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HIV latency is influenced by regions of the viral genome outside of the long terminal repeats and regulatory genes

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ABSTRACT

We have previously described an *in vitro* primary thymocyte model for HIV latency that recapitulates several important aspects of latently infected cells obtained from patients. Our original model included a truncated HIV genome expressing only Tat, Rev, and Vpu along with a reporter gene. We have now expanded these studies to include reporter viruses encoding more complete viral genomes. We show here that regions of the viral genome outside of the long terminal repeat promoter and Tat/Rev regulatory genes can substantially affect both the basal level of HIV transcription prior to stimulation, and also the level of viral expression following costimulation via CD3 and CD28 ligation. These differences in latency phenotype between truncated and more complete HIV genomes demonstrate the importance of accessory genes in the context of HIV latency and indicate that care should be taken when interpreting data derived from heavily modified HIV genomes in latency models.

Background

The ability of human immunodeficiency virus (HIV) to establish and re-emerge from a latent state in CD4+ T cells is an important barrier to eradication of the virus from infected individuals (Chun et al., 1997; Finzi et al., 1999; Wong et al., 1997). Understanding the molecular basis for HIV latency and the contribution of viral and host factors in this process is therefore a significant and pressing challenge. However, the infrequent nature of latently infected cells obtained from patients together with various difficulties inherent to their analysis has necessitated the use of model systems that allow a more thorough investigation of HIV latency (reviewed in (Han et al., 2007; Marsden and Zack, 2009, 2010)). These have included various cell-line-based approaches (Folks et al., 1987, 1989; Jordan et al., 2003), and more recently developed *in vitro* primary cell models (Bosque and Planelles, 2009; Sahu et al., 2006). Our group has also been involved in the development of *ex vivo* and *in vitro* primary cell models for HIV latency, with the goal of better understanding how HIV enters into latency and developing improved methods for activation and elimination of latently infected cells (Arlen et al., 2006; Brooks and Zack, 2002; Brooks et al., 2001, 2003a, 2003b; Burke et al., 2007; Korin et al., 2002; Scripture-Adams et al., 2002).

One mechanism by which HIV can establish a latent infection in T cells is during the process of thymopoiesis (Brooks et al., 2001). This can occur because immature CD4+CD8+ thymocytes are metabolically and transcriptionally active enough to support a productive infection by HIV. However, as these cells differentiate into naive resting T cells they also become incapable of maintaining efficient HIV expression. Hence, if a thymocyte is infected at the CD4+CD8+ stage of development then it can differentiate into a naïve T cell harboring a latent provirus. We have previously utilized the SCID-hu thymus/liver (Thy/Liv) mouse model (McCune et al., 1988; Namikawa et al., 1990) to investigate the process of thymocyte infection by HIV, and to study the molecular mechanisms associated with the establishment and reactivation of latency in this cell type (Brooks et al., 2001). This model has proved useful for a range of applications including characterizing the surface phenotype of latently infected cells, identifying stimulatory pathways that are capable of activating latent proviruses, and testing methods for selectively eliminating these cells (Brooks and Zack, 2002; Brooks et al., 2003a, 2003b; Korin et al., 2002). However the SCID-hu model is also extremely labor-intensive and involves some other limitations that can restrict its utility. For example, the requirement for active virus replication in the SCID-hu model complicates direct comparison of viruses harboring debilitating mutations, such as within the viral long terminal repeat (LTR) promoter.

To circumvent these issues, we developed an *in vitro* thymocyte model based on similar principles to the SCID-hu latency model. In this *in vitro* model, purified CD4+CD8+ fetal thymocytes are infected with

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an HIV-based reporter virus and differentiated for 1 week before costimulation by ligation of CD3 and CD28 (Burke et al., 2007). This procedure typically results in >100-fold induction of reporter expression, and when used in conjunction with LTR mutants, was used to demonstrate that the presence of NFκB binding sites within the LTR is critical for maximal stimulation of virus expression (Burke et al., 2007). Because this system was initially established to characterize the LTR, we utilized a reporter virus that expressed only Tat, Rev, and Vpu in conjunction with a reporter gene. We have now extended these studies to include viruses with more complete viral genomes. Here we report that the expression profile of HIV in both cytokine-treated and costimulated thymocytes can be substantially affected by regions outside of the viral promoter and regulatory genes. Remarkably, these differences can influence virus expression sufficiently to dictate whether HIV enters a latent or productive state in thymocyte cells.

