



# HIV-1 mutants expressing B cell clonogenic matrix protein p17 variants are increasing their prevalence worldwide

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**AIDS-defining cancers declined after combined antiretroviral therapy (cART) introduction, but lymphomas are still elevated in HIV type 1 (HIV-1)-infected patients. In particular, non-Hodgkin's lymphomas (NHLs) represent the majority of all AIDS-defining cancers and are the most frequent cause of death in these patients. We have recently demonstrated that amino acid (aa) insertions at the HIV-1 matrix protein p17 COOH-terminal region cause protein destabilization, leading to conformational changes. Misfolded p17 variants (vp17s) strongly impact clonogenic B cell growth properties that may contribute to B cell lymphomagenesis as suggested by the significantly higher frequency of detection of vp17s with COOH-terminal aa insertions in plasma of HIV-1-infected patients with NHL. Here, we expand our previous observations by assessing the prevalence of vp17s in large retrospective cohorts of patients with and without lymphoma. We confirm the significantly higher prevalence of vp17s in lymphoma patients than in HIV-1-infected individuals without lymphoma. Analysis of 3,990 sequences deposited between 1985 and 2017 allowed us to highlight a worldwide increasing prevalence of HIV-1 mutants expressing vp17s over time. Since genomic surveillance uncovered a cluster of HIV-1 expressing a B cell clonogenic vp17 dated from 2011 to 2019, we conclude that aa insertions can be fixed in HIV-1 and that mutant viruses displaying B cell clonogenic vp17s are actively spreading.**

antiretroviral therapy | HIV-1 | B cell p17 variants | proteins | lymphoma

Lymphomas represent the most frequent malignancy among HIV type 1 (HIV-1)-infected patients, with non-Hodgkin's lymphoma (NHL) as the most frequent (1–4). Despite the improved control of HIV-1 infection achieved by combined antiretroviral therapy (cART), NHLs remain a leading cause of morbidity and mortality in HIV-1-infected patients (5). The molecular events resulting in progression to NHL in the cART era are not yet well understood, and the current scenario does not explain the still high prevalence of distinct lymphoma histotypes in HIV-1-infected patients (6). Recent findings are challenging the current view of a mainly indirect role of HIV-1 in lymphoma development. Massive data have been collected on the crucial role of the circulating HIV-1 proteins in lymphoma pathogenesis since they are known to accumulate in lymphoid tissues even during successful cART (7). Indeed, the production and release of HIV-1 Gag proteins were found to be sustained by cells harboring latent viruses in the absence of virus replication (8) and even defective proviruses (9). These findings help explain the persistent expression of Gag proteins in different organs and tissues (7, 10, 11). The HIV-1 matrix protein p17 is a Gag-encoded 132-amino acid (aa)-long protein endowed with complex functions in the virus life cycle (12). HIV-1-infected cells release virion-free p17 even in the absence of active viral protease (13). Moreover, a long-term p17 accumulation in lymph nodes of cART-treated patients has been demonstrated (7), suggesting that p17 may be chronically present in the tissue microenvironment of patients, even during pharmacological control of viral replication. Extracellularly, p17 derived from clade B virus (BH10; refp17) was found to deregulate the biological activity of relevant cell populations in the context of HIV-1 pathogenesis (14). Differently from refp17, a p17 variant (vp17) derived from a Ugandan patient was found to be capable of triggering B cell growth and clonogenicity (15). This activity was also exerted by two categories of vp17s more frequently detected in the plasma of HIV-1-infected patients with than without NHL and characterized by aa insertions at positions 117 to 118 and/or at positions 125 to 126 (16). Interestingly, vp17s endowed with B cell growth-promoting activity, differently from refp17, are drastically destabilized (15, 16) and expose a functional epitope located at the p17 NH<sub>2</sub>-terminal region (17). More recently, the B cell growth-promoting activity of vp17s has been associated with the interaction of the functional epitope with the

