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Therapeutic vaccination reduces HIV sequence variability

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ABSTRACT **With HIV persisting lifelong in infected persons, therapeutic vaccination is a novel alternative concept to control virus replication. Even though CD8 and CD4 cell responses to such immunizations have been demonstrated, their effects on virus replication are still unclear. In view of this fact, we studied the impact of a therapeutic vaccination with HIV *nef* delivered by a recombinant modified vaccinia Ankara vector on viral diversity. We investigated HIV sequences derived from chronically infected persons before and after therapeutic vaccination. Before immunization the mean ± SE pairwise variability of patient-derived Nef protein sequences was 0.1527 ± 0.0041. After vaccination the respective value was 0.1249 ± 0.0042, resulting in a significant ($P<0.0001$) difference between the two time points. The genes *vif* and *5′gag* tested in parallel and *nef* sequences in control persons yielded a constant amino acid sequence variation. The data presented suggest that Nef immunization induced a selective pressure, limiting HIV sequence variability. To our knowledge this is the first report directly linking therapeutic HIV vaccination to decreasing diversity in patient-derived virus isolates.—Hoffmann, D., Seebach, J., Cosma, A., Goebel, F. D., Strimmer, K., Schätzl, H. M., Erfle, V. Therapeutic vaccination reduces HIV sequence variability. *FASEB J.* 22, 437–444 (2008)**

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INTEGRATION OF HIV, A RETROVIRUS, into the host cell genome of infected cells is a process that cannot be reversed (1). Infected cells and their progeny will therefore contain HIV proviral DNA for their lifetimes (2). To cure HIV infection, all infected cells would have to be eliminated or the proviral DNA would have to be selectively removed from the host genome. Because both approaches are not realistic given current inventional capabilities, therapeutic options are concentrated on limiting the damage HIV causes rather than clearing the virus completely from the infected organism. It is known that HIV replication directly correlates with immune deficiency. Thus, the aim of treatment approaches is to reduce the viral load as much as possible. Antiretroviral therapy (ART) can effectively lower HIV replication to undetectable levels (viral load <50 RNA copies$\times$ml^{-1}).

However, treatment must be administered lifelong, resulting in a variety of severe interrelated problems. With the high mutation rate of 10^{-3}–10^{-4}, resistant genotypes are likely to emerge during long-term therapy. Side effects, such as lipodystrophy, hepatic steatosis, or lactic acidosis (3) are important factors leading to low compliance, and thereby limiting therapeutic success. In view of the continuously increasing HIV incidence, currently affecting 40–50 million people, alternative therapeutic strategies are desperately needed (4). Among those, therapeutic vaccination has been studied as an approach to control viral replication by modulating the host's immune response to HIV (5–9). The regulatory HIV protein Nef is considered to be an interesting antigen for vaccination because of its early expression in infected cells, the presence of several cytotoxic T lymphocyte (CTL) epitopes, and its immunogenicity (10, 11). CD4 and CD8 responses to the vaccinated Nef antigen have been clearly demonstrated in both animal and human studies (12–14).

We have recently conducted a therapeutic vaccination study with a modified vaccinia Ankara (MVA)-HIV-1(LAI)-*nef* vector (13, 15, 16). Ten persons with chronic HIV infections were vaccinated at weeks 0, 2, and 16. All subjects had reactive CD8 T cells to Nef, whereas only 2 had a detectable CD4 response before vaccination. After vaccination, the CD8 response increased in 3 persons, and 8 of 10 now showed CD4 T cell reactivity to Nef. ART was interrupted 1 year after the first vaccination and resumed when the viral load reached $>10^5$ copies $\times$ ml^{-1}. Six matched control patients were not immunized but also underwent a one-cycle therapy interruption (TI) of a stable antiretroviral regimen.

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Escape mutations within CTL epitopes in Nef have been described in both HIV (14, 17) and simian immunodeficiency virus (18). HIV has been reported to react in complex ways to selective pressure (19), leading to new strains with altered drug susceptibility and replication capacity (20).

