Antibody-dependent enhancement of dengue virus infection in U937 cells requires cholesterol-rich membrane microdomains

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Dengue virus (DENV) is the causative agent of dengue fever and the more severe forms of the infection known as dengue haemorrhagic fever and dengue shock syndrome (DHF/DSS). Secondary infections with a serotype different from the primary infection are considered a risk factor for the development of DHF/DSS. One explanation for the increased risk of DHF/DSS development after heterologous secondary infections is the antibody-dependent enhancement (ADE) hypothesis. This hypothesis postulates that pre-existing non-neutralizing antibodies will form immune complexes with the new serotype-infected virus that in turn will have enhanced capacity to infect macrophages and other Fcγ receptor (FcγR)-bearing cells. Despite the evidence supporting the ADE hypothesis, the molecular mechanisms of ADE are not fully understood. In this work, we present evidence which indicates that intact lipid rafts are required for the ADE infection of U937 cells with DENV. Flow cytometry analysis to measure the percentage of infected cells showed that treatment of differentiated U937 cells with nystatin (30 μg ml−1), filipin (10 μg ml−1) or β-methyl cyclodextrin (30 mM) significantly reduces (P<0.05) the ADE of DENV-4 infection in vitro without any effect on viability or the number of FcγR-bearing cells. Later cholesterol replenishment by supplementing treated cell cultures with bovine fetal serum for 24 h re-established lipid raft integrity and reversed the alteration of the ADE in vitro (P<0.05). Our results suggest that ADE of U937 infection by DENV requires the presence of cholesterol and cholesterol-rich membrane microdomains.

INTRODUCTION

Dengue virus (DENV) belongs to the genus Flavivirus within the family Flaviviridae and comprises four serotypes (DENV1–DENV4). The virion is composed of three structural proteins: the C protein, which forms the nucleocapsid containing the viral genome, and proteins M and E, which are inserted into the lipid membrane surrounding the nucleocapsid. The genome of DENV is composed of a single-stranded positive-strand RNA of approximately 11 kb. The E protein is exposed on the virion surface and is responsible for the binding and entry of virus into the cell. In addition, the E protein is the virion neutralization antigen and is responsible for the genetic variability that leads to the four serotypes. The DENV genome additionally encodes for seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5), which, along with the virion structural proteins, are all derived from the proteolytic processing of a polyprotein (Lindenbach & Rice, 2003).

Nowadays, DENV is the most important causative agent of human viral disease transmitted by mosquitoes. The disease caused by DENV presents in three different clinical forms: dengue fever (DF), dengue haemorrhagic fever (DHF) and dengue shock syndrome (DSS). DF is characterized by high fever, headache, retro-orbital pain, myalgia and arthralgia among other symptoms. It is a self-limited illness and most of those affected recover after 5 or 6 days. However, in some patients, the disease can evolve into a more severe form, DHF/DSS. This is characterized by an increase in vascular permeability (plasma leakage), thrombocytopenia and haemorrhages which, if not controlled, can result in hypovolemia, shock and death. Due mainly to the most severe forms of the disease, it is estimated that the DENV causes more than half a million hospitalizations annually, with mortality rates ranging between 1 and 5 %, mainly in children (Gubler, 2006). Despite the disease burden, the physiological basis and mechanisms involved in the pathogenesis of the severe forms of dengue infection remain largely unknown.
A risk factor for DHF is to suffer secondary infections with a viral serotype different from the primary infection. It has been postulated that the presence of antibodies at subneutralizing concentrations, from previous infection, facilitate the infection of macrophages and other cells bearing Fcγ receptors (FcγRs), promoting viral replication (Kliks et al., 1988; Vaughn et al., 2000). This risk factor is known as antibody-dependent enhancement (ADE) and there is abundant epidemiological, experimental and clinical evidence that points to the ADE as a major risk factor for developing DHF (Huang et al., 2006; Rodrigo et al., 2006; Goncalvez et al., 2007; Chareonsirisuthigul et al., 2007; Jin, 2008).