## Significance

In the combined antiretroviral therapy era, lymphomas still represent the most frequent cause of death in HIV-1-infected patients. We expand previous observations dealing with the prevalence of HIV-1 matrix protein p17 variants (vp17s), characterized by peculiar amino acid insertions and endowed of B cell clonogenic activity, in HIV-1 patients with lymphoma as compared with patients without lymphoma. We show that the prevalence of HIV-1 mutants expressing vp17s is increasing worldwide over time. Moreover, we describe a cluster of HIV-1 mutants expressing a B cell clonogenic vp17 and highlight that insertions can be fixed and that viruses displaying clonogenic vp17s are actively spreading. This knowledge advocates for an extensive genomic surveillance program to monitor the evolution of such mutant virions worldwide.

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The authors declare no competing interest.

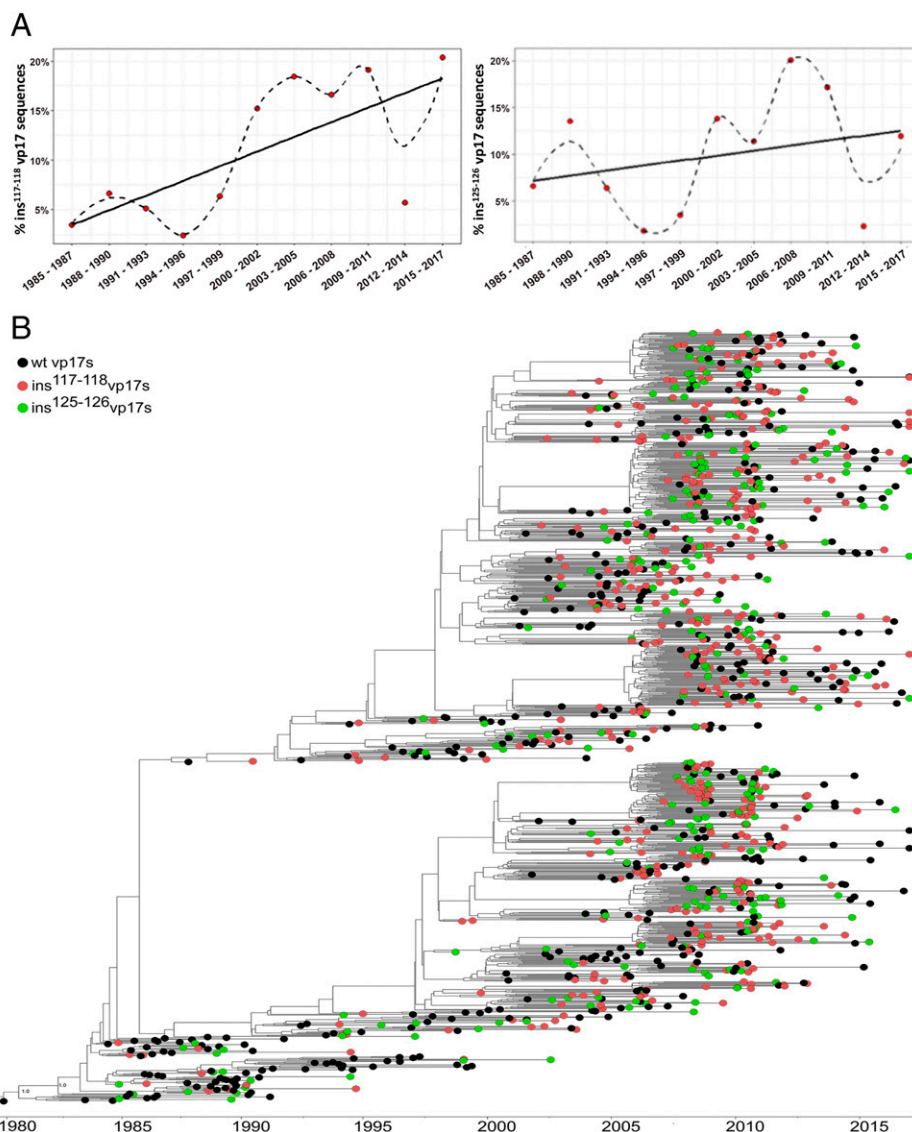
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**Fig. 2.** Insertion rate and phylogenetic analysis of vp17s. (A) Percentages of  $\text{ins}^{117-118}$  vp17 (Left) and  $\text{ins}^{125-126}$  vp17 (Right) sequences (Dataset S1). Sequences were grouped by 3-y periods (red dots). The trend in the increased frequency of  $\text{ins}^{117-118}$  and  $\text{ins}^{125-126}$  vp17s is highlighted by the local smooth (dotted lines) and the linear smooth (solid lines) with  $\alpha = 0.5$ . (B) The phylogenetic tree includes 1,221 HIV-1 clade B vp17 sequences (Dataset S2).

