Restricted replication and lysosomal trafficking of yellow fever 17D vaccine virus in human dendritic cells

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The yellow fever virus attenuated 17D vaccine strain is a safe and effective vaccine and a valuable model system for evaluating immune responses against attenuated viral variants. This study compared the in vitro interactions of the commercially available yellow fever vaccine (YF-VAX), Dengue virus and the live-attenuated dengue vaccine PDK50 with dendritic cells (DCs), the main antigen-presenting cells at the initiation of immune responses. Similar to PDK50, infection with YF-VAX generated activated DCs; however, for YF-VAX, activation occurred with limited intracellular virus replication. The majority of internalized virus co-localized with endolysosomal markers within 90 min, suggesting that YF-VAX is processed rapidly in DCs. These results indicate that restricted virus replication and lysosomal compartmentalization may be important contributing factors to the success of the YF-VAX vaccine.

INTRODUCTION

The genus Flavivirus consists of over 70 closely related, insect-vector-transmitted viruses that include important human pathogens (Kuno et al., 1998; Lakadamyali et al., 2003). This group includes Yellow fever virus (YFV), Japanese encephalitis virus (JEV), West Nile virus and Dengue virus (DENV). The significance of flavivirus diseases and their impact on society continue to increase, despite ever-increasing efforts aimed at vector control. This is particularly true in the case of DENV, of which there are four different serotypes. During the last 50 years, the number of dengue fever and dengue haemorrhagic fever (DHF) cases has increased 30-fold to include almost 100 million new cases every year, of which 500 000 are DHF, the most lethal form (Gubler, 2004; Kroeger et al., 2004). Despite the previous success of attenuated virus-based vaccines against JEV and YFV (both closely related to DENV), the development of an attenuated virus vaccine that imparts simultaneous and long-lasting immunity to all DENV serotypes has proved elusive.

We have reported previously that infection with DENV serotype 2 (DENV-2) results in reduced expression of dendritic cell (DC) surface molecules required for T-cell stimulation (Palmer et al., 2005). One implication of this study is that less-virulent forms of DENV-2 are less likely to replicate in DCs or subvert their function, whilst, ideally, remaining sufficiently immunogenic. Infection and activation of DCs by attenuated viruses probably contribute to vaccine efficacy in humans; however, pre-clinical selection strategies for candidate vaccines do not include their evaluation in DC cultures. An understanding of the nature of DC interactions with a prototypic vaccine might be useful in understanding the vaccine’s immunogenicity.

The YFV vaccine is a live-attenuated virus vaccine derived by passage in fertilized hen’s eggs. YF-VAX (Sanofi-Pasteur) is the commercially available YFV strain 17D (YFV-17D) vaccine. This study evaluated the interactions of YF-VAX and DENV-2 with DCs in an effort to understand the nature of their immunogenicity in more detail. The YFV vaccine derives from the YFV Asibi isolate and has been administered to over 400 million individuals since its development in 1936 (Theiler & Smith, 1937). A single inoculation with the vaccine induces long-lasting protection (Groot & Riberiro, 1962), with up to 97% of vaccine recipients displaying neutralizing antibodies 19 years after inoculation, and stimulates high titres of neutralizing antibodies to the envelope structural protein and...
complement-fixing antibodies to the non-structural protein NS1 ([Brandriess et al., 1990; Poland et al., 1981; Putnak & Schlesinger, 1990; Reinhardt et al., 1998; Schlesinger et al., 1986]). Additionally, this vaccine elicits CD8+ and CD4+ gamma interferon (IFN-γ)-producing T cells following primary and secondary vaccinations in human (Co et al., 2002; Reinhardt et al., 1998) and mouse models (van Kooyk & Geijtenbeek, 2003). The reasons behind the robust immunogenicity are unknown. However, it is clear that multiple passages of the parent strain in tissue culture (up to 200 times) generate a viral population that is optimally immunogenic, whilst lacking the severely debilitating and often fatal symptoms that characterize YFV infections. Only 32 aa (~0.5% of the total) separate the vaccine strain from the YFV Asibi parental strain (Hahn et al., 1987). However, further attenuation leads to poor virus immunogenicity (Fox et al., 1942).

