De novo cholesterol biosynthesis and its trafficking in LAMP-1 positive vesicles are involved in replication and spread of Marek’s disease virus

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Abstract

Marek’s disease virus (MDV) transforms CD4+ T cells and causes a deadly neoplastic disease which is associated with metabolic dysregulation leading to atherosclerosis in chickens. While MDV-infected chickens have normal serum concentrations of cholesterol, their aortic tissues were found to have elevated concentrations of free and esterified cholesterol. Here, we demonstrate that infection of chicken embryonated fibroblasts (CEFs) with the highly pathogenic MDV-RB1B increases cellular cholesterol content and upregulates the genes involved in cholesterol synthesis and cellular cholesterol homeostasis using comprehensive two-dimensional gas chromatography mass spectrometry and real-time PCR (RT-PCR), respectively. Using small pharmacological inhibitors and gene silencing, we established an association between MDV-RB1B replication and mevalonic acid, sterol and cholesterol biosynthesis and trafficking/redistribution. We propose that MDV trafficking is mediated by lysosomal associated membrane protein 1+ (LAMP-1) vesicles based on short hairpin RNA (shRNA) gene silencing and colocalization of LAMP-1, glycoprotein B (gB) of MDV and cholesterol (Filipin III) fluorescent signal intensity peaks. In conclusion, our results demonstrate that MDV hijacks cellular cholesterol biosynthesis and cholesterol trafficking to facilitate cell-to-cell spread in a LAMP-1 dependent mechanism.

Importance

MDV disrupts lipid metabolism and causes atherosclerosis in the MDV-infected chickens, however the role of cholesterol metabolism in replication and spread of MDV is unknown. The MDV-infected cells do not produce infectious cell free virus in vitro, raising the question about the mechanism involved in cell-to-cell spread of
MDV. In this report, we provide evidence that MDV replication depends on de novo cholesterol biosynthesis and uptake. Interruption of cholesterol trafficking within multivesicular bodies (MVBs) by chemical inhibitors or gene silencing reduced MDV titre and cell-to-cell spread. Lastly, we demonstrated that MDV gB colocalizes with cholesterol and LAMP-1 suggesting that the viral protein trafficking is mediated through LAMP-1 positive vesicles in association with cholesterol. These results provide new insights into the cholesterol dependence of MDV replication.

Introduction

Gallid herpesvirus 2 (GaHV-2), Marek’s disease virus (MDV), is a highly cell associated avian alphaherpesvirus, which causes deadly lymphoma and immunosuppression in chickens. Cell free virus particles are only produced by feather follicle epithelial cells and they are shed in association with skin debris into the environment which can infect chickens via their respiratory tract. Otherwise, infectious cell free particles are not produced by any other cell types in vitro or in vivo. MDV can infect many cell types; however, latency is mainly established in CD4+ T cells which are transformed into lymphoma cells (1-4). We have recently shown that MDV infection modulates cell metabolism and specifically enhances fatty acid synthesis and formation of lipid droplets (5, 6). It has been suggested that the alteration of lipid metabolism by MDV causes atherosclerosis in the infected chickens (7, 8). The influence of cholesterol synthesis in pathogenesis of Marek’s disease was confirmed in series of experiments showing that the use of a 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMG-CoA Red) inhibitor reduces...
atherosclerotic plaque area and mortality rates in MDV infected chickens (9). This study examines the mechanism involved in these in vivo observations.

Cholesterol synthesis occurs in the cytosol and endoplasmic reticulum (ER) and then the synthetized cholesterol is rapidly transported via multivesicular bodies (MVB) to other organelles including the plasma membrane. As part of de novo cholesterol biosynthesis, 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) catalyses conversion of HMG-CoA Red to mevalonic acid. Thereafter, squalene epoxidase (SqE), a rate limiting step for sterol biosynthesis, catalyzes the first oxygenation step in biosynthesis of lanosterol (10). The final stage involves conversion of lanosterol to cholesterol which is distributed via lysosomal associated membrane protein-1 (LAMP-1) positive vesicles between intracellular compartments including cell membranes (11-13). The majority of LAMP-1 is directly transported to lysosomes via trans-Golgi network (TGN), while some LAMP-1 is initially transported out to the cell membrane, and then it is internalized and delivered to lysosomes (14). It has been suggested that herpesvirus particles are transported to the extracellular space by the fusion of the plasma membrane with vesicle containing virus particles (15). MVB are suitable microenvironment for Herpes simplex virus 1 (HSV-1) replication and glycoprotein B (gB) of HSV-1 is colocalized with MVB marker LAMP-1 (16).

Interestingly, the final envelopment of varicella-zoster virus, a highly cell associated alphaherpesvirus similar to MDV, in the cytoplasm occurs at the TGN-derived vesicles (17, 18).

Very little information is available relating to the mechanism involved in transportation of MDV particles and the role of cholesterol trafficking in MDV-infected...
cells. This study examines the influence of MDV infection on cholesterol metabolism in CEFs and demonstrates that MDV replication and spread are dependent on cholesterol synthesis and uptake. Intriguingly, a key relationship between de novo cholesterol biosynthesis and MDV cell-to-cell spread was established. Interruption of trafficking from MVBs by chemical inhibitors or gene silencing reduced MDV titre and cell-to-cell spread. Lastly, we demonstrated that MDV gB was colocalized with cholesterol and LAMP-1, suggesting that the viral protein trafficking is mediated through LAMP-1 positive vesicles in association with cholesterol.

Results

Induction of de novo cholesterol biosynthesis in MDV infected CEFs

The relative levels of three metabolites (squalene, desmosterol and cholesterol), involved in the cholesterol biosynthesis, were determined in cell lysates with a total of 6 technical replicates per biological replicates obtained from the mock and MDV infected primary CEFs at 48 and 72 hpi using comprehensive two-dimensional gas chromatography mass spectrometry (GCxGC -MS). The results demonstrated that the levels of desmosterol were lower in the MDV-infected cells compared to the mock infected cells at both 48 and 72 hpi. In contrast, the cholesterol level was significantly higher in the MDV-infected cells compared to the mock infected cells at both 48 and 72 hpi (Fig.1A), suggesting that higher cellular cholesterol content may have reduced cholesterol synthesis at 48 and 72 hpi. The metabolites and the key enzymes involved in the cholesterol biosynthesis pathway are shown in Figure 1B. 3HMG-CoA Red, farnesyltransferase (FTase), SqE and 24-dehydro cholesterol reductase (DHCR24) are involved in cholesterol biosynthesis, while CYP27A1 is a gene encoding a cytochrome P450 oxidase which is involved in degradation of
cholesterol. On the other hand, ATP-binding cassette transporter 1 (ABCA1) gene, expressing the cholesterol efflux regulatory protein, is involved in cellular cholesterol homeostasis (19). Gene expression analyses of the key enzymes involved in cholesterol biosynthesis by Real Time-PCR (RT-PCR) demonstrate that HMG-CoA Red, FTase, SqE, DHCR24, CYP27A1 and ABCA1 were upregulated in the MDV-infected CEFs at 72 hpi. The upregulation of HMG-CoA Red and SqE proteins in the MDV infected CEFs were confirmed using western blot assay (Fig. 1D and 1E). The results indicate that cholesterol biosynthesis is upregulated in the MDV-infected cells.