Results

Viral activation assay

We previously described an *in vitro* HIV latency model in which CD4+CD8+ double-positive fetal thymocytes were infected with an HIV-based vector then differentiated into single-positive cells before activation (Burke et al., 2007). To determine whether unsorted thymocytes (as would be present *in vivo*) produce a similar phenotype, we isolated both total thymocytes and CD4+CD8+ double-positive thymocytes from the same donor tissue. This procedure typically resulted in a >99% pure population of CD4+CD8+ cells (Fig. 1A). The cells were infected with NLEGFPLUC virus and then differentiated in the presence of IL-2 and IL-4 for 1 week before stimulation with either anti-CD3 antibody alone, or costimulation with anti-CD3 and anti-CD28 antibodies. As previously described (Burke et al., 2007), unstimulated CD4+CD8+ thymocytes expressed little or no reporter activity without stimulation, but this activity increased >100-fold after costimulation (Fig. 1B). In the current study we found that stimulation with anti-CD3 antibody alone was also sufficient to activate virus, but to a lesser extent than costimulation (Fig. 1B). When the same procedure was performed with total thymocytes a similar pattern of activity was evident, although with uniformly higher levels of reporter expression (Fig. 1C). This is likely due to the enhanced viability of total thymocytes, which are composed of a more physiological mix of cells that have not been subjected to the same sorting procedures as the purified double-positive cells.

Comparison of NLEGFPLUC with more complete HIV genomes

The apparently latent phenotype obtained after infection of either unsorted or sorted thymocytes with NLEGFPLUC is striking, because