Thus, the aim of the present study was to elucidate whether the immune response to the therapeutic vaccination had an impact on viral genotypes in individual patients. To this end, we studied Nef nucleic acid and protein sequences before vaccination and during TI 1 year after vaccination. As a control, the structural proteins p17 and part of 24 were compared with Nef as they are also highly immunogenic (21). In addition, the regulatory HIV protein Vif was tested in parallel because of its similar natural sequence variability (2, 10). We report a significant reduction of DNA and protein sequence variability within Nef in vaccinated persons. This was not observed in control patients and also not within p17, p24, and Vif of vaccinated individuals. Taken together, our data strongly indicate that a selective pressure limiting sequence variation within the targeted gene was induced by vaccination with MVA-*nef*.

MATERIALS AND METHODS

Patient samples

Plasma samples from seven persons vaccinated with *nef* delivered by an MVA vector were tested along with plasma samples from five matched control subjects. In vaccinated patients, *nef, vif,* and *5′gag* sequences were studied before immunization and after vaccination during TI (**Fig. 1**). *Nef* sequences were tested in parallel in the five control subjects, who were managed identically except for the vaccination itself. **Table 1** presents the characteristics of vaccinated and control subjects. All individuals received a triple combination of antiretroviral drugs. There were no significant differences between the two groups in age, CD4 count, and virus load at the pre- and postvaccination blood draws. The exceptionally long period of 253 days between beginning of TI and the postvaccination sampling for vaccinated subject 03 was due to irregular follow up. In vaccinated subjects 04 and 06, however, the longer time corresponded to a slower viral load increase, with no earlier sample having a sufficient HIV load for sequencing. Even when these values were included, there was no significant difference between vaccinated subjects (64.7±36.4 days) and control subjects (24.6±5.3 days). The prevaccination specimens were selected as to ensure a similar time period between sampling of the analyzed sequences in the vaccinated and control groups (65.0±6.1 and 64.0±3.8 months, respectively).

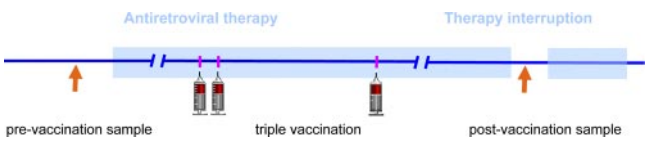

Figure 1. Triple vaccination of patients under ART. Time points of the pre- and postimmunization drawing of blood samples are symbolized by vertical arrows.

Nucleic acid extraction, polymerase chain reaction (PCR), sequencing reaction, and intracellular cytokine staining

HIV RNA was extracted from plasma samples with the High Pure Nucleic Acid Kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. The *nef* gene was amplified by reverse transcription-PCR with the primers Nef-F1 CGGA(AG)CCTGTGCCTCTTC, Nef F2 TGTGCCTCTTCAGCTACC(AG)C, and Nef-R TCCCCAG(CT)GGAAAGTCCC. Vif-F1 GAAAGGTGAAGGGGCAGTAG, Vif-F2 AGGGATTATGGAAAACAGATGG, and Vif-R TGGTCTTCTGGGG(CT)TTGTTC were used for seminested amplification of the control gene *vif*. A 5′ region of *gag* including p17 and part of p24 was amplified in a seminested PCR using Gag-F1 TTTGACTAGCGGAGGCTAG, Gag-R1 ACTCCCTGACATGCTGTC, and Gag-F2 AGATGGGTGCGAGAGCGT, as well as Gag R2 ACATGCTGTCATCATTTCTTC. All oligonucleotides were purchased from MWG Biotech (Ebersberg, Germany). Because the *5′gag* amplificate was >1000 bp long we used platinum *Taq* polymerase (Invitrogen, Karlsruhe, Germany) and extended the elongation time to 90 s. PCR products were TA cloned into the vector pCR4 (Invitrogen) according to the manufacturer's instructions. Double-sided sequencing was done at GATC (Konstanz, Germany) using vector-specific primers. Overall, ~2300 nucleotides corresponding to ~25% of the entire HIV proviral genome were analyzed at each time point in a given vaccinated individual.

