

Live attenuated yellow fever 17D infects human DCs and allows for presentation of endogenous and recombinant T cell epitopes

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The yellow fever (YF) 17D vaccine is one of the most successful live attenuated vaccines available. A single immunization induces both long-lasting neutralizing antibody and YF-specific T cell responses. Surprisingly, the mechanism for this robust immunity has not been addressed. In light of several recent reports suggesting flavivirus interaction with dendritic cells (DCs), we investigated the mechanism of YF17D interaction with DCs and the importance of this interaction in generating T cell immunity. Our results show that YF17D can infect immature and mature human DCs. Viral entry is Ca²⁺ dependent, but it is independent of DC-SIGN as well as multiple integrins expressed on the DC surface. Similar to infection of cell lines, YF infection of immature DCs is cytopathic. Although infection itself does not induce DC maturation in vitro, TNF- α -induced maturation protects DCs from YF-induced cytopathogenicity. Furthermore, we show that DCs infected with YF17D or YF17D carrying a recombinant epitope can process and present antigens for CD8⁺ T cell stimulation. These findings offer insight into the immunologic mechanisms associated with the highly capable YF17D vaccine that may guide effective vaccine design.

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The yellow fever (YF) 17D vaccine is a live attenuated vaccine that has been used for >70 yr on more than 400 million people for vaccination against YF virus with a remarkable record of safety and efficacy (1). The vaccine strain YF17D was generated from the WT strain Asibi and differs from Asibi by only 32 amino acids, 12 of which are clustered in the envelope protein (2). Viscerotropism of the parental Asibi is markedly reduced in YF17D, but the basis of the attenuation remains unknown. Vaccination generates both long-lasting neutralizing antibodies and T cell responses (3, 4). The antibody response most likely accounts for the effectiveness of the vaccine; however, a role for cell-mediated immunity in generating an effective immune response has also been suggested (4). Only recently have YF-specific human CD8⁺ T cell responses been identified (5), but the immunologic mechanisms associ-

ated with YF-specific CD8⁺ T cell priming are still unknown.

Given its safety and efficacy, YF17D has been engineered as a vaccine vector for antigens of other flaviviruses such as dengue, Japanese encephalitis, and West Nile viruses. Some of these chimeric viruses are in phase II clinical trials (6). We have recently reported that YF17D can also be engineered to deliver epitopes from unrelated pathogens, and it is able to induce protective immunity against heterologous agents (7). A better understanding of the mechanism underlying the efficacy of T cell priming by YF vectors would offer insight into vaccine design.

DCs are potent APCs that play a crucial role in regulating the adaptive immune response (8). DCs may interact with pathogens in peripheral tissue via direct infection or via phagocytosis of either infected cells or viral particles. On exposure to inflammatory signals or certain pathogens, DCs undergo a programmed phenotypic maturation program coincident with CCR7 up-regulation and migration to the

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lymph node for engagement and activation of T cells. Recent studies have demonstrated DC interaction with flaviviruses. Specifically, DC-SIGN, a DC-specific C-type lectin present most robustly on immature DCs (iDCs), mediates entry of dengue virus into DCs (9, 10) and binding of the hepatitis C virus envelope protein (11).

In this report, we explore the interaction of YF17D with human DCs. We investigate the ability of YF17D to infect DCs, the requirements for viral entry, and the cytopathic effect of YF17D in DCs. Furthermore, we evaluate the immunologic determinants of DC infection by monitoring T cell responses to endogenous and model antigens from YF17D vectors. This work provides new insight into the immunologic mechanisms associated with the highly effective YF vaccine and may provide clues relevant to effective vaccine design.

