1 **No viral evolution in the lymph nodes of SIV-infected rhesus macaques during**
2 **combined antiretroviral therapy**

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20 Running title: No SIV evolution in lymph nodes during cART.

21

22   **Abstract**

23

24   To elucidate the mode of viral persistence in primate lentivirus-infected individuals during

25   combination antiretroviral therapy (cART), four simian immunodeficiency virus

26   (SIV)239-infected monkeys were treated with cART for 1 year. The viral *env* genes prepared

27   from total RNA extracted from the mesenteric lymph nodes by single genome amplification

28   were assessed at the completion of therapy. Analyses of nucleotide substitution and

29   phylogeny revealed no viral evolution during cART.

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31   Combination anti-retroviral therapy (cART) has transformed human

32 immunodeficiency virus (HIV) infection from incurable to a manageable disease. It

33 suppresses the viral burden in patients to undetectable levels (14, 16, 27), lowers the chance

34 of viral transmission (28), increases the number of $CD4^+$ T lymphocytes (14, 16),

35 reconstitutes immunity (4, 24, 38), and extends the life expectancy of patients (1). However,

36 cART does not cure patients due to its inability to eradicate the virus from infected

37 individuals (7), suggesting the existence of a viral reservoir that is refractory to cART. Its

38 identification and eradication are, therefore, requisites for a functional cure for acquired

39 immunodeficiency syndrome (AIDS). To establish a strategy for eradication of the HIV

40 reservoir, the mechanism of persistence of the virus must be elucidated. Two mechanisms of

41 viral persistence have been proposed: one is ongoing cycles of viral replication despite the

42 presence of antivirals (37), and the other is provirus integration into long-lived cells (32).

43 Whereas preceding studies concerning this issue have been extensively conducted employing

44 clinical specimens from HIV-1-infected patients, including plasma, peripheral blood

45 mononuclear cells, and gut-associated lymphatic tissues (2, 11, 23), lymph nodes, which are

46 epicenters of virus replication in infected individuals not undergoing therapy (10, 25, 26),

47 have been subjected to scrutiny only rarely. In animal models of cART, in particular the

48 simian immunodeficiency virus (SIV)/macaque model, which allows for systemic

49 examination, the identification of the viral reservoir and the mechanism of viral holding have

50 not been studied in detail.

51   To elucidate how the virus is maintained during cART in an animal model of

52 anti-HIV chemotherapy, we administered a combination of nucleotide/nucleoside reverse

53 transcriptase inhibitors (azidothymidine [AZT], lamivudine [3TC], and tenofovir disoproxil

54   fumarate [TDF]) and protease inhibitors (lopinavir [LPV] with ritonavir [RTV]) to four

55   SIV239-infected rhesus macaques for 1 year (18). Although the plasma viral RNA loads of

56   the animals were suppressed to below the assay detection limit during the period of

57   chemotherapy, systemic analysis conducted at the completion of the therapy revealed viral

58   RNA present in lymphatic tissues, especially in mesenteric and splenic lymph nodes (MLN

59   and SLN, respectively) at high titers. Reasoning that any possible mode(s) of viral

60   persistence should be in operation in tissues with high levels of viral RNA expression, we

61   investigated viral genes in these tissues.

62   It is expected that viral genes accumulate nucleotide substitutions in proportion to

63   time post-infection in individuals not undergoing therapy due to continuous virus replication

64   mediated by the error-prone viral reverse transcriptase. Such mutation rates have indeed been

65   observed in the V3 loop of *env*, p17 of *gag* (20), and C2-C5 region of *env* (30) in HIV-1

66   infected patients as well as in the *env* gene from monkeys experimentally infected with SIV

67   (5, 19). We hypothesized that viral genes would accumulate mutations if the virus was

68   continuously replicating in the reservoir despite the presence of antivirals.

69   First, to ascertain whether such accumulation of mutations took place to a

70   detectable magnitude in our experimental system, SIV239, a molecularly cloned virus, was

71   used to infect macaques for 1 year, and we periodically sampled viral genes from the

72   untreated control animal (MM521). To reveal ongoing expression of viral genes at sampling,

73   total RNA was extracted from plasma samples collected at 8, 18, 42, and 68 weeks

74   post-infection (wpi) and examined. Single genome amplification (SGA) (29) was used to

75   amplify present viral genes and to avoid selective amplification of a particular genotype or

76   recombination between genotypes during polymerase chain reaction (PCR). Using nested

77 PCR, we amplified the entire *env* gene, which accumulates nucleotide substitutions in the

78 greatest numbers, following reverse transcription of cDNA from the extracted RNA. The

79 initial cycles of PCR were carried out utilizing the following primers: forward, SIV20F

80 (5'-ctc cag gac tag cat aaa tgg-3'); reverse, SHenv9R (5'-ggg tat cta aca tat gcc tc-3').

81 Successive PCR cycles were run with the following primers: forward, SIV21F (5'-ctc tct cag

82 cta tac cgc cc-3'); reverse, SHenv8R (5'-gcc ttc ttc ctt ttc taa g-3'). The PCR products from

83 an average of 12 independent reactions per time point were directly subjected to sequencing.

84 We computed the number of mutations in each SGA clone obtained from plasma

85 samples of an untreated monkey (MM521) through a comparison with that of the inoculum

86 virus (Fig. 1). A linear relationship with a coefficient of 1.25×10^{-4} ($r^2 = 0.8503$, $p < 0.0001$;

87 GraphPad Prism, La Jolla, CA, USA) was revealed between the number of mutations in the

88 SGA clones and the time post-infection. Using the coefficient, the cumulative mutations per

89 annum was determined to be 6.5×10^{-3} substitutions/site/year, a comparable figure to those of

90 SIV and HIV reported previously (9×10^{-3} (5, 19) and 6.0×10^{-3} (29) substitutions/site/year,

91 respectively). The accuracy of the "molecular clock" in our experimental setting prompted us

92 to examine viral RNA extracted from the lymph nodes of animals that underwent cART for 1

93 year.

94 Total RNA was extracted from the MLN of four treated animals and one untreated

95 animal as well as the SLN of one of the treated animals (MM530) at the completion of the

96 observation period and used as template for PCR; the products were subjected to sequence

97 analysis as described above. On average, 10 sequences were obtained from each sample (Fig.

98 2A and Table). The number of mutations observed in the *env* gene from MM521 (untreated)

99 was, on average, 25 of 2700 bases. In contrast, the number in treated animals was on average

5

100   1.5 of 2700 bases (Table). The difference in the number of mutations in *env* between the

101   plasma and MLN samples at 68 wpi (at necropsy) in the untreated animal, MM521, was

102   statistically insignificant ($p > 0.05$; Fig. 2A), justifying our comparison of these two distinct

103   anatomical compartments. Thus, we proceeded to compare the substitution numbers in the

104   plasma at 8 wpi, immediately before the onset of cART, with those from the lymph nodes of

105   animals treated with cART at necropsy (61-65 wpi). Nucleotide substitutions in the *env* gene

106   in both plasma and the MLN of the untreated animal (MM521) at 68 wpi increased compared

107   to that in plasma at 8 wpi ($p < 0.0001$). In contrast, those in the MLN of treated animals at the

108   completion of cART were unchanged (MM528 and SLN of MM530) or decreased

109   significantly (MM491, MM499, and MLN of MM530) (Fig. 2A). The results indicated that

110   virus did not accumulate further mutations beyond those obtained by 8 wpi.