Intracellular cytokine staining was performed as previously described (16). Briefly, 1×10^6 freshly purified peripheral blood mononuclear cells (PBMCs) were stimulated with pools of overlapping peptides spanning the Nef, p17, and p24 HIV-1 proteins. After 1 h stimulation, Brefeldin A was added, and the sample was incubated for 4 h. PBMCs were stained for the surface markers CD3, CD4, and CD8 and the intracellular marker IFN-γ. Stained cells were analyzed using a FACS Calibur flow cytometer, and the data were evaluated with Cell Quest Pro software (BD Biosciences, Heidelberg, Germany). Specific responses to Nef, p17, and p24 were reflected by the proportion of T cells reactive to the respective antigen.

Sequence analysis and statistical evaluation

The raw sequences were processed and aligned with the software Vector NTI Advance 10 (Invitrogen), Bioedit (Ibis Therapeutics Inc., Carlsbad, CA, USA), and Mega version 3.1 (22). Both nucleic acid and protein alignments were based on the Clustal W algorithm (23). First, pre-, and postimmunization protein sequences were aligned intraindividually, and the proportional sequence variation was determined. Subsequently, the pairwise alignments were analyzed for selection on the entire-gene level. The number of nonsynonymous mutations at nonsynonymous sites (dN) and the number of synonymous mutations at synonymous sites (dS) per gene were computed with SNAP (http://hcv.lanl.gov/content/hcv-db/SNAP/SNAP.html) (24), employing the Nei-Gojobori method (25). In addition, dN and dS were estimated with maximum likelihood analysis using codon substitution models codeml (26) in the PAML software package accessed *via* the PAL2NAL online interface (27). The hypothesis dN = dS, indicating the absence of selection (neutrality), was analyzed with a Z-test in Mega version 3.1. Signature amino acid positions showing the greatest variation were identified with the software VESPA (http://www.hiv.lanl.gov/content/hiv-db/P-vespa/vespa.html) (28). Site-specific selection was studied on the Selecton Server (http://selecton.bioinfo.tau.ac.il) (29) with a likelihood ratio test based on five evolution models (30).

TABLE 1. *Characteristics of vaccinated and control patients*

Patient group	Birth year	Log VL (RNA copies×ml^{-1})		CD4+ (cells×μl^{-1})		Sample time after TI (d)	Duration of ART at TI (mo)
		Pre[a]	Post[a]	Pre[a]	Post[a]		
Vaccinated							
02	1944	3.94	4.78	326	354	18	84
03	1966	4.58	5.29	888	505	253	74
04	1955	4.15	4.24	898	1193	81	50
05	1962	3.97	5.09	232	485	18	84
06	1937	4.23	5.10	449	471	49	59
07	1949	4.69	4.51	201	873	17	79
11	1962	4.96	4.20	4	395	17	40
Mean		4.36 ± 0.15	4.74 ± 0.17	428.3 ± 130.3	610.9 ± 116.2	64.7 ± 32.7	67.1 ± 6.6
Control							
01	1962	3.87	5.09	428	703	33	75
02	1970	5.44	6.28	12	484	20	74
03	1966	4.47	4.68	83	319	20	68
05	1943	5.64	4.71	420	517	27	53
06	1967	4.60	4.47	10	255	20	64
Mean		4.80 ± 0.33	5.05 ± 0.32	190.6 ± 96.2	455.6 ± 79.0	24.6 ± 2.7	66.8 ± 4.0

Pre = prevaccination; Post = postvaccination. [a]These terms are not suitable for the control patients. However, as indicated in the text, the time points are very similar in the two groups.

The pairwise variation between the individual strains was determined for Nef, Vif, and 5′-Gag using Mega. For Nef, 8–11 clones each from four vaccinated subjects were tested before and after vaccination, respectively. Sequences were aligned pairwise at both the nucleic acid and amino acid levels, and the proportional differences were calculated. Normal distribution was verified with the D'Agostino-Pearson normality test. Subsequently, the pre- and postvaccination data were compared with a *t* test. Statistic evaluations were conducted with GraphPad Prism (GraphPad Software Inc., San Diego, CA, USA).

Phylogenetic analysis of the seven pre- and postvaccination sequences was done by maximum likelihood. Specifically, we inferred branch lengths and tree topology from the postvaccination sequences, and subsequently reoptimized the number of substitutions along the branches of the tree using the prevaccination data. The decreased diversity of the postvaccination sequences is reflected by a general shortening of the estimated branch lengths.

