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Mouse Models for Studies of *In Vivo* Functions of HIV-1 Nef

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Abstract

In vitro *studies have demonstrated that HIV-1 Nef has several important activities, promoting viral replication and pathogenesis. These activities include downregulation of cell surface molecules CD4 and major histocompatibility complex class I, enhancement of viral infectivity, activation of p21-activated kinase 2, and inhibition of immunoglobulin class switching. But how important each* in vitro *activity is to* in vivo *Nef function remains elusive. To address this question, several small animal models have been developed in the past two decades, such as Nef transgenic mice, SCID-hu mice, and humanized mice. Each of those models has its own pros and cons. Easy access and relative inexpensiveness have made small animal models the favorite models for HIV research. This review will be focused on the recent progress in the understanding of the* in vivo *functions of HIV-1 Nef obtained from studies using these small animal models.*
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Key words

HIV-1. Nef.* In vivo *function. Mouse model.

Introduction

Nef is one of the six accessory proteins encoded by HIV-1 genome. Early studies with T-cell lines showed that *nef* was not required for viral growth or the production of cytopathic effects, and suggested that inactivating mutations resulted in more robust viral replication[1-3]. Based on these early findings, this gene was considered a negative factor for viral replication and thus named *nef* (standing for negative factor)[2,4]. However, subsequent studies from Greene's and Baltimore's groups, which were published side by side in the same issue of Proc Natl Acad Sci USA in 1989, argued against Nef's negative influence on viral growth and transcriptional activation[5,6]. Their work opened a new avenue for Nef research and led to more vigorous investigations into the role of Nef in viral infection. The relevance of *nef* as a critical factor for viral growth and pathogenicity was demonstrated by Kestler, et al. in the SIVmac239/rhesus macaque model[7]. The discovery of HIV-1 Nef's ability to downregulate the viral receptor, CD4, *in vitro* further suggested the importance of Nef in the survival and spread of the virus *in vivo*[8,9]. Besides downregulation of cell-surface CD4, other *in vitro* activities of Nef were soon identified, including downregulation of cell surface molecule major histocompatibility complex (MHC) class I, enhancement of viral infectivity, and activation of p21-activated kinase 2 (PAK2)[10-13]. All of the attention directed towards Nef was compellingly validated by the discovery of individuals infected by viruses with irreversibly inactivated

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nef coding sequences. In all cases, the *nef*-defective viruses had greatly attenuated phenotypes[14-16]. More recent studies have demonstrated additional functions for Nef: modulation of T-cell receptor signaling[17], exosome release[18,19], inhibition of immunoglobulin (Ig) class switching[20,21], inhibition of the antiviral effects of SERINC3 and SERINC5[22], and inhibition of lymphocyte mobility[23-25]. All these activities are potentially important for viral propagation and pathogenesis and have been extensively reviewed elsewhere[26-32]. However, the importance of these *in vitro* activities of Nef for its *in vivo* functions is largely unknown.

Table 1. Summary of mouse models used for *nef* functional study

Mouse models	Reference
Nef transgenic mouse	Chrobak, et al. 2014; Hanna, et al. 1998; Ahmed Rahim, et al. 2013; Acharjee, et al., 2014; Lindemann, et al. 1994; Brady, et al. 1993
Humanized mouse	Jamieson, et al. 1994; Zou, et al. 2012; Watkins, et al. 2013; Watkins, et al. 2015

Nef in SIV infection

Nonhuman primate (NHP)-SIV models have contributed greatly to our understanding of *in vivo* Nef activities. The milestone work of Desrosiers, et al., which first defined Nef as a pathogenic factor in SIV infection of rhesus macaques, was followed by demonstrating the feasibility of using *nef*(–)SIV as an attenuated viral vaccine[7,33]. However, limitations do exist in the use of NHP-SIV models to understand HIV infection of humans:

- Although many Nef activities are conserved across different lineages of HIV and SIV[34], some differences do exist. For instance, Nef alleles from most SIVs that do not cause disease in their natural hosts downregulate CD3 and CD28, but HIV-1 Nef does not[35] and SIV Nefs block the antiviral effect of tetherin, but HIV-1 Nef does not[36,37].
- The HIV-1 accessory genes are significantly different from SIVmac239 accessory genes in that there is no *vpx*, the 3' end of *vif* is reconstructed by recombination, and only HIV-1 has *vpu*[38-40]. In addition, the cytoplasmic tails of *env* are functionally different between HIV-1 and SIV[41]. Thus, the different activities of SIV accessory genes may confound the study of how HIV-1 Nef functions in promoting viral fitness.
- There are very few NHPs available for research and the cost associated with the studies are high.

Mouse models

To address these concerns, versatile *in vivo* models utilizing HIV-1 Nef itself were developed, which include *nef* transgenic mice and humanized mice (Table 1)[42-50]. Through these small animal models, considerable insight regarding the functions of HIV-1 Nef *in vivo* have been obtained. Despite various pros and cons, easy access and relative inexpensiveness have made these small animal models popular for HIV-1 research. In this review we will focus on recent progress in the understanding of the *in vivo* functions of HIV-1 Nef obtained from studies using these mouse models.

