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Use of a Combined Ex Vivo/In Vivo Population Approach for Screening of Human Genes Involved in the Human Immunodeficiency Virus Type 1 Life Cycle for Variants Influencing Disease Progression†

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Humans differ substantially with respect to susceptibility to human immunodeficiency virus type 1 (HIV-1). We evaluated variants of nine host genes participating in the viral life cycle for their role in modulating HIV-1 infection. Alleles were assessed ex vivo for their impact on viral replication in purified CD4 T cells from healthy blood donors ($n = 128$). Thereafter, candidate alleles were assessed in vivo in a cohort of HIV-1-infected individuals ($n = 851$) not receiving potent antiretroviral therapy. As a benchmark test, we tested 12 previously reported host genetic variants influencing HIV-1 infection as well as single nucleotide polymorphisms in the nine candidate genes. This led to the proposition of three alleles of *PML*, *TSG101*, and *PPIA* as potentially associated with differences in progression of HIV-1 disease. In a model considering the combined effects of new and previously reported gene variants, we estimated that their effect might be responsible for lengthening or shortening by up to 2.8 years the period from 500 CD4 T cells/μl to <200 CD4 T cells/μl.

The genetic makeup of an individual plays a role in determining susceptibility to infection and to progression of disease. Some of the observed variation has been attributed to immunogenetic diversity (major histocompatibility class homozygocity or specific HLA types) and polymorphism in chemokines, chemokine receptors, and cytokine genes (*CCR5*, *CCR2*, *CX3CR1*, *SDF1*, *MIP1a*/*CCL3L1*, *RANTES*, *IL-10*, and *IL-4*) (4, 36, 44). A number of genes have been identified as participating in the viral life cycle at postentry (18), and it can be speculated that polymorphism in these genes could have relevance for human immunodeficiency virus type 1 (HIV-1) susceptibility and disease progression. However, the proposition of new genetic variants is complicated by unknown environmental and genetic factors pertaining to both the human host and the virus (43). Thus, association studies may lead to identification of genetic variants that cannot be proven by subsequent studies. In addition, there is a need for statistical power to identify the small contribution of single gene effects and for the development of multigene models that take into account the combined effects of multiple genetic variants (36).

We addressed these issues by testing whether a two-step screening by an ex vivo/in vivo model of population genetics would allow identification and validation of novel host genetic variants influencing HIV-1. For this, we infected purified CD4 T cells from healthy blood donors and extracted DNA for detailed resequencing of candidate genes. Alleles identified as possibly modifying cellular permissiveness ex vivo were thereafter assessed in a cohort of HIV-1-infected individuals. Finally, selected alleles of gene candidates and of previously reported genes influencing HIV-1 infection were included in a multigene model to define their contribution to global prediction of HIV-1 disease progression.

MATERIALS AND METHODS

Cells. CD4 T cells from 128 healthy Caucasian blood donors were isolated by anti-CD4 magnetic beads (Miltenyi Biotech) and cultured ex vivo in RPMI 1640 (Gibco-Invitrogen) medium, supplemented with 20% fetal calf serum, 20 U/ml human interleukin-2 (IL-2; Roche) and 50 μg/ml gentamicin, following stimulation with 2 μg/ml phytohemagglutinin for 2 days. CD4 T cells (10^6 cells) were infected with R5 clone HIV-1 NL4-3Ba*Lenv* (1,000 pg of p24 antigen) for 2 h at 37°C in 5% CO_2 in a 1-ml final volume. Cells were washed and cultured for 7 days. Virus-containing supernatant was harvested, and p24 antigen production was monitored by enzyme-linked immunosorbent assay (Abbott). Permissiveness was defined as the ability of cells to be infected and sustain replication of HIV-1 (49) and here refers to the outcome (p24 antigen production) of a 7-day replication assay.

