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Deletion of *nef* Slows but Does Not Prevent CD4-Positive T-Cell Depletion in Human Immunodeficiency Virus Type 1-Infected Human-PBL-SCID Mice†

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The pathogenicity of four human immunodeficiency virus type 1 (HIV-1) isolates with *nef* deleted for SCID mice repopulated with human peripheral blood leukocytes (hu-PBL-SCID mice) was studied. Deletion of *nef* led to a substantial reduction in CD4-positive T-cell depletion and delayed kinetics of plasma viremia in infected hu-PBL-SCID mice. Deletion of the *nef* gene impacts both the efficiency of primary infection and the cytopathicity of virus for infected CD4-positive T cells in this animal model of HIV-1 infection.

Nef (negative effect factor) was originally described as an accessory gene product of human immunodeficiency virus type 1 (HIV-1) that reduced transcription from the viral long terminal repeat (25, 40, 41). However, the *nef* gene is essential for the induction of AIDS in simian immunodeficiency virus type 1-infected rhesus monkeys (22), and deletion of the *nef* gene has been found in some long-term HIV-infected nonprogressors (23, 28), albeit not in all such individuals (19). Deletion of *nef* also slows viral replication and thymic $CD4^+$ T-cell depletion in SCID mice grafted with human fetal thymus and liver (21). These findings confirm the importance of *nef* in HIV-1 infection in humans, but they give no insight into the function of the Nef protein.

Nef has three effects that might influence viral pathogenesis; down regulation of CD4 expression (2, 6), alteration of T-cell signaling pathways (7, 8, 14, 17, 34, 36, 38), and enhancement of viral infectivity (10, 29, 39, 43). Nef also appears to modulate the host response by altering class I major histocompatibility antigen expression (37) and cytokine production (11). It has also been reported that deletion of HIV-1 *nef* may have more impact on infection of macrophages than on that of $CD4^+$ T lymphocytes (5), so the cell tropism of the HIV-1 studied and the available target cells may influence the outcome of experimental studies in vitro or in vivo. Nef expression also appears to be more important for infection of resting T cells than for that of mitogen-stimulated, proliferating T cells (29), so the state of activation of the target cell population may influence the phenotype of mutants with *nef* deleted.

With these possibilities in mind, we assessed the impact of *nef* deletion on virus replication and pathogenicity in an animal model by using four molecularly cloned isolates of HIV-1 to infect human peripheral blood leukocyte-reconstituted severe combined immunodeficient (hu-PBL-SCID) mice. hu-PBL-SCID mice are highly susceptible to infection with both macrophage-tropic and T-cell line-tropic HIV-1 isolates, and both lead to loss of $CD4^+$ T lymphocytes (30, 32, 33). The target cells for infection include both macrophages and mature human T lymphocytes, including many activated T cells (42), in contrast to the immature $CD4^+/CD8^+$ target cells found in SCID-hu mice with fetal thymus and liver grafts (3, 9). The kinetics of viral replication can be monitored by serial measurements of viral RNA in plasma, a procedure not previously employed to study *nef* deletion mutants. The virus isolates were specifically selected to include T-cell line-tropic isolates (SF2 [35] and R9 [15], previously called R8 [2]), an isogeneic macrophage-tropic isolate (R9.BaL), and the dual-tropic 89.6 isolate (12, 13). HIV-1_{SF2} and the *nef* (orf-B) deletion mutant of SF2 have been described previously (24, 25). The R9 virus construct consists of a hybrid molecular clone derived from HXB-2 with NL4-3 coding sequences inserted between the *Bam*HI and *Xho*I restriction sites (1, 2) and is identical to the previously described R8 virus. Both the ΔN frameshift mutant and the ΔNX deletion mutant of R9 were employed in these experiments (2) with similar results, indicating that reversion of the ΔN mutant did not occur during these experiments. R9 virus with the macrophage-tropic BaL envelope (16) was generated by replacing the *Sal*I-*Bam*HI fragment of R9, extending from the distal portion of *vpr* to the middle of the gp41 coding sequence, with a corresponding fragment from the macrophage-tropic BaL clone (20). Previously described accessory gene mutants of dual-tropic HIV-1 isolate 89.6 (5) were used. The 89.6-N virus carries a stop codon inserted beginning at nucleotide 17 of *nef*. Except for the more rapidly replicating 89.6 and 89.6-N viruses, all of the other viruses used showed equivalent replication in primary human peripheral blood mononuclear cell (PBMC) cultures. PBMCs from healthy adult donors who were Epstein-Barr virus seronegative were prepared by density separation. Cells (20×10^6) were injected intraperitoneally into adult SCID mice 2 weeks prior to virus exposure, as described previously (18, 32). Each experimental group consisted of four to six mice, and all experiments were performed at least twice, with one PBMC donor used in each experiment. Plasma virus RNA copy number was determined by competitive PCR assay (Amplicor HIV-1 Monitor; Roche Molecular Systems, Somerville, N.J.). Virus infection of animals was confirmed by isolation of virus in cocultures of activated human PBMCs and cells recovered from hu-PBL-SCID mice by peritoneal lavage or of cell suspensions prepared from