RESULTS AND DISCUSSION

YF17D infects immature and mature DCs

To test the hypothesis that the immune response induced by YF17D vaccination may be generated by direct infection of DCs, we exposed both immature and mature human monocyte-derived DCs with characteristic surface phenotypes (Fig. S1, available at <http://www.jem.org/cgi/content/full/jem.20051352/DC1>) to YF17D virus. FACS analysis for the intracellular nonstructural protein NS1 showed robust expression in both iDCs and mature DCs (mDCs)

infected at 2 or 20 PFU/cell (Fig. 1 A). Detection of non-structural viral proteins not present within the virion indicates productive infection. Infection was confirmed using an antibody to nonstructural protein NS4AB (NS4; Fig. 1 B). No staining was detected when UV-inactivated virus was used for infection. In addition, virus production in both the iDC and mDC cultures was monitored by plaque assay. Results showed a 3-log increase in infectious particles within 24 h, confirming productive infection (Fig. 1 C). Notably, no substantial difference in infectious particle production was seen between iDC and mDC cultures. Infection at 20 PFU/cell peaks 48 h after infection and, though infection at 2 PFU/cell may lag behind only slightly, considerable differences in the percentage of infected cells persist (Fig. 1 A). Induction of antiviral cytokines during infection may account for lower infectivity at 2 PFU/cell. To exclude antibody-mediated enhancement of infection, DCs were generated in plasma from naive donors and, in control experiments, blocking antibodies against the Fc receptor did not block YF17D infection (unpublished data). These data demonstrate that YF17D can infect and productively replicate in human DCs, and infection occurs irrespective of their maturation state.

Other cell types were not as susceptible to YF infection. High-level virus production was not seen in T cells, B cells, or monocyte-enriched PBMCs (Fig. 1 D). In addition, infection of B cell lines (MC116, RAMOS, and Raji) did not result in productive infection (unpublished data).

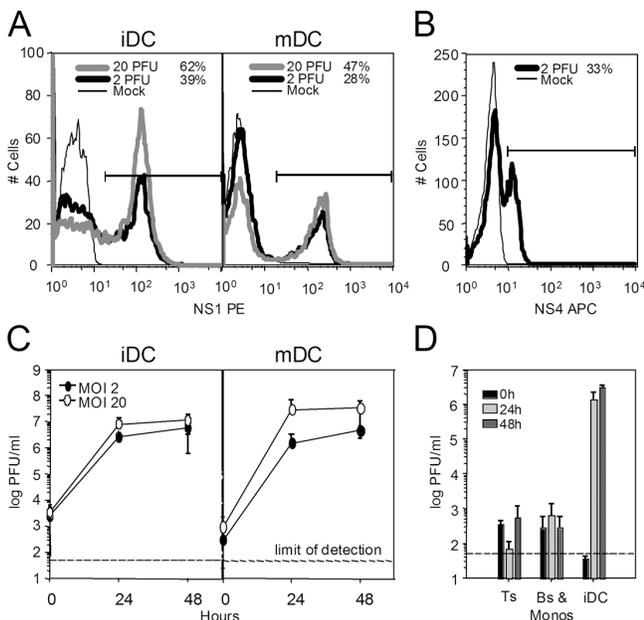


Figure 1. iDCs and mDCs are productively infected by YF17D.

(A) Intracellular FACS analysis of NS1 expression (indicated by the gate shown) in iDCs and mDCs infected with 2 or 20 PFU/cell 48 h after infection. (B) Intracellular staining of NS4 expression (indicated by the gate shown) in iDCs infected with 2 PFU/cell. (C) Virus production by iDC and mDC cultures measured by plaque assay on SW13. (D) Virus production by T cell- and monocyte-enriched PBMC fractions compared with iDCs. Values in C and D represent means \pm SD.

DC infection is Ca^{2+} dependent

The cellular receptor involved in YF17D infection is unknown. Recent reports have identified the receptors for the related flaviviruses West Nile virus and dengue virus as $\alpha\beta 3$ and DC-SIGN, respectively (9, 10, 12). In addition, the presence of an RGD motif in the ectodomain of the YF17D envelope protein suggests a mechanism for interaction with integrins (13). Both integrins and DC-SIGN require divalent cations for structural and functional integrity. Thus, to assess a possible requirement for divalent cations, iDCs were pre-treated with 10 mM EDTA and exposed to YF17D for 1 h to evaluate their role in infection. NS4AB expression at 24 h was completely blocked by EDTA (Fig. 2 A), whereas EDTA-treated DCs were still competent for infection with influenza (Fig. 2 B). EGTA treatment also blocked infection, indicating a Ca^{2+} -dependent mechanism (Fig. 2 A). Consistent with this finding, addition of Ca^{2+} , but not Mg^{2+} , was able to overcome the EDTA block (Fig. 2 A).