RESULTS

Intraindividual comparison of pre- and postvaccination sequences

To elucidate the effect of *nef* immunization on patient viral populations, we analyzed HIV sequences from vaccinated and control individuals as available from a recent clinical trial conducted at our institution (16). First, pre- and postvaccination protein sequences of the seven immunized individuals were compared. The average proportion of amino acid exchanges (excluding deletions and insertions) was 0.0508 (range 0.0192–0.1165) for Nef, 0.0477 (range 0–0.1156) for Vif, and 0.0348 (range 0.0057–0.0629) for p17/p24. Although Nef sequences accumulated the highest proportion of mutations whereas p17/p24 yielded the lowest, the difference is not significant by *t* test.

Analysis of the synonymous and nonsynonymous nucleotide substitutions in the aligned pre-and postvaccination *nef* gene yielded neutral selection in four isolates and purifying (*i.e.*, negative) selection in three genotypes. Sequence 04 yielded by far the highest dN/dS ratio among the vaccinated subjects when analyzed using SNAP (0.820) or codeml (1.12). Subject 04 is the only patient whose HIV load did not surpass 10^5 RNA copies × ml^{-1} after TI and who is still off therapy at present.

All 5′-*gag* and *vif* sequences were under neutral or negative selection. These types of selection are usually reported in HIV when dS and dN are determined for a complete gene (31, 32) and most likely obscure positive selection at specific sites (33). Thus, we focused next on detecting selection at specific codons. Comparing the seven aligned prevaccination genotypes with an alignment of seven postimmunization protein sequences, we identified the following signature amino acid positions in Nef with the prevailing genotype shifted: R8S, M10L, A23T, I114V, I153V, and Q170L. Analysis with the Selecton Server yielded positive (Darwinian) selection for all these amino acid sites. Furthermore, a signature deletion of codons 29 and 30 occurred. Five signature amino acid positions, also under positive selection according to Doron-Faigenboim *et al.* (29), were found in Vif, whereas none were detected in 5′-Gag.

Sequence variability in Nef is significantly reduced in vaccinated individuals

Assuming a common selection pressure driving the Nef sequences into a common direction, we studied genetic distances between the individual genotypes before and after vaccination. Viral loads were not significantly different at the two time points (log VL±SEM 4.36±0.15 *vs.* 4.74±0.17). Pairwise comparison of 7

individual genotypes resulted in 21 values at a time (**Table 2**), which were normally distributed according to the D'Agostino-Pearson test. The mean ± SE pairwise Nef protein sequence variabilities were 0.1527 ± 0.0041 before and 0.1249 ± 0.0042 after vaccination, respectively. This difference of 0.0278 is highly significant in the *t* test with two-tailed $P < 0.0001$ and a 95% confidence interval of 0.0160–0.0396. The variances were not significantly different (P=0.967), supporting the difference of the means in the *t* test. Pairwise sequence variability for Nef is shown in **Fig. 2*A*,** clearly demonstrating the decrease in variation between the two time points. At the nucleic acid level the results are in parallel with the respective values, decreasing from 0.1094 ± 0.0035 to 0.0895 ± 0.0023 (difference 0.0199; 95% confidence interval 0.0115–0.0283). The effect is even more obvious when deletions and insertions are also taken into account. On the other hand, protein sequence variation remained constant in Vif, used as an internal control (0.1216 ± 0.0042 *vs.* 0.1214 ± 0.0033; 95% confidence interval −0.0108 to 0.0108) (Fig. 2*B*). The 5′-Gag region also retained a practically constant variation (0.0798±0.0033 *vs.* 0.0829±0.0030; difference −0.0031; 95% confidence interval −0.0121 to 0.0060) (Fig. 2*C*). Of note, there is no overlap in the 95% confidence interval between Nef compared with Vif and 5′-Gag. Even though Nef did not accumulate significantly more mutations over time than Vif and 5′-Gag, the individual genotypes are directed to a higher genetic relatedness. In contrast, Nef sequences from control subjects retained a virtually constant protein sequence relatedness with the pairwise variability decreasing by only 0.0074 over 64 months.