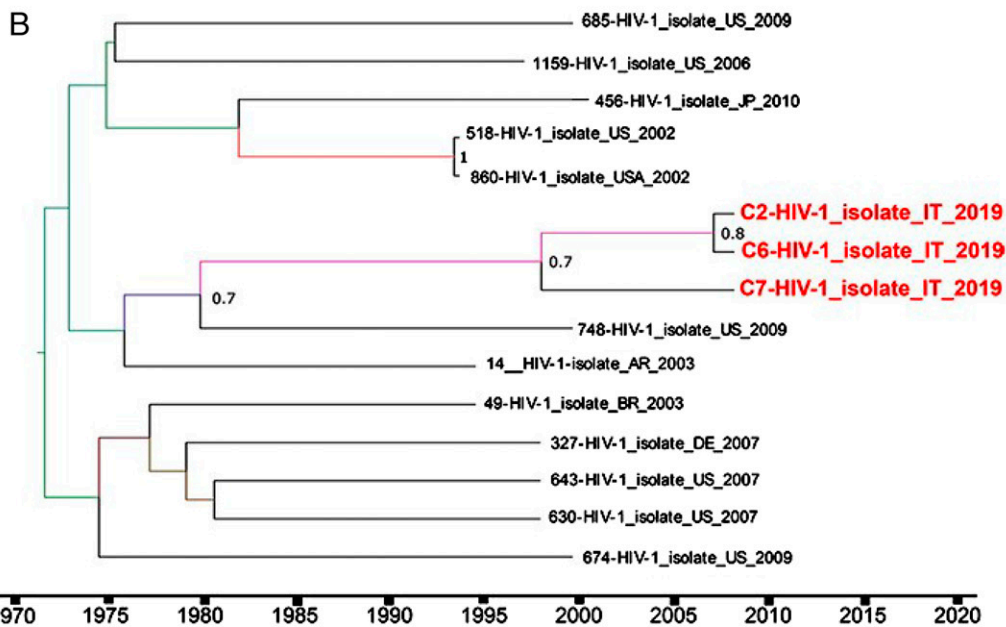
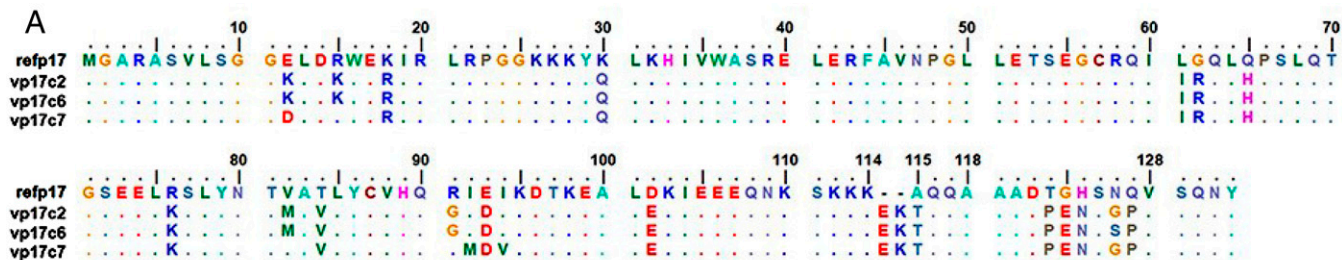
reports the absolute frequencies of wild-type (wt) p17, characterized by the absence of any aa insertion along the entire protein sequence, compared with vp17s displaying aa insertions at positions 117 to 118 ( $\text{ins}^{117-118}$  vp17s) or positions 125 to 126 ( $\text{ins}^{125-126}$  vp17s). The proportions of  $\text{ins}^{117-118}$  vp17s (Fig. 2 A, Left) and  $\text{ins}^{125-126}$  vp17s (Fig. 2 A, Right) on the total number of p17 sequences were found to increase over time. The trend in the increased frequency of  $\text{ins}^{117-118}$  vp17s over a 33-y period of observation was statistically significant ( $P = 0.0000339$ ). On the other hand, although a trend of increased frequency of  $\text{ins}^{125-126}$  vp17s was observed, this did not reach statistical significance ( $P = 0.0704$ ). This finding supports the hypothesis that mutant viruses displaying at least  $\text{ins}^{117-118}$  vp17s are spreading and progressively replacing the virus expressing wt p17.