One of the most important target cells during dengue infection are cells of the monocyte lineage (Kyle et al., 2007). These cells bear FcγRs, thus are likely to be infected via ADE. Previously, both FcγRI and FcγRIIA (CD64) have been shown to facilitate ADE infection of DENVs in human macrophage-like cells or in COS-7-transfected cells (Kontny et al., 1988; Littaua et al., 1990; Rodrigo et al., 2006). The macrophage infection via ADE results in increased levels of viral replication and viral progeny, and increased viraemia (Jin, 2008; Kliks et al., 1989). While it is clear that FcγRs are necessary to facilitate the infection of macrophages via ADE, the mechanisms are not completely understood.

It is known that FcγRs associate with lipids rafts upon IgG binding, and this association has been reported as essential for efficient signalling (García-García et al., 2007; Kono et al., 2002; Kwiatkowska & Sobota, 2001). Lipid rafts are small (10–200 nm), highly dynamic, cholesterol- and sphingolipid-enriched domains of the plasma membrane, characterized by their insolubility following cold detergent extraction and because they can act as docking sites for several intracellular signalling proteins (Hancock, 2006; Simons & Toomre, 2000). In addition, a large number of viruses invade cells using lipid rafts, where some of the raft-associated proteins function as viral receptors as well (Suzuki & Suzuki, 2006). For DENV, the infection of human macrophages in the absence of antibodies requires the presence of cholesterol, given that drugs such as methyl-β-cyclodextrin (MβCD), which sequester cholesterol from plasma membrane, inhibit this process (Reyes-del Valle et al., 2005). However, the relevance of cholesterol and lipid rafts in the ADE mechanism is unknown. In this article, we present evidence which indicates that the presence of cholesterol on the surface of macrophages is required for ADE of infection of DENV, suggesting that the presence of FcγR in cholesterol-rich membrane microdomains is required for ADE of DENV infection.

**RESULTS**

**ADE in U937 cells**

Human macrophages, among the important host cells during DENV infection in humans, can be infected by two different mechanisms. In a primary infection, DENV uses the conventional entry mechanism mediated by viral receptors and coreceptors (Reyes-del Valle et al., 2005; Chen, et al., 2008). However, during secondary infections, the virus can form virus–antibody complexes that can enter into the cell through FcγRs (Kliks et al., 1989). In vitro experimental evidence suggests that both FcγR1 and -RII are mediators of ADE of DENV infection (Rodrigo et al., 2006; Kou et al., 2008). Thus, our first step was to evaluate the presence of FcγRI and -RII on the surface of differentiated U937 cells by flow cytometry using antibodies directed against CD32 (FcγRII) and CD64 (FcγRI). Approximately 75% of cells expressed CD32 while only 4% expressed CD64 (Fig. 1), suggesting that CD32 may be the main mediator to ADE of DENV infection in differentiated U937 cells. Following this, the ADE process was induced in vitro using U937 cells and a monoclonal antibody (mAb) directed against the E protein. Differentiated U937 cells were infected with DENV type 4 at an m.o.i. of 1 and 3 in the presence of serial dilutions of anti-E protein antibodies or in the presence of an irrelevant antibody (from 10¹ to 10¹¹). Fig. 2(a) shows that at the subneutralizing concentrations of 10¹⁰, a significantly higher amount of infected cells could be observed in the presence of anti-E protein antibody compared with the amount of infected cells detected in the absence of antibody. In addition, no increase in the amount of infected cells was observed when the infection was carried out in the presence of an irrelevant antibody (data not shown). The ADE of infection was observed with both multiplicities of infection used; however, the effect was more marked when an m.o.i. of 3 was used.

To confirm that the observed augment in the infection of U937 cells was due to ADE of infection and was mediated by the FcγRII, experiments were carried out using anti-CD32 antibodies to block the receptor. A significant reduction in the amount of infected cells was observed.