111   As the samples were collected from animals at various time points post-infection,

112   the numbers depicted in Fig. 2A were converted to substitutions/site/year (Fig. 2B) for

113   further analysis. Comparison of the number of viral mutations in plasma at 8 wpi (median,

114   5.9×10^{-3} substitutions/site/year) with that in the MLN (median, 7.2×10^{-3}

115   substitutions/site/year) in the untreated animal, MM521, indicated no statistical difference (p

116    $= 0.6265$), as predicted by the analysis in Fig. 2A. Next, we compared the numbers in

117   animals that underwent chemotherapy. At 8 wpi, the treated animals were equivalent to

118   MM521 (an untreated animal) in terms of therapeutic status, since cART was started after

119   sample collection at 8 wpi. Not unexpectedly, there was no statistically significant difference

120   in plasma number between the untreated and treated animals (MM491, 8.5×10^{-3}; MM499,

121   9.8×10^{-3}; MM528, 1.6×10^{-2}; MM530, 8.5×10^{-3}; and MM521, 5.9×10^{-3} substitutions/site/year),

122   except for MM528 ($p = 0.0048$ compared to those from untreated animal). In contrast, the

123   mutations per annum in the lymph nodes of treated animals collected at necropsy (median,

124   3.4×10^{-4} substitutions/site/year) were significantly lower than those in the plasma of the

125   animals at 8 wpi (median, 9.8×10^{-3} substitutions/site/year) ($p < 0.0001$). The number of

126   mutations per year in lymph nodes between untreated and treated macaques was also

127   significantly different ($p < 0.0001$). This supports the hypothesis that ongoing viral

128   replication contributed little, if anything, to viral persistence during cART.

129   Examination of the nucleotide substitution numbers did not indicate discernible *de*

130   *novo* virus replication during cART. Therefore, we next investigated continuous viral

131   replication during cART through phylogenetic analysis of viral *env* clones. Clones were

132   obtained from the untreated animal (derived from plasma at 8, 18, 42, and 68 wpi and from

133   MLN) and from one of the treated animals (derived from plasma at 8 wpi and MLN at

134   necropsy) (Figs. 3 and S). To illustrate the accumulation and specific sites of mutations,

135   Highlighter plot analysis (<www.hiv.lanl>) was also performed. Phylogenetic analysis of the

136   viral genes from the untreated animal revealed that i) *env* clones from plasma exhibited

137   increasing genetic distance from the inoculum virus with time, ii) clones obtained at a given

138   time point branched out of the one immediately before, a clear demonstration of viral

139   evolution, and iii) clones from the lymph node formed a cluster with those from plasma

140   collected at the same time. In contrast, clones from treated animals, regardless of the tissue

141   origin or time point, formed a cluster with clones derived from plasma of the untreated

142   animal at 8 wpi and the inoculum virus (Figs. 3 and S). The results of the Highlighter plot

143   analysis were consistent with those of the phylogenetic analysis. These results clearly

144   demonstrated that viral evolution did not take place in SIV239-infected rhesus macaques

145   during cART. Analysis of the *env* genes in the peripheral blood mononuclear cells and

146   gut-associated lymphatic tissues obtained from HIV-1 patients in cART also found no

147   evidence of *de novo* viral replication (11).

148   In contrast, other studies have reported continuous virus replication during

149   combined chemotherapy (2, 37). One possible explanation of this discrepancy is the thorough

150   suppression of the plasma viral burden, < 20 copies/mL at necropsy that was achieved in this

151   study (18). *De novo* virus replication was detected in HIV-1 patients whose plasma viral

152   RNA burdens ranged from 20 to 400 copies/mL, but not in those with less than 20 copies/mL

153   (15). Our findings also indicate that the cART regimen we employed (18) was robust enough

154   to halt viral evolution nearly completely in animals.

155   Our sample size, an average of 10 sequences from each specimen, conceivably

156   limited our ability to detect minor populations with signs of ongoing replication. An analysis

157   of four animals, however, did not reveal the genotypes detailed in the current study.

158   Therefore, while our results cannot rule out possible *de novo* viral replication during cART,

159   the data indicate that it is not a major mode of viral persistence in individuals whose virus

160   replication levels are thoroughly suppressed by cART.

161   The locations of other potential viral reservoirs, in addition to resting CD4^{+} T

162   lymphocytes, an already established HIV/SIV reservoir found to be present in blood (6, 8, 9,

163   13), lymph nodes (6), and the spleen (31), remains elusive. While the cART regimen we

164   developed suppressed viral RNA levels nearly completely in the circulation and fairly well in

165   effector sites, such as the gastrointestinal tract and lung, viral RNA expression levels in

166   lymph nodes were not contained effectively (18), suggesting that the viral reservoir consists

167   of cells present in lymph nodes. We also detected CD3-positive cells, most likely CD4^{+} T

168   lymphocytes, expressing Nef protein in the follicles of the MLN of an SIV-infected animal

169   that exhibited viral rebound upon cessation of cART (18). Based on their location, these

170   might be Tfh cells, which are of the memory phenotype (21, 22, 36). The results of the

171   current study have further narrowed the location of the viral reservoir from our previous

172   study (18) to cells with longer half-lives, which retain provirus for at least 1 year. Since

173   resting CD4$^+$ T cells possess long half-lives (33), these cells satisfy this criterion for a viral

174   reservoir during cART. It is conceivable that resting CD4$^+$ T cells functioned as the

175   predominant viral reservoir in the SIV239/rhesus macaque model for patients on cART

176   employed in our study, as in preceding studies concerning the issue in the context of HIV and

177   SIV infections.

178   Lymph nodes serve as a major HIV reservoir throughout the course of infection

179   without intervention by cART (10, 25, 26). During clinical latency, the virus persists as an

180   intact provirus, which can produce infectious viral particles upon cell activation, in a

181   miniscule fraction of the resting CD4$^+$ T lymphocytes in lymph nodes (6). An extensive

182   examination of lymph node specimens from HIV patients on cART revealed an infinitesimal

183   amount of viral RNA-positive cells by *in situ* hybridization (17). Hockett *et al.* (17) revealed

184   that cART lowers the number of viral RNA-positive cells in lymph nodes but that the number

185   of viral copies in each infected cell is constant, regardless of the viral burden in the

186   circulation, suggesting the existence of virus-infected cells actively transcribing viral genes

187   under cART, as we found previously in the lymph nodes of SIV239-infected animals on

188   cART (18). Taking our current observations together with those of Hockett *et al.* (17), the

189   viral RNA-positive cells present in lymph nodes during cART may represent cells infected

190   with virus prior to the initiation of cART and transcribing viral RNA from integrated provirus

191   during therapy.

