originally isolated from the urine of an infant with congenital HCMV infection (Plotkin et al., 1989), and was kindly provided to us by Dr. E. Mocarski. In the experiments aimed at elucidating the role of US28, a more recently isolated low-passage HCMV strain VR1814 , which was originally recovered from a pregnant woman (Grazia Revello et al., 2001), was used as the basis for deletion of the US28 open reading frame. A bacterial artificial chromosomes (BAC) containing wild-type VR1814 strain of HCMV (FIX-BAC) in which the US28 open reading frame was replaced by a Kanr/LacZ cassette (FIX-BAC-ΔUS28) has been previously described (Stropes and Miller, 2008). Throughout the paper the strain of VR1814 containing US28 was referred to as HCMV(+US28), while the strain where US28 was deleted was referred to as HCMV(ΔUS28) . Finally, a clinical strain of HCMV (Merlin) was used in the experiments described in the figure 1F.

Cells

Human Foreskin Fibroblast cells (HFF, ATCC, Manassas, VA) were used in the experiment described in this study unless indicated otherwise. The cells were grown in DMEM (Life Technologies) supplemented with 10% FCS and used for infection experiments before they reached a 25^{th} passage. For all instances of HCMV infection, 80-90% confluent HFF cell monolayers were washed once with PBS and incubated with 10 mL of fresh DMEM/10% FCS containing a virus at MOI=1 for 1 hour at 37^{o}C in 5% CO_2. Media with virus was then removed; cells were washed three times with PBS and fresh media added. Unless indicated otherwise, the experiments were conducted 48 h after infection. In the experiments described

in Figs. 3H and 5H as well in the experiments analysing secretion of cytokines RAW 264.7 mouse macrophages (ATCC, Manassas, VA) were used; cells were cultured as described previously (Fu et al., 2013) .

US28 plasmid was amplified from a Merlin BAC pAL 1160 vector (Stanton et al., 2010) and ligated between restriction sites Sgf I and Mlu I on a pCMV6-AC-GFP vector (Origene) resulting in a pCMV6 plasmid encoding US28 fused with tGFP. Primer sequences for amplification were: Forward primer: 5'-GCGAGGCGATCGCCATGACACCGACGACGACG; Reverse primer: 5'-GCGCGACGCGTCGGTATAATTTGTGAGACGCGAC. Transfection with US28 was performed using Lipofectamine LTX Plus (Life Technologies) according to manufacturer's instructions. The amount of DNA added was 2.5 μg per 10^6 cells. Mock transfection refers to the transfection with a plasmid containing GFP without US28 using the same methodology. Tranfections resulted in on average 50% of the cells being tGFP-positive.

To silence ABCA1, 20nM $siRNA^{scram}$ or $siRNA^{ABCA1}$ (Life Technologies) were transfected into cells using Lipofectamine RNAiMAX (Life Technologies). To silence CDC42, 1nM $siRNA^{scram}$ or $siRNA^{CDC42}$ (Origene) were transfected into cells using Lipofectamine RNAiMAX (Life Technologies).

To activate expression of ABC transporters cells were incubated for 18 h with LXR agonist TO901317 (Sigma-Aldrich, final concentration 4μM). To block HCMV DNA synthesis, cells were incubated with phosphonoacetic acid (PAA; Sigma-Aldrich) 2 h post infection for 48 h at final concentration of 100μg/ml.

*Cholesterol and phospholipid efflu*x

High density lipoprotein (HDL) (1.083<d<1.21 g/L) was isolated from frozen human plasma (supplied by the Australian Red Cross) by sequential centrifugation in KBr solutions. Human apolipoprotein A-I was a kind gift from CSL Limited. Cholesterol efflux assay has been described previously (Low et al., 2012). In brief, cholesterol or phospholipids were labelled by incubation with respectively 1μCi of [^{3}H]cholesterol (American Radiolabeled Chemicals) or [^{3}H]-choline (Amersham-GE) for 48h in serum-containing medium. The incubation with [^{3}H]cholesterol was reduced to 24 h in the experiments where efflux was measured 24 h after infection. Cells were then incubated in serum-free DMEM for 4h prior to efflux and then for 2h with either purified human apoA-I (final concentration 30μg/ml) or isolated human HDL (final concentration 40μg/ml) or methyl-β-cyclodextrin (200 μg/ml) or for 4h with mouse plasma (final concentration 1%). Efflux was expressed as a percentage of cholesterol moved from cell to medium. Specific efflux is calculated as a difference between the efflux in the presence or absence of an acceptor.

To assess non-specific efflux, cells were grown on round coverslips, labelled and fixed with 1% paraformaldehyde for 20 minutes at 4^{o}C. Coverslips were then transferred into 24-well plates prior to incubation with cholesterol acceptors.