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spleens or local lymph nodes. Only the *nef* deletion mutant of HIV-1_{SF2} failed to infect all of the hu-PBL-SCID mice injected with 10^3 tissue culture infectious doses (TCID). Recovery of human cells and $CD4^+$ T-cell depletion were monitored by flow cytometry. Cells were doubly stained with fluorescein-labeled monoclonal antibodies to murine H-$2K^d$ and phycoerythrin-labeled antibodies to human CD45, CD3, CD4, and CD8 (Becton-Dickinson Immunocytometry Systems, Mountain View, Calif., or Pharmingen, San Diego, Calif.). Staining of 10^4 cells was evaluated by using a FACScan (Becton-Dickinson) flow cytometer, and data were analyzed with Cellquest (Becton-Dickinson) software.

***nef* deletion reverses the $CD4^+$ T-cell depletion caused by HIV-1 infection.** We infected hu-PBL-SCID mice with the four different viruses with *nef* mutated to determine the impact of a *nef* deletion on viruses with different cell tropism in the hu-PBL-SCID model. Figure 1A shows the results of infection of hu-PBL-SCID mice with T-cell line-tropic HIV-1_{SF2} or the *nef* (orf-B) deletion mutant of SF2. Two effects of the *nef* deletion in the context of HIV-1_{SF2} were obvious. First, infectivity was reduced, since only 20 to 40% of hu-PBL-SCID mice injected with 1,000 TCID of virus became infected, compared to 100% (10 of 10) of the mice infected with wild-type SF2. Second, there was no depletion of $CD4^+$ T cells in animals infected with *nef*-deficient SF2, in marked contrast to the depletion caused by infection with wild-type SF2 (Fig. 1A). By these criteria, the *nef* deletion reduced the pathogenicity of HIV-1_{SF2}.

We next infected hu-PBL-SCID mice with R9 viruses (2, 15) containing either the T-cell line-tropic NL4-3 envelope or the macrophage-tropic BaL envelope. Each virus was used either with an intact *nef* open reading frame or as a *nef* deletion mutant. All of the virus constructs were highly infectious, and virus was readily recovered from all of the animals. The results of two such experiments are shown in Fig. 1B and C. Infection with wild-type R9 caused nearly complete depletion of $CD4^+$ T cells 2 weeks after infection, whereas the *nef*-deficient R9 virus caused no $CD4^+$ T-cell depletion. The results were similar whether $CD4^+$ T cells were examined in peritoneal lavage cells or local lymph nodes. Infection of hu-PBL-SCID mice with the R9.BaL virus resulted in rapid depletion of $CD4^+$ T cells in peritoneal lavage cells and less complete depletion of $CD4^+$ T cells in local lymph nodes. Deletion of *nef* in the R9.BaL virus resulted in less $CD4^+$ T-cell depletion than observed with the R9.BaL infection, but some depletion occurred since $CD4^+$ T-cell numbers were lower than control values (Fig. 1B and C). The impact of *nef* deletion thus seemed to be less in the context of a macrophage-tropic virus than in that of a T-cell line-tropic virus, since *nef* deletion did not totally reverse $CD4^+$ T-cell depletion.

***nef* deletion delays peak viral RNA levels in hu-PBL-SCID mice.** These results were obtained at a fixed interval after HIV-1 infection. We next determined if *nef* deletion blocks $CD4^+$ T-cell depletion entirely or leads to a kinetic delay in virus replication and $CD4^+$ T-cell depletion. This experiment was performed with three virus isolates, R9, R9.BaL, and 89.6 (12), each of which was used with or without a *nef* deletion (R9) or a stop codon (89.6-N). Figure 1D depicts the results obtained 1, 2, and 3 weeks after infection of hu-PBL-SCID mice with the dual-tropic 89.6 and 89.6-N isolates. Nef deletion slowed $CD4^+$ T-cell depletion by 1 week but did not prevent it in the context of the highly virulent 89.6 isolate. Virus replication was assessed by serial plasma HIV RNA determinations in mice infected with the R9 series of viruses (Fig. 2). Plasma RNA copy number was readily detectable at 1 week after infection with R9, although plasma viremia was below the

