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HIV entry in macrophages is dependent on intact lipid rafts

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ABSTRACT

Macrophages are an important natural target cell for HIV-1, but previous studies of virus entry into these cells are limited, and the involvement of membrane cholesterol and lipid rafts is unknown. Cholesterol disruption of macrophage membranes using four pharmacological agents acting by different mechanisms: methyl-β cyclodextrin, nystatin, filipin complex and Lovastatin, all significantly inhibited productive HIV entry and reverse transcription. The inhibitory effects of these drugs resulted in decreased virus release from infected cells, and could be substantially reversed by the addition of water-soluble cholesterol. The virus bound equally to cholesterol-disrupted cells even though HIV receptor expression levels were significantly reduced. Macrophage CD4 and CCR5 were found to partition with the detergent-resistant membranes with a typical raft-associating protein flotillin-1. HIV particles were observed co-localising with a marker of lipid rafts (CTB-FITC) early post infection. These data suggest that macrophage membrane cholesterol is essential for HIV entry, and implicate lipid raft involvement.

Introduction

Virus entry into target cells is a complex process involving many cellular and viral proteins with the primary goal of overcoming the barrier of the cellular membrane. Some viruses achieve this by acquiring a lipid envelope during exit from the host cell; these enveloped viruses enter cells by fusion of their envelope with the cellular membrane at either the cell surface or within an endosome. Viruses without a lipid envelope have acquired mechanisms to penetrate the membrane barrier, entering cells mainly via an endocytic pathway using either clathrin-coated vesicles or caveolae, or by the formation of a pore at the cell surface as has been proposed for poliovirus (Hogle, 2002; Marsh and Helenius, 2006; Smith and Helenius, 2004). There is a growing amount of evidence that cholesterol, an important structural component that modulates the fluidity of biological membranes, is essential for the uptake of many viruses. Successful virus entry may require cholesterol in the host cell membrane or in the viral envelope. Many enveloped viruses, such as vaccinia virus (Chung et al., 2005), herpes simplex virus (Bender et al., 2003) and severe acute respiratory syndrome-coronavirus (Li et al., 2007) require cholesterol in the target cell membrane. The same is true for many non-enveloped viruses; SV40 (Anderson et al., 1996), foot-and-mouse disease virus and echovirus (Marjomaki et al., 2002; Pietiainen et al., 2004) require cholesterol to enter cells by the lipid raft-dependent caveolae endocytosis pathway. In the case of influenza virus, the presence of cholesterol in its viral envelope is critical, but it is not essential in the target cell (Sun and Whittaker, 2003), whereas the converse has been found for murine leukaemia virus (Lu and Silver, 2000) and duck hepatitis B virus (Funk et al., 2008). In contrast, various strains of dengue virus and yellow fever virus enter and infect cells independently of cholesterol (Umashankar et al., 2008).

Cholesterol is concentrated within the fluid mosaic bilayer of the plasma membrane, along with sphingolipids and glycerophospholipids, in specialised dynamic microdomains known as lipid rafts. These lipid assemblies are tightly packaged and ordered, allowing them to float within the rest of the fluid sea of disordered lipid bilayer (Simons and Ikonen, 1997). The presence of cholesterol in the lipid rafts confers some resistance to detergents at low temperatures, allowing their separation from detergent-sensitive membranes by ultracentrifugation (Wilflingseder and Stoiber, 2007). Due to this property, they are often referred to as detergent-resistant membranes (DRMs). Membrane rafts act as mobile platforms within the plasma membrane, and are involved in many important diverse cellular processes. Many proteins associate with lipid rafts, and these include GPI-anchored proteins, GTPases and kinases. Mounting evidence for the cholesterol dependency of productive virus entry suggests that many viruses may hijack these dynamic lipid raft platforms to utilise them as an entry portal to the cell.

Cholesterol is already known to be important at multiple stages of the HIV lifecycle. The virus assembles and buds out of lipid raft domains, and in doing so acquires a cholesterol and sphingomyelin-rich envelope (Aloia et al., 1988, 1993; Campbell et al., 2004) that is resistant to detergents but has a strikingly different composition to host cell membranes (Brugger et al., 2006; Chan et al., 2008). The HIV accessory protein, Nef, functions on many levels to manipulate cellular cholesterol, including binding to cholesterol to enhance its trafficking

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to rafts (Zheng et al., 2003), increasing cellular cholesterol biosynthesis (van 't Wout et al., 2005) and inhibiting cholesterol efflux by redistributing a major component of the cholesterol efflux pathway (Bukrinsky and Sviridov, 2006; Castrillo et al., 2003). Although in contrast, other studies have found that Nef specifically enhances the incorporation of sphingomyelin but not cholesterol into budding viral particles (Brugger et al., 2007). The importance of HIV envelope lipid composition is highlighted by the finding that depletion of viral envelope cholesterol causes a dramatic reduction in viral infectivity (Brugger et al., 2006; Campbell et al., 2004, 2002; Guyader et al., 2002; Liao et al., 2003).

The early events in HIV entry, in which the viral envelope protein, gp120–gp 41, engages with the primary receptor CD4 on target cells, are well characterised. This interaction permits conformational changes within gp120 that allow additional binding to a co-receptor, usually CXCR4 for T-lymphocyte tropic strains, or CCR5 for macrophage tropic isolates. Co-receptor binding is the trigger for virus entry, during which the fusion peptide gp41, inserts into the cellular membrane to drive the fusion event. The lipid composition of the host cell is important for HIV entry. Cholesterol depletion of target cells using pharmacological agents prevents HIV entry, and render cells resistant to infection (Kozak et al., 2002; Liao et al., 2001; Nguyen and Taub, 2002b; Popik et al., 2002). The association of CD4 with lipid raft domains is well accepted (Kozak et al., 2002; Nguyen et al., 2005; Percherancier et al., 2003; Popik et al., 2002), but whether this association is required to support productive virus entry is disputed. One study found that mutant CD4 targeted to non-raft membranes did not permit efficient HIV entry (Del Real et al., 2002), whereas alternative CD4 mutants that also prevent CD4 association with lipid rafts were found to support HIV entry (Percherancier et al., 2003; Popik and Alce, 2004). The membrane localisation of the co-receptor molecules is somewhat controversial. CCR5 has been shown to associate with lipid rafts microdomains in cell lines by its presence in DRM fractions and its co-localisation with the raft associated lipid GM1 (Manes et al., 1999; Popik et al., 2002). In contrast, studies in primary T cells identified CCR5 in the detergent-soluble membrane (DSM) fractions representing the non-raft membranes (Percherancier et al., 2003). CXCR4 has been reported to be lipid raft-associated in 293 cells (Manes et al., 2000) but non-raft-associated in T cell lines (Kozak et al., 2002; Popik et al., 2002). HIV-1 gp120 binding to CD4 molecules in lipid rafts has been proposed to cause recruitment of the HIV co-receptor into the lipid rafts or its interface, thus bringing all molecules required for HIV entry together (Kozak et al., 2002; Nguyen et al., 2005; Popik et al., 2002).

A different productive entry pathway for HIV into macrophages has been described, in which virus is taken up by an endocytic route involving macropinocytosis, a constitutive process whereby the cell takes up large quantities of the extracellular fluid (Marechal et al., 2001). Numerous intracellular pathogens are taken up into macrophages by macropinocytosis, including *Brucella* (Watarai et al., 2002) and *Afipia felis* (Schneider et al., 2007), and in these cases a requirement for lipid rafts has been established. Cholesterol is already known to play an important role in HIV replication of macrophages, with specific studies showing that Nef affects the normal function of ATP-binding cassette transporter A1 to impair cholesterol efflux from infected macrophages (Mujawar et al., 2006). Modulation of intracellular lipid metabolism in HIV-infected macrophages may facilitate virus budding from lipid raft associated membranes, but it is tempting to infer that cholesterol might also play an important role in the entry of HIV into macrophages.

