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Extension of gp120 Env Co-receptor Use to CXCR4 Is Associated with Disease Progression in LTNP Infected with Nef-Defective HIV-1

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Abstract

The LTNP condition depends on a complex interplay between host and viral factors. Infection with HIV-1 variants with attenuated replicative capacity may contribute to the beneficial course of disease in a minor group of LTNP. Among other viral genes, *nef* has been strongly associated with pathogenesis *in vivo*.
In this study, we have characterized the *nef* variants obtained from plasma RNA and PBMC-associated DNA of 6 infected hemophiliacs who were selected as LTNP in 1995 out of 112 individuals attending the Hemophilia Center in Milan, Italy. These 6 individuals naturally split into either LP or LTNP upon long-term follow up. Their *nef* alleles obtained from plasma in 1998 were analyzed in regard to their capacity to alter the infectivity/replication and to mediate CD4 and Major Histocompatibility Complex Class I (MHC-I) antigen (Ag) down-regulation once inserted in the background of the full-length infectious molecular clone NL4-3 or in the sub-genomic pcDNA3 expression vector under the control chicken β–promoter. The *nef* alleles from of 5 out of 6 individuals significantly reduced HIV infectivity/replication and impaired Nef-mediated CD4 but not Major Histocompatibility Complex Class I (MHC-I) antigen down-modulation from the cell surface. Thus, functional alterations of the *nef* gene are present in both LP and LTNP suggesting that Nef defectiveness *in vitro* is not necessarily associated with the long-term maintenance of the LTNP status. However, the viral isolates from 3 out of 3 LP showed a dual CCR5/CXCR4 co-receptor use (R5X4) in contrast to those from LTNP that were exclusively R5. Thus, *in vivo* evolution of gp120 Env to CXCR4 use appears to be associated with HIV disease progression in individuals infected with *nef*-defective viruses.

Population study

Six out of 112 (6.2 %) hemophiliacs who had contracted HIV-1 infection between 1983 and 1985 through contaminated plasma products were classified as LTNP in 1995. The clinical and virological follow-up has been carried out up to December 2006. A progressive decrease in the CD4⁺ T cell counts < 500 cells/µl has been observed in 3 individuals starting in 1996 (Figure 1A) (subject 2, 3 and 6, red lines, individuals LP). CD4+ T cells of subject 1 have been decreasing below 500 cells/µl since 2003 whereas subjects 4 and 5 maintain the LTNP status (Figure 1A, blue lines). The viremia of subjects 2, 3 and 6 has decreased as a consequence of the introduction of highly active antiretroviral therapy (HAART) in 2000. Subject 1 is not receiving antiretroviral treatment (Figure 1B).

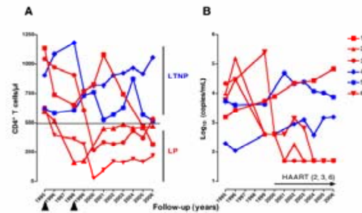


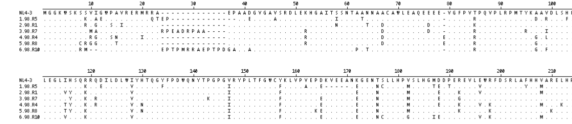
Figure 1 Levels of CD4⁺ T cells in peripheral blood (A) and viremia (B) in HIV-1 infected hemophiliacs from study entry (1995) to 2006. Arrowheads indicate sampling time.

Amplification and cloning of HIV-1 *nef* alleles

A nested PCR amplification method was adopted to amplify the *nef* gene from both proviral DNA and plasma RNA. PCR products of each sample were generated from two or more independent amplifications prior to cloning. Small aliquots (1-2 µl) were ligated into the PCR II vector. Several clones were tested for the presence of *nef* insert by *Eco RI* digestion and DNA of recombinant clones was used for sequencing. A total of 162 sequences was obtained from blood samples collected in 1995 and 1998.

Alignment of LTNP and LP predominant Nef obtained in 1998

Figure 2

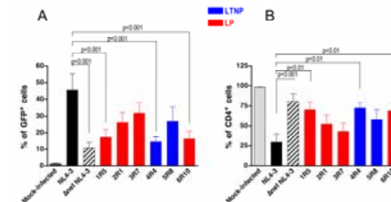


The sequencing analysis of *nef* alleles isolated in 1998 revealed that several previously defined functional regions were highly conserved in all the sequences. However, these alleles were tested for the ability to interfere in previously define Nef functions such as infectivity, CD4 and MHC-I downregulation.