![Fig. 1. Cell surface expression of FcγRI (CD64) and FcγRII (CD32) in differentiated (hatched bars) and undifferentiated (white bars) U937 cells. Non-permeabilized cells were stained with mAbs to CD32 and CD64 as primary antibodies, followed by an FITC-conjugated goat anti-mouse IgG mAb. Human macrophages were included as positive controls (black bars). Cells were assayed by FACS. Results from three different experiments (mean±s.d) are shown.](http://vir.sgmjournals.org)
when U937 cells were pre-incubated with the anti-CD32 antibodies prior to infection in the presence of facilitating antibodies (Fig. 2b), thus confirming that the increase in DENV infection observed in the presence of subneutralizing amounts of anti-E antibodies was the consequence of the ADE process.

To confirm that ADE of DENV infection in differentiated U937 cells was predominantly mediated by CD32, blockage of the ADE process was also attempted in the presence of anti-CD64 antibodies. As expected, no reduction in ADE infection was observed (Supplementary Fig. S1, available in JGV Online), supporting the prediction that CD32 is the main mediator in ADE of DENV infection in this cell type.

**Cholesterol depletion inhibits ADE of DENV**

To determine the importance of cholesterol in the ADE process, U937 cells were treated with different drugs that reduce the cholesterol concentration and inhibit raft formation, such as nystatin, filipin and MβCD. Different drug concentrations and treatment conditions were tested, but at the selected concentrations of nystatin (30 µg ml⁻¹), filipin (10 µg ml⁻¹) and MβCD (30 mM), a significant reduction in cholesterol was observed without a significant effect on cell viability (Fig. 3a). In addition, drug treatments did not affect the proliferation of infected or uninfected cells (data not shown). To confirm that drug treatment was indeed causing lipid raft disruption, untreated and treated cells were incubated with cholera toxin subunit B coupled to FITC. The toxin interacts with ganglioside GM1 present on plasma membrane lipid rafts, and allows their visualization by confocal microscopy (Blank et al., 2007). Cell treatment with any of the three drugs used inhibited the ability of cholera toxin subunit B to bind to the cell surface (Fig. 3b), thus confirming that nystatin, filipin and MβCD disrupted lipid rafts on the surface of U937 cells.

To determine the effect of raft disruption in ADE infection, U937 cells were untreated or treated with each of the three drugs and infected with DENV alone or in the presence of anti-E protein antibodies. Infection was monitored by flow cytometry. It was clearly observed that treatment with all three drugs, significantly inhibited the ADE infection in U937 cells (Fig. 4), suggesting that lipid rafts and cholesterol are important elements during DENV infection of U937 cells mediated by facilitating antibodies. Moreover, an important reduction in DENV infection was also observed in cells infected with DENV alone, confirming our previous results that indicated that cholesterol is important for viral infection in U937 cells (Reyes-del Valle et al., 2005).

Since it has been described that FcγRII is relocated to lipid rafts as it functions (Kwiatkowska & Sobota, 2001), one possible explanation for the inhibition observed in ADE infection in drug-treated cells is that the drug treatments cause a reduction in the amount of FcγRII present on the cell surface. To exclude this possibility, the amount of FcγRII on the surface of untreated and drug-treated cells was analysed by flow cytometry. Fig. 5(a, b) shows that no significant differences in either the amount of FcγRII-positive cells or in the fluorescence intensity between untreated and drug-treated cells were observed, thus indicating that the inhibition in ADE infection in drug-treated cells was not due to a reduction in the number of FcγR-bearing cells or a decrease in the amount of the receptor on the cell surface, but due to a reduction in the amount of cholesterol and lipid rafts on the cell plasma membrane.

On the other hand, to rule out the possibility that the reduction of infected cells in the presence of facilitating antibodies after drug treatment may be due to the
inhibition of another entry pathway such as receptor-mediated endocytosis, the endocytosis of transferrin, which enters into cells via clathrin-coated pits, was evaluated. No effect on the endocytosis of transferrin by U937 cells was observed after cell treatment with any of the cholesterol-disrupting drugs used (Supplementary Fig. S2).