192   Current cART is unable to eradicate the viral reservoir; or more precisely, provirus

193   integrated in the reservoir. Based on our results, it is important to establish strategies to target

194   specifically long-lived cells that harbor intact provirus while unlocking the dormant state of

195   the provirus, perhaps using histone deacetylase (3), to achieve a functional cure for AIDS.

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References

210   1.   2008. Life expectancy of individuals on combination antiretroviral therapy in high-income countries: a collaborative analysis of 14 cohort studies. Lancet **372:**293-299.

213   2.   **Anderson, J. A., N. M. Archin, W. Ince, D. Parker, A. Wiegand, J. M. Coffin, J. Kuruc, J. Eron, R. Swanstrom, and D. M. Margolis.** 2011. Clonal sequences recovered from plasma from patients with residual HIV-1 viremia and on intensified antiretroviral therapy are identical to replicating viral RNAs recovered from circulating resting CD4+ T cells. Journal of virology **85:**5220-5223.

218   3.   **Archin, N. M., A. L. Liberty, A. D. Kashuba, S. K. Choudhary, J. D. Kuruc, A. M. Crooks, D. C. Parker, E. M. Anderson, M. F. Kearney, M. C. Strain, D. D. Richman, M. G. Hudgens, R. J. Bosch, J. M. Coffin, J. J. Eron, D. J. Hazuda, and D. M. Margolis.** 2012. Administration of vorinostat disrupts HIV-1 latency in patients on antiretroviral therapy. Nature **487:**482-485.

223   4.   **Autran, B., G. Carcelain, T. S. Li, C. Blanc, D. Mathez, R. Tubiana, C. Katlama, P. Debre, and J. Leibowitch.** 1997. Positive effects of combined antiretroviral therapy on CD4+ T cell homeostasis and function in advanced HIV disease. Science **277:**112-116.

227   5.   **Burns, D. P., and R. C. Desrosiers.** 1991. Selection of genetic variants of simian immunodeficiency virus in persistently infected rhesus monkeys. Journal of virology **65:**1843-1854.

230   6.   **Chun, T. W., L. Carruth, D. Finzi, X. Shen, J. A. DiGiuseppe, H. Taylor, M. Hermankova, K. Chadwick, J. Margolick, T. C. Quinn, Y. H. Kuo, R. Brookmeyer, M. A. Zeiger, P. Barditch-Crovo, and R. F. Siliciano.** 1997. Quantification of latent tissue reservoirs and total body viral load in HIV-1 infection. Nature **387:**183-188.

235   7.   **Chun, T. W., R. T. Davey, Jr., D. Engel, H. C. Lane, and A. S. Fauci.** 1999. Re-emergence of HIV after stopping therapy. Nature **401:**874-875.

237   8.   **Chun, T. W., D. Finzi, J. Margolick, K. Chadwick, D. Schwartz, and R. F. Siliciano.** 1995. In vivo fate of HIV-1-infected T cells: quantitative analysis of the transition to stable latency. Nature medicine **1:**1284-1290.

240   9.   **Chun, T. W., L. Stuyver, S. B. Mizell, L. A. Ehler, J. A. Mican, M. Baseler, A. L. Lloyd, M. A. Nowak, and A. S. Fauci.** 1997. Presence of an inducible HIV-1 latent reservoir during highly active antiretroviral therapy. Proceedings of the National

243   Academy of Sciences of the United States of America **94:**13193-13197.

244   10. **Embretson, J., M. Zupancic, J. L. Ribas, A. Burke, P. Racz, K. Tenner-Racz,**
245   **and A. T. Haase.** 1993. Massive covert infection of helper T lymphocytes and
246   macrophages by HIV during the incubation period of AIDS. Nature **362:**359-362.

247   11. **Evering, T. H., S. Mehandru, P. Racz, K. Tenner-Racz, M. A. Poles, A. Figueroa,**
248   **H. Mohri, and M. Markowitz.** 2012. Absence of HIV-1 evolution in the
249   gut-associated lymphoid tissue from patients on combination antiviral therapy
250   initiated during primary infection. PLoS pathogens **8:**e1002506.

251   12. **Felsenstein, J.** 1981. Evolutionary trees from DNA sequences: a maximum
252   likelihood approach. Journal of molecular evolution **17:**368-376.

253   13. **Finzi, D., M. Hermankova, T. Pierson, L. M. Carruth, C. Buck, R. E. Chaisson,**
254   **T. C. Quinn, K. Chadwick, J. Margolick, R. Brookmeyer, J. Gallant, M.**
255   **Markowitz, D. D. Ho, D. D. Richman, and R. F. Siliciano.** 1997. Identification of a
256   reservoir for HIV-1 in patients on highly active antiretroviral therapy. Science
257   **278:**1295-1300.

258   14. **Gulick, R. M., J. W. Mellors, D. Havlir, J. J. Eron, C. Gonzalez, D. McMahon, D.**
259   **D. Richman, F. T. Valentine, L. Jonas, A. Meibohm, E. A. Emini, and J. A.**
260   **Chodakewitz.** 1997. Treatment with indinavir, zidovudine, and lamivudine in adults
261   with human immunodeficiency virus infection and prior antiretroviral therapy. The
262   New England journal of medicine **337:**734-739.

263   15. **Gunthard, H. F., J. K. Wong, C. C. Ignacio, J. C. Guatelli, N. L. Riggs, D. V.**
264   **Havlir, and D. D. Richman.** 1998. Human immunodeficiency virus replication and
265   genotypic resistance in blood and lymph nodes after a year of potent antiretroviral
266   therapy. Journal of virology **72:**2422-2428.

267   16. **Hammer, S. M., K. E. Squires, M. D. Hughes, J. M. Grimes, L. M. Demeter, J. S.**
268   **Currier, J. J. Eron, Jr., J. E. Feinberg, H. H. Balfour, Jr., L. R. Deyton, J. A.**
269   **Chodakewitz, and M. A. Fischl.** 1997. A controlled trial of two nucleoside
270   analogues plus indinavir in persons with human immunodeficiency virus infection
271   and CD4 cell counts of 200 per cubic millimeter or less. AIDS Clinical Trials Group
272   320 Study Team. The New England journal of medicine **337:**725-733.

273   17. **Hockett, R. D., J. M. Kilby, C. A. Derdeyn, M. S. Saag, M. Sillers, K. Squires, S.**
274   **Chiz, M. A. Nowak, G. M. Shaw, and R. P. Bucy.** 1999. Constant mean viral copy
275   number per infected cell in tissues regardless of high, low, or undetectable plasma
276   HIV RNA. The Journal of experimental medicine **189:**1545-1554.

277   18. **Horiike, M., S. Iwami, M. Kodama, A. Sato, Y. Watanabe, M. Yasui, Y. Ishida, T.**

278   **Kobayashi, T. Miura, and T. Igarashi.** 2012. Lymph nodes harbor viral reservoirs
279   that cause rebound of plasma viremia in SIV-infected macaques upon cessation of
280   combined antiretroviral therapy. Virology **423:**107-118.