**Phylogenetic Analysis of vp17s.** The evolutionary phylogenetic tree describes the temporal distribution of wt p17s,  $\text{ins}^{117-118}$  vp17s, and  $\text{ins}^{125-126}$  vp17s from 1979 to 2017. As shown in Fig. 2B,  $\text{ins}^{117-118}$  and  $\text{ins}^{125-126}$  vp17s give rise multiple times to independent evolutionary clades. It is worth noting that wt p17 and vp17 sequences obtained over time from different

countries are intermixed. This event suggests a convergent evolution that occurs when different populations acquire the same insertion at a specific position in the genome, often considered one of the most powerful lines of evidence for adaptive evolution. Our results indicate that at least  $\text{ins}^{117-118}$  and  $\text{ins}^{125-126}$  vp17s could represent an attempt of HIV-1 to better adapt to the human host. To validate this hypothesis, a maximum likelihood method was used. As shown in *SI Appendix*, Fig. S3,  $\text{ins}^{117-118}$  as well as  $\text{ins}^{125-126}$  vp17 sequences (*SI Appendix*, Fig. S4) derived from lymphoma patients are intermixed with worldwide wt p17.

**Detection of a Cluster of Sexually Transmitted HIV-1 Mutants Expressing vp17s.** In 2019, we found a cluster of three patients, two females (patients C6 and C7) and one male (patient C2), infected by a mutant HIV-1 expressing a vp17 characterized by a Glu-Lys (EK) insertion at positions 114 to 115 ( $\text{insEK}^{114-115}$  vp17) (Fig. 3A). Patient C7 was infected since 2011, but she did not receive any antiretroviral treatment at that time because lack of indication. She had sexual intercourses in 2011 with patient C2. Patient C2 had sexual intercourses with patient C6.





**Fig. 3.** Alignment and comparison among aa sequences of refp17 and vp17s isolated from three HIV-1-infected control patients. (A) Sequences are represented by the single-letter aa code; aa positions are referred to the subtype B strain BH10 (UniProtKB P04585; refp17). Each aa residue of vp17s not differing from refp17 is represented by a dot. (B) Zoom timescale phylogenetic tree. The tree includes 1,221 HIV-1 clade B sequences retrieved worldwide from the Los Alamos database (Dataset S2) and 3 HIV-1 sequences obtained in Italy in 2019, which are indicated in red.

Patient C2 became aware of being seropositive for HIV-1 in 2019, and then, patient C6, warned by patient C2, underwent HIV-1 screening test that gave a positive result. Phylogenetic inference allowed us to reconstruct the transmission history of our isolates and confirm the patients' referred dynamics of infection. As shown in Fig. 3B, the three insEK<sup>114-115</sup> vp17s form an independent cluster that assigns to patient C7 the role of ancestor at the beginning of the branch.