In light of this result, we tested specific integrin-blocking antibodies, including $\beta 1$, $\beta 3$, $\alpha\beta 3$, $\beta 5$, $\alpha\beta 5$, and $\alpha\beta 6$, as well as RGD peptides, to evaluate the role of specific integrins in viral entry. Antibodies and peptides were used at concentrations reported to be sufficient for blocking West Nile virus (12). None of the integrin-blocking antibodies considerably inhibited infection as monitored by NS4AB expression 24 h after infection (Fig. 2 C and not depicted). The lack of inhibition by RGD peptides is consistent with

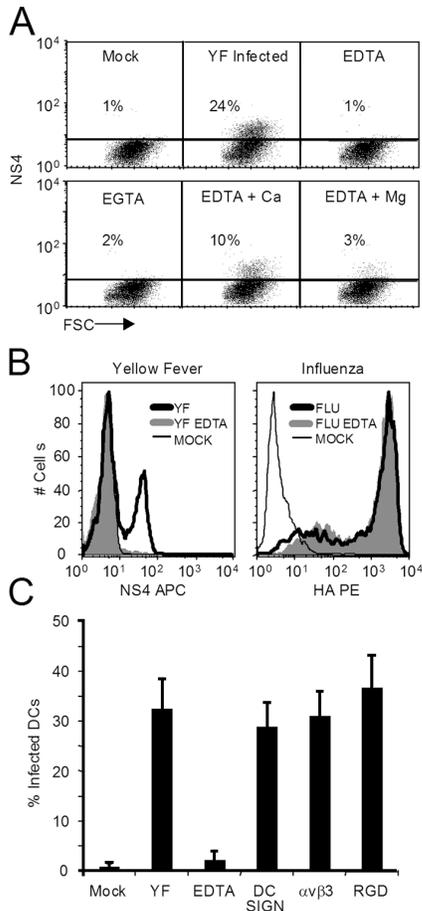


Figure 2. YF17D Infection of DCs is Ca^{2+} dependent. (A) YF17D infection of iDCs at 2 PFU/cell was monitored by intracellular FACS for YF NS4 24 h after infection. DCs were treated as indicated with 10 mM EDTA or EGTA. 6 mM Ca^{2+} or Mg^{2+} was added as indicated. The percentage of NS4-positive cells is indicated. (B) Intracellular YF NS4 or surface influenza hemagglutinin staining after 24 or 8 h, respectively. Infection was done with or without pretreatment with EDTA as indicated. (C) Bar graph represents the percentage of iDCs staining positive for YF17D NS4 24 h after infection. Samples preincubated with 10 $\mu\text{g}/\text{ml}$ DC-SIGN or $\alpha\beta 3$ blocking antibodies or 100 $\mu\text{g}/\text{ml}$ RGD peptides are indicated. Values represent means \pm SD.

previous reports suggesting YF17D infection is independent of the RGD motif (12, 13). These data, however, do not rule out the possibility that YF entry uses determinants not blocked by these antibodies or peptides.

In addition, blocking antibodies against DC-SIGN (9) did not inhibit YF17D infection (Fig. 2 C). Antibodies were used at saturation conditions, and binding to DCs was confirmed by FACS (Fig. S1). These results suggest that, in contrast to dengue, YF17D infection of DCs is not mediated by DC-SIGN. This finding is consistent with our data showing equivalent virus production in iDCs and mDCs, which differ substantially in DC-SIGN expression (high in iDCs, low in mDCs; Fig. 1 C and Fig. S1), as well as a recent report showing the inability of DC-SIGN to permit infection in THP-1 cells (10). In contrast with dengue virus

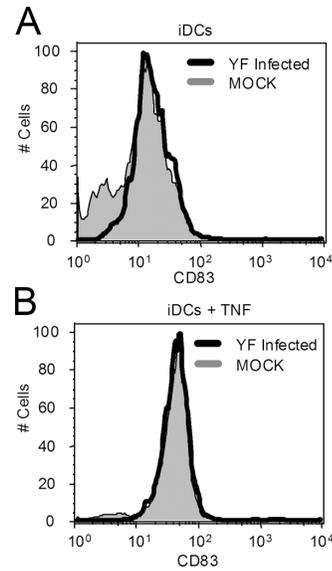


Figure 3. YF17D infection does not induce or inhibit DC maturation. (A) iDCs infected with 10 PFU/cell were analyzed for CD83 surface expression. CD83 expression of the YF17D-infected population (compared with mock infected) was analyzed 36 h after infection. (B) iDCs were infected with 10 PFU/cell and exposed to TNF- α /PGE-2 maturation cocktail. CD83 expression of the YF-infected population (compared with mock infected) was analyzed after 36 h.