281 19. **Johnson, P. R., T. E. Hamm, S. Goldstein, S. Kitov, and V. M. Hirsch.** 1991. The
282   genetic fate of molecularly cloned simian immunodeficiency virus in experimentally
283   infected macaques. Virology **185:**217-228.

284 20. **Leitner, T., and J. Albert.** 1999. The molecular clock of HIV-1 unveiled through
285   analysis of a known transmission history. Proceedings of the National Academy of
286   Sciences of the United States of America **96:**10752-10757.

287 21. **Lindqvist, M., J. van Lunzen, D. Z. Soghoian, B. D. Kuhl, S. Ranasinghe, G.**
288   **Kranias, M. D. Flanders, S. Cutler, N. Yudanin, M. I. Muller, I. Davis, D. Farber,**
289   **P. Hartjen, F. Haag, G. Alter, J. Schulze zur Wiesch, and H. Streeck.** 2012.
290   Expansion of HIV-specific T follicular helper cells in chronic HIV infection. The
291   Journal of clinical investigation **122:**3271-3280.

292 22. **Luthje, K., A. Kallies, Y. Shimohakamada, T. B. GT, A. Light, D. M. Tarlinton,**
293   **and S. L. Nutt.** 2012. The development and fate of follicular helper T cells defined
294   by an IL-21 reporter mouse. Nature immunology **13:**491-498.

295 23. **Martinez, M. A., M. Cabana, A. Ibanez, B. Clotet, A. Arno, and L. Ruiz.** 1999.
296   Human immunodeficiency virus type 1 genetic evolution in patients with prolonged
297   suppression of plasma viremia. Virology **256:**180-187.

298 24. **Pakker, N. G., D. W. Notermans, R. J. de Boer, M. T. Roos, F. de Wolf, A. Hill, J.**
299   **M. Leonard, S. A. Danner, F. Miedema, and P. T. Schellekens.** 1998. Biphasic
300   kinetics of peripheral blood T cells after triple combination therapy in HIV-1
301   infection: a composite of redistribution and proliferation. Nature medicine **4:**208-214.

302 25. **Pantaleo, G., C. Graziosi, L. Butini, P. A. Pizzo, S. M. Schnittman, D. P. Kotler,**
303   **and A. S. Fauci.** 1991. Lymphoid organs function as major reservoirs for human
304   immunodeficiency virus. Proceedings of the National Academy of Sciences of the
305   United States of America **88:**9838-9842.

306 26. **Pantaleo, G., C. Graziosi, J. F. Demarest, L. Butini, M. Montroni, C. H. Fox, J.**
307   **M. Orenstein, D. P. Kotler, and A. S. Fauci.** 1993. HIV infection is active and
308   progressive in lymphoid tissue during the clinically latent stage of disease. Nature
309   **362:**355-358.

310 27. **Perelson, A. S., P. Essunger, Y. Cao, M. Vesanen, A. Hurley, K. Saksela, M.**
311   **Markowitz, and D. D. Ho.** 1997. Decay characteristics of HIV-1-infected
312   compartments during combination therapy. Nature **387:**188-191.

313 28. **Quinn, T. C., M. J. Wawer, N. Sewankambo, D. Serwadda, C. Li, F.**
314   **Wabwire-Mangen, M. O. Meehan, T. Lutalo, and R. H. Gray.** 2000. Viral load
315   and heterosexual transmission of human immunodeficiency virus type 1. Rakai
316   Project Study Group. The New England journal of medicine **342:**921-929.

317 29. **Salazar-Gonzalez, J. F., E. Bailes, K. T. Pham, M. G. Salazar, M. B. Guffey, B. F.**
318   **Keele, C. A. Derdeyn, P. Farmer, E. Hunter, S. Allen, O. Manigart, J. Mulenga,**
319   **J. A. Anderson, R. Swanstrom, B. F. Haynes, G. S. Athreya, B. T. Korber, P. M.**
320   **Sharp, G. M. Shaw, and B. H. Hahn.** 2008. Deciphering human immunodeficiency
321   virus type 1 transmission and early envelope diversification by single-genome
322   amplification and sequencing. Journal of virology **82:**3952-3970.

323 30. **Shankarappa, R., J. B. Margolick, S. J. Gange, A. G. Rodrigo, D. Upchurch, H.**
324   **Farzadegan, P. Gupta, C. R. Rinaldo, G. H. Learn, X. He, X. L. Huang, and J. I.**
325   **Mullins.** 1999. Consistent viral evolutionary changes associated with the progression
326   of human immunodeficiency virus type 1 infection. Journal of virology
327   **73:**10489-10502.

328 31. **Shen, A., M. C. Zink, J. L. Mankowski, K. Chadwick, J. B. Margolick, L. M.**
329   **Carruth, M. Li, J. E. Clements, and R. F. Siliciano.** 2003. Resting CD4+ T
330   lymphocytes but not thymocytes provide a latent viral reservoir in a simian
331   immunodeficiency virus-Macaca nemestrina model of human immunodeficiency
332   virus type 1-infected patients on highly active antiretroviral therapy. Journal of
333   virology **77:**4938-4949.

334 32. **Shen, L., and R. F. Siliciano.** 2008. Viral reservoirs, residual viremia, and the
335   potential of highly active antiretroviral therapy to eradicate HIV infection. The
336   Journal of allergy and clinical immunology **122:**22-28.

337 33. **Siliciano, J. D., J. Kajdas, D. Finzi, T. C. Quinn, K. Chadwick, J. B. Margolick,**
338   **C. Kovacs, S. J. Gange, and R. F. Siliciano.** 2003. Long-term follow-up studies
339   confirm the stability of the latent reservoir for HIV-1 in resting CD4+ T cells. Nature
340   medicine **9:**727-728.

341 34. **Tamura, K., and M. Nei.** 1993. Estimation of the number of nucleotide substitutions
342   in the control region of mitochondrial DNA in humans and chimpanzees. Molecular
343   biology and evolution **10:**512-526.

344 35. **Tamura, K., D. Peterson, N. Peterson, G. Stecher, M. Nei, and S. Kumar.** 2011.
345   MEGA5: molecular evolutionary genetics analysis using maximum likelihood,
346   evolutionary distance, and maximum parsimony methods. Molecular biology and
347   evolution **28:**2731-2739.

348   36.   **Weber, J. P., F. Fuhrmann, and A. Hutloff.** 2012. T-follicular helper cells survive
349   as long-term memory cells. European journal of immunology **42:**1981-1988.

350   37.   **Wong, J. K., M. Hezareh, H. F. Gunthard, D. V. Havlir, C. C. Ignacio, C. A.**
351   **Spina, and D. D. Richman.** 1997. Recovery of replication-competent HIV despite
352   prolonged suppression of plasma viremia. Science **278:**1291-1295.