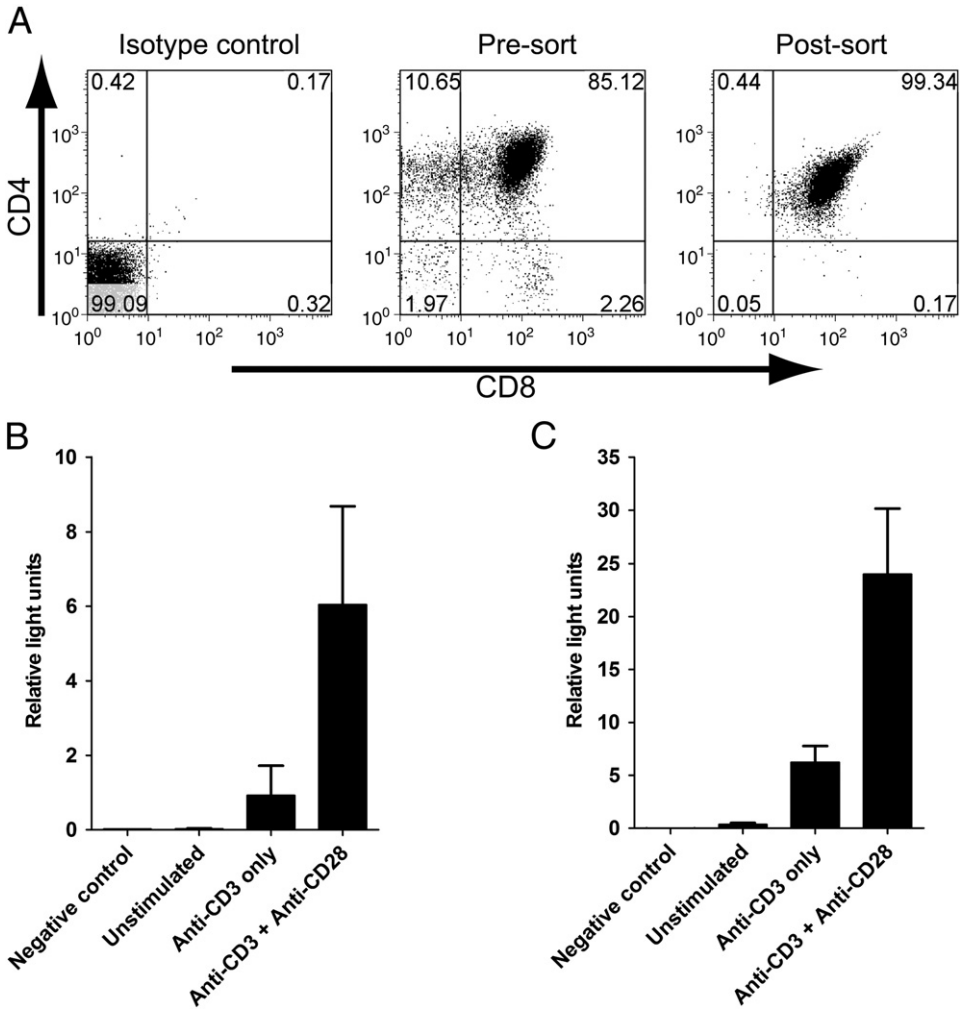

Fig. 1. Viral activation assay using sorted CD4+CD8+ thymocytes and unsorted total thymocytes. Total thymocytes were isolated from fetal thymic tissue and infected either directly or after immunomagnetic enrichment of CD4+CD8+ cells. A) Cells were stained for CD4 and CD8 surface markers both pre- and post-sort and then analyzed by flow cytometry. This isolation procedure typically resulted in a >99% purity of CD4+CD8+ cells. B) CD4+CD8+ cells were infected with NLEGFPLUC then cultured for 1 week prior to stimulation. At day 7 post-infection cells were either left unstimulated, activated with anti-CD3 antibody alone, or activated by costimulation using both anti-CD3 and anti-CD28 antibodies. Cells were then lysed and assayed for luciferase activity at day 10 post-infection. C) Total thymocytes were infected with NLEGFPLUC and assayed as described in part B.

productive infection of thymocyte cells with replication-competent HIV is common. Indeed, when HIV NL4-3 is added to thymocyte cultures under similar conditions it spreads readily (Fig. 2A) (Uittenbogaart et al., 1994). It was possible that the unusual phenotype was somehow related to the VSV-pseudotyping of NLEGFPLUC, or to the limitation of this virus to a single-round of infection. Therefore we compared total thymocyte infections performed in parallel with either NLEGFPLUC or denv(Wt) (Marsden and Zack, 2007). This latter reporter virus is also VSV-pseudotyped but is produced from a more complete HIV genome clone that expresses a protein consisting of EGFP fused to luciferase in place of env, but retains expression of all other HIV genes (Fig. 2B). Over the first 2 days of total thymocyte infection with these viruses NLEGFPLUC was indistinguishable from mock. In contrast, denv(Wt) displayed substantial gene expression (Fig. 2C). Therefore some component of the denv(Wt) virus that is absent from NLEGFPLUC is responsible for severely altering the infection characteristics of these two viruses in a manner influenced by the stimulation level of the host cell.

