Feline immunodeficiency virus dendritic cell infection and transfer

Wendy S. Sprague,1 Melissa Robbiani,2 Paul R. Avery,1 Kevin P. O’Halloran1 and Edward A. Hoover1

Correspondence
Wendy S. Sprague
wsprague@lamar.colostate.edu

1Department of Microbiology, Immunology and Pathology, College of Veterinary Medicine and Biomedical Sciences, Colorado State University, Fort Collins, CO 80523-1619, USA
2Center for Biomedical Research, Population Council, 1230 York Avenue, New York, NY 10021, USA

Feline immunodeficiency virus (FIV) interacts with dendritic cells (DC) during initiation of infection, but whether DC support or transfer FIV infection remains unclear. To address this issue, we studied the susceptibility of feline myeloid DC to FIV infection and assessed potential transfer of infection from DC to CD4+ T cells. FIV was detected in membrane-bound vesicles of DC within 2 h of inoculation, although only low concentrations of FIV DNA were found in virus-exposed isolated DC. Addition of resting CD4+ T cells increased viral DNA levels; however, addition of activated CD4+ T cells resulted in a burst of viral replication manifested by FIV p27 capsid antigen generation. To determine whether transfer of FIV infection required productively infected DC (vs virus bound to DC but not internalized), virus-exposed DC were cultured for 2 days to allow for degradation of uninternalized virus and initiation of infection in the DC, then CD4+ T blasts were added. Infection of T cells remained robust, indicating that T-cell infection is likely to be mediated by de novo viral infection of DC followed by viral transfer during normal DC/T-cell interactions. We conclude that feline DC support restricted FIV infection, which nevertheless is sufficient to efficiently transfer infection to susceptible T cells and trigger the major burst of viral replication. Feline DC/FIV/T-cell interactions (similar to those believed to occur in human immunodeficiency virus and simian immunodeficiency virus infections) highlight the means by which immunodeficiency-inducing lentiviruses exploit normal DC/T-cell interactions to transfer and amplify virus infection.

INTRODUCTION

Dendritic cells (DC) are ideally placed to play a central role in immunodeficiency virus infections and appear to be one of the first cells to encounter mucosally transmitted lentiviruses. Current evidence suggests that simian immunodeficiency virus (SIV) is passed from DC to CD4+ T cells, wherein virus amplification then occurs (Hu et al., 2000; Milush et al., 2004; Stahl-Hennig et al., 1999; Zhang et al., 1999). In human immunodeficiency virus (HIV-1) and SIV infections, there is evidence that conjugates of cutaneous DC and T cells generate virus amplification in vitro (Pope et al., 1994, 1997); and HIV-1 replication in vivo has been demonstrated in syncytia derived from DC in lymphoepithelium of the tonsil adenoid mucosa (Frankel et al., 1996, 1997). Exactly how DC serve to amplify lentivirus infection remains the subject of current research. In vitro studies involving exposure of DC to HIV-1 have yielded varying results, postulated to relate to: (i) different DC subsets used (i.e. blood vs cultured DC), (ii) different DC isolation methods (i.e. antibody purification vs metrimazide gradient), (iii) different methods of viral detection (i.e. electron microscopy vs PCR) and (iv) different virus isolates (Blauvelt et al., 1997; Cameron et al., 1994; Granelli-Piperno et al., 1998; Patterson & Knight, 1987). However, what seems least controversial is that HIV-1 amplification occurs in DC/T-cell conjugates (Cameron et al., 1992; Pope et al., 1994, 1995). Recent work has questioned whether HIV-1 and SIV are captured by or replicated in DC. It has been proposed that lentivirus transfer to T cells occurs in two phases: (i) an immediate transfer of virus captured and internalized by DC and (ii) a more efficient transfer that requires de novo virus replication in immature DC (Frank et al., 2002; Turville et al., 2004).

FIV is the feline counterpart of HIV-1 infection, and may therefore display a very similar mode of early infection pathogenesis. To investigate this point, we examined whether feline monocyte-derived DC can take up, replicate and/or transfer FIV to CD4+ T cells.