Going forward, we present a comprehensive analysis of small pharmacological inhibitors and shRNA targeting key enzymes within the cholesterol biosynthesis pathway to elucidate the relative contribution of de novo cholesterol on MDV replication and spread. All inhibitors were titrated to determine non-toxic concentrations of lovastatin (Fig 2A), mevalonic acid (Fig 2B), BIBB 515 (Fig 2C), lonafarnib (Fig 2D) and U18666A (Fig 2E) based on confluency of the cells using light microscopy and presence of live cells (7AAD-) staining using flow cytometry. In addition, shRNA targeting knockdown (GFP+ cells) of the respective enzymes HMG-CoA Red, SqE and LAMP-1 were used to validate efficiency of small pharmacological inhibitors utilized (Fig 2F).

**MDV infectivity is dependent on de novo mevalonic acid biosynthesis**

Mevalonic acid is a key metabolite in the synthesis of cholesterol, and HMG-CoA Red, rate-controlling enzyme of the mevalonate pathway, is an essential enzyme within the first stage of the cholesterol biosynthesis (19). Here, we examined the role
of HMG-CoA Red in the replication of MDV using a small pharmacological inhibitor, lovastatin, and gene silencing by shRNA (Fig. 3A). To this end, we initially determined non-toxic concentrations of lovastatin and mevalonic acid based on confluency of the cells using light microscopy and 7AAD staining using flow cytometry. MDV titres were determined in the MDV infected CEFs in the presence of different concentrations of lovastatin. The results demonstrated that non-toxic concentrations of lovastatin (lovastatin; 2.5, 10, 25 and 100 ng/ml) reduced MDV titre in a dose dependent manner (Fig. 3B). Treatments of the cells with 2.5, 10, 25 and 100 ng/ml lovastatin reduced MDV titre in a dose dependent manner. Exogenous mevalonic acid (2.5-7 μg/ml) rescued the inhibitory effects of lovastatin (100 ng/ml) on MDV titre (Fig. 3C), while it did not have any effect on MDV titre in the absence of lovastatin (Fig. 3D). RNA silencing reduced HMG-CoA Red protein levels to non-detectable levels (Fig. 3E), and reduced MDV titre (Fig. 3F), MDV plaque sizes (Fig. 3G and 3H) and MDV copy numbers (Fig. 3I).

De novo Lanosterol synthesis supports MDV replication in CEFs

The second stage in cholesterol biosynthesis is lanosterol pathway (Fig. 1B), which preferentially utilizes mevalonic acid to generate lanosterol. In this stage, squalene is converted to lanosterol by SqE (Fig. 4A), while, FTase, a cellular enzyme essential for the prenylation of cellular factors, is involved in a posttranslational protein modification process. To examine the role of SqE and FTase in MDV replication, we utilized both small pharmacological inhibitors and gene silencing using shRNA system. Non-toxic concentrations of Lonafarnib, an inhibitor of FTase, did not alter MDV titre (Fig. 4B) or plaque sizes (Fig. 4C and 4D). In contrast, non-toxic
concentrations of BIBB 515, an inhibitor of SqE, significantly reduced MDV titre (Fig. 4E). The silencing SqE by shRNA reduced protein levels (Fig. 4F), MDV titre (Fig. 4G), plaque sizes (Fig. 4H, 4I) and MDV copy numbers (Fig. 4J). Taken together, the results demonstrated that de novo synthesis of cholesterol is important for MDV replication and spread.

**Exogenous cholesterol is also required for MDV replication in CEFs**

To determine the importance of exogenous cholesterol in MDV replication, we examined cell viability of the mock or MDV-infected CEFs in cell culture medium with different concentrations of cholesterol (ranging from 0 to 2,000 nM). High levels of cholesterol (1500 and 2000 nM) were toxic to the mock infected CEFs, while these levels of cholesterol were non-toxic for the MDV-infected CEFs as determined based on cell viability using 7AAD staining (Fig. 5A), suggesting that MDV infection alters the susceptibility of the cells to exogenous cholesterol. To further interrogate the role of exogenous cholesterol on MDV replication, we analysed MDV titre in the MDV-infected CEFs which were cultured in medium containing different concentrations of cholesterol. Lower MDV titre (Fig. 5B) and plaque sizes (Fig. 5C) were observed in the MDV infected CEFs which were cultured in cell culture medium lacking exogenous cholesterol. Furthermore, the result demonstrated that add-back of cholesterol increased MDV titre (Fig. 5B). Further, the fate of exogenous cholesterol added to infection medium was determined by adding fluorescent TopFluor cholesterol to culture media of RB1B and mock infected CEFs cells. Both RB1B and mock infected CEFs cells showed uptake of the TopFluor cholesterol from the surrounding media (Fig. 5E). Further, quantification of the MFI showed significantly higher intensity and uptake of TopFluor cholesterol preferentially in CEFs cells.
infected with RB1B (average MFI of 50.9) over mock infected (average MFI of 28.4) (Fig. 5F). Also, RB1B infected CEFs cells cultured in presence of exogenous cholesterol showed significantly higher accumulation of lipid droplets (average 15 droplets per cell) compared to RB1B infected CEFs cells cultured without exogenous cholesterol (average 7 droplets per cell) (Fig. 5G & 5H). Taken together, our data demonstrate that exogenous and de novo synthesized cholesterol are involved in the MDV replication and spread.

Modulation of cholesterol trafficking impairs MDV replication and spread

U18666A, a chemical inhibitor which inhibits late endosomes (MVBs) and cholesterol transport (Fig. 6A), was utilized to study the role of cholesterol trafficking in MDV replication and spread. The results demonstrate that inhibition of cholesterol trafficking by non-toxic concentrations of U18666a significantly reduced MDV titre in MDV titre (Fig. 6B) and plaque sizes (Fig. 6C). Exogenous cholesterol (628 nM) did not rescue the inhibitory effects of U18666A (10 ng/ml) on MDV spread (Fig. 6C and 6D). Furthermore, a significant reduction in MDV genome copy number was observed in the U18666A-treated MDV-infected CEFs compared to the vehicle treated cells (Fig. 6E).

Co-localization of Filipin III, a fluorescent probe for cholesterol, and MDV (gB of MDV) was analysed using confocal immunofluorescence microscopy. Vehicle treated the MDV-infected CEFs showed a diffused distribution of cholesterol (Fig. 6F and 6G) and the peaked relative fluorescence intensity for Filipin III was associated with gB of MDV (Fig. 6G). Mander’s coefficient for Filipin III and gB of MDV confirms colocalization of cholesterol and the MDV viral protein (Fig 6H). The results also
demonstrated that U18666A disrupted both cholesterol and gB distribution in the MDV-infected CEFs (Fig. 6G).

The relative expression level and co-localization of LAMP-1, a lysosomal marker, and the gB of MDV, were analysed in the MDV-infected CEFs using confocal microscopy. The results demonstrate that the MDV-infected CEFs have greater expressions of LAMP-1 as identified by a higher fluorescence intensity in the MDV-infected cells compared to the mock-infected CEFs. Moreover, LAMP-1 and gB of MDV are colocalized in the infected cells (Fig. 6I and 6J). Mander’s coefficient for LAMP-1 and gB of MDV confirms these observations (Fig. 6K) suggesting involvement of the MVB pathway in MDV replication. The results also demonstrate a relationship between cholesterol (Filipin III), LAMP-1 and gB in the MDV infected CEFs (Fig. 6L, 6M, 6H, 6K, 6N).