Both YFV-17D and wild-type YFV replicate productively in human monocytes and macrophages including Kupffer cells (Barros et al., 2004; Liprandi & Walder, 1983; Marnaneau et al., 1999b). Replication of YFV-17D in human DCs has also been detected using plaque assays (Barba-Spaeth et al., 2005; Brandler et al., 2005) and by detection of intracellular non-structural protein NS4A and NS4B (Barba-Spaeth et al., 2005; Querec et al., 2006). Similar to previous findings (Querec et al., 2006), we have shown here that YFV-VAX-activated DCs exhibited increased surface expression of major histocompatibility complex class II (MHCII), as well as co-stimulatory molecules needed for DC stimulation of homologous T cells. However, stimulation of DCs by YF-VAX, unlike stimulation by the DENV-2 live-attenuated vaccine PDK50 and its wild-type parent, occurred with diminishing, possibly abortive, virus replication in the cells. Microscopy experiments showed that intracellular localization of YF-VAX was largely confined to lysosomal compartments in contrast to infectious wild-type DENV-2, which localized to both the trans-Golgi and lysosomes. These results suggest that YF-VAX is rapidly processed in DCs, which may constitute an important factor in promoting optimal DC/T-cell interactions.

**METHODS**

**Cell lines and viruses.** Vero cells were purchased from ATCC and expanded in complete DMEM (1 % l-glutamine, 1 % penicillin/ streptomycin and 10 % heat-inactivated fetal bovine serum). Vero cells were submitted to ATCC for Mycoplasma testing and confirmed to be negative. All viral titres were determined by limiting-dilution plaque assays on Vero cells. YF-VAX is the commercial name of the YFV vaccine, which uses the 17D strain. The titre of unpassaged YF-VAX was 3 × 10^8 p.f.u. ml^-1. DENV-2 strain S16803 was originally isolated in 1974 from a Thai patient with DHF and subsequently expanded by two passages in Toxorhynchites splendens mosquitoes and four passages in Vero cells (parental S16803). DENV-2 S16803 vaccine virus was derived from 50 further passages in primary dog kidney (PDK) cells and three more passages in fetal rhesus lung cells. Both the parental S16803 and vaccine (PDK50) were kindly provided by Dr Ken Eckels (Walter Reed Army Institute of Research, MD, USA). The initial titres of parental S16803 and PDK50 were 3 × 10^8 p.f.u. ml^-1 and 2.1 × 10^7 p.f.u. ml^-1, respectively. YF-VAX and PDK50 were propagated once in Vero cell lines to titres of 9 × 10^6 p.f.u. ml^-1 and 3.8 × 10^6 p.f.u. ml^-1, respectively. Contaminating lipopolysaccharides in viral stocks and culture supernatants were ruled out by the Limulus Amoebocyte Lysate test (BioWhittaker). Viral stocks were stored at ~80 °C until use.

**Human DCs.** DCs were generated by positive selection of CD14+ cells (Milenyi Biotech) from peripheral blood mononuclear cells collected from normal, healthy, seronegative, consenting donors in accordance with a human subject protocol approved by The Walter Reed Army Institute Human Use Research Committee. Enriched CD14+ cells were cultured for 7 days in complete RPMI (1 % l-glutamine, 1 % penicillin/streptomycin, 1 % sodium pyruvate, 1 % essential amino acids, 50 mM 2-mercaptoethanol and 10 % heat-inactivated fetal bovine serum) in the presence of recombinant human (rh) granulocyte-macrophage colony-stimulating factor (Leukine, 100 ng ml^-1; Immunex) and rhIL-4 (50 ng ml^-1; R&D Systems) at 37 °C, 5 % CO2 and 95 % relative humidity.