Candidate genes, identification of SNPs, and allelic discrimination. The genes involved in the HIV-1 life cycle selected for analysis included *TSG101* (GenBank accession no. U82130), β*TRC* (Y14153), *PPIA* (X52851), *INI1* (AJ011737), *NAF1* (AY012155), *PML*/*TRIM19* (X63131), *HP68* (X76388), *YY1* (Z14077), and *AIP1*/*ALIX* (AF151793). Single nucleotide polymorphism (SNP) discovery used single-strand conformation polymorphism and sequencing. For this, a total of 138 PCRs were designed to cover exons, putative promoter regions, and intron-exon boundaries. Selected positions were then genotyped by using TaqMan allelic discrimination (primer information available on request). The following previously reported host genetic variants influencing HIV-1 disease progression were investigated by TaqMan or restriction fragment length polymorphism analysis (primers and probes available from the authors): *CCR5* Δ32 (7, 38), *CCR5* promoter 59029G>A (28, 30), *CCR5* coding region 303T>A (37), *CCR2* coding region 64I (40), *CX3CR1* coding region 280M (8), *RANTES* promoter region −403G>A (25), *RANTES* promoter region −28C>G (16, 25), *RANTES* intron 1.1 (1), *MIP1*α intron 1 459C>T (16), and *IL-4* promoter −589C>T (35). Three additional alleles, *SDF1* 3′A (50) and *IL-10* −3575T>A

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and −592C>A (39), were assessed only in vivo. SDF1 is a ligand of CXCR4 and, thus, of limited relevance in an ex vivo analysis that used an R5-tropic strain. Although IL-10 is produced by Th2 CD4 T cells, the variants were not analyzed ex vivo because this cytokine is expected to act through macrophages and other cells not present in the purified CD4 T-cell population. The ex vivo viral replication for each genotype was represented by the median p24 antigen production.

In vivo analysis: characterizing phenotype by CD4 cell count decline. Participants (n = 851) were recruited within the genetics project of the Swiss HIV Cohort Study (SHCS) (http://www.shcs.ch/). The ethics committees of all participant centers approved the study. Patients gave written informed consent for genetic testing. DNA from peripheral blood mononuclear cells from participants was used for genotyping. The purpose of the in vivo analysis was to allocate a reliable phenotype that would be a marker of disease progression before treatment for each patient. The rate of decline in the CD4 T-cell count during the natural history of disease progression was considered to be the most appropriate marker to use as a phenotype. Patients who had at least two CD4 measurements before exposure to potent antiretroviral therapy (ART) were included in the analysis. Time origin for the CD4 T-cell measurements was the estimated date of seroconversion. This was calculated for each patient using a method proposed by Geskus et al. (14), which was applied to the entire SHCS. The method matches the first CD4 T-cell measurement of a patient with an unknown date of seroconversion with the measurements from seroconverters and uses kernel density estimation to infer the most likely date of seroconversion. The CD4 T-cell trajectories were modeled using a repeated measures hierarchical approach using Mlwin software (http://multilevel.ioe.ac.uk). Square-root-transformed CD4 T-cell counts were modeled as a linear function of time from the estimated date of seroconversion, with random effects for both the intercept and the gradient with additional terms for sex, age (16 to 29, 30 to 39, 40 to 49, 50 years, and above), and risk group (intravenous drug use [IDU]/non-IDU). For each genotype, the average square root CD4 decline per year was estimated in dominant and recessive models. Haplotypes were attributed using SNPHAP (http://www-gene.cimr.cam.ac.uk/clayton/software/). The correlation between the ex vivo phenotype of viral permissiveness and the in vivo phenotype of CD4 decline was calculated as the correlation between the coefficient from the ex vivo regression with the difference in the square-root-transformed CD4 gradient from the in vivo analysis comparing those with rare allelic presence with the common homozygous allelic group. Concordance between ex vivo and in vivo results was inspected graphically.

Multiple gene effects model. In order to estimate the combined effect of all the polymorphisms on CD4 T-cell decline, models were fitted using (i) only polymorphisms previously reported to affect disease progression and (ii) only candidate polymorphisms proposed in this paper. Finally, a stepwise procedure was used on the full model to find an optimal model by omitting the least significant term and comparing model deviations at each iteration. All models were adjusted for sex, IDU, and age and were estimated using all available pretreatment CD4 counts. Models were compared to the null model without genetic terms using the likelihood ratio test. Confidence intervals (95%) were calculated for the predicted difference in latency period due to genetic effects using the percentile simulation method with 100,000 iterations. The prognostic model was used to estimate the combined effect on disease progression of the differences in CD4 decline according to different genotypes. In order to illustrate the clinical importance of the combined effect, we estimated the range of times for the CD4 count to drop from 500 to 200 cells/μl.