METHODS

Animals and sampling. Peripheral blood mononuclear cells (PBMC) were isolated from blood of 6–24-month-old specific-pathogen-free...
(SPF) cats from a colony maintained at Colorado State University (Fort Collins, CO, USA) in accordance with Animal Care and Use Committee Regulations.

Generation of FIV supernatants. The cell-free virus inoculum (8.89 × 10⁴ TCID₅₀ ml⁻¹) was generated from co-culture of naïve feline PBMC, with PBMC from a cat intravenously infected with FIV clade C isolate Paddy Gammer (Sodora et al., 1994). Infectivity of the supernatants was determined by titration and aliquots were frozen at −70 °C.

Cell isolation and culture. DC were cultured from CD14⁺ monocytes as described previously (Sprague et al., 2005). Since purity of DC cultures was needed for the infection studies, DC harvested after 6–7 days of culture were positively selected from contaminating lymphocytes using magnetic columns (Miltenyi Biotec) and a cross-reactive canine CD11c Ab (Leukocyte Antigen Laboratory, University of California, Davis, USA). Briefly, cells were blocked with 200 μl goat serum (Sigma-Aldrich), washed with PBS + 0.5 % BSA and incubated with CD11c Ab (20 μl per 10⁵ cells) for 20 min at 4 °C. Cells were washed again with PBS + 0.5 % BSA and incubated with goat anti-mouse beads (20 μl per 10⁵ cells) and placed in a magnetic column according to manufacturer’s instructions (MS column; Miltenyi Biotec). CD4⁺ T cells were isolated from the negative fraction of the CD14⁺ selection and incubated with phycoerythrin (PE)-conjugated anti-feline CD4 (Southern Biotech) and a cross-reactive anti-human CD21 Ab (Pharmingen). Isotype-matched mouse immunoglobulins were used as controls. Cells were resuspended in flow buffer (PBS + 0.5 % sodium azide + 2 % FBS), propidium iodide was used to exclude dead cells from analysis and gating of large cells for analysis was determined by scatter properties using a Coulter Counter (Epics XL-MCL; Beckman Coulter). Data was analysed using FlowJo software (Tree Star).

Flow cytometry. Phenotypic expression of CD11c⁺-purified feline DC was evaluated by flow cytometry as described previously (Sprague et al., 2005). DC purity was assessed using anti-feline CD4, anti-feline CD8 (Southern Biotech) and a cross-reactive anti-human CD21 Ab (Pharmingen). Isotype-matched mouse immunoglobulins were used as controls. Cells were resuspended in flow buffer (PBS + 0.5 % sodium azide + 2 % FBS), propidium iodide was used to exclude dead cells from analysis and gating of large cells for analysis was determined by scatter properties using a Coulter Counter (Epics XL-MCL; Beckman Coulter). Data was analysed using FlowJo software (Tree Star).

Inoculation of DC with FIV and virus co-culture. Between 3 × 10⁵ and 7 × 10⁵ DC were placed into 15 ml centrifuge tubes (BD Falcon) in 500 μl complete RPMI. Cells were infected with 2–3 × 10⁵ 50 % TCID₅₀. DC were incubated for 2 h at 37 °C and then washed four times to remove unbound virus. DC were cultured at 6.0 × 10⁵ cells ml⁻¹ in 200 μl complete RPMI in 96-well flat-bottomed plates (BD Falcon) for an additional 7 or 9 days. Unstimulated CD4⁺ T cells or activated CD4⁺ T cells were added immediately at DC:T cell ratios of 1:10 to some wells. In some experiments, activated CD4⁺ T cells were added after 2 days of DC infection and culture to assess if infection was from de novo FIV synthesis within DC. One-half of the medium was changed and cytokines were replenished every other day during the culture period.