**Infection with YF-VAX 17D, parental S16803 and PDK50.** Unless otherwise stated, DC cultures were infected (or left uninfected) with YF-VAX, parental S16803 or PDK50 at an m.o.i. of 1 and placed in a humidified 5 % CO2 incubator at 37 °C. For DC-SIGN blocking experiments, DCs were pre-incubated with 10 μg anti-DC-SIGN antibody ml^-1 (clone 120612; R&D Systems), 1 h prior to infection. After 2.5 h, cells were washed twice to remove cell-free, unattached virus and cultured in complete RPMI (without cytokines) at a density of 1 × 10^6 cells ml^-1. Cells and/or media were analysed at different times as detailed in the figures. In some experiments, Vero cell controls were infected in the same manner as DCs. Control experiments also included uninfected DCs and DCs exposed to uninfected Vero cell supernatants.

**Detection of YF-VAX and parental S16803 infection by FACS.** DCs were infected as described above. After 48 h, cells were fixed and permeabilised with Cytofix and CytoPerm (BD Biosciences) according to the manufacturer’s recommendations. For DENV-2 detection, DCs were stained using fluorescein isothiocyanate (FITC)-labelled monoclonal antibody 2H2 (kindly provided by the Naval Medical Research Center, MD, USA). For YF-VAX detection, DCs were stained with anti-YFV-specific hyperimmune mouse ascites fluid (ATCC), followed by a secondary phycoerythrin (PE)-conjugated goat anti-mouse Ig antibody (BD Biosciences). Samples were analysed on a FACSCalibur (Becton Dickinson) using CELL QUEST software.

**Western blot analyses.** Infected DCs and Vero cells were seeded in six-well plates at a concentration of 1 × 10^6 cells per well and kept at 37 °C in a CO2 incubator. After different time periods, cells were collected in 1.5 ml centrifuge tubes and washed twice with ice-cold PBS. Cell lysates were prepared in a Triton X-100-based buffer in the presence of appropriate protease and phosphatase inhibitors. The amount of protein in the cell lysate was determined using the BCA protein detection system (Bio-Rad). An aliquot of the cell lysate (10 μg protein per sample) was analysed by SDS-PAGE and the separated proteins were transferred to nitrocellulose membranes and subjected to Western blot analyses. Membranes were probed with polyclonal antibodies from anti-DENV-2 immune ascites fluid (a generous gift of Ms M. Simmons, Naval Medical Research Center, MD, USA) or anti-YFV-specific hyperimmune ascites fluid (ATCC). Bands were visualized using SuperSignal Chemiluminescence substrate from Pierce.

**Cell-surface labelling.** To measure changes in cell-surface markers, DCs were infected with YF-VAX, parental S16803 or PDK50, or left uninfected, as described above. After 48 h incubation, cells were collected, washed and stained according to the manufacturer’s...
recommendations using PE-conjugated monoclonal antibodies specific for CD40 (clone 5C3), CD80 (1307.4) or MHCII (G46-6) (BD Biosciences). Isotype-matched PE-labelled controls were included in each experiment. For DC-SIGN experiments, DCs were stained for CD80 and CD83 (HB15c).

**Analyses of apoptotic cells.** The viability of infected DCs was compared with uninfected DCs at 48 h. Cells were stained with propidium iodide (PI) and FITC-conjugated Annexin V using an Annexin V:FITC Apoptosis Detection kit I (BD Biosciences) according to the manufacturer’s recommendations. Samples were analysed on a FACSCalibur using CELL QUEST software.

**Cytokine detection in culture supernatants.** Cells were infected with viruses as described above and cell-free media were collected and stored. IFN-α was measured by ELISA (PBL Biomedical Laboratories) according to the manufacturer’s recommendations.