RESULTS AND DISCUSSION

Permissiveness and genetic polymorphism. Infection of the various CD4 T-cell pools under standardized conditions with an R5-tropic laboratory clone of HIV-1 identified greater than 5 log differences in CD4 T-cell permissiveness (6), expressed as p24 antigen production in culture supernatant after 7 days of replication kinetics. We speculated that permissiveness could be considered as a genetic trait because previous studies demonstrated that macrophages derived from pairs of identical twins display a high concordance in the kinetics of HIV-1 replication (5, 34) and because of the reproducibility, for the same individual, of cell permissiveness and provirus transcription over time (6). Permissiveness was thereafter used as the ex vivo phenotype.

DNA from blood donors was used to identify genetic variation in nine candidate genes participating in the life cycle of HIV-1. These included *TSG101* (encoding tumor susceptibility gene 101), participating in viral budding through interaction with viral protein p6Gag (13); β*TRC* (beta-transducin repeats-containing protein), interacting with viral protein Vpu to bring CD4 to the endoplasmic reticulum degradation pathway (27); *PPIA* (peptidyl-prolyl *cis-trans* isomerase; cyclophilin A), incorporated into the viral particle through selective interaction with viral capsid (11); *INI1* (integrase interactor 1 protein), participating in viral genome transcription (46); *NAF1* (Nef-associated factor 1), interacting with viral proteins Nef and matrix (12, 19); *PML* (TRIM19; promyelocytic leukemia), proposed to act as antiviral protein (46); *HP68* (RNase L inhibitor protein) associating with Vif and Gag (52); YY1 (transcription factor ying yang 1), implicated in the down-regulation of the expression of chemokine receptors and of long terminal repeat promoter of HIV-1 (26); and *AIP1/ALIX*, (mammalian orthologue of the yeast class E vacuolar protein sorting factor, Bro1), a component of the viral budding machinery, which serves to link a distinct region in the L domain of HIV-1 p6 to ESCRT-III (42). These genes are present in different chromosomes (see Fig. S1 in the supplemental material), and thus there is no linkage.

SNP screening was performed on 96 chromosomes through single-strand conformation polymorphism and subsequent sequencing analysis of a cumulative 37,406 nucleotides per individual. For specific SNPs, analysis was extended to 256 chromosomes. Amplification success rate was 89% (33,262 nucleotides screened per donor; 37% coding, 27% promoter and 3′ untranslated region, and 36% intron-exon boundaries). A total of 34 SNPs/indels (insertion-deletions) were identified (one variant per 978 nucleotides), of which 23 were newly identified and/or validated at the time of the study (see Fig. S1 in the supplemental material). SNP frequencies ranged from 1 in 6,185 in *HP68* to 1 in 333 nucleotides for *PPIA*. We chose, for the initial steps of marker discovery, to perform association analysis on individual SNPs, not on inferred haplotypes. However, for some associations, particularly those newly detected, the precise combination of SNPs that confers the causal effect has not been established, and observations about individual SNPs are likely to be modified with further study. If a candidate SNP is not causal but is suspected to be marking a linked variant, this analysis would be followed by detailed resequencing of the region containing the associated SNPs (41).

Association of genetic variants with viral replication ex vivo. Results are presented in detail in Table S1 in the supplemental material, and relevant results are shown in Fig. 1. *TSG101* −183T>C, β*TRC* A507S, and *PML* −225C>T presented statistical significance or trend ($P < 0.1$, significance level defined post hoc), and profiles suggestive of dominant (*TSG101* and β*TRC*) or recessive (*PML*) influences. We also present *PPIA* 1650A>G in Fig. 1A, because of a suggestive profile upon visual inspection. The lack of association with differences in CD4 T-cell permissiveness for all other genetic variants of candidate genes is presented in Table S1 in the supplemental material.

In addition to the nine candidate genes, the following alleles previously reported to influence HIV-1 infection in humans were investigated in the ex vivo system: *CCR5* Δ32, *CCR5*