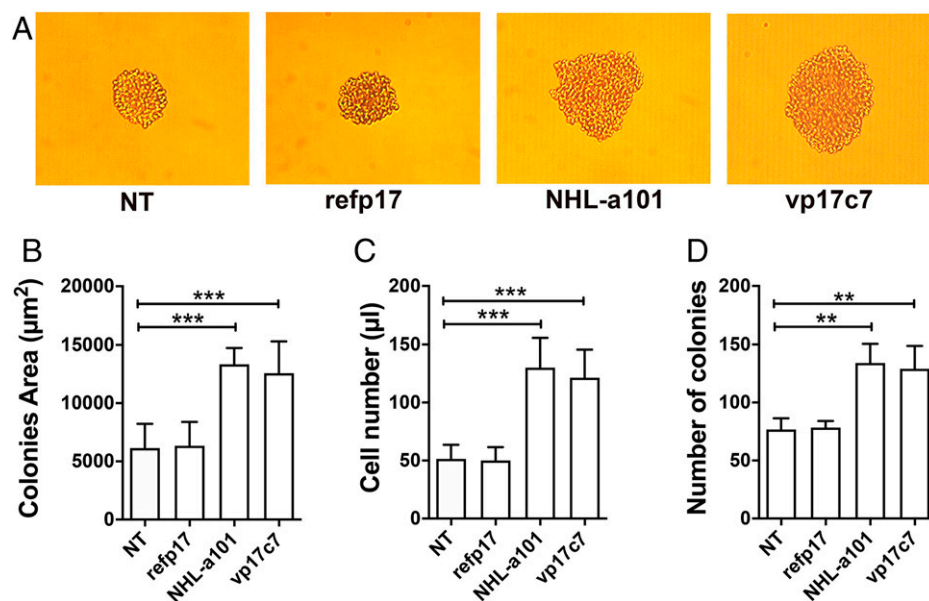
**Recombinant insEK<sup>114-115</sup> vp17 Promotes B Cell Growth and Clonogenic Activity.** Recombinant insEK<sup>114-115</sup> vp17, namely vp17c7, was obtained from the dominant sequence detected in patient C7, the ancestor sequence of the cluster, and then investigated for its ability to enhance clonogenic activity of Raji B cells. As shown in Fig. 4A and B, vp17c7 significantly increased the area of Raji cell colonies in a single-cell cloning assay (17) compared with untreated cells. A similar result was obtained with the known B cell clonogenic vp17 named NHL-a101 (16). As expected, refp17 was unable to increase the colony-forming ability of Raji cells. A cell suspension was obtained by pooling equal numbers of colonies per each experimental condition, and the absolute number of cells was evaluated. As shown in Fig. 4C, cell proliferation significantly increased when cultured in medium containing vp17c7 or NHL-a101 vp17s as compared with cells treated with refp17. The growth-promoting activity of the vp17c7 was also evaluated by the anchorage-independent growth assay. As expected,

vp17c7—as well as NHL-a101—vp17s were able to significantly increase Raji cell clonogenicity (Fig. 4D).

**Insertion of EK at Positions 114 to 115 of the refp17 Backbone Confers B Cell Clonogenic Activity to the Viral Protein.** We engineered the inactive refp17 by insertion of EK at positions 114 to 115 (insEK<sup>114-115</sup> refp17). InsEK<sup>114-115</sup> refp17, similarly to its natural counterpart vp17c7, significantly increased the area of Raji cell colonies in a single-cell cloning assay (SI Appendix, Fig. S5A and B), cell proliferation (SI Appendix, Fig. S5C), and clonogenicity (SI Appendix, Fig. S5D) compared with control cells. These data indicate that the EK insertion at positions 114 to 115 is a signature characterizing vp17s capable of promoting B cell growth and clonogenicity.