Western blot

Cultured cells or liver tissue were lysed with RIPA buffer and the lysate was subjected to SDS-PAGE, proteins were transferred to a PVDF membrane and probed. Primary antibodies used for Western Blotting analysis were monoclonal anti-ABCA1 antibody (Abcam); polyclonal anti-ABCG1 antibody (Abcam); polyclonal anti-SR-A (Novus Biologicals), polyclonal anti-ABCA3 (Novus Biologicals), monoclonal anti-tGFP antibody (Evrogen); polyclonal SR-B1 antibody (Sapphire Bioscience), monoclonal anti-GAPDH (Merck). Secondary antibodies were horseradish peroxidase-conjugated anti-IgG (anti-mouse and anti-

rabbit, GE Healthcare) and biotin-conjugated anti-IgG (anti-mouse and anti-rabbit, Merck). Horseradish peroxidase-conjugated streptavidin (Merck) was used for biotin-labelled secondary antibodies.

Cell-surface ABCA1 was assessed after biotinylation of cell-surface proteins followed by precipitation of biotinylated proteins with streptavidin Sepharose and probing with anti-ABCA1 antibody as described previously (Cui et al., 2012).

Apolipoprotein A-I binding

ApoA-I was fluorescently labelled with Alexa Fluor 350 Carboxyl succinimidyl ester (Life Technologies) according to the manufacturer's instructions. Conjugation was performed at pH 7.0 to increase specificity of Alexa Fluor 350 to the N-terminus of apoA-I. Cells were incubated with 20µg/ml fluorescent apoA-I for 2 hours at 4^{o}C in Leibovitz's L-15 media (Life Technologies). To determine the contribution of non-specific binding of fluorescent apoA-I, a separate group of cells were incubated with 20µg/ml fluorescent apoA-I in the presence of 1 mg/ml of non-fluorescent apoA-I. Cells were then harvested and apoA-I fluorescence was measured on the FLUOstar OMEGA microplate reader (BMG Labtech). Alternatively, cells were incubated as described above with Alexa Fluor 647 labelled apoA-I; live cells were observed with confocal microscopy or lysed with RIPA buffer and processed for LiCore Western blot.

Cross-linking

HFF cells seeded in 6-well plates were incubated with 60µg/ml apoA-I for 1h at 37^{o}C. DTSSP (Pierce) impermeable cross-linker was added to cells at a final concentration of 2mM for 2 hours at 4^{o}C. Quench solution (50mM Tris, 150mM NaCl, pH 7.2) was subsequently added and cells were collected in RIPA buffer for immunoprecipitation.

Polyclonal anti-apoA-I or anti-tGFP antibody was incubated with cell lysate for 18 hours at 4^{o}C. Immunoprecipitation was then performed with Protein A High-Capacity UltraLink Resin (Thermo Scientific). Incubation with resin was performed for 3 hours at 4^{o}C and the immunoprecipitate was collected in loading buffer (containing 5% β-mercaptoethanol) and incubated at room temperature for 1h. Samples were subjected to SDS-PAGE and western blot probing against ABCA1, tGFP, and apoA-I.

Lipid biosynthesis assay

Lipid biosynthesis was assessed by the incorporation of [^{3}H]acetate (Amersham-GE) into cholesterol, free fatty acids and triglycerides and phospholipids, and of [^{14}C]oleic acid (Amersham-GE) into cholesteryl esters as described previously (Fu et al., 2004). The conditions were mimicking those of the efflux experiment, i.e. 2 h incubation with the radioactive precursors in serum-free medium in the presence of 30 μg/ml of apoA-I.

Lipidomic analysis

Lipidomic analysis was performed as described previously (Weir et al., 2013). HFF were harvested 48 h after infection and resuspended in 0.5M NaCl, 20mM Tris, pH 7.0. Cell pellets were sonicated on ice and lipids were extracted using a, single phase $CHCl_3$:CH_4OH method. The analysis was performed by liquid chromatography electrospray ionization-tandem mass spectrometry (LC ESI-MS/MS) using a Agilent 1200 liquid chromatography system (Agilent Technologies), and Applied Biosystems API 4000 Q/TRAP mass spectrometer with a turbo-ion spray source (350 °C) and Analyst 1.5 and MultiQuant data systems (AB SCIEX). Lipid concentrations were calculated by relating the peak area of each species to the peak area of the corresponding internal standard (one per group).

RT-PCR

HFF cells seeded in 6-well plates were collected in cold PBS and total RNA was extracted using TRIzol (Life Technologies) according to the manufacturer's protocol. DNAse treatment of RNA was performed using DNA-*free* kit (Ambion) and cDNA was then synthesized from 2 μg of RNA with random primers using the M-MLV Reverse Transcriptase kit (Promega). Real Time PCR was performed in triplicates using Taqman Fast Advanced PCR Master mix (Applied Biosystems) with the following primers:- ABCA1(Hs01059118_m1); ABCG1 (Hs00245154_m1); NR1H3 (LXRα) (Hs00172885_m1); NR1H2 (LXRβ) (Hs01027215_g1); ABCA12 (Hs00292421_m1); ABCA3 (Hs00975530_m1); SCARB1(Hs00969821_m1); MSR1(Hs00234007_m1); LDLR(Hs00181192_m1); HMGCR (Hs00168352_m1). All Taqman primers were from Applied Biosystems. The relative amount of mRNA was calculated by using the comparative threshold cycle (ΔΔ*CT*) method and expressed as fold expression ($2^{-\Delta\Delta CT}$).