All of the studies regarding the involvement of cholesterol and lipid rafts in HIV entry have been performed using immortalised cell lines, T cell lines and primary CD4+ lymphocytes. Given their critical position in HIV pathogenesis, and their highly specialized cellular architecture, we thought it necessary to investigate the requirement of cholesterol and role of lipid rafts in HIV-1 entry into macrophages. We have assessed the effect of depleting cholesterol in the macrophage plasma membrane, using four different pharmacological agents, on virus binding, entry and HIV receptor levels. Here we show that membrane cholesterol is essential for productive HIV entry into macrophages. Virus binding to cholesterol-depleted macrophages is not reduced but there are alterations to the surface expression of the receptor molecules. We observed virus co-localising with a marker of lipid rafts early after infection, and isolated macrophage CD4 and CCR5 in the membrane fractions representing raft microdomains. This, together with the sensitivity of virus entry to membrane cholesterol depletion, implies a role for macrophage lipid rafts in HIV-1 entry.

Results

HIV co-localises with markers of lipid rafts

To investigate the involvement of lipid rafts in HIV uptake into macrophages, we first sought to visualise virus entry and lipid rafts using fluorescent Cholera toxin B subunit (CTB-FITC) the binding of which to GM1 clusters these membrane domains together into larger patches that can be observed microscopically. HIV NL4.3 pseudotyped with JRFL envelope was bound to macrophages on ice, and warmed to 37 °C in the presence of CTB-FITC and analysed at various times post entry. A representative experiment taken at 20 min post entry is shown in Figs. 1A–D, and shows virus co-localising with CTB-positive areas of the macrophage plasma membrane. The number of particles directly associated with GM1-positive membranes on the cell surface or in endocytic vesicles was quantified by taking 0.8 μm *Z*-slices through the cell and scoring the percentage of the total particles co-localising with CTB-FITC. Three consecutive *Z*-slice focal planes are shown in panels A–C. These slices start towards the bottom of the cell (A) and proceed upwards, and show many cell-associated virus particles clearly co-localising with CTB-FITC. The distribution of all virus particles and CTB-FITC is shown in a projection of *Z*-stack slices in panel D. Analysis of multiple cells ($n=10$) at 20 min post entry shows approximately 34% (±3.72 SEM) of total particles are co-localising with CTB-FITC. At later times post entry (40 and 60 min), there is a reduction in association of virus with CTB-FITC perhaps due to productive entry of most virus particles into the cytoplasm or degradation of the virus preventing its detection (Fig. 1E).

Cholesterol depletion of macrophages inhibits HIV entry but not binding

Membrane cholesterol can be disrupted by a number of pharmacological agents; the manipulation of this key component of lipid microdomains is frequently used to implicate these assemblies in virus entry. The effects of methyl-β cyclodextrin (MβCD) are well characterised. This cholesterol-binding agent is not incorporated into the membrane but selectively extracts membrane cholesterol by binding it in a central non-polar cavity (Ilangumaran and Hoessli, 1998). We incubated macrophages with various concentrations of MβCD in serum-free media, and measured the depletion of lipid rafts by detecting fluorescent CTB-FITC binding to the raft glycolipid GM1 by flow cytometry. CTB-FITC binding to drug-treated cells on ice was reduced in a dose-dependent manner compared to untreated cells confirming that MβCD effectively disrupts macrophage lipid rafts (Fig. 2A). The effect of this drug on cell viability was measured using the MTS cytotoxicity assay (Fig. 2B) and showed that 1 h treatment with MβCD had no toxic effect on the macrophages. Quantification of the amount of free cholesterol and cholesteryl esters in 10 mM MβCD-treated macrophages using Amplex Red cholesterol quantification assay revealed a significant decrease ($p=0.01$ by paired *t*-test) in all 6 donors tested with an average of 28.7% (±20.4%) reduction compared to untreated control cells.

The potential anti-viral properties of MβCD on HIV entry into macrophages was tested by measuring virus reverse transcription in

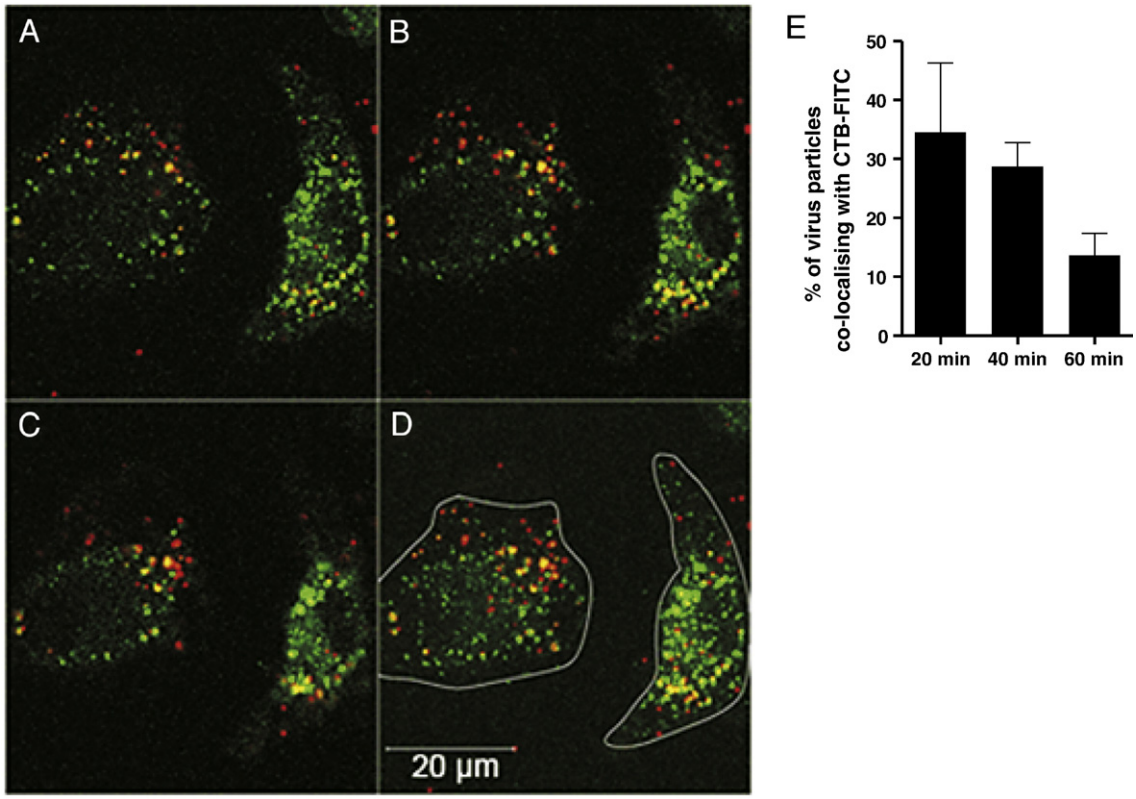

Fig. 1. Co-localisation of HIV with CTB-FITC. Replication-defective HIV generated by the transfection of 293T cells with pNL4.3luc R-E- and pJRFL envelope was spinoculated at 4 °C onto macrophage monolayers on coverslips. Cells were incubated at 37 °C for 20 min to allow absorbed virus to enter cells in the presence of 10 μg/mL of CTB-FITC. Lipid rafts were visualised by virtue of CTB-FITC fluorescence and HIV particles were detected by labelling with anti-p17 mAb. *Z* stack analysis was performed on random cells; three consecutive images (A–C) from the *Z* stack are shown with panel A towards the bottom of the cell and panel C nearer the top of the cell, panel D is a projection of all *Z* stack images. The perimeter of the cell is outlined and scale bar represents 20 μM. (E) The number of virus particles co-localising with CTB-FITC at 20, 40 and 60 min after binding was quantified by detailed *Z*-stack analysis on 10 random cells for each time point. The total number of virions per cell was counted and then the % virions associating with CTB-FITC was determined. Error bars represent mean ± SD.

drug-treated macrophages. The HIV envelope acquires cholesterol during budding, and MβCD can deplete this virion cholesterol, resulting in reduced virus infectivity (Guyader et al., 2002; Liao et al., 2003). Therefore, to avoid this potentially confounding effect, cells were pre-treated with MβCD for 1 h, and washed extensively before the addition of virus, to ensure that any anti-viral effects observed were not due to decreased particle infectivity. MβCD treatment of macrophages was found to significantly reduce the generation of reverse transcription products following challenge with HIV BaL, at both concentrations tested, in macrophages derived from six different donors (Fig. 2C). The inhibitory effect of MβCD could be partially reversed by the replenishment of cellular cholesterol with water-soluble cholesterol added for 1 h directly after MβCD treatment. Cholesterol replenishment restored virus reverse transcription to 43.5% of the untreated control (Fig. 2D). The inhibitory effects of MβCD were also observed when p24 release from infected cells was measured by ELISA (Fig. 2E). Reduced p24 release kinetics were observed in the early days post infection for all donors treated with MβCD, but the effects were less pronounced due to the longer length of this experiment and the measurement of multiple rounds of infection. Restoration of p24 release kinetics was observed upon cholesterol replenishment. Therefore, depletion of macrophage membrane cholesterol with MβCD severely inhibits the early steps of productive HIV infection of macrophages.