## Discussion

Previous studies showed that aa insertions frequently occur at the COOH-terminal region of vp17s only (16, 19), with higher prevalence in patients with than without lymphoma (16). The present study, conducted in a much larger cohort of patients with and without lymphoma than the previous ones, confirms the presence of vp17s characterized by different aa insertions in HIV-1 subtype B-infected patients. Moreover, it corroborates data obtained in previous studies showing that a higher percentage of vp17s is detected in plasma of lymphoma patients than in plasma of patients without lymphoma. Sanger sequencing spanning the



**Fig. 4.** Effect of vp17c7 on B cell clonogenicity. In the colony formation assay, Raji cells were cultured in the presence or absence of refp17, NHL-a101, or vp17c7. (A) Bright-field images represent the characteristic morphology of two-dimensional colonies. (Original magnification,  $\times 40$ .) (B) The colony area was measured using Leica Qwin image analysis software. (C) The same numbers of colonies were aseptically harvested, stained with propidium iodide to detect viable cells by flow cytometry, and counted by the counting function of the MACSQuantAnalyzer. (D) In the soft agar assay, Raji cells were incubated in medium containing or not refp17, NHL-a101, or vp17c7. The cell growth was analyzed by using 3-[4,5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT). Data are representative of three independent experiments performed in triplicate. The statistical significance was calculated using one-way ANOVA, and the Bonferroni posttest was used to compare data. NT, not treated cells.  $^{**}P < 0.01$ ;  $^{***}P < 0.001$ .

entire viral-encoded proteins allowed us to confirm the occurrence of aa insertions in specific positions, invariably confined to the COOH-terminal region of p17, between aa positions 114 and 130. The distribution of vp17s was similar among lymphoma histotypes, indicating that vp17s do not mark specific HIV-1-related NHL subtypes or HL. The finding of an increased frequency of vp17s detection in blood samples of patients with a relatively high viremia at the time of testing may likely reflect their history of chronic HIV-1 infection. In fact, it is known that higher nucleotide substitution rates within Gag are associated with higher levels of HIV-1 replication (20). However, lack of precise information on the duration of HIV-1 infection hampers the possibility to confirm this hypothesis. A limitation of this study is the lack of a deeper investigation by a next generation sequencing approach in order to describe the existence of vp17s within quasispecies in the presence of dominant wt. According to the Los Alamos database, vp17s with aa insertions in their COOH-terminal region have also been detected in HIV-1 subtype A- and C-infected patients, suggesting a common mechanism of vp17 generation among different subtypes. Conformational changes in a protein are known to alter the exposure of epitopes targeted by the host immune response, thus hampering inhibition of viral infection and replication (21, 22). Moreover, selection pressure exerted by immune response is known to shape the genetic variation of HIV-1 (23). In this perspective, generation of destabilized vp17s by aa insertions in the COOH-terminal region could represent a strategy of the virus to evade the immune system. Being that p17 is one of the main targets of both humoral and cell-mediated immune responses (14), the different pressure imposed by host immunity could favor the selection and expansion of distinct HIV-1 vp17s. This hypothesis is supported by the heterogeneous pattern of aa insertions we observed in the COOH-terminal region of vp17s obtained from different HIV-1-infected patients. On the other hand, p17 conformational changes could also contribute, at least in part, to confer resistance to protease inhibitors. In fact, mutations in p17

able to alter matrix protein structure were found to contribute to protease inhibitor susceptibility and replication capacity (24, 25); aa insertions in the COOH-terminal region may indeed extend the disordered region containing the protease cleavage site between matrix and capsid and better promote the interaction between the Gag cleavage site and viral protease. Differently from the NH<sub>2</sub>-terminal and core regions, which comprise activity constraints for p17 interaction with several cellular proteins (10, 17, 18, 26–29), the COOH terminus does not have these constraints and results partially unfolded and disordered (30, 31). This does not limit the number and location of aa insertions that can be accepted in this region during viral replication. Different biological functions can be performed by a protein that lacks ordered and/or secondary structure (32–34), and distinct vp17s fully comply with this rule (16). Whatever the aim of conformational changes in vp17s, they underlie an intrahost virus evolution and account for a dramatic change of activity of these destabilized proteins on B cell growth and clonogenicity. This identified insertion of an EK aa stretch at positions 114 to 115 has to be considered a molecular signature of vp17 proteins with enhanced B cell growth-promoting and clonogenic activity. Identifying other HIV-1-infected patients harboring HIV-1 mutants for clonogenic vp17s and longitudinal studies will contribute to our understanding of the potential of such genomic analysis to predict which p17 alterations help identify patients at risk for lymphomas.