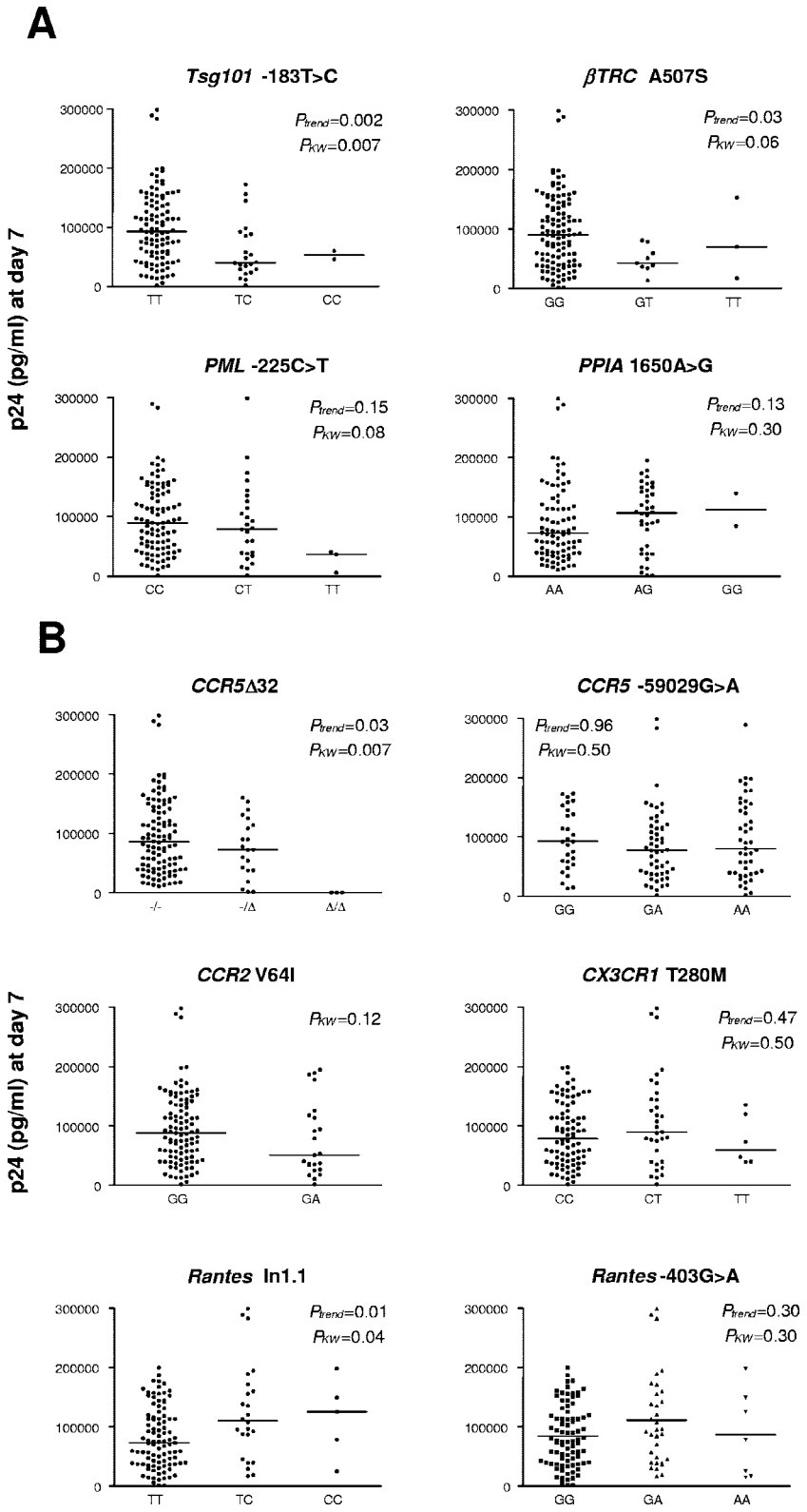

FIG. 1. Association of candidate and known alleles with HIV-1 cell permissiveness ex vivo. (A) Candidate genes. (B) A selection of previously reported host genetic variants influencing HIV-1 infection. Bars represent median values. Shown are *P* values estimated by a Kruskal-Wallis test and by a Spearman rank test for trend.