**LAMP-1 is essential for efficient replication and spread of MDV**

To corroborate the importance of LAMP-1 in MDV infection, we initially verified shRNA silencing of LAMP-1 and showed a downregulation of LAMP-1 protein in CEFs using confocal microscopy (Fig. 7A and 7B). Interestingly, shRNA silencing of LAMP-1 on the MDV-infected CEFs reduced the expression of gB in the MDV-infected CEFs, while transfection with pScramble shRNA had no effect on the gB expression in those cells (Fig. 7C and 7D). The results also demonstrate that shRNA silencing of LAMP-1 significantly reduce MDV titre, (Fig. 7E), plaque sizes (Fig. 7F and 7G) and viral copy numbers (Fig. 7H). Taken together, the results demonstrate that LAMP-1 is required for efficient replication and spread of MDV which is consistent with involvement of the MVB pathway in MDV replication.
Discussion

The salient findings of this study are that (i) MDV infection increases cholesterol content of infected cells and activates *de novo* cholesterol synthesis (ii) exogenous and de novo cholesterol biosynthesis are required for efficient MDV replication and spread (iii) MDV induces upregulation of LAMP-1, involved in cholesterol trafficking, within multivesicular bodies (MVB) (iii) glycoprotein B of MDV, an essential viral protein for replication and egress, is associated with cholesterol and LAMP-1 within MVB. Interestingly, gene silencing of LAMP-1 reduced both MDV replication and spread, highlighting the importance of LAMP-1 as a protein for transportation of gB. The results suggest that MDV hijacks cellular cholesterol biosynthesis and cholesterol trafficking to facilitate cell-to-cell spread in a LAMP-1 dependent mechanism. The results from this study could be used to design control strategies to use chemical or physiological inhibitors targeting cholesterol pathway to reduce virus replication and shedding. Alternatively, novel vaccines such as HVT vaccines expressing shRNA for silencing cholesterol pathway could be developed to reduce MDV replication *in vivo*.

MDV is a highly cell associated *alphaherpesvirus* which infects chickens and causes a deadly lymphoma and immunosuppression (1, 2, 20). Although MDV infection disturbs lipid metabolism and causes atherosclerosis (7-9), very little is known about the role of MDV-induced cholesterol metabolism in MDV replication and spread. MDV can infect various cell types, however it only transforms CD4+ T cells (1, 21). *In vitro*, MDV infection of lymphocytes occurs only after activation of B and T cells (22) which can mask the metabolic alteration under MDV infection (2, 23). Therefore, here we analysed the role of cholesterol in MDV infection in CEFs, which can be
infected with inoculum without any external activation. CEFs contain various cell populations including immune cells and fibroblasts which are used for propagation of MDV. The exact proportion of immune cells within CEFs is unknown as there are no specific antibodies to detect all different types of chicken immune cells, and many immune cells might be in their progenitor stage and do not express specific markers. Based on our earlier observation, there are only very small percentages of MHC class II positive or KULO1 positive representing macrophages and CD3+ cells representing T cells within CEFs suggesting that the majority of infected cells within CEFs are not macrophages or T cells. Therefore, it is not possible to determine the impact of immune cell infection within CEFs in the results observed in this study. Inhibition of cholesterol pathway reduces MDV replication *in vivo* and thus we believe that cholesterol pathway is involved in MDV replication even in the presence of immune system. However, further research is required to determine the role of cholesterol pathway in MDV replication in immune cells. At 72 hpi, in our MDV infection culture model, over 80% of CEFs were infected and induce maximum virus infection. At later time points, cell detachment and death occurred which can affect the cell metabolism and mask the effects of MDV infection on cholesterol biosynthesis. Thus, cholesterol biosynthesis was analysed in the samples at 48 hours and 72 hpi. We have recently observed that MDV infection increases formation of lipid droplets (5), which play an important role in sustaining the cellular cholesterol level by maintaining lipid storage, hydrolysis, and trafficking. Biosynthesis, efflux, and influx of cholesterol regulate the levels of cholesterol in cells, and together with cholesterol distribution and dynamics regulate cholesterol's function. Studies have revealed that depletion of cholesterol can inhibit herpes simplex virus entry by inhibiting cell
binding and/or changing the expression levels of receptor on the cell surfaces (24). Moreover, cholesterol and lipid rafts can also be involved in virus replication by influencing the intracellular membrane structures (25). MDV is a highly cell associated virus and the role of cholesterol in MDV cell-to-cell spread and replication needs to be further explored. This study demonstrated that genes involved in cholesterol synthesis and trafficking are upregulated and levels of cholesterol content in the MDV infected cells are increased suggesting that MDV infection increases cholesterol biosynthesis. Interestingly, MDV infected cells became resistant to cytotoxicity which is generally exerted by excessive exogenous cholesterol. Cell apoptosis is triggered by enrichment of free cholesterol in ER (26), which play a major role in protein homeostasis and the cholesterol production. It is possible that the usage and trafficking of free cholesterol remove free cholesterol from ER in the MDV infected cells. Our confocal microscopy data demonstrating colocalization of cholesterol and gB of MDV within LAMP-1 MVB support this notion. To our knowledge, this is the first report confirming the crucial role of cholesterol synthesis and trafficking in MDV replication and spread.

LAMP-1 shuttles between lysosomes, endosomes, and the plasma membrane (27) and binds to cholesterol in association with Niemann-Pick disease, type C1 (NPC1) and NPC2 proteins that export cholesterol from lysosomes (28). Our results demonstrated that MDV infection enhanced expression of LAMP-1, a marker for MVB compartment, reflecting an augmentation and involvement of the MVB pathway in MDV replication. The mechanism involved in the upregulation of LAMP-1 during MDV infection is unknown, and further work is required to establish any possible association between cholesterol synthesis and LAMP-1 expression in the MDV.
infected cells. Confocal microscopy showed colocalization of gB with cholesterol and LAMP-1 within MVB membranes. MDV encoded gB is one of the essential MDV glycoproteins, which is conserved in most herpesviruses, and here we show that gB may be sorted to the MVB compartment and confocal microscopy showed that gB was colocalized in part with the MVB membranes, which therefore represent a site of gB accumulation. Herpesvirus glycoproteins play an important role in the assembly and the production of infectious particles, and gB of herpesviruses recirculates between the plasma membrane and MVB (16, 29, 30). RNA interference with LAMP-1 reduced MDV titre and plaque sizes, highlighting the importance of LAMP-1 and MVB in MDV replication and cell-to-cell spread. It has been suggested that avian LAMP-1 is involved in degradation of avian reovirus non-structural p10 protein, involved in the induction of cell syncytium formation and apoptosis, through interacting with both p10 and the E3 ligase Siah-1 (31, 32). We are currently examining whether LAMP-1 is also involved in the degradation of MDV viral proteins, which are required for the generation of infectious cell free MDV. Feather follicle epithelial cells are the only cells capable of producing infectious cell free MDV in vivo, and we have recently generated feather follicle epithelial cell lines which will be used to differentially examine the role of LAMP-1 in degradation of MDV proteins. In conclusion, the results suggest that MDV activates LAMP-1 expression and hijacks cellular cholesterol biosynthesis and cholesterol trafficking to facilitate MDV trafficking in a LAMP-1 dependent mechanism.