**Fluorescence labelling of YF-VAX and DENV-2.** Sucrose-gradient-purified YF-VAX or parental S16803 was conjugated with Alexa Fluor 594 carboxylic acid succinimidyl ester according to the manufacturer’s instructions and placed on a shaker overnight at 4 °C. The sample was dialysed (Pierce Slide-A-Lyser, 3000 molecular weight cut-off) exhaustively with PBS for 8–12 h at 4 °C. Fluorescently labelled viruses were then stored at −80 °C in small aliquots. Once thawed, 500 μl PBS was added to the virus. The mixture was centrifuged at 4 °C for 1 h at 15000 r.p.m. using a fixed rotor to pellet the virus and remove any free dye remaining, before addition to DCs. The ratio of DC to virus particles was between 1:100 and 1:1000. Labelling did not alter viability, as DCs infected with labelled DENV-2 virus expressed viral antigens at 48 h post-infection as detected in staining experiments with FITC-labelled 2H2 monoclonal antibody (data not shown). Further confirmation of virus viability was obtained in plaque assay experiments (data not shown). As a control, mock-infected Vero cell supernatant was labelled with Alexa Fluor 594 carboxylic acid succinimidyl ester according to the manufacturer’s instructions and placed on a shaker overnight at 4 °C. The supernatant was either dialysed or column purified to remove excess free dye from the preparation before addition to DCs.

**Immunofluorescent microscopy.** DCs were washed and incubated in PBS in the presence or absence of YF-VAX or parental S16803 for 90 min at 37 °C. Cells were fixed and permeabilized with cold methanol:acetone (1:1) for 10 min at −20 °C. Cells were washed three times in PBS and non-specific sites were blocked with 5% goat serum for 30 min at 37 °C. Cells were incubated at room temperature for 1 h with FITC-conjugated CD107b (H4B4; Pharmingen) for detection of lysosomal-associated membrane protein 2 (LAMP-2) or EEA1 (clone 14; Transduction Laboratories) for detection of early endosomal antigen (5 μg ml⁻¹). In some experiments, NB-Dc₆-ceramide (2 nmol ml⁻¹; Molecular Probes) was added for detection of the trans-Golgi, as described previously (Rao et al., 1997). After incubation, cells were washed three times with PBS and mounted on slides with Vectashield mounting medium and nail polish. Cells were examined with a Bio-Rad Radiance 2000 confocal microscope using a 60× oil-immersion objective. Data were collected using LaserSharp software.

**In vitro viral replicate assay on endogenous viral RNA templates.** Vero cells or DCs (containing at least 1 × 10⁶ cells per well) were infected with YF-VAX, PDK50 or parental S16803 at an m.o.i. of 1. Cells were harvested at 48 h post-infection, washed with PBS, centrifuged at 12000 r.p.m. in a bench-top centrifuge and resuspended in buffer containing 10 mM Tris/HCl (pH 8.0), 10 mM sodium acetate and 1.5 mM MgCl₂. Cells were lysed by repeatedly passing through a 1 ml syringe fitted with a 27-gauge needle (>30 times), as described previously (Chu & Westaway, 1985; Uchil & Satchidanandam, 2003), and cell lysis was checked by microscopy. Cell lysates were centrifuged and the supernatants aliquoted, quick-frozen in ethanol/dry ice and stored at −80 °C until use. Protein concentrations were determined using a spectrophotometer (SpectraMax; Molecular Devices). Equal amounts of total protein from cell lysates were used in the replicate assays. Assays were carried out at 37 °C for 1 h in a total volume of 50 μl containing 50 mM Tris/HCl (pH 8.0), 10 mM magnesium acetate, 7.5 mM potassium acetate, 60 μg actinomycin D ml⁻¹ (to inhibit DNA-dependent RNA polymerase), 10 mM 2-mercaptoethanol, 5 mM phosphoenolpyruvate, 3 U pyruvate kinase ml⁻¹, 20 U RNAsin (Promega), 0.4 mM each of ATP, CTP and UTP (Roche Applied Science), 10 μCi [α-³²P]GTP (800 Ci mmol⁻¹) and 20 μg virus-infected cell homogenate. Reactions were terminated by the addition of 0.5 M EDTA. The RNA products were disrupted from membranes with TNE-SDS [50 mM Tris/acetate (pH 7.6), 0.1 M sodium acetate, 1 mM EDTA, 2% SDS] and extracted using an RNaseasy kit (Qiagen). RNA products were dissolved in RNA loading buffer containing 7 M urea, 1× TBE and 0.5% bromophenol blue, and viral RNA products were resolved by partially denaturing 3% PAGE containing 7 M urea. RNA bands were visualized by autoradiography.