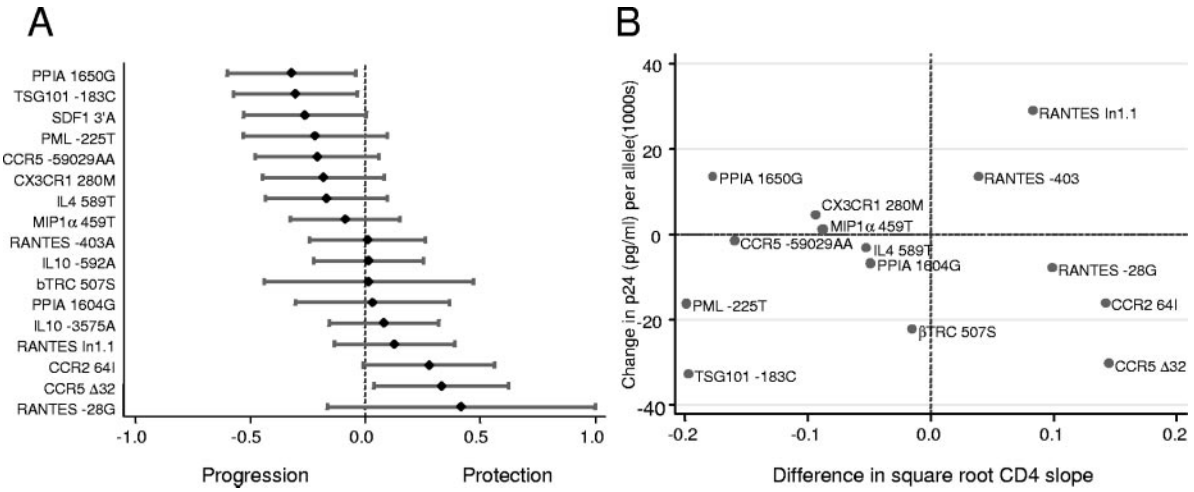

FIG. 2. Association of candidate and known alleles with HIV-1 disease progression in vivo. (A) Difference in square root CD4 gradient comparing carriers of a rare allele with patients homozygous for the common allele (whiskers show 95% confidence interval). (B) Ex vivo/in vivo correlation for markers associated with differences in permissiveness or disease progression.

59029G>A, *CCR5* 303T>A, *CCR2* 64I, *CX3CR1* 280M, *RANTES* −403G>A, *RANTES* −28C>G, *RANTES* intron 1.1, *MIP1α* 459C>T, and *IL-4* −589C>T (Fig. 1B) (see Table S1 in the supplemental material). There was an overall trend in cell permissiveness ex vivo for alleles associated with slow (*CCR5* Δ32 and *CCR2* 64I) or rapid disease progression (*RANTES* intron 1.1) in vivo.

Association of genetic variants with disease progression in vivo. Of 34 candidate alleles (i.e., SNPs and indels) identified by resequencing and tested ex vivo, 5 were thereafter assessed in the SHCS for their roles in modulating HIV-1 infection in study participants. The clinical phenotype was defined as the patient-specific rate of CD4 T-cell decline, a recognized marker of disease progression (3). We used this phenotype because of limited data on AIDS-defining illness or death in this cohort, as the progression of disease was for most patients stopped with the arrival of potent ART. Analysis excluded any CD4 T-cell values after initiation of treatment. The median follow-up time was 3.1 years; the mean was 4.0 years, during which the 851 cohort participants contributed 8,231 CD4 T-cell determinations to the analysis (median, 7 CD4 T-cell determinations per participant). Square-root-transformed CD4 cell counts were modeled as a linear function of time from the estimated date of seroconversion. Overall, we identified a hierarchy of putative effects on disease progression (Fig. 2A), where the new candidate markers exhibited effects on CD4 T-cell depletion that were comparable to a number of previously reported gene variants. Among the previously reported markers investigated, some were not informative in this data set (*IL-10* variants and *RANTES* −403G>A). The rare *CCR5* coding region 303T>A was not present in the study population. The *SDF1* 3′A genotype was associated with an accelerated progression, in agreement with most of the publications reporting on this variant (2, 20, 33, 47). In addition, other variants were at odds with some of the available literature (*RANTES* intron 1.1, *IL-4* 589T, and *CX3CR1* 280M). This reflects the ongoing controversy surrounding the net contribution of some of these variants to disease progression (44), in particular for *IL-4* 589C>T (31, 35, 48) and *CX3CR1* 280M (8, 9, 21, 24, 29). Differences between the present study and previous reports may also reflect the choice of analysis that uses, in a seroprevalent cohort, CD4 decline rather than time to AIDS or death. In single gene analysis, the time to diminish from 500 to 200 CD4 T cells was estimated to increase from 3.7 to 4.3 years ($P = 0.03$) for *CCR5* Δ32 carriers and from 3.6 to 4.2 ($P = 0.03$) for *CCR2* 64I heterozygous carriers. It decreased from 3.9 to 3.4 years ($P = 0.02$) for *PPIA* 1650A>G carriers and from 3.9 to 3.4 ($P = 0.03$) for *TSG101* −183T>C carriers (see Table S2 in the supplemental material).