Complete timecourses +/− Nef

In order to assess the potential contribution of Nef to the observed phenotype in thymocyte cells, a nef-frameshifted version of denv(Wt) termed denv(NEF-FS) was constructed. The viruses were titered in parallel in CEM cells using a limiting dilution procedure, and equivalent infectious units used to infect thymocytes at a multiplicity of infection of approximately 0.05. CD4+CD8+ thymocytes were isolated and infected with NLEGFPLUC, denv(Wt), or denv(NEF-FS) viruses and 200,000 viable cells were taken from the cultures each day for luciferase analysis. Cells were incubated for 7 days in the presence of IL-2 and IL-4 and then costimulated with anti-CD3 and anti-CD28 antibodies for a further 3 days. Results for NLEGFPLUC were similar to those obtained previously, where the virus only expressed after the cells were costimulated (Fig. 3, left panel). In contrast denv(Wt) consistently expressed both prior to and after costimulation. Reporter expression in denv(Wt) infected cells also increased following costimulation but to a much lesser extent than with NLEGFPLUC (Fig. 3, center panel). Disrupting the Nef coding sequence within denv (Wt) did not prevent background expression of the virus, and in fact the background expression of the denv(Nef-FS) virus was increased slightly with respect to the parental virus (Fig. 3, right panel). However, the overall kinetic of the denv(NEF-FS) virus in this assay was more similar to denv(Wt) than to NLEGFPLUC. Therefore the absence of Nef in NLEGFPLUC proviruses was not the reason for its lack of expression in thymocytes without overt costimulation.

The potential contribution of Vpr to the thymocyte phenotype was then assessed by constructing two new viruses. In the first virus Vpr was added back to the NLEGFPLUC virion by using a packaging construct that encodes Vpr. In the second virus a Vpr-frameshift mutation was introduced into denv(Wt), producing both virions and