Materials and Methods

Ethics Statement
Ten-day old mixed sex SPF embryonated chicken eggs which were purchased from Valo (Valo Biomedia GmbH) were used to generate primary CEFs. All embryonated chicken eggs were handled in strict accordance with the guidance and regulations of European and United Kingdom Home Office regulations under project licence number 30/3169. As part of this process the work has undergone scrutiny and approval by the ethics committee at The Pirbright Institute.

**CEFs culture and virus preparations**

CEFs were generated from mixed sex SPF Valo eggs (Valo Biomedia GmbH) incubated in a Brinsea Ova-Easy 190 incubator at 37°C until 10 days in ovo. CEFs were seeded at a rate of 1.5 x 10^5 cells /ml in 24 well plates with growth medium (E199 supplemented with 10% TBP, 5% FCS, 2.8% SQ water, amphotericin B (0.01%), Penicillin (10 U/ml) and Streptomycin (10 µg/ml)) and incubated overnight (38.5°C at 5% CO₂). Next day, 80% confluent monolayers were observed, and growth medium was replaced with maintenance medium (E199 supplemented with 10% TBP, 2.5% FCS, 3.5 % SQ water, amphotericin B (0.01%), Penicillin (10 U/ml) and Streptomycin (10 µg/ml).

**Reagents and antibodies**

*Chemicals:* Lovastatin (stock; 2.5 mg/ml), BIBB 515 (stock; 2.5 mg/ml), Lonafarnib (stock; 1 mg/ml), (Cambridge Bioscience, Cambridge, UK), U18666A (stock; 5 mg/ml) (Sigma-Aldrich/Merck, Dorset, UK) were reconstituted in DMSO. Mevalonic acid lithium salt (stock; 10 mg/ml)and Cholesterol-water soluble (stock; 300 mg/ml) (Sigma-Aldrich/Merck, Dorset, UK) were reconstituted in E199 medium. Filipin III was used as part of a kit and reconstituted in Cholesterol Detection Assay Buffer.
prior to use (Abcam, Cambridge, UK). TopFluor® cholesterol (Sigma-Aldrich/Merck, Dorset, UK) were reconstituted in ethanol. HCS LipidTOX™ (Thermo Fisher Scientific, Paisley, UK) were reconstituted in DMSO.

**Antibodies:** primary antibodies used were; mouse monoclonal antibody (mAB) to chicken α-tubulin, rabbit monoclonal antibody (mAB; IgG) to HMG-CoA Reductase (Cambridge Bioscience, Cambridge, UK), rabbit polyclonal antibody (pAB) to Squalene Epoxidase (Sigma-Aldrich/Merck, Dorset, UK). The LEP100 hybridoma (anti-LAMP-1; mouse IgG1 isotype) was obtained through the Developmental Studies Hybridoma Bank (DSHB; university of Iowa, Iowa city, USA). The HB3 hybridoma (Anti-gB; mouse IgG2b isotype) is an in house available hybridoma. Antibodies were purified in house by HPLC method. Secondary antibodies used were; goat pAB to rabbit IgG (Abcam, Cambridge, UK) goat anti-mouse IgG1-568nm and goat anti-mouse IgG2b-568nm (Thermo Fisher Scientific, Paisley, UK).

**Cells and MDV Infection**

**Metabolomics:** CEFs were either mock infected or with the very virulent RB1B strain (100 pfu per 1.5 x 10⁵ cells or MOI of 0.0006) in triplicates and harvested at 48 and 72 h post infection (hpi). The cells were washed, counted and after protein quantification using Bradford assay, the samples were sent for Metabolom analysis using GCxGC-MS (Target Discovery Institute, University of Oxford) and data analysis were performed as previously described (33). In brief, the cells were homogenized using bead beater in methanol/water (1:1), and then t-butyl methyl ether was added for phase separation. The organic phase was dried under vacuum, while methanol was added to the remaining sample and mixed in bead beater. After incubation at -80°C for 1 hr, the phase separation occurred after centrifugation, and
liquid layer was collected and dried under vacuum. Methoxyamine and MSTFA (1% TMSCI) were added to the dried samples and subsequently injected for analysis by GC/GC-MS. The lipid profiles of mock and MDV-infected cells were analysed in biological triplicates with up to six technical replicates per biological replicate. The data were adjusted and normalized based on protein content. MDV infection did not change the size of the cells as determined using light microscopy.

**Viral plaque analysis:**

*Pre-treatment of cells with pharmacological inhibitors:* lovastatin (2.5, 10, 25, and 100 ng/ml), lonafarnib (1, 10, 50, and 100 ng/ml), BIBB 515 (0.5, 1, 5, and 10 ng/ml), U18666a (1, 5.0, 7.5, and 10 ng/ml), mevalonic acid (2.5, 5, and 7.5 μg/ml), and cholesterol-water soluble (100, 200, 400, 628, 1000 and 1500 nM) were added to the cell monolayer 2 hrs prior to infection with the very virulent RB1B (100 pfu per 1.5 x 10^5 cells) and were incubated (38.5°C, 5% CO2) for 72 hrs.

*Knockdown of HMG-CoA Red, SqE and LAMP-1 expression:* Small short hairpin RNAs (shRNAs), both silencing (pshRNA) and control scrambled (pScrambled) RNA sequence were designed using the siRNA wizard (Invivogen, Toulouse, France) (Table 1) and chemically synthesized (Sigma-Aldrich/Merck, Dorset, UK).

Construction of the shRNA harbouring plasmid was performed as per the manufacturer's instruction in the psiRNA-h7SKGFPzeo plasmid. The shRNAs were annealed and ligated in the BbsI digested psiRNA-h7SK-GFPzeo plasmid vector (ksirna4-gz21). The plasmid construct with shRNAs were transformed in the chemically competent GT116 E. Coli for blue white screening and zeocin selection and amplification. The positive colonies were propagated and Maxi prep were performed for plasmid preparation. For silencing purposes both psiRNA-
h7SKGFPzeo plasmids expressing pshRNA and pScrambled were lipofected (lipofectamine 2000; Thermo Fisher Scientific, Paisley, UK) in CEFs at various DNA concentrations to determine transfection efficiency and toxicity. The transfection efficiency was observed at 24 hrs post-transfection for GFP fluorescence using an inverted fluorescent microscope and the wells showing more than 80% transfection efficiency were infected with MDV.

**Viral titre:** MDV infected cells were titrated onto fresh CEFs. Cells were fixed (1:1 ice cold acetone:methanol for 5 min) and blocked with blocking buffer (PBS + 5% FCS) for 1 hr at RT. Cells were subsequently incubated with anti-gB mAb (HB-3) and then with horse radish peroxidase-conjugated rabbit polyclonal anti-mouse IgG. After development of the plaques using AEC substrate, the cells were washed with super Q water and viral plaques were counted using a light microscopy.

**Determining non-toxic concentration of the inhibitors:** To identify non-toxic concentrations of the chemicals, mock-infected and MDV-infected CEFs were exposed to the chemicals or vehicles and cell morphology and adherence/confluency were monitored under light microscopy at different time points post treatment. Moreover, CEFs were trypsinized, stained with 7-AAD (BD Bioscience, Oxford, UK) and acquired using a MACS quant flow cytometry and FloJo software for analysis of the data. Non-toxic concentrations of the inhibitors and chemicals were selected based on flow cytometry data and confluency.