Cholesterol replenishment re-establishes the lipid raft integrity and ADE infection in U937 cells

To confirm the role of cholesterol and lipid rafts in ADE infection in U937 cells, drug-treated cells were grown in the presence of fetal bovine serum (FBS) for 24 h to re-establish cholesterol levels and lipid rafts on the cell surface. To confirm this conjecture, the lipid raft integrity was analysed by confocal microscopy after staining with cholera toxin B subunit coupled to FITC. It was observed that the cholesterol replenishment re-established the lipid raft integrity in drug-treated U937 cells (Fig. 6a).

In the drug-treated cells where cholesterol re-established ADE, infection was monitored by flow cytometry. The presence of cholesterol and the restoration of lipid rafts in drug-treated U937 cells significantly restored ADE (Fig. 6b), indicating that cholesterol and lipid rafts are necessary for ADE infection of U937 cells. Moreover, cholesterol

**Fig. 3.** (a) Cholesterol reduction after treatment of U937 cells with cholesterol-disrupting drugs. Differentiated U937 cell cultures were treated for 2 h with the cholesterol-reducing drugs nystatin, filipin and MβCD. The cholesterol concentration after treatment was determined spectrophotometrically using a commercial kit and linear regression analysis was done. The cell viability after drug treatment was determined by trypan blue exclusion assay. The cholesterol concentration relative to that in non-treated cells (grey bars) and the cell viability compared with untreated cells (white bars) are shown. Data represent the mean±SD of three individual experiments. Significant differences are indicated by an asterisk (*P<0.05). (b) Visualization of lipid rafts in U937 cells by immunofluorescence. Differentiated U937 cells were treated with cholesterol-reducing drugs as described in (a). Lipid rafts were visualized after cell staining with cholera toxin B-subunit conjugated to FITC (green). Nuclei were counter-stained with DAPI (blue). Untreated cells were used as a control of the integrity of the lipid rafts.

**Fig. 4.** Cholesterol depletion inhibits ADE of DENV infection in U937 cells. Differentiated U937 cells were treated for 2 h with the cholesterol-reducing drugs nystatin, filipin and MβCD. Afterwards, cells were infected with DENV-4 (m.o.i. of 3) complexed with 10-fold dilutions of anti-E mAb 4G2 for 2 h. Uninfected cells and cells infected without antibodies were included as controls. At 72 h p.i., the percentage of U937-infected cells was determined by FACS assay. A total of 10 000 events were counted. Data represent the mean±SD of three individual experiments. Significant differences are indicated by an asterisk (*P<0.05).
replenishment also restored DENV infection through the natural receptor (in the absence of facilitating antibodies) since the amount of cells infected in the absence of cholesterol was lower compared with the cells infected in the presence of cholesterol (Fig. 6b).

DISCUSSION

Determinants that predispose infected patients to develop DHF have been partially identified. However, since DHF/DSS often occurs in patients with second, heterotypic dengue infections or in infants with maternally transferred dengue immunity (Halstead et al., 1970; Kliks et al., 1988), ADE has been proposed as an underlying pathogenic mechanism of DHF/DSS (Halstead et al., 1970). ADE-mediated infection involves the entry of virus–antibody complexes into monocytic cells via FcγR, resulting in a significantly enhanced virus titre (Kurane et al., 1990; Huang et al., 2006; Goncalvez et al., 2007; Charoen-sirisuthigul et al., 2007; Sullivan, 2001). This mechanism has been demonstrated in vitro using dengue immune sera or mAbs and cells of monocytic and, recently, B lymphocytic lineages bearing FcγR (Littaua, et al., 1990; Morens et al., 1987; Lin et al., 2002; Brandt et al., 1982), as well as in vivo in a monkey model (Goncalvez et al., 2007). ADE of dengue infection has been detected with U937 cells, which express FcγRI and FcγRII receptors (Mady et al., 1991; Littaua et al., 1990); FcγRII is more effective than FcγRI in mediating enhancement of immune complex infectivity (Rodrigo et al., 2006).