**Plaque assay.** Viral titres in cell-culture supernatants were quantified by plaque formation on monolayers of Vero cells with an agarose double overlay, as described previously (Lambeth et al., 2005). After a 5–6 day incubation period, plaques were detected by staining the monolayers with neutral red. The virus titre (p.f.u. ml⁻¹) was calculated as the mean number of virus plaques counted at a given dilution multiplied by the dilution factor.

**Detection of YF-VAX by RT-PCR.** Approximately 1 × 10⁶ DCs or Raji cells were left uninfected or infected with YF-VAX at an m.o.i. of 2, as described above. Cells were harvested every 24 h for 5 days. Following centrifugation, cell pellets were washed twice in ice-cold PBS. Total RNA was extracted from the final pellets using an RNAqueous RNA isolation kit (Ambion). After reverse transcription, 1 μl cDNA was amplified using YF-17D-specific primers (forward: 5’-CGCAACGAGTCTGATGTGCC-3’; reverse: 5’-TGCCAAAGTTGATGCGCAT-3’). PCR products were separated by electrophoresis in 1% agarose.

**RESULTS**

**YF-VAX induces maturation and activation of DCs**

DC activation was assessed by measuring cell-surface expression levels of MHCII, CD80 and CD40. Fig. 1 shows that YF-VAX and PDK50 exposure enhanced expression of cell-surface activation markers on DCs for up to 72 h with PDK50-infected cells exhibiting higher levels of upregulation. YF-VAX-infected DCs stimulated IFN-γ production from autologous CD4⁺ T cells collected from an immune donor (data not shown), demonstrating that these cells were competent antigen-presenting cells. In contrast, parental S16803 was only capable of transient induction of MHCII (Fig. 1, top panel) and late induction of CD80 (Fig. 1, middle panel), and failed to increase expression levels of CD40 at the times points tested (Fig. 1, bottom panel).

DC-SIGN is a C-type lectin, abundant on the surface of DCs, that mediates infection by PDK50 (data not shown) and DENV (Navarro-Sanchez et al., 2003; Tassaneeitrhep et al., 2003), as well as by other viruses (Alvarez et al., 2002; Gardner et al., 2003; Halary et al., 2002). To study the role of
DC-SIGN (CD209) in activation, DCs were stimulated with YF-VAX, parental S16803 or no virus for 48 h in the presence or absence of neutralizing amounts of anti-DC-SIGN antibodies. Previous assessments of DC-SIGN engagement by YF-VAX, including confirmatory experiments in the present study, did not find evidence that YF-VAX requires DC-SIGN for these effects (Barba-Spaeth et al., 2005; Navarro-Sanchez et al., 2003; data not shown). However, to rule out DC-SIGN mediation completely, we utilized anti-DC-SIGN antibodies to block YF-VAX-induced maturation of DCs. As shown in Fig. 2, the addition of anti-DC-SIGN antibody partially blocked DENV-2-induced upregulation of CD80 and CD83. However, YF-VAX-induced upregulation of CD80 and CD83 cell-surface expression on DCs remained unaltered. These results suggest that receptors other than DC-SIGN mediate YF-VAX-induced activation of DCs.