To further pinpoint the stage at which cholesterol-containing lipid rafts are required in HIV infection, we determined the effect of cholesterol depletion on virus binding to macrophages. Equal amounts of HIV BaL were added to untreated and MβCD-treated macrophages for 2 h on ice. Unbound virions were removed by extensive washing before cells were lysed and the amount of cell-associated virus determined by p24 ELISA (Fig. 2F). A slight decrease in virus adsorption to MβCD-treated macrophages compared to untreated control cells was observed. However, this decrease was not significant and would not account for the 8-fold decrease observed in reverse transcription in MβCD-treated cells. Therefore, cholesterol and lipid rafts might play a minor role in virus binding but they are more likely to be required for entry or post-entry events.

Cholesterol depletion alters HIV receptor surface expression

Decreased entry of HIV might be attributed to alterations in receptor or co-receptor levels; therefore we sought to determine if MβCD treatment of macrophages alters the levels of CD4, CCR5 and CXCR4. Detection of these cell surface molecules using antibody labelling followed by flow cytometry shows that control macrophages express CD4, CCR5 and CXCR4 at detectable levels (Fig. 3A). Treatment of macrophages with MβCD significantly decreases the cell surface expression of all receptors; CD4 by 35%, CCR5 by 100% and CXCR4 by 59%. Both CCR5 and CXCR4 expression levels are restored upon cholesterol replenishment, implying that the decrease is due solely to cholesterol depletion. Interestingly, replenishment with cholesterol does not restore the CD4 expression levels, but reduces them further to almost undetectable levels. This further reduction in CD4 expression may explain why cholesterol replenishment only partially restores HIV reverse transcription (see above). MβCD treatment of macrophages does not reduce the expression of all membrane proteins because the expression of CD71 (transferrin receptor), a known non-raft associated protein, was not reduced. Therefore, MβCD

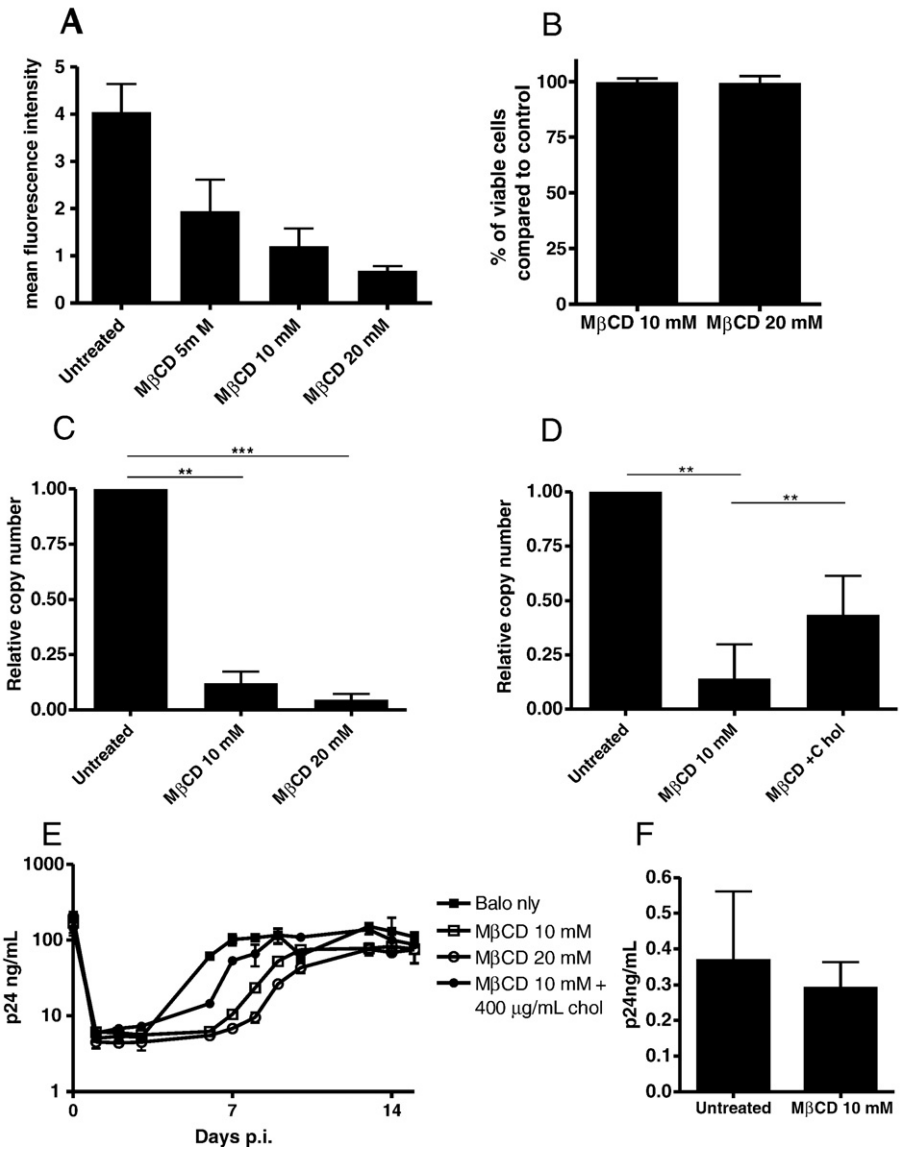

Fig. 2. Depletion of cholesterol by MβCD inhibits HIV infection of macrophages. (A) Depletion of macrophage lipid rafts was measured by detecting CTB-FITC binding to MβCD treated cells. CTB binding occurred for 30 min on ice and was measured by flow cytometry where cellular autofluorescence (without CTB-FITC) was used as the control. (B) Cell viability after 1 h treatment with MβCD was determined using the MTS cytotoxicity assay with the number of viable cells being expressed as a % of untreated control cells. (C) Productive HIV entry into MβCD treated macrophages was measured by detecting virus reverse transcription products by qPCR after 30 h of infection. The number of copies of HIV-1 DNA was normalised to the number of cells using a β-actin control. (D) MβCD treated macrophages were treated with 400 μg/mL of water-soluble cholesterol for 1 h before HIV BaL infection. After 30 h, productive HIV entry was measured by qPCR as before. (E) HIV-1 infection following MβCD treatment and cholesterol replenishment was measured by detecting released virus in supernatant samples (collected regularly from 1–15 days p.i.) by p24 ELISA. (F) Binding of HIV BaL to MβCD treated macrophages for 2 h on ice was measured by determining the amount of cell-associated virus using p24 ELISA. Data represent mean±SD of results obtained with multiple independent experiments using several donors (4 donors for CTB, viability and HIV binding assays, 4–6 donors for qPCR and p24 data is representative of 3 donors). ** Very significant difference, $p<0.01$ and *** extremely significant difference $p<0.001$ (paired t-test).

selectively alters the exposure of proteins found in the cholesterol-rich lipid raft membranes without effecting non-raft associated proteins. The combined decrease in CD4 and CCR5 receptor levels may account for the decrease in productive HIV entry.