The ex vivo (viral replication in CD4 T cells) phenotype was then plotted against the in vivo phenotype (CD4 T-cell depletion) (Fig. 2B). Visual inspection suggested concordance between ex vivo and in vivo associations for well-established host genetic variants influencing HIV-1 infection, e.g., *CCR5* Δ32 and *CCR2* 64I (lower ex vivo replication and slower disease progression), and for the new candidate *PPIA* promoter 1650A>G (greater ex vivo replication and faster disease progression). Strikingly, *TSG101* −183T>C was associated with lower CD4 T-cell permissiveness ex vivo but with a rapid loss in CD4 T cells in vivo. Extensive analysis of the role of *TSG101* variants in HIV-1 disease progression will be presented in detail elsewhere.

Multiple gene effects model. We hypothesized that inclusion of reported markers together with candidate markers of *PML*, *PPIA*, *TSG10*, and β*TRC* would improve prediction of CD4 T-cell count decline in multiple gene models. Table 1 shows the coefficients from four models, which estimate the difference in the square-root-transformed CD4 gradient comparing patients who are homozygous or carriers of the rare allele with patients homozygous for the common allele. Model 1 estimates the combined effect of the known markers compared to the null model without genetic terms ($P = 0.007$), and model 2 estimates the combined effect of the candidate markers alone ($P = 0.02$). Model 3 includes both known and candidate markers ($P = 0.001$). Model 4 was constructed using a stepwise procedure to eliminate the least predictive markers, stopping

TABLE 1. Model coefficients showing the difference in the square-root-transformed CD4 T-cell gradient comparing patients who are homozygous or carriers of rare alleles with patients who are homozygous for the common alleles[a]

Polymorphism	Gradient offset (SE)			
	Model 1	Model 2	Model 3	Model 4
Gradient of base group	−2.14 (0.17)	−2.02 (0.15)	−1.95 (0.17)	−2.24 (0.14)
Known markers				
CCR5 59029A^R	−0.42 (0.15)		−0.41 (0.15)	−0.41 (0.15)
CCR5 Δ32	0.46 (0.17)		0.49 (0.17)	0.51 (0.17)
CCR2 641	0.49 (0.16)		0.51 (0.16)	0.50 (0.16)
CX3CR1 280M	−0.21 (0.14)		−0.19 (0.14)	
RANTES −403A	−0.20 (0.24)		−0.19 (0.24)	
RANTES −28G	0.40 (0.35)		0.33 (0.35)	
RANTES ln1.1	0.24 (0.26)		0.27 (0.26)	
MIP1a 459T^R	−0.36 (0.26)		−0.40 (0.25)	−0.45 (0.25)
IL4 589T	−0.14 (0.13)		−0.12 (0.13)	
IL10 −3575A^R	0.17 (0.20)		0.13 (0.20)	
IL10 −592A^R	0.08 (0.23)		0.01 (0.23)	
SDF1 3′A	−0.23 (0.13)		−0.23 (0.13)	−0.23 (0.13)
Candidate markers				
PML −225C>T		−0.24 (0.17)	−0.27 (0.17)	−0.27 (0.16)
PPIA 1604G^R		−0.33 (0.89)	−0.19 (0.88)	
PPIA 1650A>G		−0.31 (0.14)	−0.30 (0.14)	−0.32 (0.14)
TSG101 −183T>C		−0.31 (0.14)	−0.32 (0.14)	−0.33 (0.14)
Model X^2 (df)	27 (12)	11.4 (4)	39 (16)	34 (8)
P value	0.007	0.02	0.001	0.00005

[a] Models are estimated using 8,100 CD4 T-cell measurements from 814 patients. Models are compared to the null model without genetic terms. df, degrees of freedom; R, rare allele homozygous recessive effect; In, intron.

at the model which minimized the *P* values obtained from comparing candidate models with the null model without genetic terms. The optimal model retained *CCR5* Δ32, *CCR2* 64I, *CCR5* 59029AA, *MIP1*α 495TT, *SDF1* 3′A, *PML* −225TT, *PPIA* 1650G, and *TSG101* −183C ($P = 0.00005$). The model is statistically optimized given the available data. There might be allelic variants with large effects that are excluded from the optimal model because their standard errors are large due to a small allelic frequency in the population.