Real-time DNA PCR for FIV. DNA was extracted from DC or DC/T cell co-cultures after 7 or 9 days in culture using a QIAamp Mini kit (Qiagen). Quantitative real-time PCR (qPCR) was performed using previously published FIV CENVmar primer and probe sequences (Pedersen et al., 2001) and an iCycler iQ (Bio-Rad). qPCR was performed using 2 × TaqMan Universal PCR Master Mix (Applied Biosciences) consisting of 10 mM Tris/HCl (pH 8.3), 50 mM KCl, 5 mM MgCl₂, 300 μM each of dATP, dCTP and dGTP, 600 μM dUTP, 0.625 U AmpliTaq Gold DNA polymerase and 0.25 μU uracil N-glycosylase (UNG) per reaction. Reactions were performed in a total volume of 25 μl and consisted of 12.5 μl Mastermix, 5 μl of sample or standard, 400 nM of each primer and 80 nM probe. Thermal cycling conditions were 2 min at 50 °C to induce enzymic activity of UNG, 10 min at 95 °C to reduce UNG activity and to activate AmpliTaq Gold DNA, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. A standard curve was generated with 10-fold serial dilutions of FIV C gag plasmid DNA with 1 × TE and 40 ng salmon testes DNA (Sigma-Aldrich) ml⁻¹ as a DNA carrier for each run and numbers of FIV DNA viral copies were calculated from these curves. Statistical analysis was performed on paired samples using the Wilcoxon signed-rank test using Prism version 4.0b software for Macintosh (GraphPad).

FIV p26 ELISA. Productive infection of DC or DC/T cell co-cultures was assessed by the FIV capsid antigen (Ag) p26 capture ELISA described previously by Dreitz et al. (1995). OD₅₄₀ were recorded with a Dynatech 5000MRTM microplate reader (Dynatech). A minimum OD₅₄₀ of 0.1 that was at least twice the value of negative control supernatants run in parallel defined the positive reactions. Statistical analysis was performed using the Student’s t test.

Electron microscopy. DC were pulsed with 8–9 × 10⁴ TCID₅₀ to maximize frequency of infection. DC pulsed with FIV for 2 h were washed with complete RPMI and immediately fixed with 2 % glutaraldehyde in PBS at 4 °C. Specimens were post-fixed in osmium tetroxide, dehydrated in acetone, and embedded in a 50:50 mixture of Epon 812 and Spurr resins, all using a rapid microwave technique described elsewhere (Leong & Sormunen, 1998). Ultrathin sections were mounted on nickel grids, contrasted with uranyl acetate and lead citrate, and examined using transmission electron microscopy (TEM) (JEOL 2000EXII).

RESULTS

Myeloid DC can be highly purified from blood monocytes

DC recovery using CD11c⁺ selection was approximately 51 ± 10 % and purity was between 97 and 99.5 % as measured by scattergram gating, lack of CD4⁺ and CD8⁺ staining for T cells, and CD21 staining for B cells (data not shown), resulting in a population of highly pure immature myeloid DC.

FIV viral particles are found within large DC vacuoles

DC were examined for virus uptake by TEM 2 h after inoculation and incubation at 37 °C. In the small minority of the cells that contained viral particles, virus was found within large membrane-bound intra-cytoplasmic vacuoles as seen in Fig. 1. Virus particles were similarly found in DC cultured from four different cats.
FIV DNA levels are divergent in DC versus DC-CD4+ T cell co-cultures

FIV DNA loads were lower in 7 day-cultured FIV-pulsed DC, than in paired co-cultures in which unstimulated CD4+ T cells were added to FIV-pulsed DC (P=0.0537). When activated CD4+ T cells were added to FIV-pulsed and washed DC, viral DNA increased several log fold over DC cultured alone (P=0.0156) or over DC co-cultured with unstimulated CD4+ T cells (P=0.0156) (Fig. 2).

FIV p26 capsid Ag correlates with viral DNA

FIV p26 capsid Ag in supernatants harvested from DC cultures and DC-unstimulated CD4+ T cell co-cultures could not be detected by capture ELISA (Fig. 3). Only when activated CD4+ T cells were added to the DC was Ag detected; thereafter a steady increase in viral protein was noted through 7 days post-infection (p.i.). The p26 assay results correlated with the very low FIV DNA loads detected in DC alone and in DC co-cultured with unstimulated CD4+ T cells, whereas high viral DNA levels were detected in co-cultures of virus-exposed DC with activated CD4+ T cells. Statistically significant differences distinguished FIV protein expression in DC cultures alone [5 days p.i., P=0.0302; 7 days p.i., P<0.0001] and DC-unstimulated CD4+ T cell co-cultures (5 days p.i., P=0.0353; 7 days p.i., P<0.0001) versus DC-activated CD4+ T cell cultures.