To analyse the abundance of selected miRNA, total RNAs were isolated by use of TRIzol Reagent. miRNA cDNA was generated from the total RNA using Taqman MicroRNA Reverse Transcription Kit (Applied Biosystem). The RT-PCR reaction primers for specific miRNA were purchased from Life Technologies. Real-time PCR was performed on the ABI Prism 7500 real-time PCR system (Applied Biosciences). Expression levels were normalized to U6_snRNA.

Apoptosis and necrosis

The viability of HFF cells infected with HCMV was tested using a Cell Death Detection ELISA kit (Roche). Briefly, cells were infected with HCMV for 24 or 48h and harvested. Cytoplasmic fractions from uninfected and infected cells were analysed in tandem with controls.

Analysis of cell-surface cholesterol

Abundance of cell-surface cholesterol on the plasma membrane was assessed through susceptibility to cholesterol oxidase as described previously (Cui et al., 2012). Alternatively, cells were labelled with BODIPY-cholesterol/methyl-β -cyclodextrin inclusion complex prepared according to Klein et al (Klein et al., 1995). Cells were incubated with the complex (0.17 mg/ml) at 37^{o}C for 10 min, cooled on ice for 15min, and membranes were isolated as described previously (Cui et al., 2012). Lipids were extracted from the membrane pellet and cholesterol was separated using TLC as described previously (Fu et al., 2004).

Cytokine secretion

RAW 264.7 cells were transfected with mock plasmid or with US28, stimulated or not with LPS (1 μg/ml) and conditioned medium was collected after 24-48 h incubation. Alternatively, cells treated or not with LPS were incubated with methyl-β-cyclodextrin (final concentration 1 mM) for 18 h. Concentrations of cytokines were determined using the LEGENDplex^{TM} Multi-Analyte Flow Assay kit (BioLegend) according to the manufacturer's instructions. SVEC4/VCAM-1 cells were used to assess the effect of secreted cytokines on VCAM-1 expression as described previously (D'Souza et al., 2010). Briefly, SVEC4 cells were incubated with conditioned medium from US28 transfected RAW 264.7 cells for 3h, and luciferase activity was measured.

Confocal microscopy

The abundance of lipid rafts was assessed using the Vybrant lipid rafts labelling kit (Life Technologies) according to manufacturer's instruction. Briefly, cells were seeded in an 8-well chamber (Ibidi) and transfected with pEGFP control plasmid or US28-tGFP plasmid or infected with a virus. The cells were collected by treating the cells with Accutase (Sigma) for

15 minutes at 37°C, washed and incubated with fluorescently labeled cholera toxin B (which binds to the ganglioside GM1 in the lipid rafts) for 10 minutes at 4°C. After incubation, cells were washed with PBS and then incubated with rabbit anti-cholera toxin B antibody (1/200 in PBS containing 0.5 % (w/v) BSA) for 15 minutes at 4°C. After the incubation, cells were washed and fixed with 4 % (w/v) paraformaldehyde for 15 minutes at room temperature. For flotillin staining, cells were permeabilized with 0.1 % (v/v) Triton X-100 for 10 minutes and blocked with 10 % (v/v) goat serum for 30 minutes at room temperature. After blocking, cells were incubated with rabbit anti-flotillin monoclonal antibody (Abcam), followed by Alexa 594 conjugated anti-rabbit IgG (Life Technologies) for 1 hour at room temperature. Confocal microscopy was performed using a Nikon A1r imaging system. All quantifications were performed using ImageJ software. Total fluorescence was measured and calculated according to the following formula as described by Gavet et al (Gavet and Pines, 2010): corrected total cell fluorescence (CTCF) = whole-cell signal − (number of pixels for the selected cell = surface selected × background).

MCMV infection of mice

Balb/c mice were infected intraperitoneally with 1.8x 10^6 pfu of MCMV (Smith strain) or injected with PBS with the same volume (100µl). Mice were anesthetized using Morbital at 3 weeks post infection; plasma and liver samples were collected and stored at -20^{o}C. All animal experiments were carried out in accordance with the Declaration of Helsinki and conformed to the institutional guidelines for animal care; the experimental protocol was approved by the ethical committee in Warsaw Medical University (#93/2013).

Colorimetric microassays (Sigma) were used to analyse lipoproteins in mouse plasma.

Total RNA was extracted from the liver using a standard TRIzol (Life Technologies) protocol following homogenization of the liver with gentleMACS tissue dissociator (Miltenyi Biotec) using M tubes as per manufacturer's instructions.

Statistics

All data is shown as mean ± SD unless stated otherwise. Statistical significance of the differences was assessed in GraphPad Prism software package by Student's *t*-test when data followed normal distribution or Mann-Whitney U test on ranks.

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