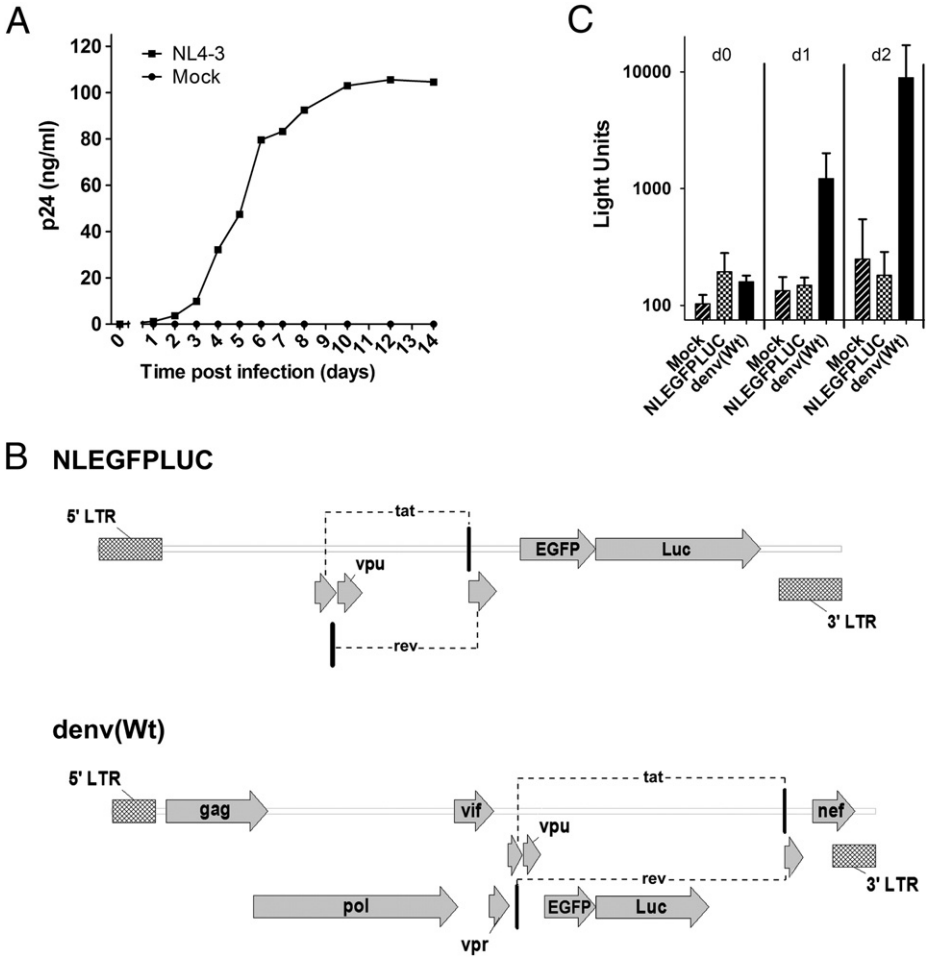

Fig. 2. Replication and expression kinetics in primary thymocytes. A) Fetal thymocytes were isolated and infected with replication competent HIV (NL4-3). Cells were incubated in the presence of IL-2 and IL-4, (without costimulation) and monitored by ELISA for HIV p24 protein accumulation in the supernatant. Under these conditions HIV spreads rapidly in thymocyte culture. B) Schematic diagram of the NLEGFPLUC and denv(Wt) HIV vectors. denv(Wt) contains a GFP–luciferase reporter gene in place of HIV env. C) Total thymocytes were infected with either NLEGFPLUC or denv(Wt), and cells were assayed for luciferase activity at days 0, 1, and 2 post-infection. Cells were cultured in media containing IL-2 and IL-4 without costimulation.

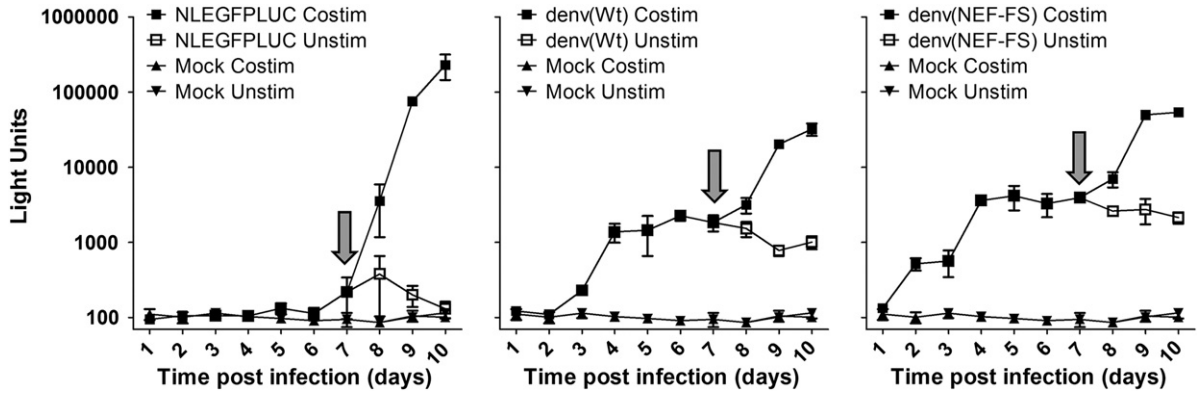

Fig. 3. Timecourse in CD4+CD8+ thymocytes. CD4+CD8+ thymocytes were infected with equivalent infectious units of either NLEGFPLUC, denv(Wt), or denv(NEF-FS). At 7 days post-infection cells were divided into costimulated or unstimulated conditions (indicated with arrow). Quadruplicate samples of 200,000 viable cells were harvested at each timepoint and assayed for luciferase activity. For clarity, results for each virus are shown on a separate panel with the same mock control values.

resultant proviruses that are Vpr-deficient. These viruses were used to infect CD4+CD8+ thymocytes and samples were taken immediately post-infection and again at d6 post-infection. Addition of vpr to the NLEGFPLUC virus and removal from denv(Wt) did not alter their expression characteristics with respect to the parental viruses (Fig. 4). Therefore Vpr was not responsible for the increased basal expression levels in these cells.