**YF-VAX undergoes limited replication in DCs**

As both YF-VAX and PDK50 readily activated DCs, we wanted to verify replication inside the cells. Attenuated DENV readily replicates in DC cultures (unpublished data), although less efficiently than the parental strain. Fig. 3(a) shows that YF-VAX virus production increased with time in Vero cells, but not in DC cultures, where a decline was observed after an initial increase in YF-VAX virus production at 24 h. Examination of viral RNA in infected cells by RT-PCR showed expression at 24 and 48 h time points (Fig. 3b). The outcome of these experiments, although suggestive of virus replication, could also reflect viral persistence in the absence of infection, as viral plaques were detected several days after inoculation of YF-VAX into cell-free cultures (Fig. 3a) and RT-PCR signals were detected after incubating YF-VAX with Raji cells (Fig. 3b), which have previously been reported to be refractory to infection (Barba-Spaeth et al., 2005). In further experiments using flow cytometry to evaluate viral protein expression in DCs and Vero cells incubated with YF-VAX and DENV-2 (m.o.i. = 1) for 48 h, we detected YF-VAX replication in Vero cells (Fig. 4a, dotted line, right panel), but not in DCs (Fig. 4a, left panel), suggesting the absence or low expression levels of YF-VAX proteins in DCs. The specificity of the YFV-17D-specific hyperimmune mouse ascites fluid was tested in DCs and Vero cells infected with parental S16803 (Fig. 4a, shaded areas). Western blot analyses of DCs infected with YF-VAX (m.o.i. = 1) confirmed the paucity of viral protein in YF-VAX-infected DC cultures (Fig. 4b). In contrast, protein expression was detected in Vero cell control cultures and in similar experiments with parental S16803 and PDK50 utilizing anti-DENV-2 immune ascites fluid. We also evaluated viral RNA intermediates in DCs and Vero cells infected with YF-VAX, PDK50 or parental S16803 (m.o.i. = 1) for 48 h. The RNA replicase assay measures RNA-dependent RNA polymerase activity and detects three viral replicative RNA species: genomic plus-strand RNA (vRNA), double-stranded replicative-form RNA (RF) and replicative intermediates (RIs). Fig. 5 shows that, although the three intracellular replicative forms of YF-VAX could be detected in Vero cell cultures, they were not detectable in DCs. PDK50- and parental S16803-derived viral replicative RNA species were found in Vero cells as well as in DCs.
Additional experiments using higher YF-VAX virus loads (m.o.i. = 10) and longer time periods (72 h) yielded similar results (data not shown). These results suggested a low level RNA-dependent RNA polymerase activity or its absence. Taken together, the data on YF-VAX replication in DCs are suggestive of an early replication cycle that becomes restricted, possibly abortive, at later time points. An alternative explanation is that a rapid induction of antiviral cytokines following the initial infection of a subpopulation of cells suppresses subsequent infection of neighbouring cells. To address this possibility, we measured levels of secreted IFN-α, a likely candidate for mediating immediate antiviral effects. YF-VAX readily induced IFN-α after 48 h in DC cultures. At this time point, the mean level of secreted IFN-α was 344 ± 243 pg ml⁻¹ for three separate experiments. No IFN-α was detectable at 12 and 24 h, the time period coinciding with peak virus production and RNA expression levels.

**YF-VAX-exposed DCs undergo minimal apoptosis**

Apoptotic cell death may be induced by intracellular virus activity including virus replication and/or accumulation of viral products (Despres et al., 1996). Here, we compared the induction of apoptosis in YF-VAX-, PDK50- and parental S16803-infected DCs by assessing Annexin V–PI staining of DC cultures. The withdrawal of cytokines from culture media on day 7 prior to infection was reflected by the percentage of apoptotic cells in uninfected control cultures (Fig. 6). The percentage of apoptotic cells increased marginally in YF-VAX-infected cultures; however, a higher degree of apoptosis was caused by PDK50 and parental S16803.
YF-VAX localizes to lysosomes in DCs