Phagocytosis through FcγR is a very important process in phagocytic cells and is characterized by the dramatic, actin-dependent extension of the plasma membrane around particles. It is followed by secondary activity such as the production of superoxide and the release of inflammatory cytokines from the phagocyte (Aderem & Underhill, 1999).

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Fig. 5. Cholesterol depletion does not alter the FcγR expression on U937 cells. Differentiated U937 cells were treated or not with cholesterol-reducing drugs as indicated in the legend to Fig. 3. Afterwards, non-permeabilized cells were stained with an anti-human FcγRIIA (CD32) mAb as primary antibody and an anti IgG-FITC conjugate as secondary antibody in order to quantify the number of FcγR expressing cells by FACS assay. Human macrophages obtained from peripheral blood and undifferentiated U937 cells were included as controls. A total of 10 000 events were counted. (a) Histograms of one experiment to show cell fluorescence levels. (b) Percentage of cells expressing FcγRIIA (CD32). Results of three individual experiments are shown. Data represent the mean ± SD.
The interaction of FcR and IgG triggers rapid phosphorylation of specific tyrosine residues in the receptor within immunoreceptor tyrosine-based activation motifs (ITAMs) (Garcia-Garcia & Rosales, 2002). All of these signals can converge to locally reorganize the actin cytoskeleton at a phagosome. Recent advances have demonstrated the complexity of phagocytic signalling, such as the involvement of phosphoinositide lipids, movement of FcR to lipid rafts and multicomponent signalling complexes in transducing signals from the phagocytic receptor to the cytoskeleton (Diakonova et al., 2002; May & Machesky, 2001; Bourmazos et al., 2009). Since one of the elements that has been described as important in the FcR function is the translocation of the FcR to lipid rafts, the importance of rafts and cholesterol in ADE infection in U937 cells was evaluated. Our data clearly show that ADE infection of U937 cells with DENV requires the presence of cholesterol, given that treatment with drugs that sequester cholesterol and avoid lipid raft formation inhibit ADE infection. In support of this statement, we confirm that cholesterol replenishment to the cells and re-establishment of lipid rafts on the surface of U937 cells restored ADE infection, confirming that ADE infection requires cholesterol and lipid raft formation. Additionally, we corroborate that the inhibition of ADE infection after cholesterol-reducing drug treatment was not due to a reduction in the number of FcR-IIIR present on the cell surface or to a reduction in the number of FcR-IIIR-bearing cells, supporting our conclusion. Since the drugs were added before DENV, it is logical to expect that early events were mainly affected; however, the treatment may also affect internal membranes, used during DENV replication and morphogenesis (Welsch et al., 2009).

It is likely that the reduction in cholesterol on the cell surface induced by the drugs impaired lipid raft formation. This process would in turn prevent translocation of FcR-IIIR to lipid rafts, a process that is required for FcR-IIIR function, because the components necessary for phagosome signalling are recruited to lipid rafts. Although it has been described that DENV uses the clathrin-mediated endocytosis pathway in several cell types in the absence of facilitating antibodies (Acosta et al., 2009; Mosso et al., 2008), this pathway seems to not be relevant for immunocomplexes because endocytosis of opsonized particles by FcR-IIIR (CD32) involves lipid raft-induced receptor clustering which leads to signalling through ITAM (Moi et al., 2010).