The *nef* transgenic mouse model

Mouse cells are not susceptible to HIV infection. To study the *in vivo* functions of HIV-1 Nef, this limitation has been overcome by expression of *nef* or HIV proviral genomes in mice[45-47,51,52]. A summary of these transgenic mouse models is shown in table 2. Consistent with the findings in human infection[14-16], results from these models indicate that Nef harbors a major determinant of HIV pathogenicity.

Different regulatory elements have been used in transgenic mice to target *nef* expression in T-cells, including the regulatory elements of murine CD3δ, human CD2, and TCR β chain gene[46,47,51]. Although severe immunodeficiency, along with T-cell loss and alteration of T-cell activation, is observed in these models, none of these models has exhibited multiorgan syndromes and a disease similar to human AIDS. However, when *nef* is expressed under the regulatory sequences of the human CD4 gene in $CD4^+$ T-cells and cells of the monocyte/macrophage lineage (CD4C/HIV-1^{Nef} Tg mice), T-cell loss, alteration of T-cell activation, and pathological lesions in various mouse organs such as heart and kidney have developed and are strikingly similar to human AIDS, especially to pediatric AIDS. In addition, disease latency and progression are correlated with *nef* expression levels[43]. Although $CD4^+$ T-cell loss has been detected in all the *nef*-expressing mice, a slightly elevated number of regulatory T-cells is seen in CD4C/HIV-1^{Nef} mice. Interestingly, regulatory T-cells also retain some important suppressive functions[42], which is probably related to decreased apoptosis, enhanced cell proliferation, and increased generation

Table 2. Summary of *nef* transgenic mouse models

Promoter used	*Nef* expressing cells	T-cell loss	$CD4^+CD8^+$ thymocyte loss	T-cell activation status	Multiple organ syndrome	Immune response to antigen stimulation	References
Mouse CD3δ	T-cells	Yes	No	Increased	NR	NR	Skowronski, et al. 1993
Human CD2	T-cells	Yes	Yes	Decreased	NR	NR	Brady, et al. 1993
Mouse TCR β chain	T-cells	Yes	Yes	Increased	Lymphadenophaty, splenomegaly	Decreased	Lindemann, et al. 1994
Human CD4	$CD4^+$ T-cells, cells for the monocyte/macrophage lineage	Yes	Yes	Increased	Yes	Decreased	Hanna, et al. 1998
C-fms	Microglia	NR	NR	NR	NR	NR	Acharjee, et al. 2014

NR: not reported.

from precursors. These findings suggest that Nef may differentially affect different $CD4^+$ T-cell subsets. In addition, periphery $CD4^+$ single positive (SP) cells are more readily depleted than thymic $CD4^+$ SP cells, indicating that the mechanisms underlying the depletion of the $CD4^+$ SP cells in these two sites are different. In contrast to $CD4^+$ T-cells, $CD8^+$ T-cells in CD4C/HIV-1^{Nef} mice show a normal development and could mount a strong response against lymphocytic choriomeningitis virus (LCMV) infection. However, these $CD8^+$ T-cells could not maintain a memory phenotype after virus clearance, which can be partially rescued by adoptive transfer of non-transgenic $CD4^+$ helper T-cells[44]. These results imply that Nef affects the development and function of $CD4^+$ and $CD8^+$ T-cells in distinct ways, which is similar to what is observed in HIV infection. However, expression of Nef in developmental precursors to mature $CD4^+$ T-cells complicates the interpretation of these studies. To address this concern, an inducible TRE/HIV *nef* transgenic strain and an inducer CD4C/rtTA Tg mouse strain were generated. Expression of *nef* in the double (TRE/HIV *nef* x CD4C/rtTA) Tg mice was induced with doxycycline administration, and *nef* expression led to AIDS-like diseases in older mice, which is the same as those observed in CD4C/HIV-1^{Nef} Tg mice, indicating that the diseases in CD4C/HIV-1^{Nef} Tg mice are not due to developmental defects caused by *nef* expression[53,54]. To find out which population of *nef*-expressing immune cells is responsible for the phenotypes observed in CD4C/HIV-1^{Nef} mice, five novel transgenic mice were established to express *nef* in different immune cell populations. Distinct AIDS-like phenotypes are found to be associated with cell-specific *nef* expression, as shown in table 3[55]. Even though the cell population responsible for all of the observed phenotypes in CD4C/HIV-1^{Nef} Tg mice was not identified, the results suggest pleiotropic effects of *nef* expression in both $CD4^+$ T-cells and cells of the monocyte/macrophage lineage are required for the full pathology.

Another type of *nef* transgenic mouse model was generated by expressing *nef* in the microglia of CNS, allowing the study of the effect of Nef on the development of HIV-1-associated neurological disorders (HAND). Expression of *nef* in microglia results in the hyperactive neurobehavioral phenotype in male mice, which is pertinent to CCL2 induction and accompanying dysfunction of the dopaminergic system, indicating the role of Nef in HAND pathogenesis[45].

