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Homodimerization is Required for HIV-1 Nef Function in Humanized Mice

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Abstract

Background: The HIV-1 Nef accessory factor is essential for efficient viral replication and immune evasion in vivo. Dimerization of Nef has been linked to its interaction with multiple host cell binding partners, including the endocytic trafficking adaptor protein, AP2. Internalization of both CD4 and the SERINC5 restriction factor by Nef requires AP2 and clathrin-mediated endocytosis in order to enhance infectivity and avoid immune surveillance. Here we explored the role of Nef dimerization and AP2 recruitment in HIV-1 infectivity in vitro as well as viral replication and CD4$^+$ T cell loss in humanized mice.

Methods: To test the importance of Nef dimerization and AP2 recruitment in HIV-1 pathogenesis, we generated mutants that are deficient for Nef dimerization (Ile109/Leu112/Tyr115/Phe121 to Asp; 4D mutant) and AP2 binding (Asp 174/175 to Ala; DDAA or DD mutant, and Arg 134 to Glu; R134E or RE mutant). A virus defective for Nef expression was also included (ΔNef mutant). Viruses were produced in transfected 293T cells with or without amplification in MT2 T cells. Infectivity and replication were compared using TZM-bl reporter cells and PBMCs cells, respectively. BLT (bone marrow-liver-thymus) and hPBMC-NSG humanized mice were infected with each virus, and replication measured by real-time quantitative RT-PCR or p24 alpha assays in plasma and tissues. Human CD4$^+$ T cell counts were followed in PBMCs and tissues by flow cytometry as a surrogate for HIV-1 pathogenesis.

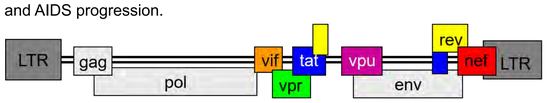
Results: In vitro, HIV-1 infectivity was reduced with the 4D, R134E and ΔNef viruses, and the most profound effect was observed with the 4D mutant. Humanized BLT mice infected with ΔNef viruses in either an X4 (NL4-3) or R5 (p81a) background showed significantly lower viral loads and reduced CD4 depletion compared to wild-type. hPBMC-NSG mice infected with the 4D and R134E mutant viruses showed decreased viral loads and displayed CD4$^+$ T cell counts comparable to uninfected mice. Viral loads and tissue CD4 counts reflected the levels found in plasma.

Conclusions: Our results demonstrate that Nef dimerization and interaction with AP2 are both important for HIV-1 pathogenesis in humanized mouse models of HIV/AIDS. These data support a strategy to disrupt Nef dimerization as a new path to antiretroviral drug discovery.

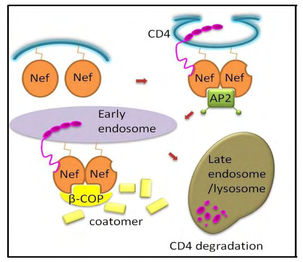
Introduction

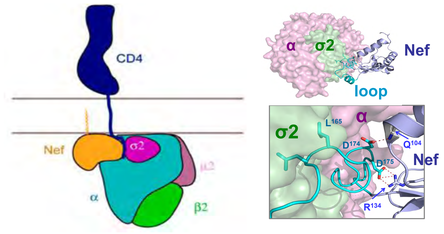
HIV-1 Nef dimers interact with CD4 and the AP2 complex

- The HIV accessory protein Nef is a virulence factor required for high titer viral replication, immune escape, and AIDS progression.



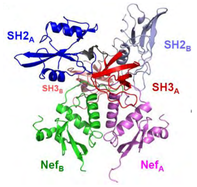
- Nef interacts with CD4 and induces endocytosis through the Adaptor Protein 2 (AP2) complex. Polar contacts involving Nef D174, D175, and R134 stabilize the Nef internal loop for AP2 interaction.



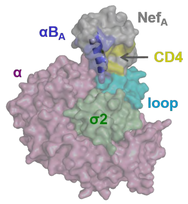


Ren et al., eLife 2014

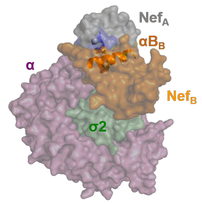
- Structure alignment, biochemical and cell-based data indicate that Nef dimerization may play an important role in its interaction with AP2 and HIV pathogenesis.