In this respect, it is well-known that after recognition and binding of IgG complexes to FcR, the receptor becomes clustered and recruited to lipid raft microdomains (Barabé et al., 2002; Rollet-Labelle et al., 2004; Kwiatkowska et al., 2003). The association of a number of immune receptors with lipid rafts is necessary for receptor signalling and functional responses upon ligand binding (Simons, & Ikonen, 1997). Moreover, it has been demonstrated previously that FcR–lipid raft interactions are essential for efficient signalling events initiated following receptor cross-linking (Katsumata, et al. 2001; Kwiatkowska & Sobota, 2001). Mutations in key residues within the transmembrane region of FcR that alter its association with lipid rafts have been reported to have a negative impact on its ability to transduce intracellular signals (Garcia-Garcia et al., 2007). Thus, it is reasonable to propose that translocation of FcR to lipid rafts is an important step for ADE infection. Moreover, this translocation may also be important for the binding of virus–antibody complexes to U937 cells. Enhancement of the DENV immune complex infectivity appears to be mediated mainly through FcR-IIIR (Rodrigo et al., 2006). Recently, it
has been reported that disruption of the lipid raft structure following depletion or sequestration of membrane cholesterol greatly inhibited FcγRII-mediated membrane IgG binding. Furthermore, specific FcγR mutants, which show reduced association with lipid rafts, displayed decreased levels of IgG binding compared with wild-type FcγR. In contrast, constitutively lipid raft-associated (GPI-anchored CD32a) exhibited an increased capacity for IgG binding compared with the full-length transmembrane FcγR (Bournazos et al., 2009). Thus, in the absence of lipid rafts, the initial low affinity interactions between DENV immune complexes and FcγR may never become stable enough, leading to complex dissociation. Additionally, deterrence of receptor clustering within the lipid raft will prevent activation of signalling pathways required for complex internalization. These two mechanisms are not mutually exclusive and may provide an explanation for the observed reduction in ADE in U937 cells after lipid raft disruption.

The role of lipid rafts in DENV infection in the absence of facilitating antibodies was also observed in our assays, as described previously (Reyes-del Valle et al., 2005). Moreover, lipid rafts are also required for different viruses in processes such as entry, signalling, replication or morphogenesis (Kapadia et al., 2007; Mackenzie et al., 2007; Lee et al., 2008; Liao et al., 2001; Barman et al., 2004). Thus, it is possible that DENV uses rafts to recruit some molecules required for viral entry or signalling. It is remarkable that the proteins identified so far as dengue attachment and receptor molecules, such as DC-SIGN, heparan sulphate, GRP78, HSP90 and HSP70, are all associated with lipid rafts (Chu et al., 2004; Cambi et al., 2004; Philippova et al., 2008; Reyes-del Valle et al., 2005). Thus, characterization of the molecules that are relocated to lipid raft platforms during viral entry through both its natural receptor or FcγR will be very helpful to determine the events and signalling pathways activated at the early stages of dengue infection.

Recently, it has become evident that cholesterol plays an important role in the replicative cycle of several flaviviruses. For DENV, it was reported that cholesterol metabolism is required for genome replication, (Rothwell et al., 2009). On the other hand, levels of total plasma cholesterol, and high- and low-density lipoproteins were found to be significantly decreased in patients with severe cases of DHF (van Gorp et al., 2002). Statins are currently used as part of the treatment of hepatitis C virus and human immunodeficiency virus infections (del Real et al., 2004; Ikeda et al., 2006). It will be of considerable interest to investigate whether the administration of statins or other drugs that affect cholesterol biosynthesis can modulate disease outcome in DENV infections.

In summary, our results suggest that cholesterol and lipid rafts present on the plasma membrane play an important role during ADE infection of DENVs in macrophages. However, the molecular basis of the cholesterol requirement in ADE of DENV infection is not yet fully understood. Future experiments are directed to dissect the steps followed by immune complexes binding to FcγR and the activated cellular signalling pathways.

**METHODS**

**Cell cultures and viral strain.** The human myelomonocytic cell line U937 (ATCC: CRL-1593.2) was grown in 1 x RPMI 1640 reduced serum medium (Invitrogen) supplemented with nonessential amino acids, 110 mg sodium pyruvate l−1, 4 mM l-glutamine, 5 % FBS (Invitrogen), 5 U penicillin ml−1 and 5 μg streptomycin ml−1 at 37 °C in 5 % CO2. The U937 cells were differentiated to macrophages by the addition of phorbol 12-myristate 13-acetate (PMA; Sigma Aldrich) at 160 nM final concentration, 48 h prior to infection. Treatment resulted in the differentiation of more than 60 % of U937 cells, as determined by measuring the surface expression of CD14, as a differentiation antigen of monocytes/macrophages, by flow cytometry (FACS) assay (data not shown). Propagation of the prototype DENV serotype 4 (H241 strain) was done in BALB/c suckling mice brains and its titre was determined by plaque assay in BHK-21 cells, as described previously (Mosso et al., 2008).