Loss of infectivity and CD4 down-modulation capacity of *nef* alleles from LTNP and LP

The *nef* variants obtained in 1998 were inserted in place of Nef of the infectious molecular clone NL4-3. Chimeric viruses were generated by transfection in 293 cells. The infectivity/early stage replication of chimeric infectious molecular clones was examined by incubating of equal amounts of RT activity on CEM-GFP cells that express GFP under the control of a subtype B HIV-1 LTR. The extent GFP⁺ infected cells was evaluated after staining with a CD4-specific mAb by FACS analysis 5 days post-infection.

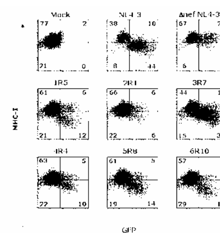
Figure 3



The *nef* alleles of subjects 1 (1R5), 4 (4R4) and 6 (6R10) significantly diminished the proportion of GFP⁺ infected cells as compared to WT virus ($p < 0.001$ by repeated measures ANOVA, panel A). Conversely, the percentage of CD4⁺ cells infected with the chimeric viruses 1R5, 4R4 and 6R10 was significantly higher than that of cells infected with NL4-3 and was comparable to that of Δ*nef* NL4-3 (panel B).

nef alleles from LTNP and LP maintain MHC-I Ag down-modulation capacity

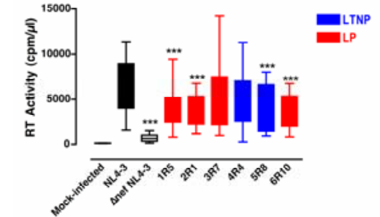
Figure 4



HLA-ABC staining and Facs analysis of CEM-GFP infected with chimeric viruses. In contrast to CD4 down-modulation, the infection with chimeric viruses expressing *nef* alleles caused a reduction of MHC-I Ag membrane expression comparable to that of NL4-3.

nef alleles of LTNP and LP reduce the efficiency of virus replication in PBMC

Figure 5



Equal amounts of RT activity were loaded onto resting PBMC that were subsequently activated by PHA 24 h post-infection. The levels of viral replication of each *nef* chimeric virus were compared to those of NL4-3 and Δ*nef* virus at their peaks of replication, usually 9-10 days post-infection. The replication of the chimeric viruses 1R5, 2R1, 5R8 and 6R10 was significantly impaired as compared to that of NL4-3 ($n = 4$, $p < 0.001$, repeated measures ANOVA).

nef alleles obtained from HIV-1 infected hemophilic LTNP and LP are equally defective in HIV infection/replication and CD4 down-regulation capacities and maintain MHC Class I downregulation.
Since evolution of gp41 Env in macaques infected with *nef*-deleted SIV *(Alexander et al 2003)* and evolution towards CXCR4 usage was reported in individuals infected with *nef*-deleted viruses*(Jeckle et al 2002),* the coreceptor usage was determined on viral isolates obtained in 1995 and 1998.

Table 1

Pt/year	Coreceptor use
LTNP-4/95	No isolation
LTNP-4/98	No isolation
LTNP-5/95	R5
LTNP-5/98	R5
LP-1/95	No isolation
LP-1/98	R5
LP-2/95	R5X4
LP-2/98	R5X4
LP-3/95	R5
LP-3/98	R5X4
LP-6/95	R5
LP-6/98	R5X4

R5 viruses are present in LTNP whereas LP are characterized by the presence of either R5 to X4 switch or X4 viruses.

Summary and Conclusion

Functional alterations of the *nef* gene have been present in most LTNP and LP in our cohort of HIV infected hemophiliacs. Three out of 6 individuals who were LTNP in 1995 have progressed to HIV disease during the follow-up by the year 2000, and an additional one (LTNP-1) too by 2003. This clinical observation suggests that alterations in Nef functions are likely necessary but not sufficient to maintain the LTNP status, as previously reported by independent investigators. Functional alterations in other accessory genes such as Vif, Vpu and Vpr, together with the evolution of the HIV-specific immune responses might therefore contribute to maintain low levels of viral replication and delay disease progression. In addition, the detection of CXCR4 co-receptor use in primary isolates of LP, but not LTNP, indicates that the evolution of the gp120 Env gene towards higher virulence may occur and overcome the presence of *nef*-defective HIV suggesting that LTNP harbor HIV which they control immunologically. Thus, a constant follow-up of these individuals and their viruses will contribute to dissecting out both viral and host factors that could provide novel insights for designing better strategies for efficient long-term viral suppression.