HIV receptors are associated with lipid rafts in macrophages

The reduction in HIV receptor levels in cholesterol-depleted macrophages and the co-localisation of virus with a marker of lipid rafts may imply that the receptors are localised in these micro-domains. Traditional methods to isolate lipid rafts rely on cholesterol's property of favouring the formation of membranes that are resistant to detergent at low temperature, and can be separated from soluble membranes by ultracentrifugation. We adapted a procedure for isolating macrophage DRMs by disrupting cells by a combination of nitrogen cavitation and homogenisation followed by fractionation using an Optiprep density gradient. Western blot analysis of equal volumes of each fraction shows the expected localisation of the control proteins: flotillin-1 is located in fractions 4 and 5 representing the DRMs, and transferrin receptor is in fractions 8 and 9 indicative of the DSMs (Fig. 3B). The receptor CD4 is present in the detergent-resistant fractions 4 and 5, and has a similar distribution to that of flotillin-1, although it is also present at low levels in fractions 6 and 7. CCR5 is present in detergent-resistant fractions 4–5 only. Therefore, the receptors required for macrophage-tropic HIV entry reside in the DRM fractions of macrophage membranes, which have properties of lipid rafts and contain raft-associated protein flotillin-1.

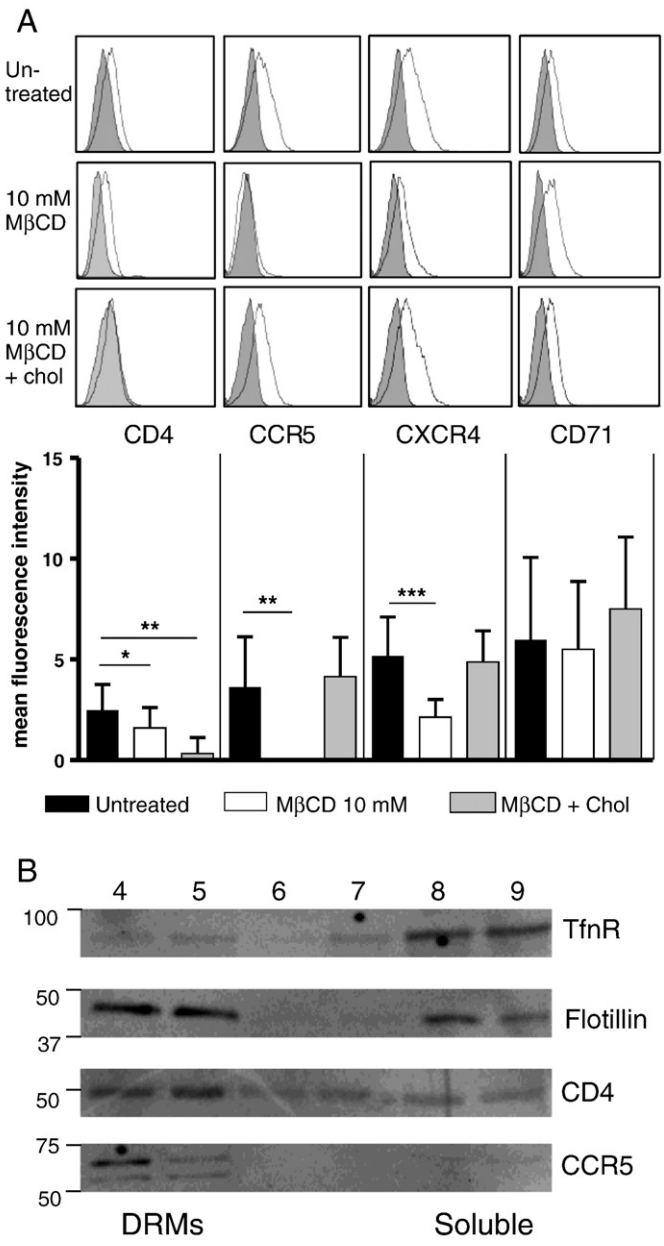

Fig. 3. HIV receptors are associated with DRMs in macrophages and their levels are altered by MβCD treatment.(A) The surface expression of HIV receptors after MβCD and cholesterol treatment was determined by staining macrophages with fluorescent monoclonal anti-human antibodies to CD4, CCR5, CXCR4, CD71, or an appropriate isotype control. Cell surface binding was measured by flow cytometry, and mean fluorescence intensity values were calculated by subtracting the isotype control fluorescence from the specific antibody fluorescence. Representative FACS plots of macrophages from one donor are shown, and graphs show mean ± SD of data acquired from five different donors. * Significant difference $p<0.05$, ** very significant difference, $p<0.01$ and *** extremely significant difference $p<0.001$ (paired *t*-test). (B) Macrophage membranes from lysed cells were incubated in 1% Triton X-100 and then fractionated using an Optiprep step gradient by ultracentrifugation. Fractions were collected, concentrated by TCA precipitation and 20 μL samples of each fraction were analysed by SDS-PAGE using antibodies to CD4, CCR5, flotillin and CD71.

Chelating cholesterol with nystatin and filipin complex inhibits HIV entry

To exclude any drug-specific effects of MβCD, we used two other compounds that can bind to cholesterol and disrupt lipid rafts by directly inserting into membranes and sequestering cholesterol into complexes. Both nystatin and filipin complex, at concentrations significantly inhibiting CTB-FITC binding (Fig. 4A) and not compromising cell viability (Fig. 4B), inhibited HIV infection in a dose-dependent manner. Nystatin at concentrations of 66 and 50 μg/mL significantly reduced reverse transcription in infected cells by 8.4- and 4.1-fold respectively compared to those treated with the same concentration of DMSO vehicle (Fig. 4C). Filipin complex significantly inhibited the number of HIV reverse transcripts by 3.2-fold compared to the untreated control (Fig. 4D). Similarly, these inhibitory effects can be observed as reduced p24 release kinetics at early time points post infection (Fig. 4E). Both nystatin and filipin complex do not reduce virus binding to macrophages (Fig. 4F). This supports the findings obtained by MβCD treatment, implying that cholesterol and lipid rafts are likely to be required for entry and/or post-entry events and not binding.

Studies with MβCD indicate that the HIV receptors are sensitive to cholesterol depletion so we sought to determine if receptor levels would also be altered when cholesterol organisation is disrupted by another mechanism (Fig. 4G). Nystatin significantly decreased CD4 and CCR5 expression by 59% and 91%, respectively. The expression level of CXCR4 was only slightly decreased. However, nystatin did significantly