Discussion

In addition to directly contributing to HIV pathogenesis, infection of thymocyte cells by HIV can also influence viral persistence by providing a mechanism for the establishment of latency (Brooks et al., 2001). In the current work we have expanded our investigation of this postintegration latency in thymocytes. We previously demonstrated that *in vitro* infection of cytokine-stimulated CD4+CD8+ thymocytes with a truncated HIV-based reporter virus (NLEGFPLUC) produces cells harboring an integrated, but tightly latent, provirus (Burke et al., 2007). These cells express little or no reporter activity over the course of a 10 day assay period unless the cells are first allowed to differentiate for several days and are then costimulated with anti-CD3 and anti-CD28 antibodies. Here we show that this pattern of activity is emulated in total thymocyte cell cultures that have been treated in the same manner (Fig. 1). However, when replication-competent HIV is used in this procedure, a productive infection ensues. Moreover, a reporter virus that is Env-deficient but encodes all other HIV genes (denv[Wt]) is also capable of expressing in total thymocytes without costimulation (Fig. 2). When performing a complete 10-day timecourse experiment, in which the cells are costimulated for the final 3 days of the assay, the expression profiles of NLEGFPLUC and denv(Wt) are quite distinct. NLEGFPLUC expresses at a much lower level prior to stimulation and a consistently higher level after stimulation than denv(Wt) (Fig. 3). These observations indicate that the establishment of a latent or productive infection in thymocyte cells can be strongly influenced by factors outside of the LTR promoter and Tat/Rev regulatory loops.

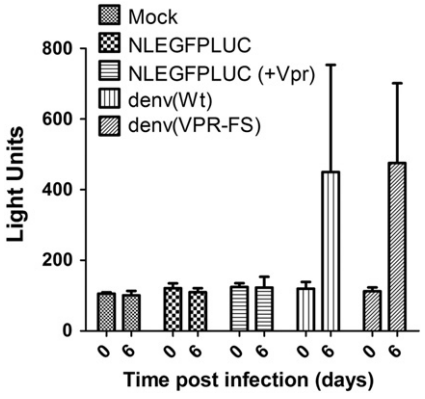

Fig. 4. Reporter expression in thymocytes after infection with virions +/− Vpr. Thymocytes were infected with virions either containing or deficient in Vpr protein. These cells were assayed both immediately post-infection and at day 6 post-infection.

Several potential mechanisms could underlie the differences in expression profile between NLEGFPLUC and more complete viral genomes. One possibility is that differing virion compositions could influence virus expression in thymocytes. In particular, the NLEGFPLUC virions were originally produced using the vpr-deficient packaging construct pCMVΔR8.2DVPR (An et al., 1999; Naldini et al., 1996), resulting in virions that do not contain the Vpr protein. Vpr has been shown to be important for infection of some primary cell types (Connor et al., 1995). We therefore produced NLEGFPLUC virions with a packaging construct that contains vpr. A vpr-deficient version of denv(Wt) was also constructed and included for comparison. The addition of Vpr to NLEGFPLUC or removal of Vpr from denv(Wt) did not alter the expression profiles of these viruses in thymocytes (Fig. 4), illustrating that Vpr was not the source of the different virus characteristics. A second possibility is that *de novo* expression of an HIV accessory/regulatory protein by the denv(Wt) provirus in the newly infected target cell enhanced expression sufficiently to allow detectable reporter activity. This is unlikely to be due to differences in expression of Tat, Rev, or Vpu because these genes are encoded by the packaged RNA in both viruses, and therefore they would be present and expressed by each provirus in the newly infected thymocyte cells. Vif was also present in both NLEGFPLUC and denv(Wt) virions, and because this viral protein functions primarily in a producer-cell-dependent fashion (Sheehy et al., 2002), or prior to integration in a newly infected host cell (Chiu et al., 2005), it is unlikely to modulate viral gene expression in the manner observed here. In contrast, the nef open reading frame is not functional in the NLEGFPLUC provirus. Nef has been shown to be capable of interacting with cellular kinases and modulating the state of the cell in multiple ways that enhance viral expression (Baur et al., 1997; Renkema et al., 2001; Simmons et al., 2001). To determine whether Nef is the factor that allows expression of denv(Wt) in thymocytes prior to costimulation, we introduced an inactivating mutation into the nef open reading frame of denv(Wt) to produce denv(NEF-FS). This virus was similar in function to that of the denv(Wt) (Fig. 4), demonstrating that Nef only modestly influences expression in this system, and the lack of Nef in NLEGFPLUC is unlikely to explain its latent phenotype in cytokine stimulated thymocytes. It therefore appears that the difference in phenotype may be a product of multiple viral accessory proteins acting in concert, internal

enhancer elements (Van Lint et al., 1994), or a more complex interaction involving other components of the viral genome such as splice sites.