Finally, an alternate transgenic mouse model, Tg26, has implicated Nef in the induction of HIV-1-related B-cell lymphomas[56] and HIV-1-associated nephropathy[57,58].

Despite the significant knowledge that has been gained from the studies of *nef* transgenic mice during the past two decades, limitations of this approach need to be noted. First, since it only expresses *nef*, the transgenic approach does not allow studies on combinational effects of Nef with other viral proteins; second, since there is no viral replication in these mice, the role of Nef in AIDS pathogenesis cannot be evaluated in the context of a systemic infection; third, since HIV

Table 3. Phenotypes associated with cell-specific *Nef* expression in nef transgenic mouse models

Promoter used	Regulatory element used	*Nef* expressing cells	T-cell loss	$CD4^+CD8^+$ thymocyte loss	T-cell activation	Multiple organ syndrome
Mouse CD4	Mouse CD4 enhancer and regulatory element	Thymic and peripheral $CD4^+$ T-cells	Yes	Yes	Yes	No
Partial human CD4 promoter with mouse silencer	Mouse CD4 enhancer and human CD4 regulatory element	$CD4^+$ T-cells and lymphoid DC	Yes	Yes	Yes	Much milder disease only seen in older mice (> 12 months old)
Human CD4	Mouse CD4 enhancer and regulatory element	$CD4^+$ T-cells and lymphoid and myeloid DC	Yes	Yes	Yes	No
Mouse CD11c	Mouse CD11c regulatory element	DC	Slightly depleted	No	Slighted activated	Occasional mild lung disease and pulmonary hypertension, splenomegaly
Human CD68	Human CD68 regulatory element	Myeloid cells	Slightly depleted	No	No	Occasional mild lung disease and pulmonary hypertension

DC: dendritic cells.

cannot productively infect mouse cells due to block of viral entry and other steps of viral life cycle[59,60], results obtained from the transgenic studies in relation to human infection need to be interpreted with caution; finally, Nef is expressed in all $CD4^+$ cells in CD4C/HIV-1 *nef* transgenic mice, whereas there is only a small fraction of $CD4^+$ cells that are infected in human infection. To overcome these limitations, a more robust model system needs to be developed.

Humanized mouse model

The term "humanized mice" can refer to one of the followings: (i) mice in a normal background that are transgenic with one or several human genes; (ii) immunodeficient mice that are either transplanted with human cells or implanted with human tissues; (iii) immunodeficient mice that are transplanted with human stem/progenitor cells; and (iv) combinations of any two above, or three above[48,61-63]. All these humanized mouse models have been exploited in the past three decades in order to develop a better model system to study HIV pathogenesis and to test new preventive and therapeutic strategies. This section will be focused on the recent progress obtained about *in vivo* functions of HIV-1 Nef through use of the second, third and forth humanized mice abovementioned. The results from those studies are summarized in table 4.

SCID-hu mice

The first paper on using humanized mice to study HIV-1 Nef was published in 1994 by Zack, et al. In this study, human fetal thymus and liver were implanted under the kidney capsule of SCID mice and allowed to grow into a thymic organoid. Wild-type and *nef*-defective HIV-$1_{JR\text{-}CSF}$ or HIV-$1_{NL4\text{-}3}$ were directly injected into the thymic organoid, and both *nef*-defective viruses showed attenuated growth properties relative to wild-type. CCR5-tropic HIV-$1_{JR\text{-}CSF}$ was less cytotoxic than CXCR4-tropic HIV-$1_{NL4\text{-}3}$[48]. Besides, *nef*-defective HIV-$1_{NL4\text{-}3}$ lost the ability to deplete thymocytes, which was in sharp contrast to the wild-type virus that depleted thymocytes within six weeks of infection[48]. These results indicate that HIV-1 Nef is required for efficient *in vivo* viral replication and pathogenicity in human thymus. However, this model is not an optimal system to study a systemic viral infection because it only contains

Table 4. Summary of the studies on the *in vivo* functions of HIV-1 *nef* in humanized mouse models

Mouse model*	Viral strain	Tropism	*Nef* mutation	Route of infection	Viral replication *in vivo*	Periphery T-cell loss	$CD4^+CD8^+$ thymocyte loss	Mutation reverted	References
1	JRCSF	CCR5	Frameshift	Intra-implant injection	Reduced	ND	No†	ND	Jamieson, et al. 1994
1	NL4-3	CXCR4	71 aa deletion	Intra-implant injection	Reduce	ND	No	ND	Jamieson, et al. 1994
2	LAI	CXCR4	Deletion	Intravenous	Reduced	No	No	No	Zou, et al. 2012
2	LAI	CXCR4	Frameshift	Intravenous	Reduced	Yes	Yes	Yes‡	Watkins, et al. 2013
2	LAI	CXCR4	1 bp deletion plus 4 base insertion	Intravenous	Reduced	50% loss	50% loss	No	Watkins, et al. 2013
2	LAI	CXCR4	13 bp deletion plus 4 base insertion	Intravenous	Reduced	50% loss	50% loss	No	Watkins, et al. 2013
2	LAI	CXCR4	P72A/P75A	Intravenous	Reduced	Yes	Yes	No	Watkins, et al. 2013
2	JRCSF	CCR5	Deletion	Intravenous	Reduced	Yes	No	ND	Watkins, et al. 2015