**Antibodies and reagents.** The flavivirus group-reactive mAb 4G2 (Henchal et al., 1985), directed against the E protein, was used to induce ADE of DENV infection and to detect infected cells by FACS. The FITC-conjugated F(ab′)2 goat anti-mouse IgG mAb (ZYMED) was used as secondary antibody to detect DENV infection by FACS. mAb type IgG2a anti-β-actin (Sigma-Aldrich) was used as a negative control of ADE in vitro. Mouse mAb anti-human CD14 (Santa Cruz Biotechnology) was used to evaluate the U937 differentiation. Anti-human FcγRII (CD32) mAb IV.3 (Mederax) and FcγRI (CD64) mAb 32.2 (Mederax) were a donation from Dr Carlos Rosales (Instituto de Investigaciones Biomédicas, UNAM, Mexico). Cholecalciferol subunit B FITC conjugate (Sigma-Aldrich) was used as a lipid raft marker according to the manufacturer’s recommendations. Transferrin Alexa Fluor 680 conjugate (Molecular probes) was used to evaluate the clathrin-endocytic pathway by immunofluorescence assays. Finally, MβCD (30 mM), nystatin (30 μg ml−1; Sigma-Aldrich) and filipin (10 μg ml−1; Invitrogen) were used as cholesterol-reducing drugs.

**Expression of FcγR on U937 cells.** The cell surface expression of FcγRII (CD32) and FcγRI (CD64) in differentiated and undifferentiated U937 cells was evaluated by flow cytometry. Briefly, cells were fixed with 4 % paraformaldehyde in cold PBS for 30 min at 4 °C and blocked with 10 % FBS in PBS, for 30 min at 4 °C. Cells were stained with mAbs to CD32 and CD64 for 1 h at 4 °C as primary antibodies, followed by an FITC-conjugated goat anti-mouse IgG mAb. Cells were assayed by FACS in a FACS Calibur (BD Immunocytometry Systems). The results were analysed using the software WinMDI, version 2.8. Ten thousand events were counted per sample of three independent trials. For some experiments, differentiated U937 cells were treated with cholesterol-disrupting drugs prior to evaluating the presence of CD32.

**ADE of DENV infection.** Virus–mAb complexes were pre-formed by mixing 10-fold dilutions of the mAb 4G2 with a constant amount of virus (m.o.i. of 1 or 3) for 30 min at 37 °C, in slow oscillation. The mixture was added to clumps of adherent, previously differentiated, U937 cells grown in six-well plates (2 x 105 cells per well), and left for 2 h. After infection, the cell cultures were washed once with acid glycine (pH=3) and twice with PBS, to remove non-internalized complexes. Finally, cells were supplemented with RPMI medium, incubated at 37 °C and 5 % CO2, and the infection was allowed to proceed for 72 h. Infections with viruses incubated with anti-β-actin...
mAbs were run in parallel as a negative control. To evaluate the participation of the FcRs during ADE in vitro, cell cultures were pretreated with anti-human FcRRII (CD32) or FcRRI (CD64) mAbs (Garcia-Garcia et al., 2007) for 30 min at 4 °C, before being infected with the virus–mAb complexes. The number of infected cells was determined by FACS.

Detection of infected U937 cells by flow cytomtery assay. To determine the number of U937 cells infected with DENV, cells were harvested and fixed with 4% paraformaldehyde in cold PBS for 30 min at 4 °C. After fixation, cells were permeabilized for 30 min at 4 °C with 0.1% Triton X-100 in PBS and blocked with 10% FBS in PBS, also for 30 min at 4 °C. After each step, cells were washed once with cold PBS by centrifugation at 1200 g for 6 min. Cells were stained with mAb 4G2 for 1 h at 4 °C as primary antibody, followed by an FITC-conjugated goat anti-mouse IgG mAb. Finally, cells were washed, resuspended in PBS and assayed by FACS in a FACS Calibur (BD Immuno cytometry Systems). The results were analysed using the software WinMDI, version 2.8. Ten thousand events were counted per sample of three independent trials.