To confirm that DCs internalize YF-VAX, we exposed DCs to Alexa Fluor 488-labelled infectious virus (infectivity was confirmed in Vero cell cultures; data not shown). Co-labelling experiments with organelle-specific markers permitted us to track viral transport in DCs by confocal microscopy. Within 90 min, the majority of internalized YF-VAX co-localized with the lysosomal membrane protein LAMP-2 (Fig. 7) and to a lesser extent with the endosomal antigen EEA1 (data not shown), indicating that the virus was targeted to lysosomes. Similar to YF-VAX, Alexa Fluor-labelled parental S16803 localized with endolysosomal markers, but in addition, localization to the trans-Golgi was also detected, as visualized by the Golgi-specific marker NBD-C6-ceramide.

DISCUSSION

Confirming earlier studies (Querec et al., 2006), our results provide evidence for a potent activation of DCs by YF-VAX, but suggest limited and possibly abortive virus replication. We detected an increase in the number of infectious particles and viral RNA expression by RT-PCR in DC cultures, supporting previous indications of YF-VAX replication in DCs (Barba-Spaeth et al., 2005; Brandler et al., 2005). However, plaque assay experiments showed a decline in virus production at time points later than 24 h, and additional studies assessing RNA-dependent RNA polymerase activity using an RNA replicase assay and protein expression failed to detect RNA RIs or viral proteins in infected cultures at 48 h and beyond this time period. Although limitations in assay sensitivity cannot be ruled out, our results are suggestive of a reduced YF-VAX replicative cycle in DCs. Furthermore, our studies suggested that internalization and presumably degradation within lysosomal compartments contributed to the curtailment of YF-VAX replication. Lysosomal sequestration of YF-VAX in DCs is reminiscent of phagocytic uptake of DENV by Kupffer cells, which results in virus degradation (Marianneau et al., 1999a, b) and is atypical for viruses that proceed through an infective life cycle. Many viruses that enter cells by receptor-mediated endocytosis undergo a pH-dependent endosomal/virus fusion that allows virus uncoating and escape of viral nucleocapsids into cytoplasmic sites for initiation of the replication cycle (Gruenberg, 2001; Lakadamyali et al., 2003). In marked contrast to DCs, but similar to Vero cells, replication of YF-VAX in monocytes and the macrophage-like cell line U937 is highly productive (Barros et al., 2004; Liprandi & Walder, 1983; Marianneau et al., 1999b) and we speculate that the difference between these cell types and DCs is related to uptake and processing of YF-VAX. An additional consideration is that the disproportionate number of envelope protein mutations carried by YF-VAX in comparison with the parental Asibi strain (Hahn et al., 1987) alters host binding and penetration properties, as has been described previously for attenuated variants of JEV and Murray Valley encephalitis virus (Lee & Lobigs, 2002), with implications for their intracellular distribution sites. Evaluations of the DC localization sites of viruses that retain only the non-structural components of YF-VAX and express the envelope
gene of DENV or other flaviviruses have been described and may be useful in future evaluation of mutant viral trafficking (e.g. ChimeriVax; Brandler et al., 2005).

The limitation of YF-VAX replication in DCs might also have resulted from early production of IFN-α/β by a subpopulation of infected DCs that suppressed virus replication in neighbouring cells. The antiviral activity of IFN-α/β might also account for the rapid and productive infection detected in Vero cells, which do not produce IFN (Diaz et al., 1988). However, detectable levels of IFN-α/β were apparent only after 48 h, a time point beyond peak viral activity, as detected by plaque assays and PCR. It is possible that small amounts of secreted IFN-α/β, undetectable by ELISA, participated in limiting early virus replication; however, this is unlikely to be the sole mechanism of suppression.