The importance of this work also lies in the finding that aa insertions in vp17s have been fixed and transmitted to progeny virions. We have analyzed a total of 3,990 sequences retrieved from the Los Alamos HIV database that were deposited between 1985 and 2017. Here, we highlight the increased worldwide prevalence of HIV-1 mutants expressing vp17s with aa insertions at positions 117 to 118. It is worth noting that genomic surveillance started in 2019 in our laboratory uncovered the presence of B cell clonogenic insAA<sup>117-118</sup> vp17s in 4% of the control cohort patients, whereas these vp17s were

never detected previously in such cohorts. This finding suggests that HIV-1 mutant strains carrying clonogenic vp17s are spreading worldwide and increasing their prevalence over time. Detection of a cluster of HIV-1 expressing a B cell clonogenic insEK<sup>114-115</sup> vp17 definitively attests that aa insertions can be fixed in HIV-1 and that mutant viruses displaying B cell clonogenic vp17s are actively spreading. This finding indicates the occurrence of constraints during the still ongoing evolutionary trajectory of HIV-1. Whether these HIV-1 mutants display a better fitness than the wt ones to the human host and/or represent an adaptation to cART constitutes an attractive hypothesis for further study.

The possible role of B cell clonogenic vp17s in AIDS-defining cancers has been previously highlighted (6, 16, 35). Recent evidence showed the persistence of defective proviruses in HIV-1-infected individuals during cART capable of producing and releasing HIV Gag proteins in the microenvironment (36). Indeed, the presence of vp17s characterized by aa insertions at the COOH-terminal region in the proviral DNA of peripheral blood mononuclear cells has been recognized (37). We have recently shown that B cell clonogenic vp17s are secreted by HIV-1-infected cells in the microenvironment (38). Secretion of vp17s was found to occur even in the absence of an active viral protease (38). This finding suggests that vp17s may also persist in lymphoid tissues, where they may exert a chronic B cell stimulation. If so, the impact of vp17s on B cell growth and transformation is likely to persist. Recent data have highlighted the capability of vp17s to also promote angiogenesis and lymphangiogenesis (16, 37, 39, 40), which are essential in supporting proliferation and survival of lymphoma, as well as tumor cell dissemination. Accumulation of p17 in different organs and tissues (6, 8, 10, 41) even in the central nervous system of patients under successful cART (11), possibly by blood-brain barrier crossing (42), has been documented. This evidence supports the hypothesis that actively secreted vp17s may be stored within a variety of organs and tissues, where they may likely function as promoters of cell growth and transformation. These findings call for inhibiting secretion and/or biological activity of vp17s. This may be achieved in the microenvironment by using a specific therapeutic vaccine aimed to generate a long-lasting and potent

neutralizing antibody response (43, 44). It may also be possible by avoiding Pr55<sup>Gag</sup>/PI(4,5)P<sub>2</sub> interaction through small molecule inhibitors able to engage the PI(4,5)P<sub>2</sub> binding pocket of the viral protein (45).

In conclusion, our results show that HIV-1 mutants expressing vp17s are circulating and spreading. This knowledge advocates for an extensive genomic surveillance program to monitor the evolution of such mutant virions worldwide. Early identification of clonogenic vp17s would make it possible to implement therapeutic or preventive strategies to avoid their long-term cancer-promoting activity.

## Materials and Methods

The study was conducted in accordance with the Declaration of Helsinki and national standards and was approved by the Brescia Ethics Committee (NP 3163). A complete description of the source of materials and our methods is in *SI Appendix, SI Materials and Methods*. It includes a description of the patients and procedures for Sanger sequencing; bioinformatic, phylogenetic, and statistical analyses; production of recombinant proteins; and clonogenic assays.

**Data Availability.** All study data are included in the article and/or supporting information.

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