Cholesterol depletion and replenishment of U937 cells. In order to evaluate the participation of the cholesterol-enriched membrane microdomains in the ADE of DENV, U937 cells grown in six-well plates were washed once with PBS and treated with cholesterol-reducing drugs as follows: MβCD (30 mM), nystatin (30 μg ml⁻¹) and filipin (10 μg ml⁻¹) for 2 h, at 37 °C and 5% CO₂. Drugs were diluted in DMSO. After treatment, cells were washed three times with PBS and infected with DENV (m.o.i. of 3) either complexed or not with mAbs for 2 h. Infection was allowed to proceed for 72 h and the number of infected cells was determined by FACS. In addition, to confirm the participation of cholesterol during ADE of DENV infection, cholesterol replenishment in cholesterol-depleted U937 cells was also carried out. After drug treatment, U937 cells were washed three times with PBS and incubated with RPMI medium supplemented with 5% FBS for 24 h at 37 °C and 5% CO₂. The replenished cells were infected with virus–mAb complexes as described above and the percentage of infected cells was evaluated by FACS.

Prior to the ADE experiments in cholesterol-depleted or -reconstituted cells, the effect of the drug treatments on total cholesterol levels and cell viability was assayed in uninfected U937 cells. Total cholesterol was quantified in treated and non-treated cells using a cholesterol oxidase/peroxidase assay kit following the manufacturer’s recommendations (BioSystems). Briefly, pelleted U937 differentiated cells (2 x 10⁶ cells), treated or untreated with the drugs, were treated with lysis buffer RSB-NP-40 (MgCl₂, 1.5 mM, Tris/HCl 10 mM and Nonidet P-40 15 mM) for 5 min on ice. Then, 20 μl aliquots of the cellular lysates were mixed with 1 ml cholesterol reagent and incubated for 5 min at 37 °C. A calibration curve was made with cholesterol standards (R²=0.9974); absorbance was obtained at 500 nm and regression analysis was used to determine the cholesterol concentration of the sample. The viability of treated and non-treated cells was analysed by trypan blue exclusion assays.

Immunofluorescence microscopy of lipid raft integrity and transferrin internalization. To visualize the lipid rafts, U937 cells were grown on coverslips placed in 24-well plates, differentiated for 48 h, and treated with cholesterol-reducing drugs as described above. Immediately afterwards, cells were washed twice with PBS, fixed in 1% paraformaldehyde for 30 min at room temperature and blocked in PBS containing 10% FBS, 3% BSA and 10 mM glycine. Then, cells were incubated for 30 min at 4 °C with 1 μg FITC-conjugated CT-B ml⁻¹. Finally, the cells were washed extensively (at least eight times) with 50 mM ammonium chloride in PBS and the coverslips were mounted onto glass slides in Vectashield mountant with DAPI to stain the nuclei (Vector Laboratories). The images were acquired under a Leica LSM-SPC-5 Mo inverted confocal microscope, fitted with HXCP Lavo lambda blue 63 x 1.4 oil immersion lens and these were processed using the LAS AF software confocal microscope (Leica). Cells were replenished with cholesterol as described previously and the cells were processed for immunofluorescence in an identical way.

Endocytosis of transferrin was evaluated by immunofluorescence assay as described by Chu & Ng (2004). Briefly, U937 cells were treated with cholesterol-reducing drugs and then 0.25 μg ml⁻¹ human transferrin reagent coupled to AlexaFluor was added to cells and the transferrin was allowed to bind for 20 min at 4 °C. Cells were then shifted to 37 °C for 1 h to allow transferrin entry and were fixed and processed for immunofluorescence.

Statistical analysis. Microsoft Excel was used for graphical representations and statistical data analyses. Values were expressed as the arithmetic mean ± SD. Samples were compared by using an unpaired Student’s t test and P-values <0.05 were considered statistically significant.

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