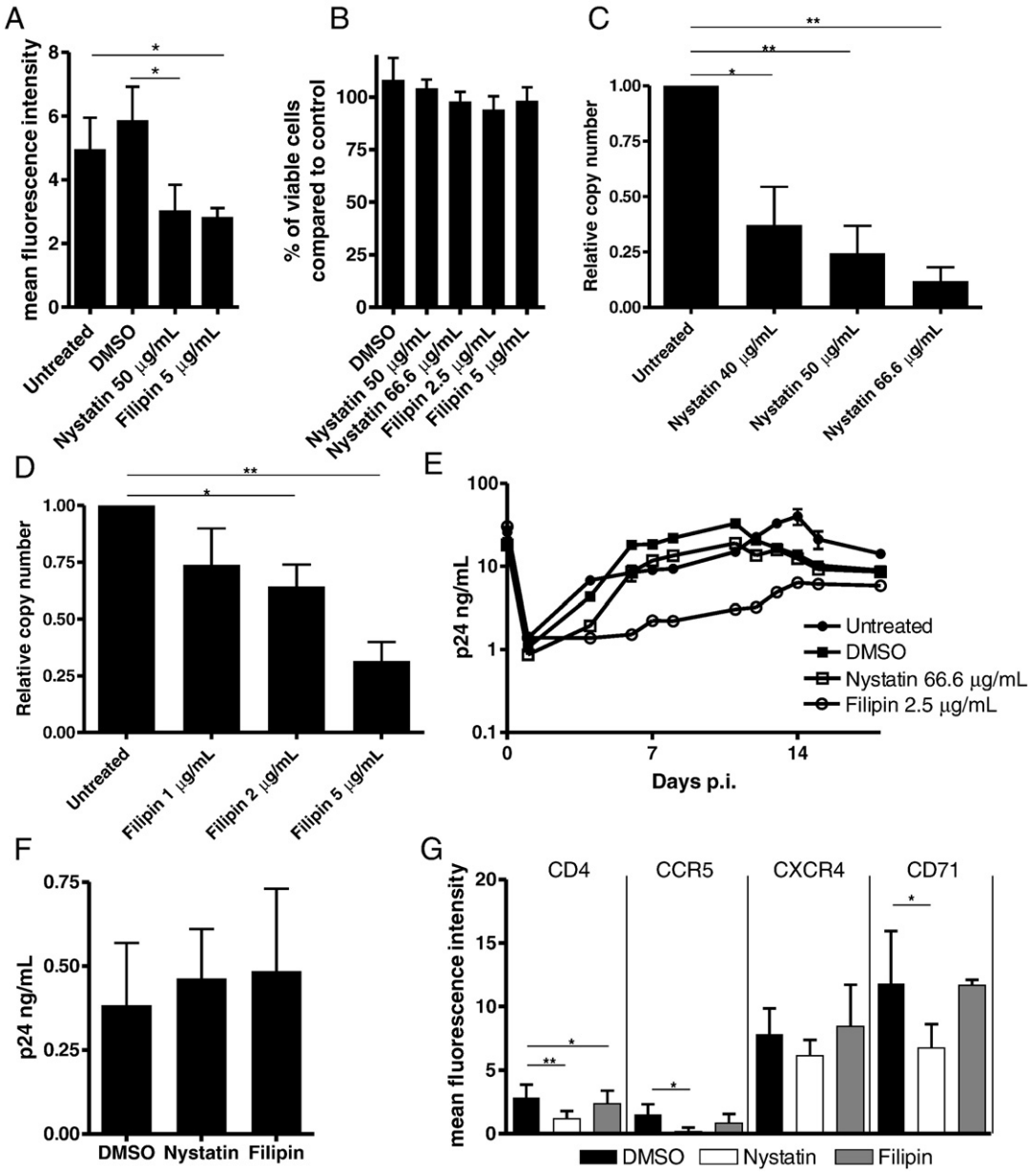

Fig. 4. Nystatin and filipin complex inhibit HIV entry into macrophages. (A) CTB-FITC binding to nystatin and filipin treated cells for 30 min on ice was measured by flow cytometry, with cellular autofluorescence (without CTB-FITC) used as the control. (B) Cell viability after 1 h treatment with nystatin or filipin was determined using the MTS cytotoxicity assay with the number of viable cells being expressed as a % of untreated control cells. (C, D) Productive HIV entry into nystatin (C) or filipin complex (D) treated macrophages was detected after 30 h of infection with HIV BaL by qPCR. (E) HIV-1 infection following nystatin and filipin complex treatment was measured by detecting released virus in supernatant samples by p24 ELISA. (F) Binding of HIV BaL to nystatin (50 μg/mL) and filipin complex (5 μg/mL) treated macrophages for 2 h on ice was measured by p24 ELISA. (G) The surface expression of HIV receptors after nystatin or filipin complex treatment was determined by staining macrophages with fluorescent antibodies to CD4, CCR5, CXCR4, CD71 or an appropriate isotype control. Cell surface binding was measured by flow cytometry, and mean fluorescence intensity values were calculated by subtracting the isotype control fluorescence from the specific antibody fluorescence. Data represent mean ± SD of results obtained with multiple independent experiments (3 donors for qPCR, CTB and p24 binding assays, 4 donors for receptor expression, viability and p24 data is representative of at least 2 donors). * Significant difference $p<0.05$, ** very significant difference, $p<0.01$ (paired t-test).

reduce levels of CD71 by 43% indicating that this drug might reduce the cell surface expression of many proteins including those outside lipid rafts. Conversely, filipin complex marginally decreased CD4 and CCR5 but had no effect on the levels of CXCR4 or CD71. The anti-viral effects of nystatin might be explained by the lower levels of CD4 and CCR5 but it is likely that filipin complex might inhibit infection by a different mechanism than decreased receptor levels.

Inhibition of cholesterol synthesis inhibits HIV entry

The cholesterol-binding drugs, MβCD, nystatin and filipin, mechanically disrupt lipid rafts either by actively removing or by sequestering cholesterol. Lipid rafts can also be disrupted using statins, such as Lovastatin. These drugs inhibit the 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase enzyme responsible for the production of mevalonate, a precursor in cholesterol biosynthesis. Cells were treated with Lovastatin for 4 days in the presence of serum-free medium supplemented with lipoprotein-deficient serum after which cell viability was unaffected (Fig. 5A). The binding of CTB-FITC to Lovastatin-treated cells was reduced by 33% (±11.1%) compared to the untreated cells (Fig. 5B). Lovastatin treatment of macrophages very significantly inhibited HIV reverse transcription in a dose-dependent manner by 16.6- and 5-fold with 12.5 μM and 5 μM Lovastatin, respectively (Fig. 5C). A marked decrease