To take intraindividual variation of viral subpopulations into account, we tested 8–11 clones each derived from pre- and postimmunization samples from 4 individuals. The respective variability was $6.21 \times 10^{-3} \pm 5.4 \times 10^{-4}$ and $12.6 \times 10^{-3} \pm 1.18 \times 10^{-3}$ for patient 04, $4.5 \times 10^{-3} \pm 0.62 \times 10^{-3}$ and $0.85 \times 10^{-3} \pm 0.28 \times 10^{-3}$ for patient 05, $18.9 \times 10^{-3} \pm 2.3 \times 10^{-3}$ and $5.0 \times 10^{-3} \pm 0.81 \times 10^{-3}$ for patient 07, and $36.5 \times 10^{-3} \pm 3.1 \times 10^{-3}$ and $6.0 \times 10^{-3} \pm 1.2 \times 10^{-3}$ for patient 11. The absolute values are more than 10× smaller than the corresponding interindividual data. Thus, the presence of different subpopulations does not account for the increased genetic relatedness between the individual isolates. However, the average intrapatient variation between the tested clones diminished significantly (P<0.0001) in patients 05, 07, and 11, corresponding to the interindividual data.

TABLE 2. *Pairwise protein sequence variability pre- (left bottom) and postvaccination (right top) with HIV MVA-nef*

Patient	02	03	04	05	06	07	11
02		0.1317	0.1317	0.152	0.1471	0.1415	0.1220
03	0.1171		0.1311	0.0829	0.1122	0.1408	0.0971
04	0.1512	0.1584		0.1268	0.1410	0.1408	0.1116
05	0.1520	0.1538	0.1707		0.1366	0.1415	0.1073
06	0.1373	0.1324	0.1320	0.1683		0.1202	0.0878
07	0.1469	0.1500	0.1408	0.1531	0.1346		0.1165
11	0.1610	0.1650	0.1408	0.1914	0.1538	0.1952	

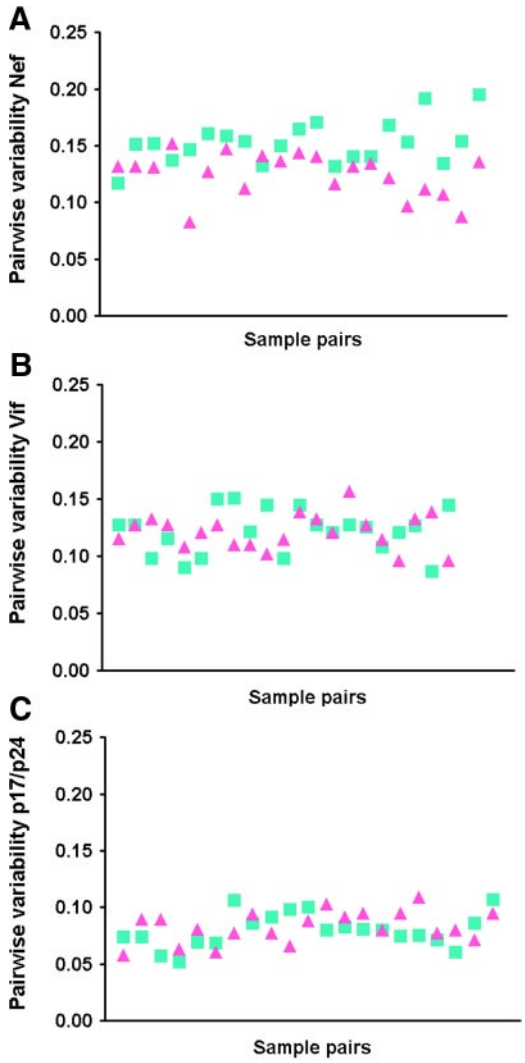

Figure 2. Protein sequence variability of Nef (*A*), Vif (*B*), and 5′-Gag (*C*) in 7 vaccinated persons. The *y* axis depicts the proportion of amino acid differences for the respective pairwise alignment (*x* axis). Evaluation of 7 individuals yielded 21 values, before (cyan squares) and after vaccination (magenta triangles), respectively. The graphs clearly show that Nef variation decreased from pre- to postvaccination time points in all but three sample pairs, whereas Vif and 5′Gag variability remains virtually constant.