353   38.   **Zhang, Z. Q., D. W. Notermans, G. Sedgewick, W. Cavert, S. Wietgrefe, M.**
354   **Zupancic, K. Gebhard, K. Henry, L. Boies, Z. Chen, M. Jenkins, R. Mills, H.**
355   **McDade, C. Goodwin, C. M. Schuwirth, S. A. Danner, and A. T. Haase.** 1998.
356   Kinetics of CD4+ T cell repopulation of lymphoid tissues after treatment of HIV-1
357   infection. Proceedings of the National Academy of Sciences of the United States of
358   America **95:**1154-1159.

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Table. Origins and numbers of *env* clones.

ANIMAL ID	cART*	SPECIMEN	SAMPLE COLLECTION (WPI)	No. SGA** CLONES	No. NUCLEOTIDE SUBSTITUTIONS	
					MINIMUM/MAXIMUM	MEAN ± SD
MM521	UNTREATED	PLASMA	8	10	1 / 8	3.3 ± 2.2
		PLASMA	68	10	20 / 29	24.9 ± 3.2
		MLN[#]	68	12	20 / 33	25.0 ± 3.4
MM491	TREATED	PLASMA	8	10	1 / 7	3.5 ± 1.8
		MLN[#]	63	10	0 / 2	0.6 ± 0.8
MM499	TREATED	PLASMA	8	11	2 / 7	4.2 ± 1.7
		MLN[#]	64	10	0 / 4	0.7 ± 1.3
MM528	TREATED	PLASMA	8	10	4 / 11	6.6 ± 2.4
		MLN[#]	61	11	0 / 3 (12[†])	1.7 ± 1.1[††]
MM530	TREATED	PLASMA	8	10	2 / 8	4.0 ± 1.7
		MLN[#]	65	10	0 / 4	2.1 ± 1.2
		SLN[##]	65	10	0 / 6	2.4 ± 1.9

*Combination anti-retroviral therapy; **single genome amplification; [#]mesenteric lymph node; [##]splenic lymph node; [†]interpreted as a hypermutant driven by APOBEC3G/F (Hypermut 2.0, <www.hiv.lanl>); [††]computed excluding the clone with hypermutation.
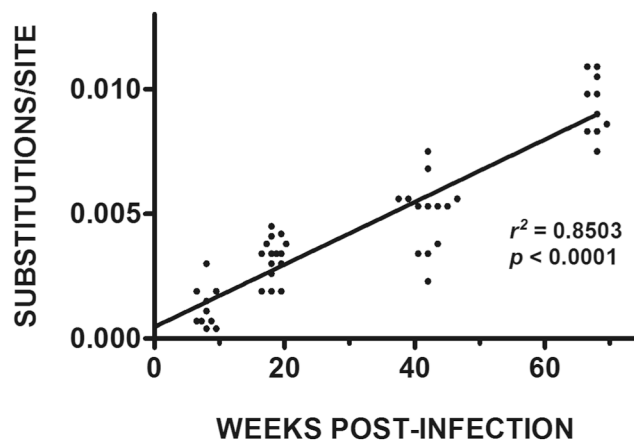
Fig. 1.

Time-dependent accumulation of nucleotide substitutions in SIV genomes circulating in an infected and untreated rhesus macaque. The sequences of viral *env* genes in circulation collected at 8, 18, 42, and 68 wpi from SIV239-infected animals and an untreated animal (MM521) were determined. Tamura-Nei distances (34) of the sequences were computed with the MEGA5 software (35), and the number of nucleotide substitutions per site were plotted against the number of wpi. Each symbol represents a single genomic amplicon derived from plasma samples collected at the time points designated above.
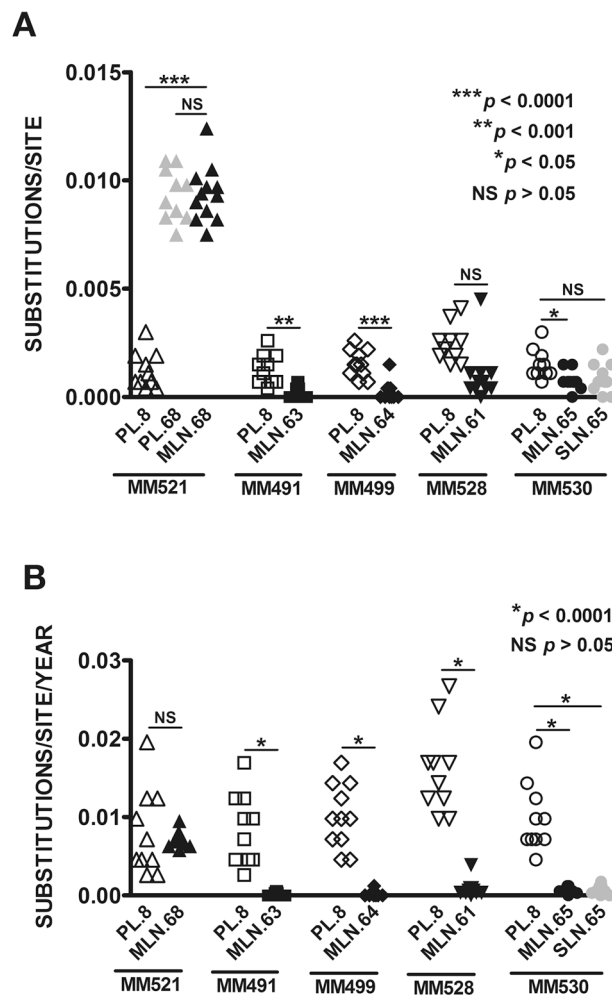
Fig. 2.