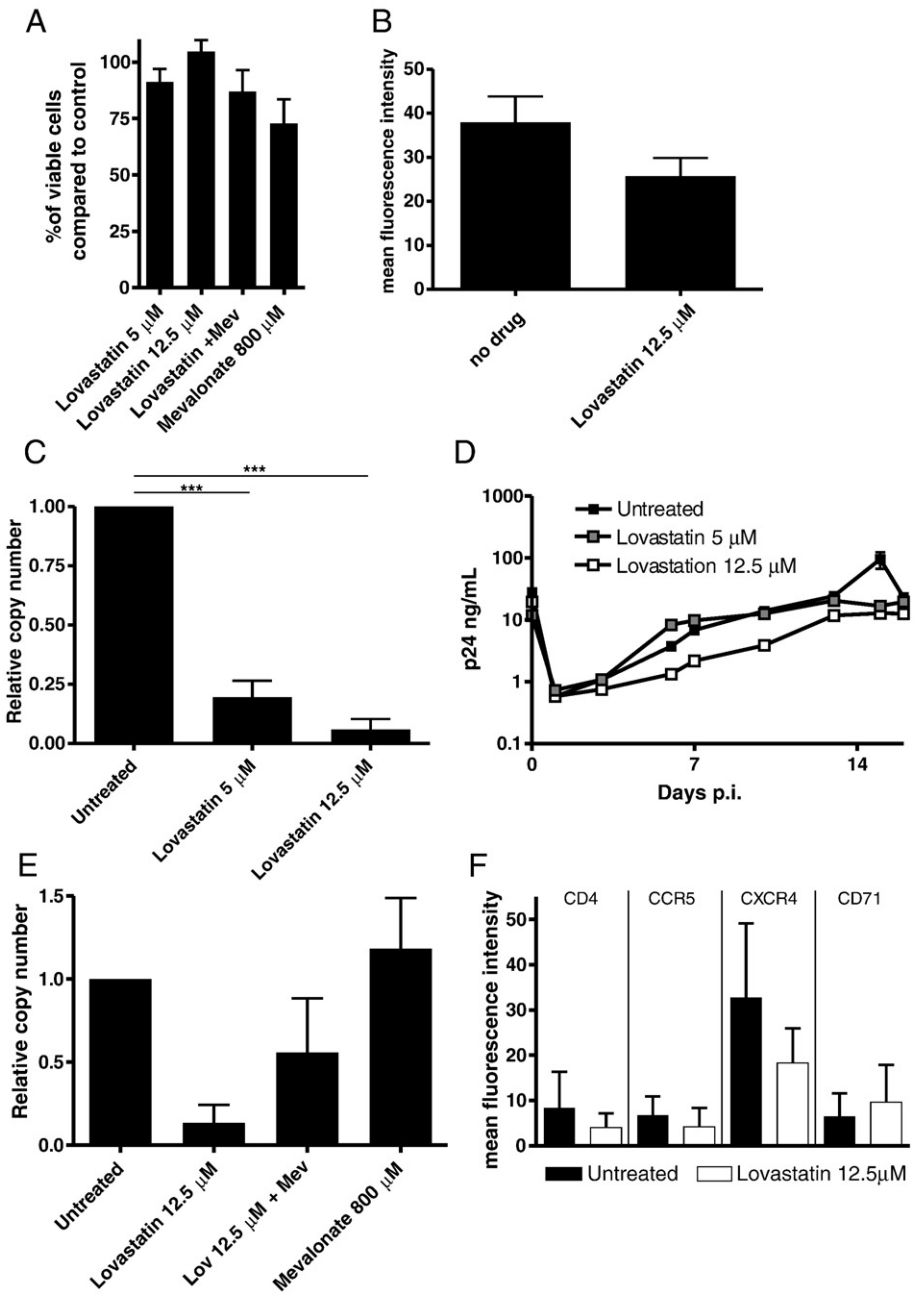

Fig. 5. Inhibiting cholesterol synthesis inhibits HIV entry into macrophages. (A) Cell viability after 4 days treatment with Lovastatin and/or mevalonate was determined by MTS cytotoxicity assay with the number of viable cells being expressed as a % of untreated control cells. (B) CTB-FITC was bound to Lovastatin-treated macrophages for 30 min at 37 °C and was measured by flow cytometry, with CTB-FITC binding on ice used as the control. (C) Productive HIV BaL entry into macrophages pre-treated with Lovastatin for 4 days was measured after 30 h of infection by qPCR. (D) HIV-1 infection following Lovastatin treatment was measured by detecting released virus in supernatant samples by p24 ELISA. (E) Macrophages were pre-treated with 12.5 μM Lovastatin and/or 800 μg/mL mevalonate (whose production is blocked by Lovastatin) for 4 days. Productive HIV entry was measured by qPCR detection of HIV BaL reverse transcription after 30 h of infection. (F) The surface expression of HIV receptors after Lovastatin treatment was determined by staining macrophages with fluorescent antibodies to CD4, CCR5, CXCR4, CD71 or an appropriate isotype control. Data represent mean ± SD of results obtained with multiple independent experiments (2 donors for CTB and viability assays, 2–5 donors for qPCR, 5–6 donors for receptor expression and p24 data are representative for 2 donors). *** Extremely significant difference $p<0.001$ (paired t-test).

in p24 release kinetics compared to untreated control was also observed (Fig. 5D). The effects of Lovastatin on HIV reverse transcription were partially reversed by the addition of mevalonate, the product of HMG-CoA reductase, to the culture medium (Fig. 5E). The surface expression levels of all HIV receptors (but not CD71) were decreased after Lovastatin treatment but not to significant levels (Fig. 5F). Although these effects consistently implicate normal cholesterol synthesis for productive virus entry and receptor expression levels, we were unable to detect significant changes in cholesterol abundance *per se* in Lovastatin-treated cells using the standard assay.

Discussion

Here we have demonstrated the critical importance of cholesterol for entry of HIV-1 into macrophages. Firstly, we showed that productive virus entry is significantly reduced when the macrophage target cells are treated with MβCD, a cholesterol-depleting drug. By incubating cells with this drug before the addition of virus, only cholesterol from the target cells would be removed allowing virus particle cholesterol and infectivity to remain intact. The addition of exogenous cholesterol to macrophage membranes directly after

MβCD treatment substantially restored HIV infection, indicating that the decreased infectivity was at least in part due to the depletion of cholesterol from the cell membrane. Secondly, we modified the properties of cholesterol-rich macrophage membranes using nystatin and filipin complex to sequester cholesterol into large aggregates. Treatment of macrophages with these drugs significantly inhibited productive HIV entry in a concentration-dependent manner. Thirdly, depletion of macrophage cholesterol using Lovastatin, a compound that inhibits HMG-CoA reductase, the rate limiting enzyme in cholesterol biosynthesis, significantly inhibited HIV productive entry after a prolonged 4 day treatment. Incubation of macrophages with mevalonate, whose production is prevented by Lovastatin, partially restored productive HIV entry into macrophages. Therefore, modification of macrophage membrane cholesterol content using 4 different pharmacological inhibitors acting on cholesterol by different mechanisms all significantly inhibited HIV entry.

Cholesterol is a major component of lipid raft microdomains and manipulation of its localisation or presence within membranes is frequently used to implicate these lipid assemblies in virus entry. Here we provide additional evidence for the involvement of macrophage lipid raft microdomains in HIV-1 entry by observing virus co-localisation with a marker (CTB-FITC) of these microdomains at an early time post-infection. Furthermore, we have isolated the receptor molecules CD4 and CCR5, the receptors typically utilised by macrophage-tropic viruses, in DRM fractions partitioning with a known-raft associated protein flotillin-1. These results confirm observations regarding the importance of target cell plasma membrane cholesterol and lipid raft microdomains in virus entry into T cell lines and primary CD4+ T cells (Liao et al., 2001; Manes et al., 2000; Percherancier et al., 2003; Popik et al., 2002) and extend them to macrophages, a critical natural target cell for HIV-1.

For viruses to infect target cells, they have to firstly bind to attachment factors or receptor molecules on the cell surface. A decrease in productive virus entry may reflect a reduction in virus binding to cells. We confirmed which stage of infection was being affected by cholesterol depletion by investigating virus binding to the macrophage membranes. No significant decrease in the amount of bound virus after a 2 h incubation on ice was observed between drug-treated and control untreated macrophages. This indicates that cholesterol depletion of macrophages does not perturb the attachment of virus, perhaps to alternative molecules such as heparan sulphate glycosaminoglycans (Saphire et al., 2001), but must alter virus entry events after attachment.

The surface expression levels of the HIV receptors were found to be altered to differing extents by the 4 pharmacological agents. MβCD treatment had the most pronounced effect on the receptors, with CD4 and CXCR4 being significantly reduced and CCR5 surface levels disappearing to almost undetectable levels. Cholesterol replenishment restored the levels of CCR5 and CXCR4 to similar levels as the untreated macrophages. Interestingly, CD4 expression was decreased further by cholesterol replenishment to almost undetectable levels. This complete knockdown in CD4 surface expression may explain why only a partial restoration of HIV reverse transcription was observed upon cholesterol replenishment of MβCD treatment macrophages. The addition of cholesterol may reduce CD4 expression to a level that is not favourable for HIV infection. The mechanism by which cholesterol further decreases CD4 surface expression is unknown but as these cholesterol-treated macrophages have a more granular appearance by flow cytometry, we can speculate that these macrophages actively take up cholesterol via an endocytic mechanism that may simultaneously internalise CD4. It is not uncommon for cholesterol replenishment to give rise to only partial restoration of infection; it has been reported for other viruses with the suggested explanation being that only one form of cholesterol may have been restored (Tang et al., 2008).

The reduction in surface expression of HIV receptors following depletion of membrane cholesterol contrasts with findings using different cells. Treatment with similar concentrations of MβCD caused no reduction in plasma membrane expression of CD4 on primary T-cells (Percherancier et al., 2003), PBLs (Viard et al., 2002) or PM1 T cell line (Popik et al., 2002). The reduction of CD4 surface expression upon MβCD and cholesterol treatment, to the best of our knowledge, seems to be unique to macrophages. This observation may result from the differences in CD4 expression and internalisation on macrophages compared to T lymphocytes. In macrophages, CD4 expression levels are 10 to 20-fold lower than they are in CD4+ T cells (Collman et al., 1990; Kazazi et al., 1989; Lee et al., 1999), and CD4 internalisation rates are enhanced, likely due to the absence of Lck (Pelchen-Matthews et al., 1998). Reduction in CXCR4 and CCR5 surface expression has been reported after treatment with a cyclodextrin derivative BCD in PM1 and primary T cells (Liao et al., 2001). More importantly, cholesterol has been shown to be essential for the conformational integrity of CCR5 and CXCR4, and in these studies BCD treatment prevented the cell surface binding of antibodies specific for distinct CCR5 epitopes (Nguyen and Taub, 2002a, 2002b). Molecular simulations, and work with model membranes of defined composition show that the integrity of lipid raft microdomains is very sensitive to minor changes in overall cholesterol concentrations (Risselada and Marrink, 2008). Most likely, cholesterol depletion in macrophages disrupts lipid rafts, thereby redistributing raft-associated proteins such as CCR5 to other membrane domains. It is possible that, in this new context, their conformation is altered to one that is no longer recognised by antibodies, and unable to sustain HIV entry.