DCs express pathogen-recognition molecules, including the C-type lectin DC-SIGN, that recognize and internalize pathogens (van Kooyk & Geijtenbeek, 2003). DC-SIGN mediates the internalization of a number of viruses including DENV, a flavivirus closely related to YFV (Navarro-Sanchez et al., 2003; Tassaneetrithep et al., 2003), many of which subvert DC antigen-processing and presenting functions (Alvarez et al., 2002; Gardner et al., 2003; Halary et al., 2002). In contrast to these viruses, previous work has shown that YF-VAX is not internalized by DC-SIGN-expressing THP-1 cells (Navarro-Sanchez et al., 2003) and our data on YF-VAX confirm this finding. Additionally, we found that expression of DC activation markers on DENV-2-infected DCs decreased in the presence of DC-SIGN-blocking antibody, but remained unaffected in YF-VAX-infected cultures, supporting the hypothesis that YF-VAX activation of DCs is independent of DC-SIGN engagement. Recent work has reported the involvement of Toll-like receptors 2, 7, 8 and 9 in YF-VAX activation of DCs (Querec et al., 2006). Activation resulted in increased DC expression of cell-surface molecules. Moreover, we found enhanced secretion of IFN-α and stimulation of IFN-γ-producing CD4+ T cells (data not shown). Of interest is the fact that, similar to PDK50, increased expression of cell-surface molecules on YF-VAX-infected DCs was sustained, even at 72 h, in contrast to cultures infected with DENV-2 parental S16803, where cell-surface molecule expression was suppressed or delayed. Although our results point to

Fig. 7. YF-VAX is targeted to lysosomal compartments in immature DCs. The upper panels show the localization of YF-VAX (red) and lysosomal compartments (LAMP-2) (green), and co-localization (yellow), after uptake for 90 min. The middle panels show that YF-VAX (red) does not co-localize with the trans-Golgi (green) after uptake for 90 min. The lower panels show co-localization (yellow) of parental S16803 DENV-2 (red) with the trans-Golgi (green). Cells were visualized with a Bio-Rad confocal microscope.
clear-cut DC maturation outcomes for attenuated and wild-type viruses, it is probable that attenuated viral strains will exhibit varying outcomes depending on the source of the viral stocks. To minimize virus modification, we utilized the commercial YF-VAX vaccine virus, which was propagated only once in Vero cells. Markedly different outcomes were found for DCs infected with virus stocks derived from infection of SW13 cells with full-length infectious YFV-17D plasmids, where lack of DC maturation has been reported (Barba-Spaeth et al., 2005).

Our data showed that exposure to YF-VAX did not induce significant levels of DC apoptosis, lending further support to our conclusion on its restricted replication in DCs. In contrast, PDK50 and parental S16803 induced increased staining of Annexin V- and PI-positive cells. Apoptosis is a common outcome for replicating/virulent viruses including YFV (Catteau et al., 2003; Despres et al., 1996; Quaresma et al., 2005) and is triggered by many factors including intracellular viral protein accumulation and the release of cytokine mediators that interfere with virus replication and/or initiate an inflammatory process. Virus replication and its potentially damaging consequences might impose limitations on the antigen-presenting functions of infected DCs, and we have demonstrated previously that induction of apoptosis in DENV-2-infected DC cultures interferes with their immunostimulatory functions (Palmer et al., 2005), with important implications for the initiation of anti-DENV immune responses (Mathew et al., 1999).

In summary, we compared DC infection by YF-VAX, the live-attenuated DENV vaccine PDK50 and the DENV-2 parental S16803 virus. Both YF-VAX and PDK50 effectively induced DC activation, but there were important differences between the viruses. In contrast to PDK50 and parental S16803, YF-VAX exhibited restricted replication in DCs, and DC-SIGN was not required for internalization. Additionally, most YF-VAX localized to lysosomes within DCs. We propose that these features permit the rapid processing of YF-VAX in DCs, contributing to its overall success as a vaccine.

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