Conclusion

Our data show that infection of primary thymocytes with a truncated HIV-based vector leads to markedly different expression profiles than occurs during wild-type HIV infection, or during infections using vectors based on more complete viral genomes. The mechanism for establishment of latency using NLEGFPLUC is different than in cell line models such as U1 (Folks et al., 1987) and ACH-2 (Folks et al., 1989), which are latent primarily because of mutations in Tat or the trans-activator response region (TAR) respectively (Emiliani et al., 1996, 1998). This indicates that the observed differences in post-integration latency in thymocytes are due to factors outside of the LTR and Tat/Rev regulatory protein coding sequences. One important feature of these results relates to the more general use of HIV vectors in latency experiments. Latency models utilizing truncated or minimal HIV genomes have produced valuable information as experimental systems (Kim et al., 2006; Weinberger et al., 2005). However our data suggest that latency using certain truncated reporter viruses may not accurately reflect the phenotype obtained using complete virus. Thus one must take care not to over-interpret these types of system.

Methods

Molecular cloning and plasmid construction

NLEGFPLUC was originally constructed by replacing the enhanced green fluorescent protein (EGFP) sequence in DAEGFP (An et al., 1999) with an EGFP–luciferase (EGFPLuc) fusion protein as previously described (Burke et al., 2007). This plasmid contains a truncated HIV-1 genome that encodes only Tat, Rev, and Vpu in addition to the EGFPLuc reporter gene. denv(Wt) encodes the EGFPLuc reporter gene in place of Env, but expresses all other HIV genes (Marsden and Zack, 2007). The nef-frameshifted mutant denv(NEF-FS) was constructed by first performing a partial digest of denv(Wt) to cleave the *Xho* I restriction site within the *nef* open reading frame. The product of this digest was then used in an end-filling reaction using T4 DNA polymerase, followed by ligation of the resultant blunt-end DNA fragment. denv (VPR-FS) was created by partial digest of denv(Wt) to cleave only the *Eco*RI site within Vpr, followed by a T4 DNA polymerase catalyzed end-filling reaction and ligation. All plasmids were expanded in the Stbl3 strain of *Escherichia coli* (Invitrogen).

Virus production

Viruses pseudotyped with vesicular stomatitis virus G (VSV-G) protein were generated by transfection of 293FT cells using Lipofectamine 2000 reagent (Invitrogen). The day before transfection cells were seeded at a concentration of 6.6×10^6 cells/well in 10 cm dishes. To generate denv(Wt), denv(NEF-FS), and denv(VPR-FS) viruses, each plate was cotransfected with 10 μg of the relevant virus-encoding plasmid and 2 μg of pHCMVG. For generation of NLEGFPLUC virus, 5 μg of NLEGFPLUC, 2 μg of pHCMVG, and 5 μg of either pCMVΔR8.2DVPR (An et al., 1999; Naldini et al., 1996) (for Vpr− virions) or pCMVΔR8.2 (Naldini et al., 1996) (for Vpr+ virions) were added per plate. The cells were incubated for 20 hours with Lipofectamine/DNA complexes then incubated for a further 30 hours with fresh media before virus harvesting. After this incubation, the media was recovered and centrifuged at 800*g* for 5 minutes to remove any floating cells. The supernatant was then filtered (0.45 μm) and the virus was concentrated by ultracentrifugation at 50,000*g* for 90 minutes at 4 °C. Aliquots were stored, frozen at −80 °C and thawed immediately before use. HIV p24 protein concentration was assayed using an HIV p24 antigen ELISA kit (Beckman Coulter) according to manufacturer's instructions. Viral titrations were performed by infection of 293FT cells as previously described (Burke et al., 2007).