Nystatin and filipin complex modify cholesterol organisation within cells, but do not affect overall cholesterol concentration, and have a differing effect on HIV receptor levels. Nystatin significantly decreases CD4 and CCR5 surface expression but also significantly decreased non-raft associated CD71, implying that effect of nystatin may not be restricted to proteins located in cholesterol rich domains. Conversely, filipin complex treatment significantly reduced CD4 expression levels and slightly decreased CCR5 levels but did not appear to have such a dramatic effect on the expression of other membrane proteins. The effects of cholesterol sequesteration on the surface expression of the HIV receptors, after nystatin and filipin treatment, have not been investigated before. Disruption of cholesterol biosynthesis with Lovastatin also reduced the surface expression of the HIV receptors on macrophages but not by significant levels. This is in contrast to observations with CD4 T lymphocytes, in which Lovastatin down-modulated the mRNA and cell surface protein expression of CCR5 (but not CD4 or CXCR4), resulting in reduced HIV-1 infection (Nabatov et al., 2007).

The sensitivity of virus entry to depletion of cellular cholesterol could be explained by the association of the viral receptors, CD4 and CCR5, with lipid rafts. The tightly structured cholesterol-rich lipid rafts, known as DRMs, can be isolated in the presence of ice cold 1% Triton X-100 and separated by ultracentrifugation. Macrophage CD4 and CCR5 were found in the DRM fractions along with flotillin-1 whose location in these membranes is well known. In contrast, CD71 was found in the soluble fractions reflecting its association with the non-raft membranes. Decreased HIV receptor, but not CD71, expression after MβCD treatment supports the notion that the HIV receptors are located in cholesterol-rich domains in macrophages. We provide additional evidence for the involvement of lipid rafts in virus entry by visualising virus uptake in parallel with a fluorescent marker of lipid rafts. At 20 min post infection, approximately a third of all cell-associated virions were co-localised with lipid raft membranes in punctate structures that appear to have been internalised and may resemble an endosomal compartment.

CD4 association with DRMs has been established in many different cell types and we can show this to be true for macrophage CD4 (Kozak et al., 2002; Liao et al., 2001; Nguyen et al., 2005; Percherancier et al.,

2003; Popik et al., 2002). CCR5 has been reported to be present in lipid rafts in cell lines artificially over-expressing this receptor (Manes et al., 1999; Popik et al., 2002), but not in primary CD4 T lymphocytes, where this receptor was found in non-raft membrane domains (Percher-ancier et al., 2003). Here, we show that in macrophages, CCR5 is also present in or associated with lipid rafts, and both receptors required for macrophage-tropic virus entry are localised in similar membrane domains. This is in agreement with electron micrographs showing CD4 and CCR5 (but not CXCR4) localised on the outer membranes of microvilli and blebs in macrophages, often in closely apposed microdomains (Singer et al., 2001). However, despite existing together in DRMs it is possible that further aggregation of receptors is still required and may occur upon gp120 binding.

The most likely explanation for the inhibition of productive HIV entry into cholesterol-depleted macrophages is that redistribution of (or conformational changes to) the HIV receptors render the macrophages unsusceptible to HIV infection. It is plausible that manipulation of membrane cholesterol may inhibit entry by other mechanisms, of which we suggest five possibilities. 1) Depletion of macrophage cholesterol may alter the lipid composition of the cellular plasma membrane so that it is no longer competent for fusion with the HIV lipid envelope. 2) Disruption of membrane rafts may prevent receptor migration and co-localisation. Both CD4 and CCR5 are mobile within cellular membranes (shown in CHO cells) with the chemokine receptor being significantly more mobile than CD4 and requiring membrane cholesterol for this mobility (Steffens and Hope, 2004). 3) Signal transduction pathways induced upon CCR5 and envelope engagement may be required for virus entry (Harmon and Ratner, 2008). Cholesterol has been shown to be essential for CCR5 signalling and its depletion may prevent these important signalling events in macrophages (Cardaba et al., 2008). 4) HIV entry into macrophages may proceed by macropinocytosis, and cholesterol-depletion may block this uptake pathway in macrophages, as has been reported in brain microvascular endothelia (Liu et al., 2002). 5) Lovastatin may inhibit HIV entry by another mechanism, as it has been shown that blockade of HMG CoA reductase impedes prenylation of small Rho GTPases that may be involved in post-entry signalling events (del Real et al., 2004), or by diminishing HIV-1 attachment to target cells by suppressing ICAM1 LFA1 interactions (Giguere and Tremblay, 2004). However, addressing these possibilities to determine the exact role of cholesterol will be challenging, especially using infectious macro-phage-tropic virus and primary macrophages. Our study highlights the importance of cholesterol and lipid rafts in macrophage HIV infection. This provides further support for the use of cholesterol lowering pharmacological agents as anti-retroviral therapy by con-firming their anti-viral effect in another pathogenically significant HIV target cell, the macrophage.

Materials and methods

Cell culture and virus stocks

Peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll-Paque Plus (GE Healthcare) density gradient centrifugation from the blood of healthy donors. Monocytes were isolated by CD14 positive selection using anti-CD14 magnetic beads (Miltenyi Biotec) according to the manufacturer's instructions. Monocytes were seeded at a density of 1.5×10^5 cells/cm^2 in 6-well plates, 12-well plates, 24-well plates or T75 flasks. Culture media referred to as RPMI FCS M-CSF: consisted of RPMI 1640 (PAA) with 10% Fetal Calf Serum (FCS; PAA), 2 mM L-glutamine (PAA), 100 U/mL penicillin and 100 μg/mL streptomycin (PAA), supplemented with 100 ng/mL (approximately 1.7×10^4 U/mL) recombinant human M-CSF (R&D Systems). Monocytes were differentiated for 7–9 days prior to use. HIV-1 BaL was obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH, from S. Gartner (Gartner et al., 1986) and viral stocks were generated in differen-tiated, unstimulated PBMCs. Infected cell supernatants were har-vested 14–21 days after infection and frozen for use in infectivity assays. Virus stocks with proviral DNA removed were generated for qPCR experiments by treatment with 100 μg/mL DNase I (Sigma). To generate single round replication competent virus, 293T cells were regularly passaged in DMEM (PAA) with 10% FCS, 2 mM L-glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin. Cells seeded into T75 flasks were transfected with pNL4.3lucR-E- (2 μg) and pJRFL Env (1.5 μg) using Fugene 6 according to manufacturers instructions. The NL4.3 JRFL virus was harvested 48 h later, passed through a 0.44 μm filter and frozen.

Treatment with cholesterol disrupting agents

Macrophages were washed once with serum-free RPMI, and treated for 1 h at 37 °C with varying concentrations of MβCD, filipin complex, or nystatin (all from Sigma) diluted in serum-free RPMI. As Nystatin is dissolved in DMSO (MβCD and Filipin in water), to exclude for any effects of this solvent the control cells were incubated in the same dilution of DMSO as used for Nystatin. After incubation, cells were washed once with serum-free RPMI prior to infection or flow cytometry analysis. Following infection, cells were cultured in RPMI FCS M-CSF. Water soluble cholesterol (400 μg/mL, Sigma) was added for a further 1 h where stated. For Lovastatin treatment, macrophages were washed once with serum-free RPMI, before the addition of varying concentrations of Lovastatin (Sigma) with or without mevalonate (Sigma), diluted in RPMI with 10% lipoprotein-deficient serum (Sigma). Cells were left for 4 days at 37 °C before infection or flow cytometry analysis. Infected cells were cultured in RPMI FCS M-CSF following the infection procedure.