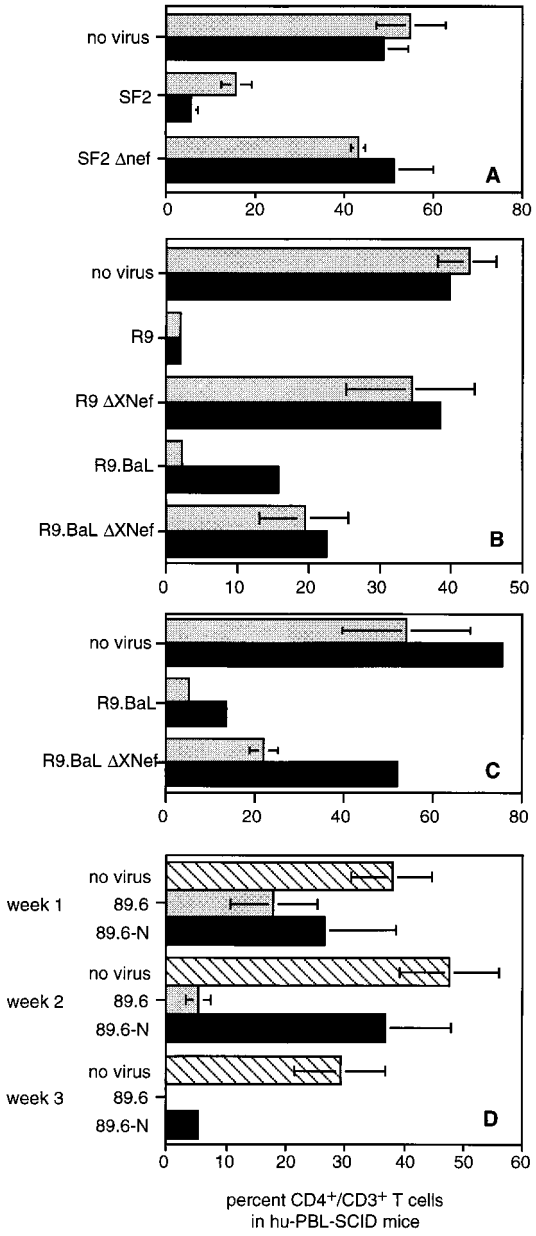

FIG. 1. Infection of hu-PBL-SCID mice with *nef*-deficient HIV-1 isolates slows $CD4^+$ T-cell depletion. (A) Mice were infected with 10^3 TCID of either wild-type SF2 or *nef*-deficient SF2 (SF2Δnef) at 2 weeks after PBL reconstitution, and residual $CD4^+$ T cells were enumerated in peritoneal lavage cells (light fill) or periportal lymph nodes (dark fill) after 2 weeks of infection. Only two of the five mice injected with SF2Δnef were infected in this experiment (data reported are for infected mice only), and in a replicate experiment, only one of the five hu-PBL-SCID mice was infected. (B) Mice were infected as in experiment A, with the R9 virus (2) expressing either the T-cell line-tropic NL4-3 envelope or the macrophage-tropic BaL envelope and with or without the NX deletion of *nef*. Each error bar indicates the standard error for five replicate mice; lymph node samples were pooled prior to analysis by flow cytometry, so there is no measure of mouse-to-mouse variation. Bars without error bars have a standard error of <2%. (C) Replicate of experiment B in which only R9.BaL and its *nef* deletion counterpart were studied. (D) Recovery of $CD4^+$ T cells at 1, 2, and 3 weeks after infection of hu-PBL-SCID mice with the dual-tropic 89.6 isolate or a *nef* stop codon variant (5, 12).