envelope protein, which contains one or two N-linked glycans, the envelope protein of YF17D-204 used in these experiments is not glycosylated (14). Although other YF17D substrains (YF17DD and YF17D-213) may be glycosylated, our data indicate that DC-SIGN interaction is not necessary for infection and that YF17D interacts with DCs via mechanisms distinct from related flaviviruses. Although YF17D infection of DCs is Ca^{2+} dependent, the receptor for YF17D remains elusive.

YF17D infection does not alter DC maturation in vitro

Several studies indicate that viral infection of DCs promotes or inhibits maturation (15–17). To evaluate DC maturation as a result of YF17D infection, surface expression levels of CD83, CD86, and MHC class II were assayed 36 h after infection. Interestingly, infection alone did not induce robust DC maturation. Although slight up-regulation of CD86 and MHC class II was observed (unpublished data), conventional maturation markers such as CD83 were not expressed (Fig. 3 A). However, in contrast to inhibition of maturation by several infectious pathogens (16, 17), YF17D infection did not inhibit TNF- α -mediated DC maturation (Fig. 3 B). These results suggest, therefore, that in the context of YF17D vaccination, release of inflammatory cytokines in peripheral tissue may play an important role in triggering DC maturation. This will allow for DC migration to the lymph node and subsequent T cell engagement and priming. Consistent with this model, an increase in TNF- α has been detected in subjects vaccinated with YF17D (18).

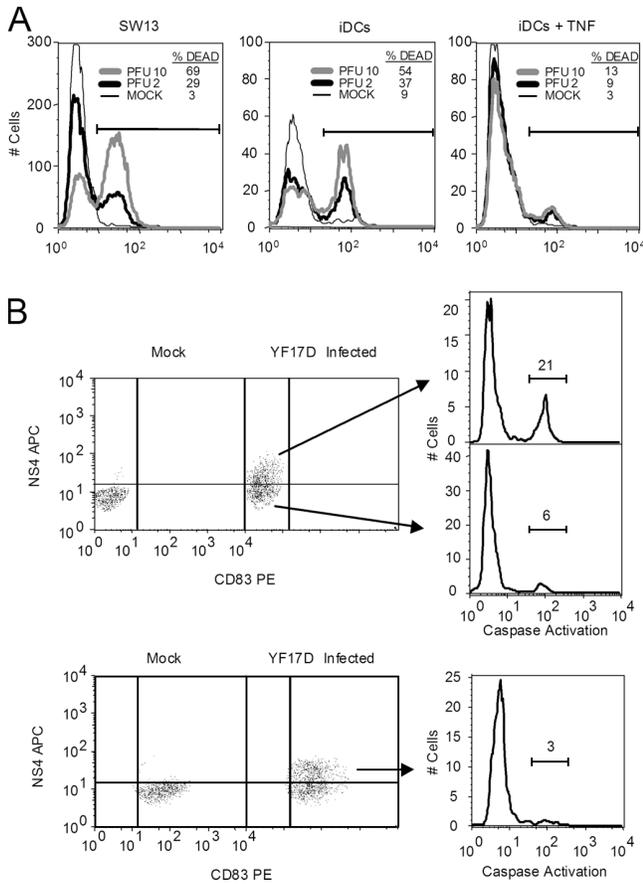


Figure 4. Maturation protects iDCs from YF cytopathogenicity. (A) CaspaTag is measured using CaspaTag-FITC in SW13, iDCs, or iDCs + TNF- α , exposed to the indicated PFU of YF17D virus at 48 h. Tables indicate the percentage of cells that were CaspaTag positive. (B) YF17D-infected iDCs or iDCs + TNF- α were identified by intracellular staining with NS4. CD83 staining was used to distinguish iDCs versus mDCs. CaspaTag activation of the indicated cell populations is indicated by histograms, and the percentage that is CaspaTag positive is indicated.