Nucleotide substitutions in *env* genes from SIV239-infected animals. The number of mutations in *env* from the plasma (PL, at 8 and 68 wpi) and mesenteric lymph node (MLN, at 68 wpi) of an SIV-infected but untreated animal (MM521) and from the plasma (at 8 wpi) and MLN (at necropsy, 61, 63, 64, or 65 wpi) plus splenic lymph node (SLN, at necropsy, 65 wpi) from SIV-infected and treated monkeys (MM491, MM499, MM528, and MM530) were assessed as described in the legend to Fig. 1. (A) Nucleotide substitutions per site are shown. The statistical significance of differences between substitution numbers was evaluated by Student's *t*-test using GraphPad Prism. *$p < 0.05$; **$p < 0.001$; ***$p < 0.0001$; NS, $p > 0.05$. (B) Nucleotide substitutions per annum are shown. *$p < 0.001$; **$p > 0.05$.
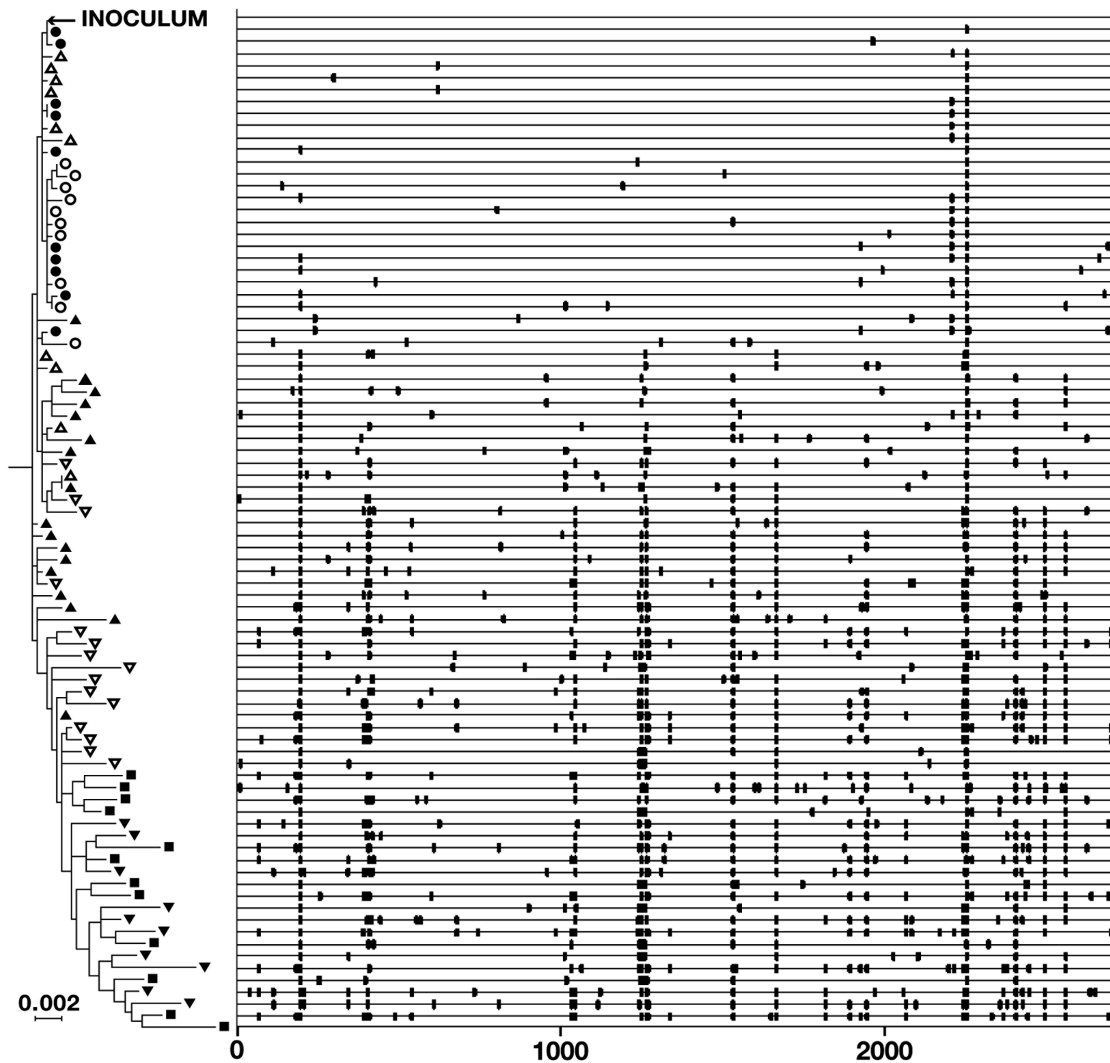
Fig. 3.

       Phylogenetic relationship of *env* sequences from treated (MM530) and untreated (MM521) SIV-infected animals. Sequences of the entire *env* gene from both animals were subjected to phylogenetic analysis. Phylogenetic trees were constructed using the Maximum Likelihood method (12). Open circles, sequences in the plasma of MM530 at 8 wpi; closed circles, those from the MLN of MM530 at 65 wpi; open triangles, those from plasma of MM521 at 8 wpi; closed triangles, those from plasma of MM521 at 18 wpi; open inverse triangles, those from plasma of MM521 at 42 wpi; closed inverse triangles, those from plasma of MM521 at 68 wpi; closed rectangles, those from MLN of MM521 at 68 wpi. The scale represents a genetic distance equivalent to 0.002 substitutions per site. The corresponding sequence of SIVmac251 32H (GenBank accession no. D01065) was applied as an outgroup.
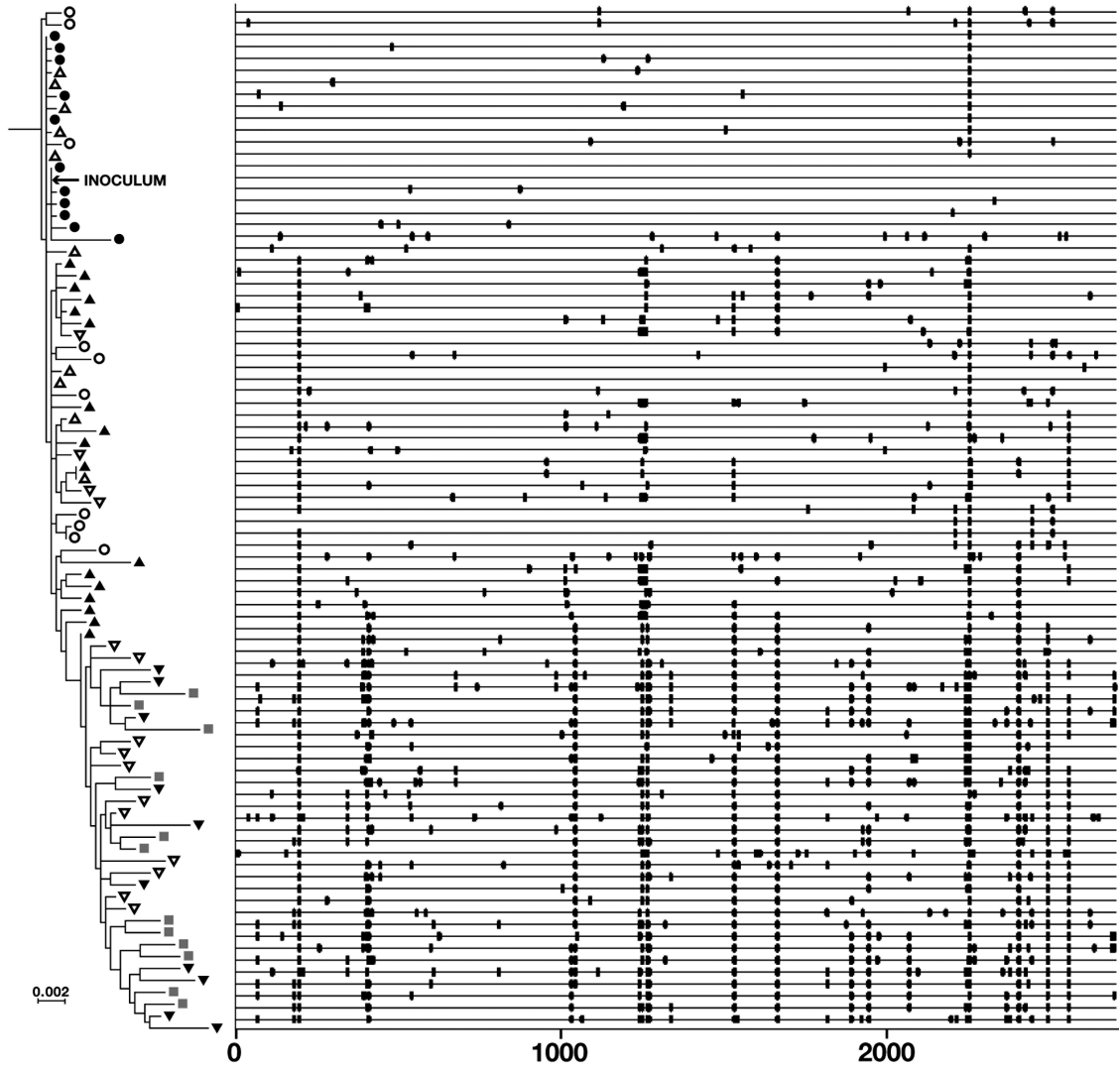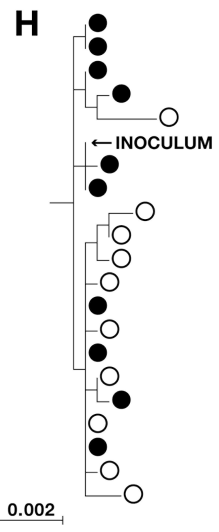
A