In summary, we show that the sequence variability is significantly reduced in Nef of vaccinated individuals but remains virtually constant in the internal control proteins Vif and p17/p24, as well as in the control group.

Phylogenetic distance decreases in vaccinated patients

Our previous observations indicated that Nef vaccination selectively reduced the variability of Nef in the group of vaccinated individuals. Next, we studied the phylogenetic relationships between the viral isolates. Analysis of the postvaccination Nef sequences with the maximum likelihood algorithm resulted in the unrooted tree as shown in **Fig. 3*B***. The same tree archi-

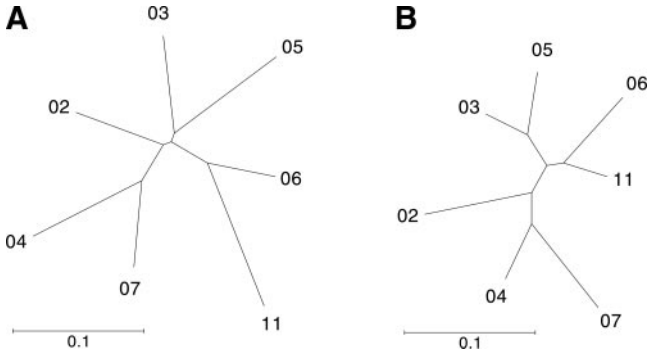

Figure 3. Phylogenetic analysis of Nef protein sequences before (*A*) and after (*B*) vaccination. Both trees were constructed with the same topology using the maximum likelihood algorithm. Branch lengths directly reflect genetic distances.

tecture was applied to prevaccination sequences (Fig. 3*A*) to compare branch lengths as a measure for genetic distances. Although they increased slightly in patients 02, 06, and 07, the remaining sequences showed substantially diminished branch lengths when comparing post- to prevaccination samples. This was most pronounced in strain 11, in agreement with the respective pairwise variation and T cell response.

T cell response and correlation to genetic variation

Immunogenicity data were available for 6 vaccinated individuals. Before immunization, all but one individual showed measurable CD8 T cell response to Nef (mean 0.26%, range 0.01–0.42%) and also to p17 and p24. During the three administrations of the vaccine, these values increased modestly to 0.35% (range 0.04–0.60%). The prevaccination CD4 immune response to Nef was minimal, with a mean of 0.01% (range 0–0.01%). However, administration of the MVA-*nef* vaccine stimulated the maximum CD4 Nef reactivity, reaching a mean of 0.08% (range 0.01–0.25%). No similar increase was observed for the CD4 T cell response directed to p17 and p24. During the TI study, we observed a strong boost of the CD8 T cell response directed to Nef (the mean of the frequency increased from 0.26 to 1.32%), whereas the responses specific to p17 and p24 increased only modestly. CD4 and CD8 responses were higher in the case samples compared to the controls, but not significantly different.

Of note, the proportions of both CD8 and CD4 Nef-positive T cells during TI correlated to the respective data during vaccination but not to the prevaccination values. Along these lines, there was a good correlation between diminishing pairwise variation and increasing CD8 (Pearson $r=0.685$; $P=0.135$) as well as CD4 ($r=0.707$, $P=0.116$) response during immunization. With only 6 data pairs available, the P values did not reach the widely used threshold value 0.05. Patient 11 had the highest T cell responses coupled with the most pronounced decrease in pairwise variation, while 02 exhibited the lowest respective data. The branch lengths presented in the preceding section support these findings very well.

Figure 4 illustrates the correlation between CD8 T cell responses and decreased variation for Nef (Fig. 4*A*) compared to virtually no correlation for p17/p24 (Fig. 4*B*).

Taken together, these data indicate a vaccine-specific CD8 and CD4 immune response that is boosted during TI and correlates to the diminished variability in the patients' autologous strains.