*Mouse model used in each study: 1 represents SCID mice implanted with human fetal liver and thymus under the kidney capsule; 2 represents humanized BLT mice.
†JRCSF with intact *nef* does not deplete $CD4^+CD8^+$ thymocytes, and *nef* defective JRSCF does not, either.
‡The ORF of *nef* with frame-shift mutation was repaired in bone marrow/liver/thymus mice via either 1 base or 13 base deletion in *nef* sequence, which are shown in the following two rows below in this table.
ND: not done.

human thymocytes and lacks all other immune cells known to be involved in HIV replication and pathogenesis such as T-cells, monocytes/macrophages, and dendritic cells.

Bone marrow/liver/thymus humanized mice

Several humanized mouse models have been developed and they have been reviewed by Denton and Garcia[61]. However, the study of Nef in HIV-1 infection has been largely restricted to the bone marrow/liver/thymus (BLT) humanized mice. This model was constructed by first implanting a piece of fetal liver sandwiched between two pieces of thymus under the kidney capsule of an immunodeficient mouse, followed by autologous bone marrow transplantation of human hematopoietic stem cells. Virtually all subsets of myeloid and lymphoid lineages were found in the peripheral blood and organs in this model[62], which makes it a valuable tool to study human immune-related diseases. Since its creation, this model has been widely used in various areas of biomedical research, including the study of human innate and adaptive immune responses, HIV replication and pathogenesis, and evaluation of approaches to prevent HIV transmission[50,64-70].

Infection of bone marrow/liver/thymus humanized mice with CXCR4 tropic HIV-1

Consistent with the findings in SCID-hu mice, intravenous infection of humanized BLT mice with low, medium, and high doses of $HIV\text{-}1_{LAI}$ (LAI, CXCR4 tropic) caused robust viral replication and the accompanying depletion of $CD4^+$ T-cells and $CD4^+CD8^+$ thymocytes. However, regardless of the dose of inoculums, *nef*-defective LAI showed reduced and delayed viral replication and failure to systemically deplete CD4+ T-cells in BLT mice. Importantly, *nef*-defective LAI lost the capacity to deplete $CD4^+CD8^+$ thymocytes, suggesting that Nef is the only viral factor responsible for $CD4^+CD8^+$ thymocyte killing in this model[50]. Collectively, these findings indicate that Nef contributes to T-cell loss during HIV infection via direct killing resulting from elevated viral replication, and diminished replenishment resulting from defective thymocyte development. In another study from the same group, the authors infected BLT mice with the LAI harboring a frame-shifted (fs, via four base insertion) *nef* and later found the recovery of *nef* ORF via 1 base or 13 base deletions (LAINeffsΔ-1 and LAINeffsΔ-13) downstream of the mutated site during

the course of infection, indicating the presence of selective pressure to restore *nef* ORF *in vivo*[68]. These two derived Nef mutants (LAINeffsΔ-1 and LAINeffsΔ-13) were assayed for their *in vitro* activities, including CD4 and MHC class I downregulation, PAK2 activation, and enhancement of infectivity, and they were both found defective for CD4 downregulation activity, but with other Nef activities intact. Compared with wild-type virus, infection of BLT mice with LAINeffsΔ-1 and LAINeffsΔ-13 led to threefold reduction of peak viral load and about 50% reduced capacity of the viruses to deplete peripheral $CD4^+$ T-cells and $CD4^+CD8^+$ thymocytes, suggesting that CD4 downregulation activity of Nef is important for HIV-1 pathogenesis. In addition, no other changes in the rest of the *nef* sequences of both LAINeffsΔ-1 and LAINeffsΔ-13 were observed, implying that the two expressed proteins were stable *in vivo*[68].

The role of the SH3 binding domain-dependent activity of Nef in pathogenesis was also addressed[68]. This highly conserved Nef proline motif is critical for the activation of PAK2[71]. The authors infected BLT humanized mice with the LAI containing the P72A/P75A mutation in Nef and found that the double mutation did not affect the capacity of viral replication and pathogenesis. Further, the mutations had only a weak tendency to revert back to wild-type sequences during the course of experiments[68], clearly indicating that SH3 domain binding activity of HIV-1 Nef is dispensable for viral replication and pathogenesis in BLT mice.