PDB: 4U5W (Nef:Nef:Hck SH3-SH2)
JJ Alvarado et al., JBC 2014



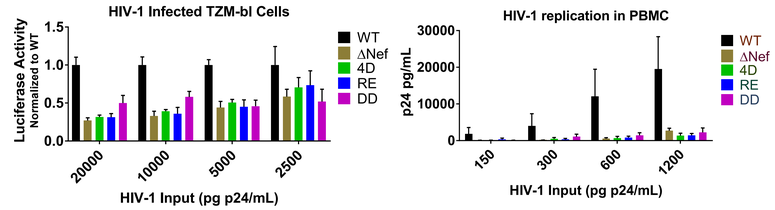
PDB: 4NEE (Nef:AP2 α-σ2)



PDB: 4U5W (Nef:Nef:Hck SH3-SH2) +
PDB: 4NEE (Nef:AP2 α-σ2)

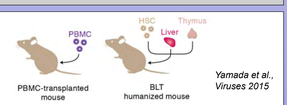
Mutations that disrupt Nef dimerization and interaction with CD4/AP-2 decrease HIV-1 infectivity

- Nef dimerization and AP2 interaction mutants decrease HIV-1 infectivity and replication. (ΔNef: stop codon prevents Nef expression; 4D: Nef dimerization interface mutant; R134E/DD: defective for AP2 recruitment).



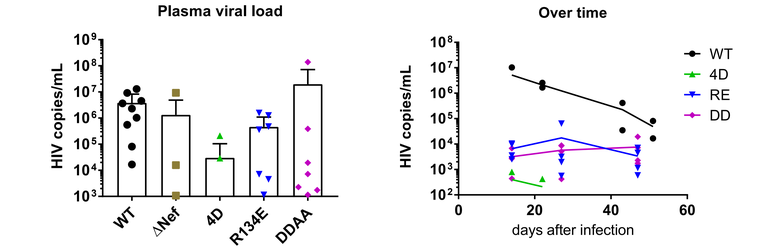
Nef mutations reduce viral loads and CD4$^+$ T cell depletion in HIV-infected mice

- Immunocompromised NSG mice engrafted with donor PBMCs or human tissues plus CD34$^+$ human HSCs support HIV-1 replication *in vivo.*



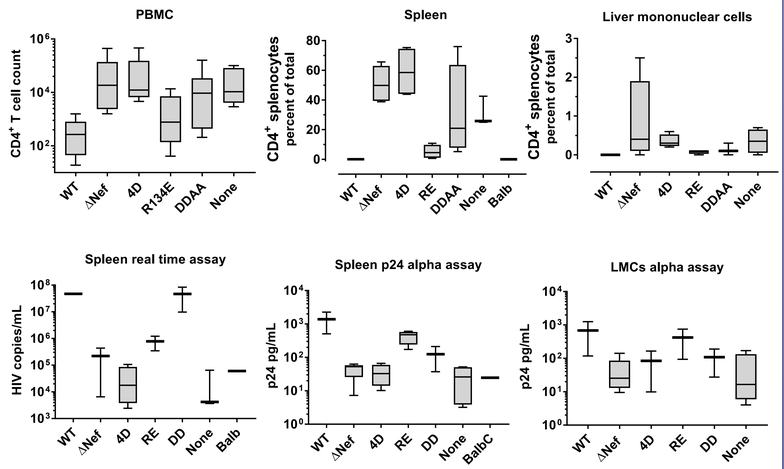
Yamada et al., Viruses 2015

- Decreased viral load and CD4$^+$ T cell depletion in humanized mice (NSG-PBMC) infected with HIV NL4-3 harboring Nef mutants (2000 $TCID_{50}$).