**qPCR to amplify MDV genes**
DNA samples were isolated from $5 \times 10^6$ cells using the DNeasy-96 kit (Qiagen, Manchester, UK), according to the manufacturer’s instructions. A master-mix was prepared: primers Meq-FP and Meq-RP (0.4 μM), Meq probes (0.2 μM), ovo forward and reverse primers (0.4 μM), and ovo probe (0.2 μM, 5’Yakima Yellow-3’TAMRA, Eurogentec) and ABsolute Blue® q-PCR Low Rox master-mix (Thermo Fisher Scientific, Paisley, UK). A standard curve generated for both Meq (10-fold serial dilutions prepared from plasmid construct with Meq target) and ovo gene (10-fold serial dilutions prepared from plasmid construct with ovo target) were used to normalise DNA samples and to quantify MDV genomes per $10^4$ cells. All reactions were performed in triplicates to detect both Meq and the chicken ovotransferrin (ovo) gene on an ABI7500® system (Applied Biosystems) using standard conditions. MDV genomes were normalised and reported as viral genome per $10^4$ cells.

Plaque size measurement
There were approximately 70 to 100 plaques in each well, and many plaques were fused with their adjacent plaques or were located at the edge of the wells, which made measuring their sizes very difficult. Therefore, in earlier studies, we standardized our methods and measured the sizes of all the plaques which could be measured correctly and compared that with the results from 20 plaques from each well. The data indicated that the size of 20 plaques from each well represents accurate average sizes of measurable plaques. Therefore at 72 hpi, the treated CEFs were fixed and virus plaques were visualized using AEC substrate (as listed above). Viral plaques were imaged (object lens magnification; 10X) with an inverted light microscope and the pictures were processed using Adobe Photoshop software. All viral plaques were measured using the ImageJ software area tool. (NIH, USA).
The free hand tool was used to measure the defined plaque area (μm²) based on anti-gB staining as described previously (5, 6). Data were corrected and exported as plaque size area (mm²).

**Real-Time Polymerase Chain Reaction (Real Time-PCR)**

*RNA extraction and cDNA:* Total RNA was extracted from mock and MDV infected CEFs using TRIzol (Thermo Fisher Scientific, Paisley, UK) according to the manufacturer’s protocol and the purified RNA was reverse transcribed to cDNA using Superscript® III First Strand Synthesis kit (Thermo Fisher Scientific, Paisley, UK) and oligo-dT primers according to the manufacturer’s recommended protocol. *SYBR green Real Time-PCR:* Quantitative real-time PCR using SYBR Green was performed on cDNA using the LightCycler® 480 II (Roche Diagnostics GmbH, Mannheim, GER) as previously described (6). Briefly, each reaction involved a pre-incubation at 95 °C for 5 min, followed by 40 cycles of 95°C for 20 sec, 55°C–64°C (Tₘ as per primer) for 15 s, and elongation at 72°C for 10 s. Subsequent melt curve analysis was performed by heating to 95°C for 10 sec, cooling to 65°C for 1 min, and heating to 97°C. Primers sequences and accession numbers are outlined in Table 2. Relative expression levels of all genes were calculated relative to the housekeeping gene β-actin using the LightCycler® 480 Software (Roche Diagnostics GmbH, Mannheim, GER). Data represent mean of 6 biological replicates in duplicates.

**Western blot**

Samples were lysed in a lysis buffer in the presence of protease inhibitors (Thermo Fisher Scientific, Paisley, UK). The lysates were suspended in sample loading buffer (Sigma-Aldrich/Merck, USA) and loaded in 10% SDS-PAGE. After semi-dry transfer
of SDS-PAGE, nitrocellulose membranes were blocked (5% skimmed milk powder in PBS for 2 hrs at RT). Membranes were incubated overnight (4°C) with the following primary antibodies: rabbit monoclonal antibody to chicken tubulin, rabbit monoclonal antibody to SqE and rabbit monoclonal antibody to HMG-CoA Red, in the respective blots. Next day, membranes were washed (0.5% skimmed milk powder in PBS) and incubated (5% skimmed milk powder in PBS for 2 hrs at RT) with the following secondary antibody; donkey pAB to goat IgG Red, in the respective blots. Finally, blots were probed with odyssey CLx imaging system (Li-Cor, USA), bands were quantified with image studio lite software and images were processed using Adobe Photoshop software.

**Flow Cytometry**

Viability of CEFs treated with the pharmacological inhibitors or diluent (vehicles) were determined to identify their non-toxic concentrations. Each pharmacological inhibitor was titrated and CEFs were exposed to the inhibitors for 72 hrs. 

**Viability assay:** Trypsinized CEFs were stained with 7-AAD (BD Bioscience, Oxford, UK) and acquired using a MACS quant flow cytometry and cell viability was analysed using FloJo software. Non-toxic concentrations of the inhibitors and chemicals were selected based on cell viability determined using flow cytometry data and confluency. CEFs were acquired in triplicates from 4 independent experiments using a MACS quant flow cytometry and the results were analysed using FloJo software.

**Fluorescence confocal microscopy**

At 72h post mock or infection with the RB1B virus in the presence/absence of U18666A or shRNA and pScrambled overexpressing CEFs targeting LAMP-1, the
samples were prepared for imaging. CEFs trypsinized and seeded in 24 well plates that contained 12 mm diameter round coverslips at a rate of $1.0 \times 10^5$ cells per well. In brief, mock or infected CEFs were fixed with 4% formaldehyde for 30 min at RT and washed twice with PBS. Cells were subsequently permeabilized (where indicated) with 0.1% Triton X buffer solution, blocked (1 hr in 0.5% BSA/PBS) and incubated overnight (4°C) with the anti-gB (mouse IgG2b; 1 µg/500 µl in 0.5% BSA/PBS) or anti-LAMP-1 (mouse IgG1; 1 µg/ml in 0.5% BSA/PBS). Cells were washed twice with PBS and again incubated overnight (4°C) with the anti-IgG2b-568nm (goat anti-mouse: 0.5 µg/500 µl in 0.5% BSA/PBS) or goat anti-IgG1-568nm (goat anti-mouse: 0.5 µg/500 µl in 0.5% BSA/PBS). Next day nuclei were labelled with DAPI. Coverslips were mounted in vectashield mounting medium for fluorescence imaging.

Cholesterol staining: Cellular localization of cholesterol based on Fillipin III staining was performed according to manufactures recommendation (Abcam Ltd; Cambridge, UK). In brief, CEFs were fixed in the Cell-Based Assay Fixative Solution for 10 minutes (RT in the dark). Cells were subsequently washed and stained (1 hr; RT in the dark) with Fillipin III (1:100 of Fillipin III stock solution in Cholesterol Detection Assay Buffer). After a final wash, coverslips were mounted in vectashield mounting medium for fluorescence imaging (excitation of 340-380 nm and emission of 385-470 nm).

TopFluor® cholesterol staining: The CEFs cells were infected either with mock or RB1B virus in maintenance media containing 628 nM of TopFluor® cholesterol for 72h and samples were prepared for confocal imaging. Lipid droplets staining: The CEFs cells were infected either with mock or wRB1B virus in maintenance media containing 628 nM cholesterol for 72h and samples were prepared for confocal imaging.
microscopy. TopFluor® cholesterol and lipid droplets groups were stained with Alexa-Fluor™-568 goat-anti mouse IgG2b and Alexa-Fluor™-488 goat-anti mouse IgG2b™ (Thermo Fisher Scientific, Paisley, UK) secondary antibody respectively. Further lipid droplets group were stained with HCS LipidTOX™ red neutral lipid stain. 