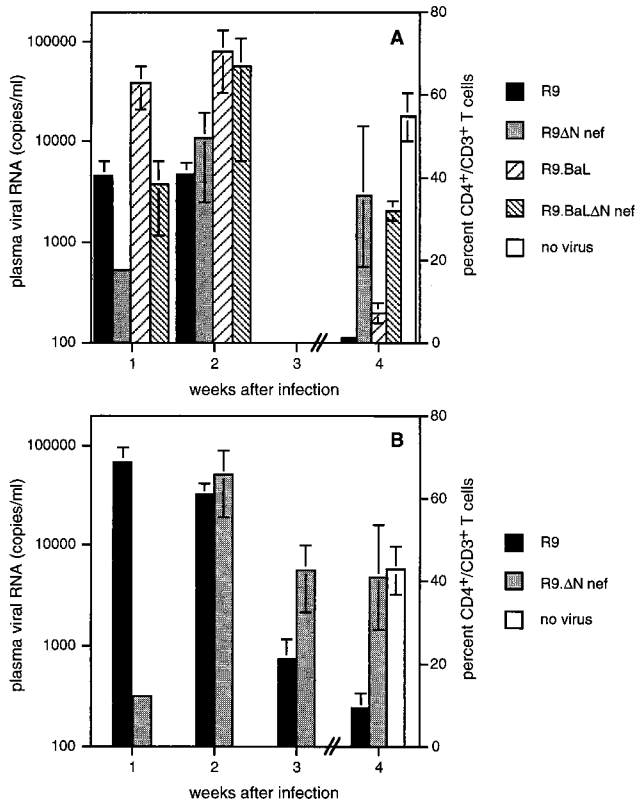

FIG. 2. Kinetics of plasma viral RNA and $CD4^+$ T-cell depletion after infection with wild-type and *nef*-deficient R9 and R9.BaL viruses. Plasma viral RNA load was determined with the Roche Amplicor competitive PCR assay, which has a lower limit of detection of 400 copies/ml with the sample volume available. A and B represent two replicate experiments, each performed with a single PBMC donor. HIV RNA levels were measured weekly in plasma samples from three to five mice, and survival of $CD4^+$ T cells in peritoneal lavage cells was determined at 4 weeks after infection. Values determined at 1 to 3 weeks after infection are for plasma viral RNA, and those determined at 4 weeks represent percent $CD4^+$ T cells.

lower limit of detection in mice infected with the R9.ΔNnef virus. However, RNA levels in plasma were comparable in mice infected with both the wild-type and *nef*-deficient viruses at 2 weeks after infection and declined thereafter (Fig. 2B). $CD4^+$ T cells were not depleted at 4 weeks after infection with the R9.ΔNnef virus in two replicate experiments (Fig. 2A and B). Deletion of *nef* in the context of the T-cell line-tropic R9 virus thus delayed peak viremia by 1 week and reduced the extent of $CD4^+$ T-cell depletion at all of the time points examined. Infection of hu-PBL-SCID mice with the macrophage-tropic R9.BaL virus led to higher RNAs level in plasma at 1 and 2 weeks after infection than did infection with the R9 virus. Deletion of *nef* in the R9.BaL virus led to a significant reduction ($P = 0.04$ by t test) of RNA levels in plasma at 1 week after infection but not at 2 weeks (Fig. 2A). Depletion of $CD4^+$ T cells by R9.BaL infection was partially reversed by *nef* deletion. The deletion of *nef* thus led to a kinetic delay in the increase in viral RNA levels following infection with both T-cell line-tropic and macrophage-tropic HIV-1 strains and prevented most or all of the $CD4^+$ T-cell depletion associated with infection with the wild-type virus.

These studies show that deletion of the *nef* accessory gene from four different HIV-1 isolates that differed in cell tropism, cytopathicity, and replication kinetics led, in each isolate, to a slower course of virus replication and a delay in $CD4^+$ T-cell depletion in infected hu-PBL-SCID mice. Deletion of *nef* caused a major reduction in the infectivity in only one of the four HIV-1 isolates examined, SF2. This result contrasts with those of in vitro experiments in which the *nef*-deleted SF2 virus replicated to higher levels than the wild-type virus (25). Our results indicate that expression of *nef* contributes to the efficiency of virus infection and spread in this in vivo model of HIV-1 infection. The effect of *nef* deletion was seen with HIV-1 isolates that were T-cell line tropic, macrophage tropic, or dual tropic, although the impact of *nef* deletion appeared to be greatest in the T-cell line-tropic SF2 and R9 viruses (Fig. 1 and 2). However, *nef* deletion resulted in a kinetic delay in peak levels of viral RNA of only 1 to 2 weeks (Fig. 1 and 2), although $CD4^+$ T-cell depletion appeared to be delayed for a longer time (Fig. 2). These results could be explained by two effects of *nef* deletion: reduction in the efficiency of primary infection and less cytopathic effects (direct or indirect) for $CD4^+$ T cells of *nef*-deficient viruses.

Our results are similar to those observed following infection of SCID mice with human fetal thymus and liver transplants with *nef*-deleted HIV-1 (4, 21), despite important differences in target cells between the two SCID mouse models of HIV-1 infection. If a critical *nef* function is activation of target cells (14), then one might expect less effect of *nef* deletion in the hu-PBL-SCID model, but the results obtained with the two animal models seem comparable. The importance of *nef* in both of these models seems to be less than that implied by the finding of long-term survivors with spontaneous *nef* deletions (23, 26, 27) or the absence of simian AIDS in monkeys infected with *nef*-deficient simian immunodeficiency virus type 1 (22). A vigorous and sustained immune response to *nef*-defective viruses following an attenuated primary infection of humans or primates may explain these apparently discordant results. In contrast, no detectable immune response to HIV-1 infection has been observed in hu-PBL-SCID mice in the absence of PBL donor immunization (31), so neither the wild-type nor the *nef*-deficient virus should be subject to immune control in these experiments.

We conclude that the impact of *nef* deletion varies according to the virus isolate and that Nef contributes to both the success of initial infection and the rate of $CD4^+$ T-cell depletion. Nef function may be less critical for highly pathogenic, dual-tropic isolates like 89.6.

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