Cell culture and isolation procedures

All cells were cultured in 5% CO2 at 37 °C and cell lines were split every 2–3 days as needed. 293FT cells were cultured in Dulbecco's modified Eagle medium (Invitrogen) containing 10% fetal bovine serum (FBS, Omega Scientific), 100 μg/ml of G418 (Invitrogen), and 100 units/ml of penicillin + 100 μg/ml of streptomycin (Pen/Strep, Invitrogen). Primary thymocytes were cultured in thymocyte culture media composed of Iscove's modified Dulbecco's medium (IMDM, Irvine Scientific) supplemented with 10% human AB serum (Sigma), 4 mM L-glutamine (Invitrogen), Pen/Strep, 20 units/ml of interleukin (IL)-2 (Roche), and 20 ng/ml of IL-4 (Invitrogen).

All cell sorting was performed by using immunomagnetic beads and an autoMACS cell sorter (Miltenyi Biotec) according to the manufacturer's instructions. Fetal thymic tissues of 18 to 24 weeks gestational age were obtained from Advanced Bioscience Resources with appropriate Institutional Review Board permission. Total thymocytes were extracted by suspending thymic tissue in IMDM containing 10% FBS, 4 mM L-glutamine, and Pen/Strep, shredding it through a stainless steel screen, and then passing the resultant cell suspension through a 40 μm filter. For isolation of CD4+CD8+ thymocytes, CD8+ cells were first isolated by positive selection using a human CD8 MultiSort kit. The magnetic beads were then removed and the CD4+CD8+ (double positive) cells were isolated by positive selection using human CD4 MicroBeads. During costimulation, cells were activated for 3 days using plate-bound anti-CD3 and soluble anti-CD28 antibodies as previously described (Brooks et al., 2001; Burke et al., 2007).

Flow cytometry

Cells were stained for surface marker expression using mouse anti-CD4 phyco-erythrin, and CD8 peridinin-chlorophyll protein complex antibodies (Beckman Coulter). Data were acquired using a FACSCalibur (Becton Dickinson) flow cytometer and analyzed with FlowJo (v7) software.

Luciferase assays

Expression of firefly luciferase was quantified using the Luciferase Assay System (Promega) according to the manufacturer's instructions. Cells were washed with 1× phosphate buffered saline, pelleted, resuspended in 100 μl of 1× cell lysis buffer, vortexed briefly, and then stored at −80 °C. During analysis, cells were thawed and mixed with 100 μl of luciferase assay reagent. Lysates were analyzed using a Monolight 2010 (Analytical Luminescence Laboratories), a FLUOstar OPTIMA (BMG LABTECH), or a Lmax luminometer (Molecular Devices).

Infection procedures

All infections were performed by incubating cells at 37 °C for 2 hours with virus in media containing 10 μg/ml of polybrene. Thymocytes were infected in a total volume of 1 ml, and after infections cells were washed and resuspended in 1 ml of thymocyte culture media at a concentration of between 2×10^6 cells/ml and 2×10^7 cells/ml in round-bottomed tissue culture tubes. For cells infected with reporter viruses, aliquots of 200,000 viable cells were removed at various times post-infection and processed in a luciferase assay. Infection of thymocytes with replication-competent virus was performed as above using 100 ng of HIV NL4-3 and 2×10^7 thymocyte cells. Aliquots of cell-free supernatant were then removed from the cultures at various times and assayed using an ELISA assay.

Competing interests

The authors' laboratory has received funding at various times from Amgen, Johnson and Johnson Research, and Merck.

Authors' contributions

M.M., B.B. and J.Z. designed the experiments. M.M. performed the experiments. M.M. and J.Z. wrote the paper. All authors read and approved the final manuscript.

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