Cell viability assays and cholesterol quantification

To determine cell viability following treatment with cholesterol disrupting agents, macrophages were incubated with CellTiter 96® AQueous One Solution Cell Proliferation Assay reagent (Promega) diluted 1/6 in serum-free RPMI or RPMI with lipoprotein-deficient serum containing Lovastatin or mevalonate, for 1 h at 37 °C. In triplicate, 100 μL samples of the supernatant were then transferred to a 96 well plate and the absorbance read at 490 nm. Background absorbance readings from reagent and media alone were deducted and the values expressed as a percentage of untreated cells. To quantify cellular cholesterol levels, drug treated or control macro-phages were detached by pipetting with PBS containing 5 mM EDTA and 12 mM lidocaine (Sigma), on ice. Cells were lysed and the cholesterol was extracted as previously described (Chung et al., 2005). The amount of cholesterol was assayed with Amplex Red cholesterol assay kit (Molecular Probes) according to the manufacturer's instruc-tions and the concentrations were determined from a standard curve and normalised to the number of cells.

Quantitative PCR

Macrophages were infected in 12-well plates with 500 μL of DNase I treated HIV-1 BaL by spinoculation in a sealed plate spinning centrifuge at 2000 ×g for 90 min at 37 °C. The virus inoculum was removed, cells were overlaid with RPMI FCS M-CSF and infections were left to proceed for 28–30 h. Cells were harvested by scraping and DNA was extracted using DNeasy Blood and Tissue Kit (Qiagen) according to the manufacturer's instructions. qPCR reactions to detect late stage reverse transcription products were carried out as previously described (Butler et al., 2001; Cohen et al., 2008). HIV-1 standards consisting of pNL4.3LucR^-E^- HIV-1 backbone plasmid diluted with 30 ng/μL HeLa DNA were used in duplicate at dilutions ranging from 10 to 1×10^7 copies, and actin standards consisting of

human Xsomal DNA (Eurogentec) were used at 10-fold dilutions from 80 ng.

ELISA for p24 antigen

To measure multiple rounds of HIV infection, macrophages were infected in 12-well plates with HIV-1 BaL for 2 h at 37 °C. The inoculum was removed and replaced with RPMI FCS M-CSF. Over a 14–16 day period, supernatant samples were taken at intervals and kept at −80 °C. For analysis of HIV-1 binding, macrophages were incubated with HIV BaL for 2 h on ice. The cells were then washed 5 times in ice-cold PBS to remove unbound virus, and lysed in 200 μL TES (1×Tris buffered saline [TBS], 1% Empigen [Fluka], 10% FCS, 0.05% Tween 20) before heat inactivation. For analysis of HIV-1 production, samples from infected cells were diluted in TES and heat inactivated. To create a standard curve, recombinant p24 protein (Aalto) was diluted in doubling dilutions in TES. Costar EIA/RIA flat bottom high binding 96-well plates (Corning) were pre-coated with 10 μg/mL sheep anti-HIV-1-p24 Gag antibody (clone D7320, Aalto) diluted in 0.05 M carbonate-bicarbonate buffer pH 9.6 (Sigma) and blocked with 2% BSA (Sigma) in TES. Diluted samples or standards were added to the plates and after overnight incubation at room temperature, bound p24 was detected using a biotinylated mouse anti-HIV-1-p24 Gag antibody (clone EH12EI, Centre for AIDS reagents, NIBSC) at 0.23 μg/mL in TT/SS (TBS, 20% FCS, 0.05% Tween 20) followed by 0.625 μg/mL streptavidin-HRP (Pierce). Plates were developed using TMB substrate (Pierce), the reaction was stopped with 1 M H_2SO_4 (Sigma) and plates were then read using a Molecular Devices *E max* precision microplate reader and SoftMax Pro software version 4.0. GraphPad Prism software version 4 was used to create standard curves (2-site binding equation) and to calculate unknown values.

Antibody staining and flow cytometry

Detached macrophages were washed once with FACS buffer (PBS, 0.01% NaN_3, 1% FCS, 10 μg/mL human IgG), and 1×10^5 cells were incubated with 5 μg/mL directly conjugated mouse anti-human antibodies to CD4 (clone 1180), CCR5 (clone 45531), CXCR4 (clone 44717.111) or appropriate isotype control (all R & D systems). As a control, the expression of the non-raft associated protein transferrin receptor was also measured using mouse anti-human CD71 (clone RVS10) with isotype control mouse IgG1 (clone 203) (both diluted 1/10 and from Immunotools). Cells were fixed in 4% formaldehyde. Flow cytometry was carried out using a Becton-Dickinson FACSCalibur flow cytometer with 10000 events collected and data analysed using FlowJo software, version 7.1.3.

Cholera toxin B binding

Macrophages were washed once in serum-free RPMI, and incubated with CTB-FITC (Sigma) at 10 μg/mL in serum-free RPMI, for 30 min on ice or at 37 °C. Detached cells were then analysed by flow cytometry as described. To visualise virus and CTB, HIV-1 capable of a single round of infection NL4.3 JRFL was spinoculated (1 h at 4 °C at 700 ×*g*) onto day 7 macrophage monolayers seeded onto glass coverslips in 24 well plates. Cells were washed with ice-cold PBS and incubated in RPMI containing 10 μg/mL CTB-FITC for 20 min at 37 °C. Cells were fixed in 4% formaldehyde, quenched in 80 mM glycine in PBS and permeabilised in buffer containing 1% FCS, 0.1% saponin and human IgG 10 μg/mL in PBS. To detect virus, cells were stained with mouse anti-HIV p17 diluted 1/500 (clone 4C9, Centre for AIDS Reagents, NIBSC) followed by Alexa Fluor 647 conjugated goat anti-mouse IgG2a antibody diluted 1/400 (clone A21131, Invitrogen). Coverslips were mounted on slides using mowiol and images collected using a Zeiss Pascal Microscope.

Membrane fractionation and western blotting

Macrophages (1×10^7) were harvested by scraping, pelleted and suspended in 1 mL H buffer (10 mM sodium Hepes, pH 7.2, 250 mM sucrose, 2 mM $MgCl_2$, 10 mM NaF, and 1 mM vanadate) containing protease inhibitors (Roche). The cells were nitrogen-cavitated using a nitrogen cavitation bomb (model 4639; Parr Instrument Company) equilibrated at 4 °C, 50 bar for 10 min (Harder and Kuhn, 2000). Cells were further disrupted by adding 1 mL HNE buffer (10 mM Hepes, pH 7.0, 150 mM NaCl, and 5 mM EDTA), passaged 15 times through a 23G syringe and 1% Triton X-100 was added before cells were incubated for 1 h at 4 °C. A 200 μL extract containing approximately 1×10^6 cells was adjusted to 45% Optiprep™ (Nycomed Pharma) and transferred to an SW55 centrifuge tube (Beckman Coulter) and overlaid with another 200 μL 45% Optiprep to make a 1 mL layer. This was overlaid with 2 mL of 35% and 0% Optiprep in HNE and gradients were spun at 40,000 rpm for 3 h at 4 °C. Nine fractions of 550 μL were collected and precipitated in an equal vol of 20% trichloroacetic acid, washed twice in ice-cold acetone and resuspended in 8 M urea and SDS sample buffer. Equal volumes of each fraction were run on a 10% SDS-PAGE protein gel with Biorad precision plus protein standards. Protein was transferred onto 0.2 μm PVDF membranes, and membranes were incubated with primary mouse anti-human antibodies to CD4 (clone 34915, 2.5 μg/mL R & D systems) CCR5 (clone CTC5, 2.5 μg/mL R & D systems), flotillin-1 (clone 18, 1.25 μg/mL, BD biosciences) and transferrin receptor (clone 2, 1.25 μg/mL, BD biosciences). To detect primary antibody binding, membranes were incubated with goat anti-mouse IgG (Fab specific) antibody conjugated to alkaline phosphatase (Sigma) diluted 1/30,000. Protein was detected using ECF substrate (Pierce), and membranes were imaged using STORM imager.

Statistical analysis

Statistical analysis was performed by two-tailed paired *t*-test (unpaired with 2 replicates) using GraphPad Prism version 4. Stars indicate the *p* value: *=significant $p=0.05$–0.01, **=very significant $p=0.01$–0.001, ***=extremely significant $p<0.001$.

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