Infection of bone marrow/liver/thymus humanized mice with CCR5 tropic HIV-1

Since CCR5 tropic viruses are most frequently seen during transmission[72], Watkins, et al. investigated the role of $HIV\text{-}1_{JR\text{-}CSF}$ (JRCSF, CCR5-tropic) Nef in HIV-1 infection of BLT mice with *nef*-defective virus (JRCSF-Nef*dd*). Although peak viral loads in peripheral blood for both JRCSF and JRCSFNef*dd* were comparable, the replication levels of JRCSFNef*dd* displayed considerable variation over time. The JRCSF infection resulted in $CD4^+$ T-cell loss in the peripheral blood, bone marrow, spleen, lymph nodes, lung, and liver of infected mice, while JRCSFNef*dd* induced no loss of $CD4^+$ T-cells in peripheral blood, but a small but significant reduction of $CD4^+$ T-cells in the previously mentioned organs. Further analysis revealed that $CD4^+$ T-cell loss in peripheral blood coincided with $CD8^+$ T-cell activation, which was consistent with the findings in human infection of CCR5 tropic viruses. Nef was found to be required for both systemic T-cell activation and significant $CD4^+$ T-cell loss to occur. Interestingly, the authors found that the BLT mice reconstituted with cells and tissue expressing HLA B42:01 had viral loads in JRCSFNef*dd*-infected mice suppressed about 200-fold compared to JRCSF-infected mice. This observation may be relevant to studies of large populations of HIV-1-infected individuals expressing B42:01 where viral burdens were also reduced[73]. These results indicate host-specific suppression of viral replication in a small animal model exists and suggest the possibility that absence of Nef greatly enhances the anti-HIV effect of B42:01 in BLT mice[70]. In comparing infections of BLT mice with CXCR4 tropic LAI and CCR5 tropic JRCSF, two further differences are noted. One is the ability to deplete $CD4^+CD8^+$ thymocytes; in contrast to LAI, neither wild-type nor *nef*-defective JRCSF caused $CD4^+CD8^+$ thymocyte depletion, which is probably due to a lower number of CCR5-expressing cells in the thymus[74]. However, because the lack of $CD4^+CD8^+$ thymocyte depletion in *nef*-defective LAI-infected BLT mice is so prominent, further study on the underlying mechanisms of the different killing phenotypes of double-positive thymocytes by LAI or JRCSF Nef is warranted. Another difference is the status of systemic T-cell activation; there is no difference in $CD8^+$ T-cell activation between LAI and *nef*-defective LAI infection, while a large difference existed for JRCSF infection. One explanation is that a relatively low level of viral replication is more likely to induce T-cell activation, but not cell killing that is more likely induced by high viral load[50].

Conclusions

Because HIV-1 infects and causes diseases only in humans and chimpanzees[75,76], there is no perfect animal model to completely mimic human infection, and thus to be used to study the role of Nef in viral replication and pathogenesis *in vivo.* Even so, in the past three decades significant progress has been made in the development of improved animal models to replicate HIV infection *in vivo*, and each of these models has its own pros and cons. Because of easy access and relative inexpensiveness, small animal models have become one of the favorite models for HIV research. This review summarized recent progress on the understanding of the *in vivo* functions of Nef gained from the studies using these small animal models. Results from these studies emphasize the contribution of Nef to HIV replication and pathogenesis *in vivo*[77-79]. Despite the progress, more unsolved questions regarding Nef in viral infection still lie ahead. For example, in most of these

studies Nef was derived from lab-adapted HIV-1 strains such as NL4-3 and LAI. So, future studies on the *in vivo* functions of Nef derived from primary isolates, such as transmissible founder viruses, should be carried out in these small animal models[80]. In addition, the contribution of individual Nef activity in viral infection also needs to be clarified. Another important question worthy of study is the mechanism of the cytopathic effect of LAI Nef on thymocytes, especially on $CD4^+CD8^+$ thymocytes. The answers to these questions will help to further define the *in vivo* functions of Nef in viral infection, and more importantly, based on these results Nef inhibitors can then be developed and tested in these small mouse models.