DISCUSSION

Although effective antiretroviral therapies with the potential to minimize HIV replication in infected persons are available, there is no cure for HIV infection in sight (34). A major drawback is that medical treatment must be administered for the duration of the patient's life, with resistant viral variants frequently arising (35). To increase the reactivity of the host immune system to HIV, the concept of therapeutic vaccination was introduced (36). The HIV accessory protein Nef is one of the candidates used in this scenario (37). Nef, a small (25–34 kDa) myristoylated protein, is expressed by all primate and simian lentiviruses (38). Its expression in early stages of the viral life cycle ensures T cell activation and the establishment of a persistent state of infection (39). Nef has a positive effect on viral infec-

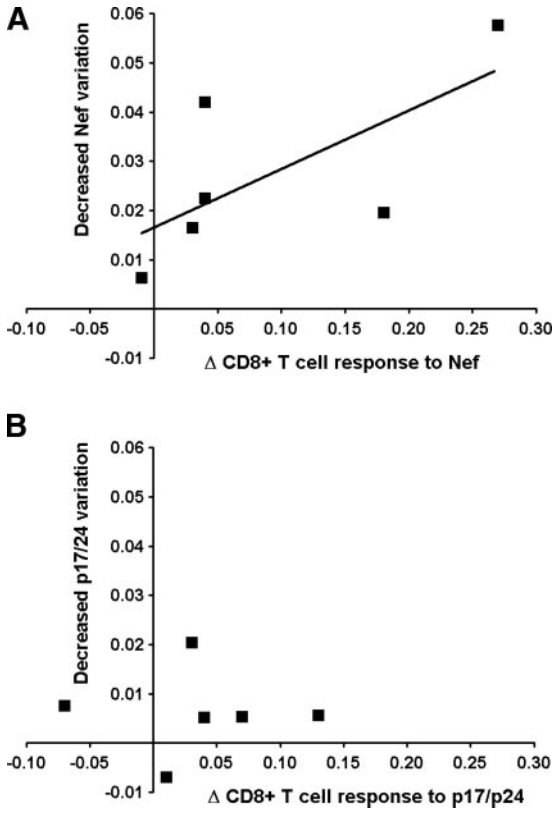

Figure 4. Correlation between CD8+ T cell responses and genetic variation for Nef (*A*) and p17/p24 (*B*). *x* axes: augmented proportion of reactive CD8+ T cells during vaccination; *y* axes: diminished pairwise protein sequence variability.

tion and promotes the survival of infected cells (40). Its role in HIV persistence is based largely on its ability to down-modulate the surface levels of important molecules at the immune synapse, such as major histocompatability complex (MHC) I and MHC II on antigen-presenting cells and target cells and CD4 and CD28 on helper T cells (41). Furthermore, Nef has been reported to alter the function of dendritic cells (42) and by that to dysregulate the CD8 response (43). A Nef-induced increase of monocyte migration has been linked to HIV-associated dementia (44, 45). Studies from transgenic mouse models suggest an important role in the pathogenicity of AIDS (46, 47), and a marked neurotoxicity has been reported for rats (48). As a major HIV-encoded determinant in its escape from the host immune surveillance (49), Nef represents also a promising target for anti-HIV approaches focusing on activation of antiviral immunity. In line with this hypothesis, Nef-compromised wild-type viruses have a reduced pathogenicity as seen in individuals with long-term nonprogressive disease (50, 51).

Reduction of Nef variation subsequent to vaccination

We were interested in genetic correlates of the immune response to an MVA-*nef* therapeutic vaccination. To this end, we analyzed HIV sequences from vaccinated and control individuals as available from a recent clinical trial conducted by us (16).

With all autologous HIV isolates in the vaccinated individuals subjected to similar selective pressure, we hypothesized that the respective Nef sequences might evolve in the same direction. Assuming that this would alter their genetic diversity, we analyzed the pairwise genetic variation before and after vaccination. We found that variability between pre- and postvaccination Nef sequences decreased significantly in the seven immunized patients. In parallel, the variation within intraindividual viral subpopulations diminished in 3 of 4 individuals.

We applied both internal and external controls to reliably link our results to the immunization with MVA-*nef*: Vif and 5′-Gag sequences in the vaccinated group as well as Nef genotypes in the nonvaccinated group yielded a virtually constant pairwise mean variation over the time period of ~5 yr. This argues against general selection processes in the course of chronic HIV infection accounting for the diminishing Nef variation. In total, by analyzing the entire *nef* (624 nucleotides) as well as part of *vif* (500 nucleotides) and *gag* (1050 nucleotides), we have characterized ~25% of the total HIV genome.