Maturation protects DCs from YF17D-induced apoptosis

Because YF17D has been shown to be cytopathic in mammalian cells, we evaluated potential cytopathogenicity in human DCs using a FACS-based assay for monitoring apoptotic cell death. Similar to infection by several other viruses, YF17D induces cleavage of caspase-3 in iDCs and triggers cell death. In a dataset representative of three individual experiments, we show 37% (2 PFU/cell) and 54% (10 PFU/cell) CaspaTag-positive iDCs at 48 h after infection (Fig. 4 A). These values were similar to the ones obtained in the SW13 cell line (29% at 2 PFU/cell and 69% at 10 PFU/cell). Interestingly, when we exposed infected iDCs to inflammatory stimuli capable of inducing maturation, there was a considerable reduction in the YF17D-induced cytopathogenicity: 9% (2 PFU/cell) and 13% (10 PFU/cell) of the total cells had activated caspase-3 48 h after infection (Fig. 4 A).

To confirm that iDC cytopathogenicity was YF17D specific and that CaspaTag-negative mDCs were indeed

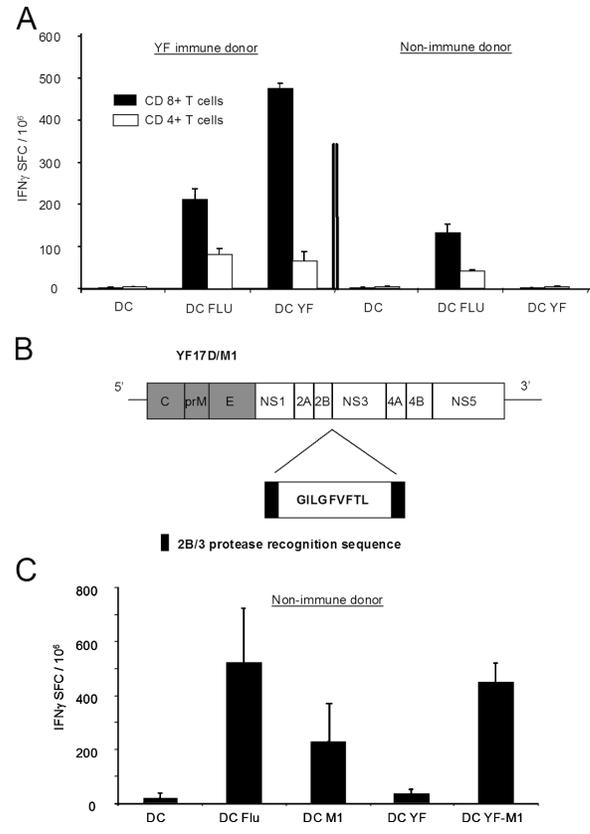


Figure 5. YF17D infection of DCs allows for processing and presentation of endogenous and model T cell epitopes. (A) DCs infected with influenza or YF17D were used to stimulate purified CD8⁺ T cells from YF17D-immunized donor 1 or a naive donor. Stimulation was measured using IFN- γ ELISPOT, and data are shown as spot-forming cells/10⁶ cells. (B) Schematic showing the insertion site of the M1 epitope at the 2B-3 junction. (C) DCs infected with influenza, YF17D, YF17D-M1, or pulsed with 10 μ M M1 peptide were used to stimulate CD8⁺ T cells from a naive donor. T cell stimulation was measured by IFN- γ ELISPOT, as mentioned in Materials and methods. Values represent means \pm SD.

YF17D infected, we stained the infected iDCs for CD83, YF NS4AB, and activated caspase-3. Again, in a representative dataset, gating on the YF-infected CD83⁻ cells showed 21% caspase activation compared with 6% in the YF17D-negative population (Fig. 4 B). In contrast, mature YF17D-infected DCs (CD83⁺/NS4⁺) showed only 3% caspase activation (Fig. 4 B). Plaque assays performed on the supernatants from both cultures confirmed comparable levels of virus production (unpublished data). Based on this data, we conclude that the resistance to YF17D-induced cytopathogenicity is dependent on DC maturation. This resistance to cytopathogenicity could play an important role in allowing infected DCs to remain alive, permitting trafficking to lymph node and priming of T cell responses. This finding is consistent with data from the influenza model in which DC maturation confers resistance to cytopathogenicity, thereby facilitating T cell priming (15).