Declaration of interest

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References

1. Terwilliger E, Sodroski JG, Rosen CA, Haseltine WA. Effects of mutations within the 3' orf open reading frame region of human T-cell lymphotropic virus type III (HTLV-III/LAV) on replication and cytopathogenicity. J Virol. 1986;60:754-60.
2. Ahmad N, Venkatesan S. Nef protein of HIV-1 is a transcriptional repressor of HIV-1 LTR. Science. 1988;241:1481-5.
3. Niederman TM, Thielan BJ, Ratner L. Human immunodeficiency virus type 1 negative factor is a transcriptional silencer. Proc Natl Acad Sci U S A. 1989;86:1128-32.
4. Gallo R, Wong-Staal F, Montagnier L, Haseltine WA, Yoshida M. HIV/HTLV gene nomenclature. Nature. 1988;333:504.
5. Hammes SR, Dixon EP, Malim MH, Cullen BR, Greene WC. Nef protein of human immunodeficiency virus type 1: evidence against its role as a transcriptional inhibitor. Proc Natl Acad Sci U S A. 1989;86:9549-53.
6. Kim S, Ikeuchi K, Byrn R, Groopman J, Baltimore D. Lack of a negative influence on viral growth by the nef gene of human immunodeficiency virus type 1. Proc Natl Acad Sci U S A. 1989;86:9544-8.
7. Kestler HW, Ringler DJ, Mori K, et al. Importance of the nef gene for maintenance of high virus loads and for development of AIDS. Cell. 1991;65:651-62.
8. Guy B, Kieny MP, Riviere Y, et al. HIV F/3' orf encodes a phosphorylated GTP-binding protein resembling an oncogene product. Nature. 1987;330:266-9.
9. Garcia JV, Miller AD. Serine phosphorylation-independent downregulation of cell-surface CD4 by nef. Nature. 1991;350:508-11.
10. Schwartz O, Marechal V, Le Gall S, Lemonnier F, Heard JM. Endocytosis of major histocompatibility complex class I molecules is induced by the HIV-1 Nef protein. Nat Med. 1996;2:338-42.
11. Goldsmith MA, Warmerdam MT, Atchison RE, Miller MD, Greene WC. Dissociation of the CD4 downregulation and viral infectivity enhancement functions of human immunodeficiency virus type 1 Nef. J Virol. 1995;69:4112-21.
12. Chowers MY, Spina CA, Kwoh TJ, Fitch NJ, Richman DD, Guatelli JC. Optimal infectivity in vitro of human immunodeficiency virus type 1 requires an intact nef gene. J Virol. 1994;68:2906-14.
13. Sawai ET, Baur A, Struble H, Peterlin BM, Levy JA, Cheng-Mayer C. Human immunodeficiency virus type 1 Nef associates with a cellular serine kinase in T lymphocytes. Proc Natl Acad Sci U S A. 1994;91:1539-43.
14. Gorry PR, McPhee DA, Verity E, et al. Pathogenicity and immunogenicity of attenuated, nef-deleted HIV-1 strains in vivo. Retrovirology. 2007;4:66.
15. Calugi G, Montella F, Favalli C, Benedetto A. Entire genome of a strain of human immunodeficiency virus type 1 with a deletion of nef that was recovered 20 years after primary infection: large pool of proviruses with deletions of env. J Virol. 2006;80:11892-6.
16. Kirchhoff F, Greenough TC, Brettler DB, Sullivan JL, Desrosiers RC. Brief report: absence of intact nef sequences in a long-term survivor with nonprogressive HIV-1 infection. N Engl J Med. 1995;332:228-32.
17. Abraham L, Fackler OT. HIV-1 Nef: a multifaceted modulator of T cell receptor signaling. Cell Commun Signal. 2012;10:39.
18. Shelton MN, Huang MB, Ali SA, Powell MD, Bond VC. Secretion modification region-derived peptide disrupts HIV-1 Nef's interaction with mortalin and blocks virus and Nef exosome release. J Virol. 2012;86:406-19.
19. Lenassi M, Cagney G, Liao M, et al. HIV Nef is secreted in exosomes and triggers apoptosis in bystander CD4+ T cells. Traffic. 2010;11:110-22.
20. Qiao X, He B, Chiu A, Knowles DM, Chadburn A, Cerutti A. Human immunodeficiency virus 1 Nef suppresses CD40-dependent immunoglobulin class switching in bystander B cells. Nat Immunol. 2006;7:302-10.
21. Xu W, Santini PA, Sullivan JS, et al. HIV-1 evades virus-specific IgG2 and IgA responses by targeting systemic and intestinal B cells via long-range intercellular conduits. Nat Immunol. 2009;10:1008-17.
22. Usami Y, Wu Y, Gottlinger HG. SERINC3 and SERINC5 restrict HIV-1 infectivity and are counteracted by Nef. Nature. 2015;526:218-23.