Activated CD4+ T-cell infection arises from de novo FIV replication in DC

Infectious lentivirus captured by DC does not survive more than 24 h (Turville et al., 2004) in vitro. Therefore, to
determine if transfer of infection was induced by *de novo* replication of FIV within DC, FIV-pulsed and washed DC were cultured for an additional 48 h prior to adding activated CD4$^+$ T cells. Amplification of viral DNA was still apparent when activated CD4$^+$ T cells were added 48 h after initial FIV-DC exposure, strongly suggesting that *de novo* replication of FIV had occurred (Fig. 4). Statistical analysis revealed a trend towards significance ($P=0.0625$) between FIV DNA viral loads in DC cultured alone versus those cultured with activated CD4$^+$ T cells.

**FIV p26 capsid Ag is detectable after *de novo* replication**

FIV p26 capsid Ag was detectable in culture supernatants of DC-CD4$^+$ T cells 7 days after addition of activated CD4$^+$ T cells, again correlating with FIV DNA viral loads (Fig. 5). In contrast, a minimum of 9 days was required before p26 was detectable in supernatants of DC cultured alone, suggesting that the virus replicates slowly at low levels in these cells. Increase in DC FIV DNA loads over time supports *de novo* replication.

FIV DNA loads increased in DC cultured alone 9 days after DC were exposed to FIV (Fig. 6). This correlates with the detection of p26 in culture supernatants by day 9 noted in Fig. 5 and further supports low level *de novo* replication. However, statistical significance was not reached and this was likely to be due to low sample numbers ($n=4$).

**DISCUSSION**

The results of this study support the tenet that the exploitation of DC by lentiviruses is the pivotal event in the undermining of the immune response by lentiviruses (Frank & Pope, 2002; Lore *et al.*, 2005; McDonald *et al.*, 2003; Turville *et al.*, 2004; Wiley & Gummuluru, 2006). To understand the events involved in the initial contact of FIV with purified immature feline DC, FIV-pulsed and washed DC were examined for virus uptake. Uptake of FIV viral particles into large cytoplasmic vacuoles was evident by 2 h post-incubation, comparable to that seen in SIV and HIV-1 (Blom *et al.*, 1993; Frank *et al.*, 2002; Wiley & Gummuluru, 2006). In addition, the low viral DNA levels found in feline DC exposed to FIV for 2 h, then washed and recultured for 7 days strongly parallel the results reported by Turville *et al.* (2004, 2005) regarding HIV-1-exposure of immature human DC. We did observe higher DC viral DNA levels in a few animals. While this could have been due to
lymphocyte contamination, lymphocytes were not detected by flow cytometry or light microscopy, and the differing DNA levels could reflect individual variation among cats.

When unstimulated CD4+ T cells were added to the FIV-exposed and washed DC followed by co-culture for 7 days, only slight amplification of viral DNA was detected. While it has been shown previously that highly purified unstimulated CD4+ T cells cannot be infected with HIV-1 in vitro (Ignatius et al., 1998; Stevenson et al., 1990; Zack et al., 1992), modest enhancement of virus replication was noted in DC-unstimulated CD4+ T cell co-cultures (Weissman et al., 1996). In addition, it has been shown that IL-4, which was added to our co-cultures, enhances replication in both purified unstimulated CD4+ T cells and in DC-unstimulated CD4+ T cell co-cultures (Unutmaz et al., 1999; Weissman et al., 1996). Most prominent in our studies was the marked increase in viral DNA loads observed after co-culture of activated CD4+ T cells with FIV-pulsed DC. This result correlates with previous in vitro and in vivo studies demonstrating amplified HIV and SIV production in activated CD4+ T cells (Cameron et al., 1992; Granelli-Piperno et al., 1998; Zhang et al., 1999).