Infection of DCs allows for processing and presentation of endogenous and model antigens

In light of the direct infection of DCs and the resistance to YF17D-induced cell death on maturation, we evaluated the ability of mDCs to process and present endogenous antigens produced by YF17D. mDCs generated from subjects vaccinated with YF17D were infected and used to stimulate autologous CD8⁺ and CD4⁺ T cells. IFN- γ ELISPOT results showed that infected DCs were able to process and present YF17D antigen for antigen-specific CD8⁺ and CD4⁺ T cell stimulation (Fig. 5 A). Although the mechanism of processing and presentation of endogenous CD4 epitopes remains poorly characterized, antigen may be derived from the exogenous capture of the small percentage of dying infected cells or by alternative mechanisms of processing (19). No T cell response was detected in naive donors. Influenza was used as a positive control to show T cell responses in both immune and naive donors.

Because of the efficacy of the YF17D vaccine, many groups have proposed its use as a vaccine vector for generating CD8⁺ T cell immunity. We propose that these vectors may directly infect DCs, allowing for processing and presentation of CD8⁺ T cell epitopes. We tested this hypothesis by constructing a YF17D vector carrying the immunodominant HLA-A2 M1 CTL epitope from influenza matrix protein. The M1 CTL epitope was inserted between NS2B and NS3 proteins. This site has been shown previously to tolerate a small insertion of foreign sequences (20). mDCs from naive donors were infected with YF17D or chimeric YF17D-M1 virus at 2 PFU/cell as described in Materials and methods and used to stimulate autologous CD8⁺ T cells. IFN- γ ELISPOT results showed that YF17D-M1-infected DCs stimulate robust M1-specific CD8⁺ T cell responses similar to M1 peptide-pulsed or influenza-infected DCs (Fig. 5 C), whereas DCs infected with YF17D alone did not stimulate IFN- γ secretion in the naive donor. Importantly, this is the first demonstration of a YF17D 2B/3 chimeric virus presenting an HLA A2 CTL epitope for T cell stimulation, and it offers proof of principle for a possible vaccine approach.

These data indicate that the YF17D vector is capable of delivering endogenous and recombinant epitopes to the surface of the DC enabling specific T cell activation. In the presence of a maturation stimulus produced in infected tissue, we therefore propose that Ca²⁺-dependent direct infection of DCs may offer a mechanism for the robust and long-lasting immunity associated with the YF17D vaccine.

MATERIALS AND METHODS

Isolation and preparation of cells. PBMCs, DCs, and T cells were prepared as previously described (21). PBMCs were isolated from whole blood by sedimentation over a Ficoll-Hypaque gradient (GE Healthcare). T cell-enriched and T cell-depleted fractions were prepared by adherence to plastic in 1% single donor plasma. iDCs were prepared from the T cell-depleted fraction by culturing cells in the presence of 1,000 U/ml GM-CSF (Berlex) and 500–1,000 U/ml IL-4 (R&D Systems) for 6 d (22). Cultured cells consisted of >75% CD14⁺ CD83⁺ HLA-DR⁺ DCs, with contaminating cells being B cells and dying myeloid cells. To generate mature DCs, cultures

were stimulated on day 6 with 50 ng/ml TNF- α (Qbiogene) and 10 mM PGE-2 (Sigma-Aldrich) for 36–48 h (23). At that time, cells were >85% CD14⁺ CD83⁺ HLA-DR⁺ DCs. Patient material was obtained as per protocol approved by the Institutional Review Board of the Rockefeller University Hospital (JKR-0397), and all patients gave informed written consent. Immunized donor 1 was vaccinated in 2000, and donor 2 was vaccinated in 2001. “Naive donor” refers to healthy blood donors that have not received any flavivirus vaccine.