INOCULUM

0.002

0   1000   2000

B

INOCULUM

0.002

0   1000   2000

C

INOCULUM
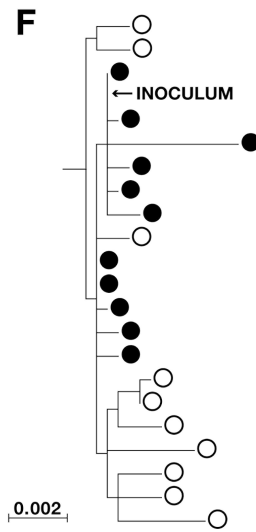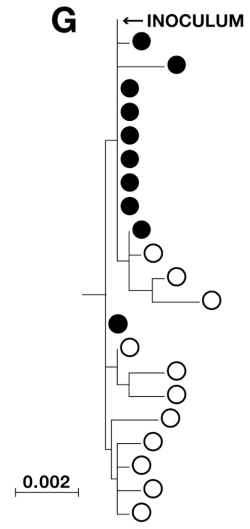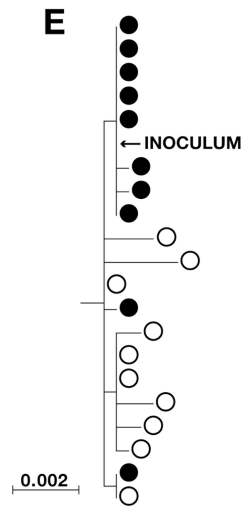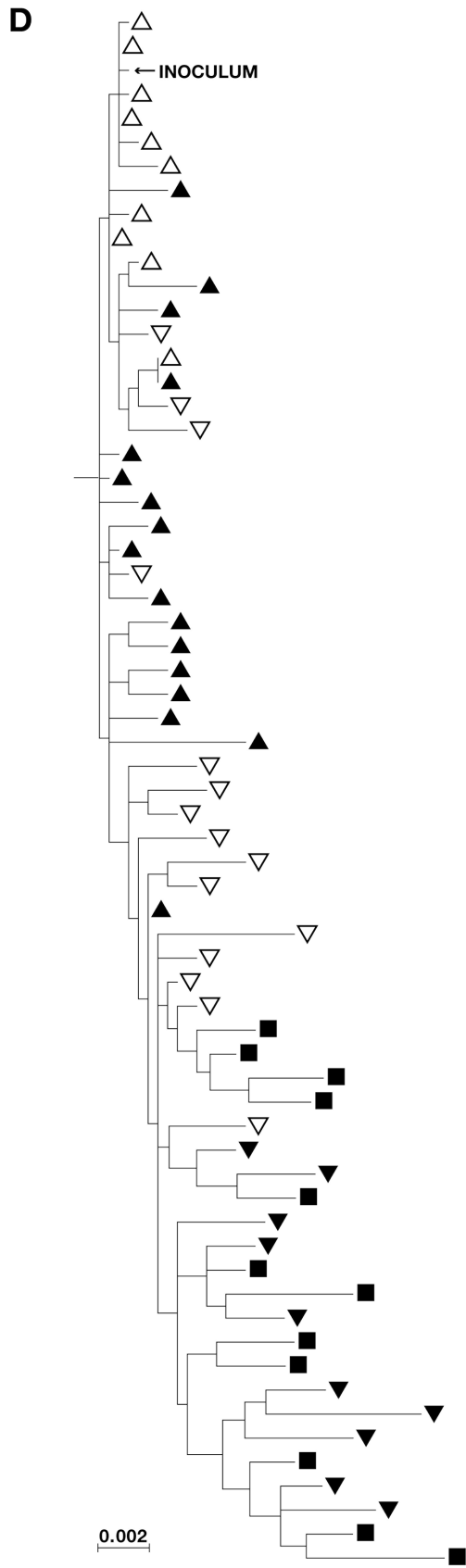
0.002