Visualization: Cells were viewed using a Leica SP2 laser-scanning confocal microscope and optical sections recorded using either the 663 or 640 oil-immersion objective with a numerical aperture of 1.4 and 1.25, respectively. All data were collected sequentially to minimize cross-talk between fluorescent signals. The data are presented as maximum projections of z-stacks (23-25 sections; spacing 0.3 mm). Maximum projections of z-stacks were analysed in the LAS AF lite software for localization of relative fluorescent intensity across a straight line based

Manders’ colocalization coefficient: All images were processed using the FIJI software, and Manders’ colocalization coefficient was calculated using the colocalization function (COLOC) for a specified region of interest (ROI). Manders’ M1 and M2 weighted coefficients were calculated to determine the extent of colocalization between a pair of fluorescent signals and imaged in two channels. Manders M1 determines the degree of channel colocalizing (gB with Filipin II, gB with LAMP-1, and LAMP-1 with Filipin III) and M2 determines the reverse. The uptake of the fluorescent cholesterol by mock and wRB1B infected CEFs were analyzed by ImageJ (Fiji software, USA) and estimated as mean fluorescence intensity (MFI) per cell. For estimation of lipid droplet formation, in total 15 Z-stack cells from mock and wRB1b infected cells were analyzed by IMARIS (Bitplane Scientific Software).

Statistical Analysis
All data are presented as mean ± standard deviation (SD) from at least three independent experiments. Quantification was performed using Graph Pad Prism 7 for windows. The differences between groups, in each experiment, were analysed by one-way analysis of variance (ANOVA), followed by Tukey multiple comparisons to identify those which groups differed if the ANOVA was significant. Results were considered statistically significant at $P < 0.05$ (*).

**Competing Interests**

The authors have declared that no competing interests exist.

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**Author Contributions**

Conceptualization: SB, NB. Data curation: NB, SB. Formal analysis: NB, SB. Funding acquisition: SB. Investigation: NB, SB. Methodology: NB, SB. Supervision: SB. Visualization: NB, SB. Writing – original draft: NB, SB. Writing – review & editing: SB.

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Figure legends

Figure 1: Induction of de novo cholesterol synthesis in MDV infected CEFs.

Alteration of cholesterol and intermediates in MDV-infected CEFs. Metabolomics analysis of relative ratio (ratio of area to internal standard) of cholesterol metabolites from mock (non-infected; no-inf)- and MDV-infected (RB1B; Inf) CEFs are shown at 48 hpi and 72 hpi. (A) Box and whisker plots showing minimum and maximum relative levels of the named metabolites identified by Metabolom analysis using GCxGC-MS in the no-inf or MDV infected CEFs at 48 and 72 hpi. (B) Schematic of cholesterol biosynthesis pathways with summary of major metabolites outlining
preferential induction of *de novo* cholesterol biosynthesis. Arrows indicate changes in the respective metabolites to demonstrate flux through pathways at 72hrs post infection. (C) Fold change gene (HMG-CoA R; 3-hydroxy-3-methyl-glutaryl-CoA reductase, squalene epoxidase; SqE and Farnesyl transferase; FTase, DHCR24; 24-dehydrocholesterol reductase, ABCA1; ATP-binding cassette transporter 1) expression based on RT-PCR in CEFs infected with MDV over no-inf cells at 72 hpi. (D) Relative expression levels of HMG-CoA Red and SqE proteins in the no-inf and MDV-infected CEFs at 72 hpi (E) presented as relative fluorescence intensity over α-tubulin. Non-parametric Wilcoxon tests (Mann-Whitney) was used to assess normal distribution and test significance with the results shown as mean ± SD. *** (p = 0.0005) and **** (p = 0.0001) indicates a statistically significant difference compared to control. The experiment was performed in biological triplicates with six technical replicates per biological replicates.

**Figure 2: titration of pharmacological inhibitors and shRNA targeting rate limiting enzyme of cholesterol biosynthesis pathways**

Analysis by MACS quant demonstrating percentages of live CEFs (7AAD−) after 72 h treatment with various concentrations of small pharmacological inhibitors including (A) lovastatin (inhibitor of HMG-CoA Red), (B) mevalonic acid, (C) BIBB 515 (inhibitor of SqE), (D) lonafarnib (inhibitor of FTase) and (E) U18666a (inhibitor of MVB). Bar graphs with single bars in black represent concentrations of the inhibitors which did not induce cell death compared to vehicle treated. Transfection efficiency of (F) psiRNA-h7SKGFPzeo plasmid expressing pshRNA or ps scrambled as identified by GFP fluorescence 24 hrs post transfection and targeting HMG-CoA Red, SqE and LAMP-1 in CEFs. All experiments were performed in triplicates and data is
Figure 3: Mevalonate pathway is required for MDV infection of CEFs.

(A) Schematic mevalonate biosynthesis pathway highlighting the relevant pharmacological inhibitor (red) and the respective enzyme (yellow box) as well as metabolite (green box) studied based on metabolites identified by Metabolom analysis using GCxGC-MS as primary committed step to de novo cholesterol biosynthesis. Analysis of MDV titre (PFU/ml) in CEFs in the presence of (B) Lovastatin (2.5, 10, 25 and 100 ng/ml) or in combination with (C) mevalonic acid (2.5, 5 and 7.5 µg/ml) or (D) only mevalonic acid (2.5, 5 and 7.5 µg/ml) at 72 hpi. (E) Relative expression levels of HMG-CoA Red protein (~64 kd) by western blot in the MDV-infected CEFs transfected with pshRNA or pScrambled at 72 hpi presented as relative fluorescence intensity over α-tubulin. (F) MDV titre in CEFs transfected with pshRNA or pScrambled RNA at 72 hpi. (G) Analysis of MDV spread based on plaque size area (mm²) in the presence of Lovastatin (100 ng/ml), or in combination with Mevalonic acid (7.5 µg/ml), Mevalonic acid only (7.5 µg/ml) or pshRNA knockdown of HMG-CoA Red. (H) Representative pictures of MDV plaques taken at 10X magnification using an inverted light microscope. (I) MDV genome copy numbers per 10⁴ cells (MEQ gene with reference ovotransferrin gene) were determined using qPCR on DNA samples extracted in CEFs treated with Lovastatin (100 ng/ml) or pshRNA at 72 hpi. *** (p = 0.0002) **** (p < 0.0001) indicates a statistically significant difference compared to control (no treat) or vehicle. Vehicle
indicates Lovastatin carrier (DMSO; maximum of 1:500 ratio of culture medium) and Mevalonic acid carrier (RPMI 1640). All viral titre experiments were performed in 6 replicates and data is representative of 3 independent experiments.