Preparation of virus stocks. YF17D viral stocks were derived from pACNR/FLYF plasmid containing the full-length infectious YF17D genome under an SP6 promoter (24). In vitro-generated RNA transcripts were electroporated in SW13 cells as previously described (25). Virus stocks were harvested at 48 h after transfection with typical yields of 10⁷–10⁸ PFU/ml as determined by plaque assay on SW13. Single-use aliquots were stored frozen at –80°C until use. YF17D/M1 was constructed by inserting the influenza HLA-A2 CTL epitope of matrix protein (GILGFVFTL) (26) between YF NS2B and NS3 proteins. Two specific oligonucleotides containing the M1 epitope sequence (forward, 5'-aggggagcgcgcagaagtgaatttaggattcgtgttcacgctcggcaccggagaagt-3' and reverse, 5'-acttctccggtagc-gagcgtgaacacgaatcctaaaattccattctgcgcgctccct-3') and two YF17D-specific primers were used to generate two PCR fragments containing the influenza M1 epitope and a portion of the NS2B or NS3 gene, respectively. 1/50 μ l of each PCR reaction was mixed together and used as a template for PCR with YF17D-specific primers lying in NS2B and in NS3. The final product was digested with BssHII and BstEII and cloned in YF17D2B/3 as described previously (7). The recombinant virus was recovered 48 h after transfection of SW13 cells with infectious RNA and tested for stability by PCR and sequence analysis of the region overlapping the insertion. UV inactivation was performed with a UV chamber (GS Gene-Linker; Bio-Rad Laboratories) using the sterilizing program.

Infection of cells. DCs were washed in RPMI 1640 and infected for 1 h at 37°C using the PFUs indicated in the figures. UV-inactivated virus was used as a negative control. The infection was quenched with 5% pooled human serum and washed twice to remove excess virus. For EDTA/EGTA blocking, DCs were washed twice in PBS without Ca²⁺ and Mg²⁺, and 10 mM EDTA or EGTA was added before infection. For antibody blocking, DCs were washed in RPMI 1640 and resuspended in 1% FBS/PBS. DCs were incubated with 10 μ g/ml of antibody (DC-SIGN [m612; R&D Systems] or α v β 3 [Chemicon]) or 100 μ g/ml RGD tripeptides (Sigma-Aldrich) for 15 min at room temperature before infection. After 1 h of infection, DCs were resuspended in conditioned media with or without TNF- α /PGE-2 maturation stimulus, as indicated in the figures, and incubated 24 h before monitoring for infection.

Immunostaining for FACS analysis. Surface staining was done in serum containing media at 4°C. Anti-CD14, CD25, CD40, CD83, CD86, HLA-DR, and isotype control were obtained from BD Biosciences. Cytoperm/CytoFix Kit from BD Biosciences was used for fixation and permeabilization. mAb 1A5 is a mouse mAb against the nonstructural protein NS1 (27), and C12 is a rabbit polyclonal antisera that recognizes the nonstructural proteins NS4A and NS4B (28). Secondary antibodies used were PE (Jackson ImmunoResearch Laboratories) or APC (Invitrogen).

Plaque assay. For plaque titration, serial 10-fold dilutions were used to infect monolayers of SW13 for 1 h at 37°C. After infection, cells were overlaid with 0.6% agarose-containing medium, and plaques were allowed to develop at 37°C for 4 d. Plaques were fixed in 7% formaldehyde for 1 h and stained with crystal violet (1.25% in 20% ETOH) (25).

Detection of influenza-specific T cells by ELISPOT. DCs and T cells were plated in 96-well Millipore plates coated with 5 μ g/ml of α -IFN- γ mAb (Mab-1-D1K; Mabtech). Cultures were incubated for 24–

36 h at 37°C, washed with mild detergent, and incubated with 1 µg/ml biotin-conjugated α-IFN-γ mAb (Mab 7BG-1; Mabtech). Spots were visualized using Vectastain Elite Kit (Vector Laboratories). ELISPOT reagents were provided by R. Darnell (The Rockefeller University, New York, NY). Evaluation was performed in a blinded fashion by an independent service (Zellnet Consulting, Inc.) using an automated ELISPOT reader (Carl Zeiss MicroImaging, Inc.). Spots represent IFN-γ production by single cells and are reported as spot-forming cells/10⁶ cells.

Online supplemental material. Fig. S1 shows purity and phenotype of iDC and mDC cultures. Online supplemental material is available at <http://www.jem.org/cgi/content/full/jem.20051352/DC1>.

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