All newly emerged Nef signature mutations had been reported in a compilation of 1643 subtype B sequences (52). The total number of amino acid exchanges accumulating between pre- and postvaccination samples did not differ significantly between Nef, Vif, and 5′-Gag. Vif yielded five signature amino acid mutations, compared to eight in Nef. Thus, our main finding is not a generally higher rate of mutations induced in Nef but the fact of the lowered sequence diversity.

T cell activity is characterized as a major driving force for HIV evolution and is also linked to the limitation of viral diversity by positive selection (53, 54). In the present study, the increased Nef-specific CD4 T cell response during vaccination and the boost in Nef-specific CD8 response during TI in vaccinated patients correlated well with the observed decrease in genetic variability. With CD4 and CD8 immunity being closely interrelated, we hypothesize that both T cell populations were exercising the immune pressure in the context of Nef vaccination, leading to the observed effects on the Nef sequences.

Clinical implications

Ganeshan *et al.* (55) reported higher dN/dS ratios in *env* sequences among perinatally infected children for those with slow disease progression compared with those with higher viral loads and rapid disease progression. Positive selection apparently is a correlate of vigorous and clinically relevant immune responses in these patients.

The high mutation rate and genetic variability of HIV are major obstacles in terms of controlling viral replication both by the host's immune response and by ART and thus have immediate clinical implications. Genetic variation promotes resistance to ART and escape from the host's immune response. Although high sequence diversity can be expected to be advantageous for any virus in contest with the host's immune system, this is particularly true for HIV, with lifelong replication even under an optimal ART regimen. Because genotypes are archived even when not detected as circulating clones (56), a highly variable virus population has many more genotypes ready for changing environmental conditions. Phenotypic HIV *in vitro* replication capacity (fitness) has been correlated with viral genetic diversity of intraindividual subpopulations and with the stage of HIV infection (57). The data have been confirmed *in vivo*, where SIV strains resembling HIV isolates from late-stage infection showed greater pathogenicity than those corresponding to earlier and intermediate stages (58).

Accumulating data from *in vivo* human clinical studies also underscore the clinical implications of HIV diversity: Long-term nonprogressing patients have been shown to have lower interindividual variation in Nef and in the LTR region (59). Sagar *et al.* (60) reported higher viral loads and lower CD4 counts in women initially infected with multiple HIV genotypes. Along these lines, the presence of more than one genotype has been associated with disease progression (61). On one hand, genetic diversity promotes the emergence of highly adapted and thus fit genotypes. On the other hand, enhanced replication efficiency leads in turn to greater genetic variation. These two related features both influence disease progression and HIV transmission on the population level. One can envision that the risk of acquiring multiple HIV strains simultaneously or subsequently increases with the intra- and interindividual variation in the index population, respectively. *Vice versa*, multiple genotypes present in one individual can be transmitted to others, thereby increasing interindividual variation.

Variation in ART target genes has particular clinical implications with regard to promoting the emergence of resistance mutations. We envision a new approach derived from the results presented here: therapeutic vaccination with ART target genes with the goal of hampering the accumulation of resistance mutations in the patient's viral populations.

CONCLUSIONS

We present here a new approach of interpreting viral protein sequence data as an addition to focusing solely on described escape mutations. We found both inter- and intraindividual diminished Nef variation in patients immunized with MVA-*nef*. Correspondingly, dN/dS analysis of postvaccination n*ef* sequences indicated positive selection. Variability remained virtually constant in Vif and 5′-Gag sequences analyzed in parallel *vs.* the matched control groups, clearly attributing our results to the therapeutic vaccination.

Our findings suggest a general effect, applicable to other HIV proteins used as antigens for vaccinations or even to different viruses that cause chronic infections (*e.g.,* hepatitis C). We plan further studies to better characterize how sequence variability reacts to changing selection pressures, driven both by the host's immunologic response and by antiviral therapy. Advances in this field would be promising in view of the long-term goal of improving therapeutic options and ultimately the overall prognosis of persons living with HIV and other chronic viral infections. FJ

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