23. Stolp B, Imle A, Coelho FM, et al. HIV-1 Nef interferes with T-lymphocyte circulation through confined environments in vivo. Proc Natl Acad Sci USA. 2012;109:18541-6.
24. Murooka TT, Deruaz M, Marangoni F, et al. HIV-infected T cells are migratory vehicles for viral dissemination. Nature. 2012;490:283-7.
25. Stolp B, Reichman-Fried M, Abraham L, et al. HIV-1 Nef interferes with host cell motility by deregulation of Cofilin. Cell Host Microbe. 2009;6:174-86.
26. Fackler OT, Murooka TT, Imle A, Mempel TR. Adding new dimensions: towards an integrative understanding of HIV-1 spread. Nat Rev Microbiol. 2014;12:563-74.
27. Foster JL, Garcia JV. Role of Nef in HIV-1 replication and pathogenesis. Adv Pharmacol. 2007;55:389-409.
28. Foster JL, Garcia JV. HIV pathogenesis: Nef loses control. Cell. 2006; 125:1034-5.
29. Foster JL, Garcia JV. HIV-1 Nef: at the crossroads. Retrovirology. 2008; 5:84.
30. Foster JL, Denial SJ, Temple BR, Garcia JV. Mechanisms of HIV-1 Nef function and intracellular signaling. J Neuroimmune Pharmacol. 2011; 6:230-46.
31. Kirchhoff F, Schindler M, Specht A, Arhel N, Munch J. Role of Nef in primate lentiviral immunopathogenesis. Cell Mol Life Sci. 2008;65:2621-36.
32. Tokarev A, Guatelli J. Misdirection of membrane trafficking by HIV-1 Vpu and Nef: Keys to viral virulence and persistence. Cell Logist. 2011;1:90-102.
33. Daniel MD, Kirchhoff F, Czajak SC, Sehgal PK, Desrosiers RC. Protective effects of a live attenuated SIV vaccine with a deletion in the nef gene. Science. 1992;258:1938-41.
34. Kirchhoff F, Munch J, Carl S, et al. The human immunodeficiency virus type 1 nef gene can to a large extent replace simian immunodeficiency virus nef in vivo. J Virol. 1999;73:8371-83.
35. Schindler M, Schmokel J, Specht A, et al. Inefficient Nef-mediated downmodulation of CD3 and MHC-I correlates with loss of CD4+T cells in natural SIV infection. PLoS Pathog. 2008;4:e1000107.
36. Gustin JK, Douglas JL. BST-2/tetherin: viral tether, viral sensor or both? Future Virol. 2013;8.
37. Sauter D, Schindler M, Specht A, et al. Tetherin-driven adaptation of Vpu and Nef function and the evolution of pandemic and nonpandemic HIV-1 strains. Cell Host Microbe. 2009;6:409-21.
38. Etienne L, Hahn BH, Sharp PM, Matsen FA, Emerman M. Gene loss and adaptation to hominids underlie the ancient origin of HIV-1. Cell Host Microbe. 2013;14:85-92.
39. Zhang F, Wilson SJ, Landford WC, et al. Nef proteins from simian immunodeficiency viruses are tetherin antagonists. Cell Host Microbe. 2009;6:54-67.
40. Jia B, Serra-Moreno R, Neidermyer W, et al. Species-specific activity of SIV Nef and HIV-1 Vpu in overcoming restriction by tetherin/BST2. PLoS Pathog. 2009;5:e1000429.
41. Postler TS, Desrosiers RC. The tale of the long tail: the cytoplasmic domain of HIV-1 gp41. J Virol. 2013;87:2-15.
42. Chrobak P, Afkhami S, Priceputu E, et al. HIV Nef expression favors the relative preservation of CD4+ T regulatory cells that retain some important suppressive functions. J Immunol. 2014;192:1681-92.
43. Hanna Z, Kay DG, Rebai N, Guimond A, Jothy S, Jolicoeur P. Nef harbors a major determinant of pathogenicity for an AIDS-like disease induced by HIV-1 in transgenic mice. Cell. 1998;95:163-75.
44. Ahmed Rahim MM, Chrobak P, Priceputu E, Hanna Z, Jolicoeur P. Normal development and function but impaired memory phenotype of CD8(+) T cells in transgenic mice expressing HIV-1 Nef in its natural target cells. Virology. 2013;438:84-97.
45. Acharjee S, Branton WG, Vivithanaporn P, et al. HIV-1 Nef expression in microglia disrupts dopaminergic and immune functions with associated mania-like behaviors. Brain Behav Immun. 2014;40:74-84.
46. Lindemann D, Wilhelm R, Renard P, Althage A, Zinkernagel R, Mous J. Severe immunodeficiency associated with a human immunodeficiency virus 1 NEF/3'-long terminal repeat transgene. J Exp Med. 1994;179: 797-807.
47. Brady HJ, Pennington DJ, Miles CG, Dzierzak EA. CD4 cell surface downregulation in HIV-1 Nef transgenic mice is a consequence of intracellular sequestration. Embo J. 1993;12:4923-32.