**Figure 4:** Lanosterol synthesis pathway is required for MDV infection of CEFs.

(A) Schematic lanosterol biosynthesis pathway highlighting the relevant pharmacological inhibitor (red) and the respective enzyme (yellow box) studied based on metabolites identified by Metabolom analysis using GCxGC-MS. Analysis of MDV (B) titre (PFU/ml) and (C) plaque size area (mm²) in the presence of different non-toxic concentrations of the chemical inhibitor of FTase, Lonafarnib, or vehicle at 72 hpi. (D) Representative pictures of MDV plaques taken at 10X magnification using an inverted light microscope at 72 hpi. Analysis of MDV (E and F) titre (PFU/ml), relative expression levels of SqE protein (~95 kd) by western blot in the MDV-infected CEFs transfected with pshRNA or pScrambled at 72 hpi presented as relative fluorescence intensity over α-tubulin and (G) plaque size area (mm²) in the presence of different non-toxic concentrations of the chemical inhibitor of SqE, BIBB 515, or in SqE gene knockdown CEFs using shRNA. (H) Representative pictures of MDV plaques in CEFs treated with BIBB515 or SqE knockout CEFs taken at 10X magnification using an inverted light microscope at 72 hpi. (I) MDV genome copy numbers per 10⁶ cells (MEQ gene with reference ovotransferrin gene) were determined using qPCR in MDV-infected CEFs treated with Lonafarnib (0.1 µg/ml), BIBB 515 (10 ng/ml) or psiRNA. * (p = 0.01) and **** (p < 0.0001) indicates a statistically significant difference compared to control (no treat). Vehicle indicates lonafarnib carrier (DMSO; maximum of 1:500 ratio of culture medium) and BIBB 515 carrier (DMSO; maximum of 1:500 ratio of culture medium). All viral titre experiments...
were performed in 6 replicates and data is representative of 3 independent experiments.

**Figure 5: Cholesterol is essential for MDV replication.**

Analysis for the contribution of exogenous cholesterol on MDV replication (A) The mock and MDV-infected CEFs viability based on 7AAD- cells as analysed by FACS at 72 hpi in the presence of varying concentrations of cholesterol. (B) Analysis of MDV titre (PFU/ml) in CEFs cultured in the presence of exogenous cholesterol (0, 100, 200, 400, 628, 1000, 1500 and complete nM). (C) Analysis of MDV plaque size area (mm$^2$) in the presence/absence of cholesterol (628 nM). Representative pictures, taken at 10X magnification using an inverted light microscope, demonstrating differential plaque sizes for the various treatments performed at 72 hpi. (E) Representative pictures showing uptake of TopFluor-cholesterol (green) in mock and wRB1B infected (red) CEFs cells. (F) MFI per cell of TopFluor-cholesterol in mock infected versus wRB1B infected CEFs cells in cholesterol containing (628 nM) and knock-out media. (G) Representative pictures demonstrating maximum projection of Z-stacks for each individual channel showing lipid droplets (red) in mock and wRB1B infected (green) individual CEF cell. (H) IMARIS panel shows the three-dimensional representative picture of lipid droplet surrounding DAPI (blue) stained nucleus and were used to calculate lipid droplet per cell in mock and wRB1B infected CEFs cells. *** (p = 0.0002) **** (p < 0.0001) indicates a statistically significant difference compared to control (no treat). Vehicle indicates cholesterol carrier (Milli Q water). Maintenance medium (MAINT) used for optimal viral replication. All viral titre experiments were performed in 6 replicates from 3 independent experiments.
**Figure 6: Inhibition of intracellular vesicle transport inhibits MDV spread**

Targeted interference of cholesterol trafficking in MVB by the U18666a small pharmacological inhibitor and shRNA. Analysis of MDV (B) titre (PFU/ml) and (C) plaque size area (mm²) in the presence of varying non-toxic concentrations of U18666A (0.75, 1.0, 5.0, 7.5 AND 10 ng/ml) or in combination with cholesterol (628 nM) at 72 hpi. (D) Representative pictures, taken at 10X magnification using an inverted light microscope, demonstrating differential plaque sizes from the treatment groups at 72 hpi. Confocal microscopy imaging with maximum projection of Z-stacks for each channel demonstrating (F, G, I, J, L, M) cytoplasmic distribution in MDV-RB1B (gB; green) and mock infected CEFs either treated with U18666a (10 ng/ml) or vehicle or for cholesterol (Filipin III; Blue) and Lysosomal-membrane associated protein-1 (LAMP-1; Red). Validation of colocalization efficiency by Mander’s colocalization coefficient as analysed using image J at 72 hpi with MDV.

Colocalization between (H) gB (M1; green) and Filipin III (M2; blue) (K) gB (M1; green) and LAMP-1 (M2; red) (N) LAMP-1 (M1; red) and Filipin III (M2; blue) which, respectively, represent the overlap with M1 as the denominator and vice versa (n=30). Maximum projection of all combined channels (MERGE) were analysed on an LAS AF Lite software to determine the relative fluorescent intensity (Arbitrary Unit; A.U) profiles from selected (white dash box) non-infected and MDV-infected CEFs either treated with U18666a or vehicle only for (G) gB (green) and Filipin III (Blue) (J) gB (green) and LAMP-1 (Red) and (M) gB (green), Filipin III (Blue) and LAMP-1 (Red) along the white line path (µm) indicated in the corresponding ROIs (Merge) demonstrating overlapping signals. *** (P = 0.0002) **** (P < 0.0001) indicates a statistically significant difference compared to control (no treat) or vehicle. Vehicle indicates U18666a carrier (DMSO; maximum of 1:500...
ratio of culture medium). All viral titre experiments were performed in 6 replicates and the data are representative of 3 independent experiments.

Figure 7: LAMP-1 facilitate MDV cell-cell spread

Targeted avian LAMP-1 knockdown using an shRNA expressing psiRNA-h7SKGFPZeo plasmid (GFP⁺) demonstrating lack of MDV spread. (A, C, E)

Representative and visualisation, at 72 hpi, by confocal microscopy imaging with maximum projection of Z-stacks for each channel demonstrating cytoplasmic distribution of LAMP-1 in (A) MDV-RB1B (gB; green and LAMP-1; red) or pshRNA expressing plasmid targeting silencing (LAMP-1⁻⁺) or scrambled (Scr) in (C) non-infected (pshRNA; green and LAMP-1; red) and (E) MDV-infected CEF (pshRNA; green gB; red). Maximum projection of all combined channels (MERGE) were analysed on an LAS AF Lite software to determine the relative fluorescent intensity (Arbitrary Unit; A.U) profiles from selected (white dash box) non-infected and MDV-infected CEFs for (B) MDV-RB1B gB (green) and LAMP-1 (red), (D) pshRNA expressing plasmid (LAMP-1⁻⁺ or Scr (green) and LAMP-1 (red) in non-infected CEFs and (F) pshRNA expressing plasmid (LAMP-1⁻⁺ or Scr (green) in MDV-RB1B gB (red) infected CEFs along the white line path (µm) indicated in the corresponding ROIs (Merge) demonstrating overlapping signals. Analysis, at 72 hpi, of MDV (G) titre (PFU/ml) and (H) plaque size area (mm²) in CEFs expressing shRNA for targeted LAMP-1 silencing (pshRNA) or scrambled (pScramble). (I) Representative pictures, taken at 10X magnification using an inverted light microscope, demonstrating differential plaque sizes for the various treatments performed at 72 hpi. (J) MDV genome copy numbers per 10⁴ cells (MEQ gene with reference ovotransferrin gene) were determined using qPCR in CEFs expressing shRNA for targeted LAMP-1.
silencing (pshRNA) or scrambled (pScramble). **** (P < 0.0001) indicates a statistically significant difference compared to vehicle or pScramble. All viral titre experiments were performed in 6 replicates and the data are representative of 3 independent experiments.
### Table 1: List of shRNA sequence used in the study