From the models, where effects are maximized by looking at the differences in progression time between a hypothetical patient with all the favorable markers and one with the least favorable markers, we could speculate that the genetic effects of these markers might be responsible for lengthening or shortening the time from 500 CD4 T cells/μl to < 200 CD4 T cells/μl by up to 2.8 years. However, this analysis will likely represent an overestimate of the effect, given that the best-fitting model will be overfitted to the specific data set and would not necessarily be reproduced in another data set.

In sensitivity analyses, we assessed a model limited to using the first six measurements for each patient. This approach suggested that *CCR5* 59029AA, *CCR5* Δ32/*CCR2* 64I, *MIP1*α 459TT, *RANTES* −28G, and the candidate allele *PPIA* 1604GG have substantially greater effects when the measurements are restricted to earlier times in the disease process. These estimates are consistent with temporal variation in the effect alleles have on the course of infection, as previously reported for variants of the chemokine receptors CCR5 and CCR2 (23, 32, 51). The model did not include data on HLA typing, a major genetic influence (4), the recently described gene dose effect of *CCL3L1* (17), or an extensive analysis of the CCR5 region to better define the contribution of *CCR5* 59029G>A in the context of the haplotypic structure of the gene cluster (15). Cross-validation with other cohorts remains mandatory before the proposed alleles of *PML*, *TSG101*, and *PPIA* can be defined as true host genetic variants influencing HIV-1 infection.

Only 10% of patients included in the study had known dates of seroconversion; thus, we estimated seroconversion dates (14). The effects of known influences (i.e., *CCR5* and *CCR2* alleles) found in this patient population support the validity of the effects estimated for other allelic variants. The study population investigated contributed substantial data on CD4 counts but insufficient data on viral load and limited data on AIDS-defining illness or death, as the progression of disease was for most patients controlled with the availability of potent ART. Analysis excluded any values after the initiation of treatment. We believe it is a strength of this work to define disease progression by CD4 loss rather than by AIDS or death, as this is an approach that can be proposed to other seroprevalent cohorts where disease progression is halted by treatment.

Conclusions. We are aware that an ex vivo system of population genetics will not capture genes linked to mechanisms of pathogenesis that are not relevant to a CD4-only model, in particular, those involving immunogenetic determinants, or other cell types involved in the pathogenesis of AIDS. This system also represents a reductionist approach to a highly complex process involving host and viral factors. However, we reasoned that the analysis of a simplified, more homogenous surrogate assay might overcome some of the obstacles in the analysis of complex traits by providing a method to single out potential candidate genes which could then be tested for their role in HIV-1 disease in vivo. The population included 128 donors, approximately 1 log less than the number of participants probably needed in association studies in vivo (22), consistent with the speculation that a standardized, simplified sys-

tem may magnify the consequences of specific alleles. Association studies do not define causality between certain SNPs and clinical/biological outcomes. The functional relevance of a marker allele needs biological plausibility and validation. The ex vivo system can be used for functional analyses such as the detailed analysis of cellular restriction blocks (6) and analysis of mRNA expression or splicing of selected genes (10). Alternatively, the ex vivo system can also be seen as an independent confirmation of in vivo findings, thus increasing the likelihood of identifying true genetic associations.

Overall, from eight previously reported genes associated with disease progression, the ex vivo/in vivo data were in agreement for two genes, one gene was identified only in vivo, and one gene that could not be evaluated ex vivo was associated with an effect in vivo. The data were in agreement with the literature for three genes, and one remains controversial among several publications. In particular, there was a correlation between the ex vivo and in vivo phenotype for well-established genetic variants of *CCR5* and *CCR2*. For nine candidate genes, five were dropped during the ex vivo assessment, one gene presented consistent data ex vivo/in vivo, while two were retained by both approaches; however, the attributed effect was of contrary sign between the two testing strategies. The paradoxical nature of the TSG101 and PML allele effects (less ex vivo viral production and faster progression in vivo) would imply mechanisms of pathogenesis for which there is no biological support at this time. The sequential approach for the selection of markers cannot exclude the possibility that valid markers were excluded that would have been identified in vivo. In the absence of a gold standard, and in the context of the complexity of making genetic associations on solid ground, a sequential strategy for screening ex vivo and validating in vivo cannot be presented as satisfactory or as suboptimal; rather, it is a potential approach to examining host genes participating in the viral life cycle and therefore intuitively amenable to testing in a cellular system. These are critical issues in the field of complex trait genetics where only 30% of reported associations can be considered proven (22, 45).

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