48. Jamieson BD, Aldrovandi GM, Planelles V, et al. Requirement of human immunodeficiency virus type 1 nef for in vivo replication and pathogenicity. J Virol. 1994;68:3478-85.
49. Watkins RL, Zou W, Denton PW, Krisko JF, Foster JL, Garcia JV. In vivo analysis of highly conserved Nef activities in HIV-1 replication and pathogenesis. Retrovirology. 2013;10:125.
50. Zou W, Denton PW, Watkins RL, et al. Nef functions in BLT mice to enhance HIV-1 replication and deplete CD4+CD8+ thymocytes. Retrovirology. 2012;9:44.
51. Skowronski J, Parks D, Mariani R. Altered T cell activation and development in transgenic mice expressing the HIV-1 nef gene. Embo J. 1993; 12:703-13.
52. Hanna Z, Kay DG, Cool M, Jothy S, Rebai N, Jolicoeur P. Transgenic mice expressing human immunodeficiency virus type 1 in immune cells develop a severe AIDS-like disease. J Virol. 1998;72:121-32.
53. Jolicoeur P. The CD4C/HIV(Nef)transgenic model of AIDS. Curr HIV Res. 2011;9:524-30.
54. Rahim MM, Chrobak P, Hu C, Hanna Z, Jolicoeur P. Adult AIDS-like disease in a novel inducible human immunodeficiency virus type 1 Nef transgenic mouse model: CD4+ T-cell activation is Nef dependent and can occur in the absence of lymphophenia. J Virol. 2009;83:11830-46.
55. Hanna Z, Priceputu E, Chrobak P, et al. Selective expression of human immunodeficiency virus Nef in specific immune cell populations of transgenic mice is associated with distinct AIDS-like phenotypes. J Virol. 2009;83:9743-58.
56. Curreli S, Krishnan S, Reitz M, et al. B cell lymphoma in HIV transgenic mice. Retrovirology. 2013;10:92.
57. Medapalli RK, He JC, Klotman PE. HIV-associated nephropathy: pathogenesis. Curr Opin Nephrol Hypertens. 2011;20:306-11.
58. Kaufman L, Collins SE, Klotman PE. The pathogenesis of HIV-associated nephropathy. Adv Chronic Kidney Dis. 2010;17:36-43.
59. Tervo HM, Goffinet C, Keppler OT. Mouse T-cells restrict replication of human immunodeficiency virus at the level of integration. Retrovirology. 2008;5:58.
60. Bieniasz PD, Cullen BR. Multiple blocks to human immunodeficiency virus type 1 replication in rodent cells. J Virol. 2000;74:9868-77.
61. Denton PW, Garcia JV. Humanized mouse models of HIV infection. AIDS Rev. 2011;13:135-48.
62. Melkus MW, Estes JD, Padgett-Thomas A, et al. Humanized mice mount specific adaptive and innate immune responses to EBV and TSST-1. Nat Med. 2006;12:1316-22.
63. Van Duyne R, Pedati C, Guendel I, et al. The utilization of humanized mouse models for the study of human retroviral infections. Retrovirology. 2009;6:76.
64. Karpel ME, Boutwell CL, Allen TM. BLT humanized mice as a small animal model of HIV infection. Curr Opin Virol. 2015;13:75-80.
65. Sepulveda-Crespo D, Serramia MJ, Tager AM, et al. Prevention vaginally of HIV-1 transmission in humanized BLT mice and mode of antiviral action of polyanionic carbosilane dendrimer G2-S16. Nanomedicine. 2015;11:1299-308.
66. Akkina R. Humanized mice for studying human immune responses and generating human monoclonal antibodies. Microbiol Spectr. 2014;2.
67. Denton PW, Long JM, Wietgrefe SW, et al. Targeted cytotoxic therapy kills persisting HIV infected cells during ART. PLoS Pathog. 2014;10:e1003872.
68. Watkins RL, Zou W, Denton PW, Krisko JF, Foster JL, Garcia JV. In vivo analysis of highly conserved Nef activities in HIV-1 replication and pathogenesis. Retrovirology. 2013;10:125.
69. Yamada E, Yoshikawa R, Nakano Y, Misawa N, Koyanagi Y, Sato K. Impacts of humanized mouse models on the investigation of HIV-1 infection: illuminating the roles of viral accessory proteins in vivo. Viruses. 2015;7:1373-90.
70. Watkins RL, Foster JL, Garcia JV. In vivo analysis of Nef's role in HIV-1 replication, systemic T cell activation and CD4(+) T cell loss. Retrovirology. 2015;12:61.
71. Kuo LS, Baugh LL, Denial SJ, et al. Overlapping effector interfaces define the multiple functions of the HIV-1 Nef polyproline helix. Retrovirology. 2012;9:47.
72. Denton PW, Estes JD, Sun Z, et al. Antiretroviral pre-exposure prophylaxis prevents vaginal transmission of HIV-1 in humanized BLT mice. PLoS Med. 2008;5:e16.
73. Leslie A, Mathews PC, Listgarten J, et al. Additive contribution of HLA class I alleles in the immune control of HIV-1 infection. J Virol. 2010;84:9879-88.
74. Denton PW Estes JD, Sun Z, et al. Antiretroviral pre-exposure prophylaxis prevents vaginal transmission of HIV-1 in humanized BLT mice. PLoS Med. 2008;5:e16.
75. Novembre FJ, Saucier M, Anderson DC, et al. Development of AIDS in a chimpanzee infected with human immunodeficiency virus type 1. J Virol. 1997;71:4086-91.
76. Juompan LY, Hutchinson K, Montefiori DC, Nidtha S, Villinger F, Novembre FJ. Analysis of the immune responses in chimpanzees infected with HIV type 1 isolates. AIDS Res Hum Retroviruses. 2008;24:573-86.
77. Arhel NJ, Kirchhoff F. Implications of Nef: host cell interactions in viral persistence and progression to AIDS. Curr Top Microbiol Immunol. 2009;339:147-75.
78. Kirchhoff F. Immune evasion and counteraction of restriction factors by HIV-1 and other primate lentiviruses. Cell Host Microbe. 2010;8:55-67.
79. Laguette N, Bregnard C, Benichou S, Basmaciogullari S. Human immunodeficiency virus (HIV) type-1, HIV-2 and simian immunodeficiency virus Nef proteins. Mol Aspects Med. 2010;31:418-33.
80. Ochsenbauer C, Edmonds TG, Ding H, et al. Generation of transmitted/founder HIV-1 infectious molecular clones and characterization of their replication capacity in CD4 T lymphocytes and monocyte-derived macrophages. J Virol. 2012;86:2715-28.