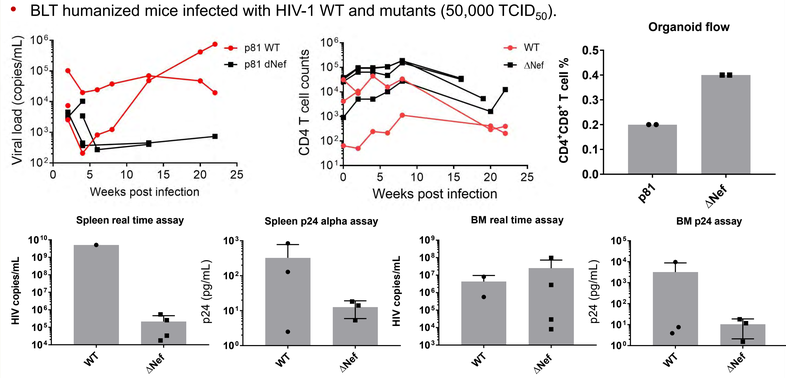
2 x ΔNef and 4 x 4D mice below limit of detection

2 x 4D mice below limit of detection after 22 days

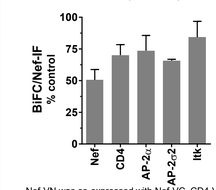


Using BLT humanized mice to investigate Nef functions during acute HIV-1 infection

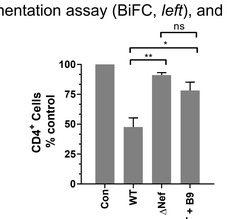
- BLT humanized mice infected with HIV-1 WT and mutants (50,000 $TCID_{50}$).



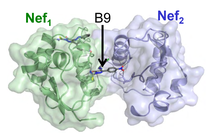
- Small molecule Nef inhibitor 'B9' partially disrupts Nef interaction with CD4 and AP2 α and σ2 subunits by Bimolecular Fluorescence Complementation assay (BiFC, *left*), and inhibits CD4 downregulation in infected cells *(middle)*.



Nef-VN was co-expressed with Nef-VC, CD4-VC, AP-2α-VC, and AP-2α2-V, in 293T cells in the presence or absence of B9 (3 μM). Cells were also transfected with Nef-VC plus Itk-VN in the presence of B9 as a control.



CEM-GFP cells were infected with wild-type HIV-1 (*WT*), a Nef-defective mutant (Δ*Nef*), or with WT in the presence of B9 (3 μM).



Docking of B9 on crystal structure of Nef dimer, PDB: 1EFN
Emert-Sedlak et al. Chem Biol 2013

- Pharmacokinetics and oral bioavailability of selected B9 analogs. No significant toxicity after 5 consecutive single daily intraperitoneal (2 mg/kg) or oral (30 mg/kg) administrations of analog **2** (*data not shown*).

Compound	Route	Vehicle	Dose (mg/kg)	T_{max} (h)	C_{max} (ng/mL)	AUC_{last} (h ng/mL)	$T_{1/2}$ (h)	V_{ss} (L/kg)	Cl (mL/min/kg)	%F
1 (B9)	iv	Solution	2		3959	9784	2.13	0.37	3.41	2
	po	Solution	10	0.5	418	963				
2	iv	Solution	2		11215	2398	2.7	0.83	13.49	53
	po	Solution	10	0.25	2369	6302				
	Iv	Solution	2		4416	2134	1.53	0.91	15.48	85
	po	Suspension	10	0.5	5611	9034				

Emert-Sedlak et al., Bioorg Med Chem Lett. 2016

Summary and future directions

- Humanized mice infected with HIV-1 lacking Nef expression (ΔNef) or expressing a homodimerization-defective Nef mutant (4D) had lower viral loads and reduced CD4$^+$ T cell depletion. Results with a Nef mutant defective for AP2 interaction (Nef-RE) suggest that the interaction between Nef and AP2 may also contribute to viral pathogenesis *in vivo*.
- The effect of these Nef mutations on HIV-1 latency and persistence in humanized mice is currently under investigation.
- Small molecules targeting Nef dimers and disrupting Nef:AP2 interaction may provide a new strategy for anti-retroviral drug development.

Acknowledgements

- Reagents and technical support: Drs. Lori Emert-Sedlak, Jerrod Poe, John Jeff Alvarado (School of Medicine), Moses Bility (Graduate School of Public Health) University of Pittsburgh
- Funding: NIH K01 OD018431 (PI: STS) and R01 AI057083/AI102724 (PI: TES)