| Gene                                      | Sequence (5'-3')                                                                 | Tm  |
|-------------------------------------------|---------------------------------------------------------------------------------|-----|
| 3-hydroxy-3-methylglutaryl-CoA reductase  | siRNA#1: F ACCTCGCACGCAATCCACGCTTTCAAGAGAAATGGAAGCGTGGATCGGTTCTT  
 R CAAAAGAGCCAATCCACGCTTTCAAGAGAAATGGAAGCGTGGATCGGTTCTT | 47.62 |
|                                          | scRNA#1: F ACCTCGTCCCTCAATGGTTGCAATGGAAGCGTGGATCGGTTCTT  
 R CAAAAGAGCCAATCCACGCTTTCAAGAGAAATGGAAGCGTGGATCGGTTCTT | 52.38 |
|                                          | siRNA#2: F ACCTCGGAAGCAAGAGCGGTGATATCTTGTGAAAGAGTTTGGATGAGATGAG  
 R CAAAAGAGCCAATCCACGCTTTCAAGAGAAATGGAAGCGTGGATCGGTTCTT | 52.38 |
|                                          | scRNA#2: F ACCTCGCAATCGCGACGTTCATGGGATATCTTGTGAAAGAGTTTGGATGAGATGAG  
 R CAAAAGAGCCAATCCACGCTTTCAAGAGAAATGGAAGCGTGGATCGGTTCTT | 52.38 |
|                                          | siRNA#3: F ACCTCGAAGGGAGGCGTGGATATCTTGTGAAAGAGTTTGGATGAGATGAG  
 R CAAAAGAGCCAATCCACGCTTTCAAGAGAAATGGAAGCGTGGATCGGTTCTT | 52.38 |
|                                          | scRNA#3: F ACCTCGCAATCGCGACGTTCATGGGATATCTTGTGAAAGAGTTTGGATGAGATGAG  
 R CAAAAGAGCCAATCCACGCTTTCAAGAGAAATGGAAGCGTGGATCGGTTCTT | 52.38 |
| Lysosomal-associated membrane protein 1   | siRNA#1: F ACCTCGCATCTCAATGGGATATCTTGTGAAAGAGTTTGGATGAGATGAG  
 R CAAAAGAGCCAATCCACGCTTTCAAGAGAAATGGAAGCGTGGATCGGTTCTT | 42.86 |
| (LAMP-1)                                  | scRNA#1: F ACCTCGCAATCGCGACGTTCATGGGATATCTTGTGAAAGAGTTTGGATGAGATGAG  
 R CAAAAGAGCCAATCCACGCTTTCAAGAGAAATGGAAGCGTGGATCGGTTCTT | 42.86 |
|                                          | siRNA#2: F ACCTCGAAGGGAGGCGTGGATATCTTGTGAAAGAGTTTGGATGAGATGAG  
 R CAAAAGAGCCAATCCACGCTTTCAAGAGAAATGGAAGCGTGGATCGGTTCTT | 33.33 |
|                                          | scRNA#2: F ACCTCGCAATCGCGACGTTCATGGGATATCTTGTGAAAGAGTTTGGATGAGATGAG  
 R CAAAAGAGCCAATCCACGCTTTCAAGAGAAATGGAAGCGTGGATCGGTTCTT | 33.33 |
|                                          | siRNA#3: F ACCTCGAAGGGAGGCGTGGATATCTTGTGAAAGAGTTTGGATGAGATGAG  
 R CAAAAGAGCCAATCCACGCTTTCAAGAGAAATGGAAGCGTGGATCGGTTCTT | 33.33 |
|                                          | scRNA#3: F ACCTCGCAATCGCGACGTTCATGGGATATCTTGTGAAAGAGTTTGGATGAGATGAG  
 R CAAAAGAGCCAATCCACGCTTTCAAGAGAAATGGAAGCGTGGATCGGTTCTT | 33.33 |
| Squalene epoxidase (SQLE)                 | siRNA#1: F ACCTCGGAAGGGAGGCGTGGATATCTTGTGAAAGAGTTTGGATGAGATGAG  
 R CAAAAGAGCCAATCCACGCTTTCAAGAGAAATGGAAGCGTGGATCGGTTCTT | 52.38 |
|                                          | scRNA#1: F ACCTCGCAATCGCGACGTTCATGGGATATCTTGTGAAAGAGTTTGGATGAGATGAG  
 R CAAAAGAGCCAATCCACGCTTTCAAGAGAAATGGAAGCGTGGATCGGTTCTT | 52.38 |
|                                          | siRNA#2: F ACCTCGAAGGGAGGCGTGGATATCTTGTGAAAGAGTTTGGATGAGATGAG  
 R CAAAAGAGCCAATCCACGCTTTCAAGAGAAATGGAAGCGTGGATCGGTTCTT | 52.38 |
|                                          | scRNA#2: F ACCTCGCAATCGCGACGTTCATGGGATATCTTGTGAAAGAGTTTGGATGAGATGAG  
 R CAAAAGAGCCAATCCACGCTTTCAAGAGAAATGGAAGCGTGGATCGGTTCTT | 52.38 |
|                                          | siRNA#3: F ACCTCGAAGGGAGGCGTGGATATCTTGTGAAAGAGTTTGGATGAGATGAG  
 R CAAAAGAGCCAATCCACGCTTTCAAGAGAAATGGAAGCGTGGATCGGTTCTT | 52.38 |
|                                          | scRNA#3: F ACCTCGCAATCGCGACGTTCATGGGATATCTTGTGAAAGAGTTTGGATGAGATGAG  
 R CAAAAGAGCCAATCCACGCTTTCAAGAGAAATGGAAGCGTGGATCGGTTCTT | 52.38 |
| Gene name                                                                 | Accession no         | Primers                      | Tm (°C) | Product size |
|--------------------------------------------------------------------------|----------------------|------------------------------|---------|--------------|
| Lysosomal-associated membrane protein 1 (LAMP-1)                          | NM_205283.2          | FWD CTGCCGGTGCTGTCTCTGAT     | 60      | 166 bp       |
|                                                                          |                      | REV GGGAGAGATAGGGGTGGTT      |         |              |
| Squalene epoxidase (SQLE)                                                | NM_001194927.1       | FWD CGCTGACGTTGTTAGCTGAT     | 60      | 200 bp       |
|                                                                          |                      | REV AAGGACGCAGTCTGTTG        |         |              |
| Farnesyl-diphosphate farnesyltransferase 1 (FDFT1)                       | NM_001039294.1       | FWD CTACCCTCTGCTCAAGGTC      | 60      | 195 bp       |
|                                                                          |                      | REV TTAGAAACGTTGGCCACTCG     |         |              |
| 3-hydroxy-3-methyl-glutaryl-CoA reductase (3-HMG-CoA reductase)           | AB109635.1           | FWD GCTATGCTGTTAGCATAGGT     | 60      | 145 bp       |
|                                                                          |                      | REV TCACTGGGGAACAGTACGCT     |         |              |
| Cytoplasmic Beta Actin                                                   | X00182               | FWD TGCTGTTCCCATCTATCG       | 60      | 150 bp       |
|                                                                          |                      | REV TTGGTGACAATACCGTTCA      |         |              |