0   1000   2000
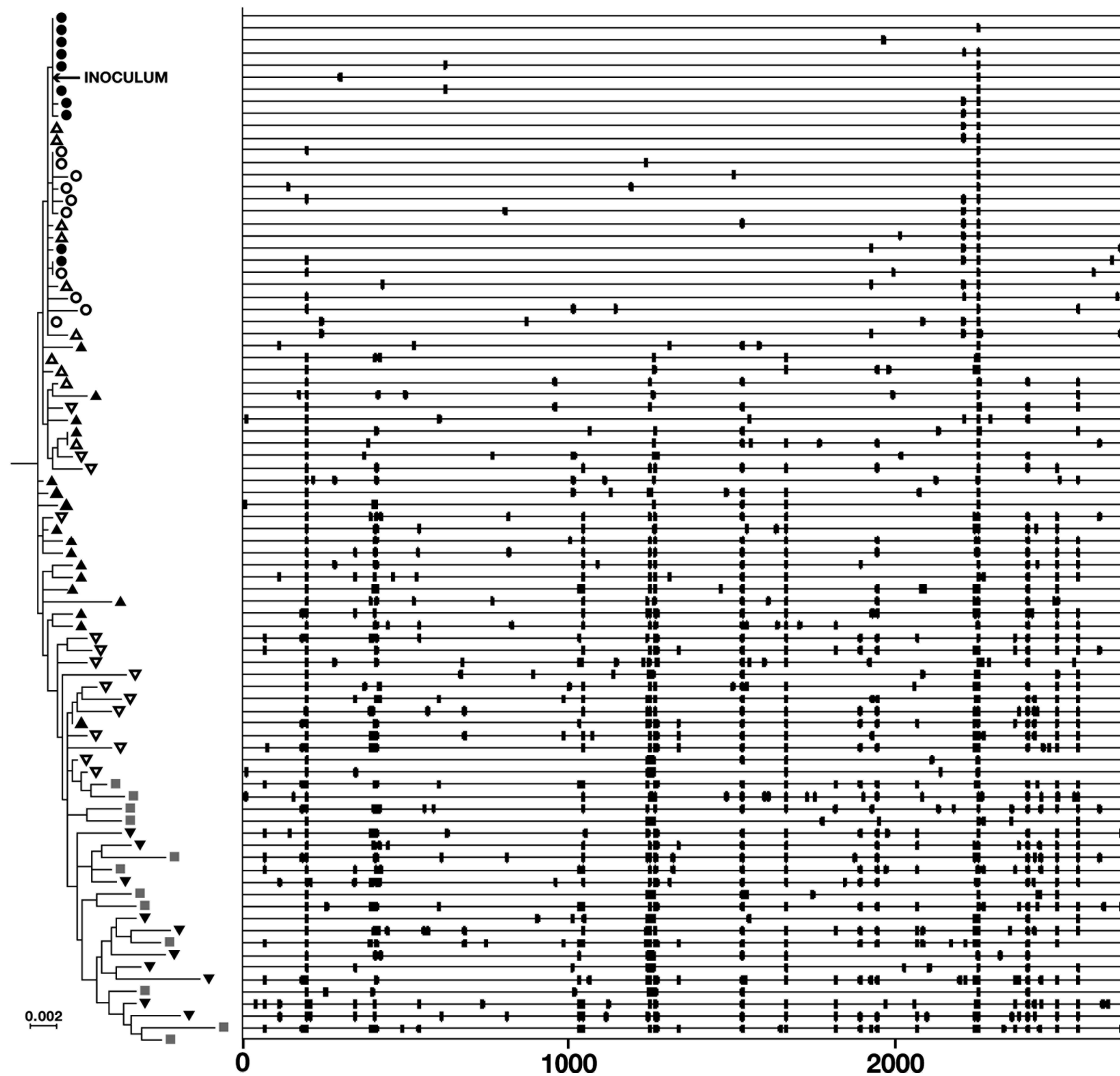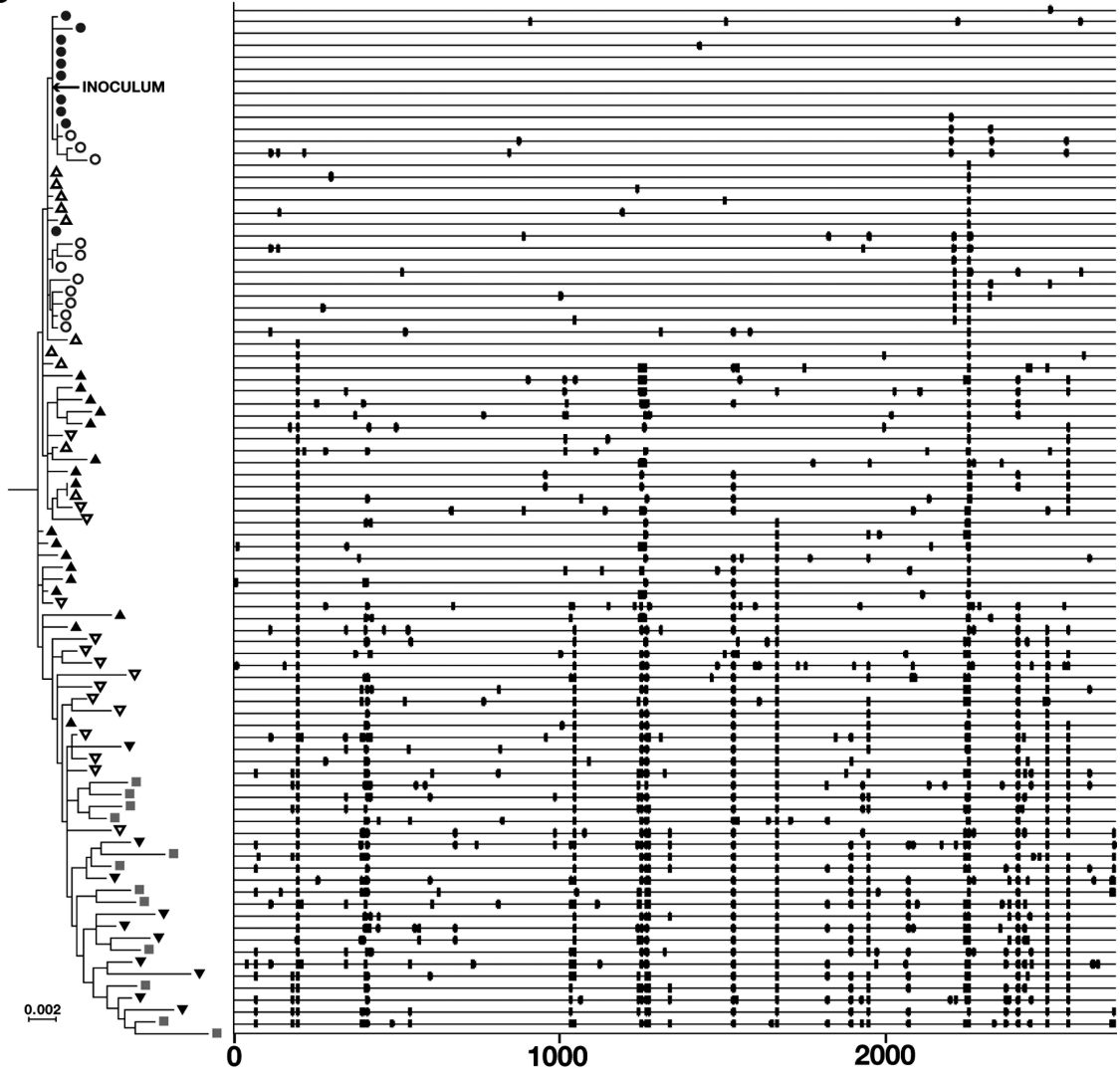
Fig. S.

   Phylogenetic relationships of *env* sequences from treated and untreated animals. Sequences were subjected to phylogenetic analysis as described in the legend to Fig. 3. Sequences from MM521 (untreated) and (A) MM491 (treated), (B) MM499 (treated), and (C) MM528 (treated) are shown. Phylogenetic trees generated using the sequences from an individual animal are also shown (D, MM521; E, MM491; F, MM499; G, MM528; and H, MM530). Open circles, sequences in the plasma of treated animals at 8 wpi; closed circles, those from MLN of treated animals at the completion of cART; open triangles, those from plasma of MM521 at 8 wpi; closed triangles, those from plasma of MM521 at 18 wpi; open inverse triangles, those from plasma of MM521 at 42 wpi; closed inverse triangles, those from plasma of MM521 at 68 wpi; closed rectangles, those from MLN of MM521 at 68 wpi. The scale represents a genetic distance equivalent to 0.002 substitutions per site. The corresponding sequence of SIVmac251 32H (GenBank accession no. D01065) was employed as an outgroup.