The lack of detectable FIV capsid protein and low viral DNA levels in immature DC cultured alone and in DC-unstimulated CD4+/T cell co-cultures is consistent with the results of a study by Messmer et al. (2000) that demonstrated the absence of SIV p27 Ag in immature DC cultured alone and the lack of detectable viral levels in immature DC/unstimulated CD4+ T cell co-cultures until cells had been in culture for more than 7 days. FIV capsid protein was also undetected in feline bone-marrow-derived DC and DC/unstimulated PBMC co-cultures infected with FIV and cultured for 6 days (van der Meer et al., 2007). The low viral DNA levels in the DC/unstimulated CD4+ T cell co-cultures could reflect a lack of viral DNA integration into genomic DNA (Stevenson et al., 1990) and/or a block in reverse transcription, phenomena demonstrated in HIV-1 infection of unstimulated CD4+ T cells (Zack et al., 1992). Blocks in reverse transcription could also be due to the presence of the enzymically active, low-molecular-mass cytidine deaminase, APOBEC3G, which has been shown to inhibit HIV-1 infection in CD4+ T cells by an as yet undetermined cytidine-deaminase-independent mechanism (Chiu et al., 2005), and/or to the presence of Mur1, a gene product that has been shown to inhibit HIV-1 replication in unstimulated CD4+ T cells (Ganesh et al., 2003). It has also recently been shown that APOBEC3G inhibits HIV replication in immature DC through a cytidine-deaminase-dependent mechanism (Pion et al., 2006). In addition, upregulation of APOBEC3G in mature DC has been demonstrated, which could explain the comparatively decreased HIV replicative capacity in mature DC. FIV p26 capsid Ag was detected by 5 days after addition of activated CD4+ T cells to DC cultures. This result was as expected, since IL-2-stimulated cellular activation and proliferation have been shown to be necessary for productive HIV-1 infection (Zack et al., 1992; Oswald-Richter et al., 2004; Weissman et al., 1996).

The means by which DC transfer infection to T cells remains controversial. In order to test the possibilities that the transfer of FIV from feline DC to CD4+ T cells was by uptake of DC-replicated or endocytosed virus particles or by transfer of input virus bound to or endocytosed by DC, we recultured FIV-pulsed DC for 48 h before the addition of activated CD4+ T cells. FIV infection of CD4+ T cells remained unobstructed and productive infection ensued. While it is not known with certainty that the originally pulsed virus was completely degraded in the 48 h time period, de novo replication of FIV in these immature DC presents the most likely scenario for viral transfer to CD4+ T cells (Turville et al., 2004, 2005). This scenario is supported by an increase in FIV DNA and p26 capsid protein in FIV-pulsed DC after 9 days in culture compared with 0 or 2 days in culture. In addition, we attempted to block infection using the reverse transcriptase inhibitor, zidovudine (AZT); however, results were not interpretable due to toxicity of AZT, at antiviral concentrations, to DC. Our provisional conclusions are that immature feline DC transfer FIV infection to CD4+ T cells by immediate transfer of virus via exosomes and/or through endolysosomal pathways and that delayed transfer of virus occurs via de novo viral replication in DC, thus revealing observations parallel to those obtained with HIV-1 and immature human DC (Turville et al., 2004; Wiley & Gummeluru, 2006).

In summary, we have demonstrated that FIV can capitalize on initial DC/CD4+ T-cell interactions to transmit infection to amplifying CD4+ T cells, consistent with observations made with HIV-1 and SIV. While it may not be surprising that the interactions between immune deficiency viruses from different species and the innate immune system are similar, that these interactions are conserved illustrates the importance of understanding the mechanisms at work in this early stage of pathogenesis. That immunodeficiency-inducing lentiviruses are remarkably efficient in transferring infection to CD4+ T cells after DC capture reinforces a focus on mucosal vaccination and microbicide strategies to contain and/or prevent these infections.

ACKNOWLEDGEMENTS

This work was supported by National Institutes of Health (NIH) grants RO1-AI-033773 and KO8-AI-01784.

REFERENCES


