Summary and Conclusion
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- Nef, a Negative factor, is 25-30 kd myristoylated early HIV protein and is known to be involved in downregulation of cell surface molecules such as CD4, MHC-1, CD28 etc. of infected cell. Nef also interfere with cellular signal transduction processes and is also known to delay apoptosis of infected cells.

- More than 30 putative Nef targets and associated functions have already been identified but the molecular mechanisms involved and their contribution to viral pathogenesis or fitness are not fully elucidated.

- Multiple reports discussing biological role of Nef are available but, majority of them are based on subtype B viruses predominant in Western world while in India subtype C infection is dominant.

- In India, over 5.28 million individuals are estimated to be HIV infected and more than 80% of the strains tested belong to subtype C. But extensive characterization of subtype C isolates is still lacking.

- Many reports on genotypic differences for structural genes like env and gag are available. But data on regulatory gene, nef in infected
Indians is scarce. Hence in the present study, we aimed at critically evaluating Nef from Indian samples with following objectives

- To analyze genetic diversity in nef from HIV-1 Indian subtype C virus.
- To characterize Nef for its role in influencing rate of disease progression.
- To study effect of clade-specific Nef on viral fitness.
- To identify crucial functional domains in Nef using systematic site-directed mutagenesis.

**Genetic analysis of Indian HIV-1 nef: Subtyping, variability and implications**

- We analyzed Nef for its genetic diversity and potential CTL epitopes in five individuals at early seroconversion stage, five long-term survivors and four subjects with normally progressive HIV-1 infection.
- Major functional domains in Nef were conserved in all our sequence, although few variations in seroconverter sequences were observed in juxtapositions to some functional domains.
- In phylogenetic tree, our seroconverter sequences segregated away from those reported from India earlier for 411-428bp and 478-522bp,
(Lole et al., 1999). This suggests that, the currently circulating virus has undergone evolutionary changes in last 6 years, especially in these conserved regions of Nef

- Indian Nef sequences clustered away from other African subtype C sequences, consensus C, ancestral C and representative sequences from other clades. These results strongly support the view that Indian nef sequences tend to form a distinct subclade within the clade C. The difference between Indian subtype C and African subtype C sequences was as high as 25%, especially for the C terminal region.

- All the LTS enrolled in the present study gave no evidence of deletion in nef gene. Truncation in nef gene is known to be associated with slow progression of disease.

- Putative CTL epitopes for Indian Nef sequences were predicted using computer assigned algorithm. Seroprevalent sequences showed substitutions within epitopes predicted based on seroconverter sequences. Mutations in Nef led to altered intensity of epitope binding which may eventually alter the outcome of immune response.

- Epitope prediction data would be useful in identifying Nef epitopes to be included in multi-epitope HIV-1 vaccine for Indian population and would also be helpful in HIV-1 evolutionary studies.
Sequence analysis for *nef* also suggests the possibility of using regulatory gene sequences for viral subtyping.

**Generation and characterization of HIV-1 clones chimeric for subtypes B and C nef**

- This study was aimed at evaluating the influence of subtype specific sequence diversity in Nef on its biological role and in-turn viral fitness.
- Nef from Indian subtype C was compared with extensively studied subtype B.
- Nef chimeras were constructed using pNL432 (subtype B) and pIndieC-1 (Indian subtype C) infectious clones. The ability of chimeras to multiply in activated human PBMCs was studied.
- The proviral infectious clones pNL432 (subtype B) and pIndie-C1 (Indian subtype C) with distinct viral growth phenotype and having considerable sequence heterogeneity in *nef* were used to construct infectious proviral cassette vector reporter system which precisely
facilitated alteration in \( nef \). To ascertain the Nef-dependency of viral infectivity, frame-shift \( nef \)-deficient mutants were also constructed.

- The chimeras were monitored for their replication kinetics and infectivity in activated human peripheral blood mononuclear cells.
- For NL-derived clones, \( Nef \)-positive parental and its chimeric clone grew quite similarly but for Indie-derived clones, chimeric clone showed 46\% increase in peak RT production as compared to wild type.
- For NL virus, the difference between wild type and Nef deficient virus was approximately 30\% along with a shift in peak RT production from 6\textsuperscript{th} day to 12\textsuperscript{th} day post infection while for Indie virus the difference between wt and Nef deficient virus was about 54\%.
- NL-viruses grew much better than Indie-viruses. RT production reached peak on day 6 post-infection for NL-viruses, whereas on day 15 for Indie viruses post infection.
- Our results here clearly highlight the indispensability of Nef in viral life cycle, although the influence of subtype specific sequence variability was not very profound.
Generation and characterization of HIV-1 Nef mutants

- This study was aimed at identifying crucial functional domains in Nef across all major clades of HIV influencing its activity and in-turn viral fitness.
- We constructed 55 viral strains with single or multiple point mutations in Nef using proviral parental clone pNL432.
- The mutants were designed based on analysis of sequence conservation across all major clades of HIV-1 and published structure-function reports.
- These single or multiple point mutants were analyzed for their ability to alter infectivity in Nef dependent MAGI cells and for replication kinetics in stimulated human PBMCs. Nef expression levels were studied for all the mutants using western blot analysis.
- Of 55 mutants constructed, 14 mutants had markedly reduced infectivity (less than 50%) in MAGI cells.
- Out of these 14 mutants, 11 mutants had impaired growth kinetics in stimulated human PBMCs as compared to wild type.
- Out of 55 mutants constructed 12 mutants showed substantially reduced Nef expression level as compared to wt.
• Few mutants with impaired infectivity and growth kinetics had reduced Nef expression level. Majority of these mutations; nG119L, nP136A, nW141A and nL189A, responsible for lowering Nef expression have not been reported previously. Correlation between low Nef expression level and decreased viral fitness was never reported and our results strongly suggest this new possibility.

• We also report new crucial amino acid positions in Nef previously unreported influencing viral fitness. Knowledge of these newly identified important domains together with those already reported in literature will enable to comprehensively understand Nef structure-function relationship.

Briefly, in this study, we analyzed genetic diversity in Nef from HIV-1 Indian subtype C virus. Nef from seroconverters and long term survivors were analyzed for sequence diversity and were also compared with representative sequences from other clades. Genetic diversity was observed for Nef between Indian subtype C and sequences from other clades. This genetic diversity was further evaluated in context with viral fitness *in vitro*. But, no major difference was observed in ability of Nef from two different subtypes (subtype B and C) to influence viral fitness. These results clearly suggest that sequences conserved in Nef across subtypes may be more
crucial for its function than subtype associated variations. Hence, finally, we constructed a large panel of single or multiple point mutants for conserved amino acids in Nef across all major clades of HIV and these mutants were evaluated for basic biological properties like infectivity, growth kinetics and Nef expression level.